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Standardization of concentrated antibodies for use in automated immunohistochemistry

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Abstract

Background: Immunohistochemistry (IHC) is a method of identifying proteins in cells or tissues that is useful for diagnosis and research. In modern pathology, it has assumed an important supporting role in the molecular diagnosis of certain neoplasia, with an exponential contribution in personalized medicine. Automation in immunohistochemistry contributes to reduced test variability through standardization. The transition to automation is a process, and for a laboratory that already has a collection of stock-concentrated primary antibodies, ideally, these antibodies will be compatible with the chosen automated method, as antibodies are of high value to be discarded as a consequence of migration to automated immunohistochemistry.

Methods: 78 concentrated antibodies were tested for use in Ventana Medical Systems' Benchmark XT automation platform. Thirty-one human tissue samples containing the antigens of interest were used as positive control.

Results: All antibodies tested showed good performance, indicating the feasibility of using these antibodies concentrated in the automation platform in question. The protocol most frequently used was the one with antigenic retrieval with Cell Conditioning 1 for 60 minutes and incubation in the primary antibody for 32 minutes at 42ºC. The dilutions of the primary antibodies in automation ranged from 1:20 to 1:4000.

Conclusion: Under the aforementioned conditions, it was possible to take advantage of the portfolio of concentrated antibodies present in the laboratory at the time of transition from manual to automated immunohistochemistry.

Keywords: Automation, immunohistochemistry, standardization, concentrated antibodies

Introduction

Immunohistochemistry (IHC) is a technique that is used to identify proteins in cells or tissue constituents through antigen–antibody interaction, and it represents an important tool in the identification and localization of a variety of antigens [1]. IHC stands at the interface between traditional pathology, pathology recognition based on microscopic analysis of morphology, and molecular diagnosis. In addition to the invaluable utility of IHC as a tool for classifying neoplasms in the era of personalized medicine, the method has become essential for the correct stratification of oncology patients for target-specific [2,3].

In modern pathology, IHC has at least three main contributions: (1) diagnostic-mainly, the use of antibodies for the diagnosis of undifferentiated neoplasms, determination of primary site in metastatic diseases, and subtyping of neoplasias; (2) genetics-analysis of gain or loss of protein expression due to changes in genes and the mutational state of certain biomarkers; and (3) therapeutic-through the analysis and quantification of tissue expression, IHC results can determine the best treatment option and predict response to a target-specific [2,4].

Although IHC is a widely used method in diagnosis and research, there is a lack of standardization that may contribute to intra- and interlaboratory divergences [4,5]. Among the

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challenges permeating IHC are pre-analytical factors such as fixation of the samples, analytical factors such as selection of the antibody (whether monoclonal or polyclonal), choice of detection system, use of controls, and endogenous protein and enzyme blockade [6-8].

It is not possible to standardize a single protocol in all laboratories, but it should be possible to standardize the protocols within a single laboratory, in order to ensure reproducibility. This requires strict adherence to the protocol during the performance of the technique. It is clear that performance consistency, just as in clinical analysis labs, can only be achieved by automation, given its inherent consistency and control [6,9].

Among the main advantages of implementing IHC automation are the standardization of protocols and the possibility of increasing workload without compromising quality. Monitoring errors in processes with alarms for adverse situations, such as inadequate temperature and reagent volumes, expiration date control, and reagent inventory are also important [10,11].

The kinetics during incubation in automation, heating, and evaporation control guarantee a uniform environment that leads to reproducible results, optimizing and accelerating the reactions [10]. Variations in reagent use flexibility in automation platforms gave rise to the terms "open" or "closed" system. Closed systems offer greater standardization but with less flexibility. Open-system platforms, in contrast, enable easy migration from a manual routine to an automated one through the flexibility of reagents, to include the use of manual routine antibodies and protocol customization; thus, open platforms are preferable in research [10,11]. Laboratories incorporating manual immunohistochemistry have concentrated antibodies in stock; however, in the transition to automation, these financially valuable reagents would be discarded. In this study, the laboratory already had a portfolio of antibodies and opted for a platform that accepts antibodies from other suppliers, prompting us to ask whether a good immunostaining with concentrated antibodies from other suppliers in the Benchmark automation platform XT-Ventana Medical Systems could be obtained. Thus, we will test the hypothesis that it will indeed be possible to use concentrated antibodies from other manufacturers, with satisfactory immunostaining, on Benchmark XT automation platform.

Methods

In this experimental study stock-concentrated antibodies from manufacturers ARP, Calbiochem, Dako, Diagnostic BioSystems (DBS) and Novocastra (Leica Biosystems) were tested on the Benchmark Ventana automation platform.

Tissue samples

The study was approved by the Ethics Committee in Research at the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), opinion no. 332.213. Thirty one human samples (Table 1) fixed in 10% neutral buffered formalin and embedded in paraffin were selected from the Pathology Department, Santa Casa de Misericórdia de Porto Alegre, based on the presence of the antigen of interest for antibody testing. All tissue samples used in this standardization were selected five years after the issuance of the report containing the anatomopathological diagnosis, without prejudice to the conclusion of the case. Samples were already part of a bank of positive tissue controls in the laboratory.

To test the protocols, the samples were cut to 2-µm thickness, placed on StarFrost slides (Knittel Glass, Bielefeld, GER, ref. 9589), and individually labeled. The concentrated antibodies were stored, according to the manufacturer's instructions, at 2-8°C or in a freezer with automatic defrost from -30°C to -20°C. The manufacturer's indication in the data sheet for antigen retrieval and dilution was verified, and an algorithm (Figure 1) was followed. Antigen retrieval was divided into three groups: (1) heat-induced epitope retrieval; (2) enzymatic antigen retrieval with Protease I (0.38 mg/mL of alkaline protease in sodium azide of 0.01%; Ventana Medical Systems, Oro Valley, AZ, EUA, ref. 760-2018); and (3) antigen retrieval would not be required.

Manual technique

The protocol that follows was used for all antibodies until the implementation of automation. Deparaffinization (65°C) and antigen retrieval were performed using PT Link, Pre-Treatment Module for Tissue Specimens (Agilent), at a temperature of 97°C without using pressure, with appropriate Target Retrieval Solution for 30 minutes. Endogenous peroxidase blockade was done for all antibodies by Dual Endogenous Enzyme Block, contained in the Dako EnVision Dual Link System-HRP (DAB) detection kit (K4065), for 10 minutes. Primary antibodies were diluted in Antibody Diluent, Background

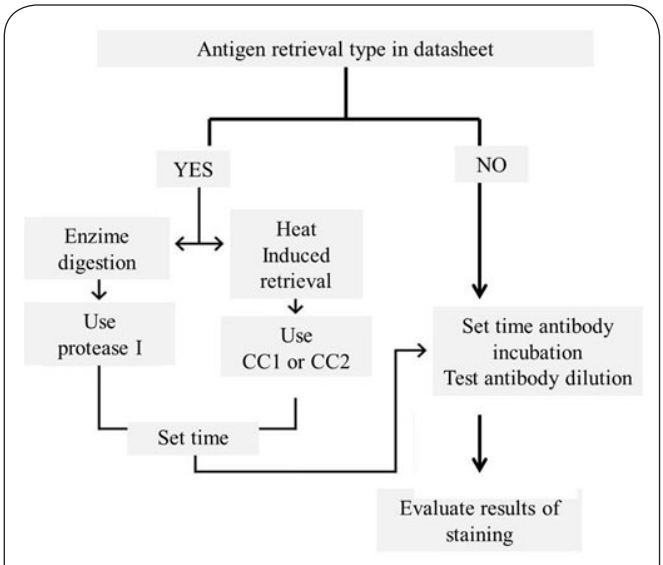

Figure 1. Decision algorithm for determination of antigen retrieval.

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Table 1. Samples used as a positive control.

ID	Sample	Antibody
1	Appendix	Desmin, Muscle actin, Smooth Muscle actin, Vimentin
2	Breast	Ck7, Ck8/18, E-cadherin, Epithelial Membrane Antigen (EMA)
3	Bone (Plasmocytoma)	Cd 38, Kappa Light Chains (KLC), Lambda Light chains (LLC)
4	Breast carcinoma	Her2, Estrogen receptor , P53
5	Infected lung	Cytomegalovírus (CMV)
6	Infected lymph node	Epstein Barr Vírus (EBV)
7	Infected kidney	Polioma vírus
8	Kidney	Collagen IV
9	Large intestine	Cdx2, Carcinoembryonic Antigen (CEA), Ck20, Villin
10	Liver	Alpha-1-fetoprotein, Hepatocyte specific antigen (HSA)
11	Lung	Cd68
12	Lung adenocarcinoma	Napsin A
13	Lymph node	Blc2, Blc6, Cd7, Cd8, Cd10, Cd20, Cd21, Cd23, Granzime B, Leucocyte common antigens (LCA)
14	Lymph node (Anaplastic large cell lymphoma)	Alk 1
15	Lymph node (Lymphoblastic cell lymphoma)	Terminal Deoxynucleotidyl transferase (TDT)
16	Lymph node (Hodgkin's lymphoma)	Cd15, Cd30
17	Medullary thyroid carcinoma	Calcitonin
18	Neuroendocrine pancreatic carcinoma	Cd57, Chromogranin A, Neuron specific enolase (NSE), Synaptophysin
19	Nerve (Peripheral nervous system)	Glial fibrillary acidic protein (GFAP), Neurofilament protein (NFP), S100
20	Ovarian serous papilliferous adenocarcinoma	Wilm´s tumor 1 (WT1)
21	Pancreas	Glucagon
22	Placenta	Annexin A1, Cd 31, Cd34, Chorionic Gonadotropin (HCG) Placental Alkaline Phosphatase (PLAP), Von Villenbrand Factor (VWF)
23	Prostate	Alpha-methylacyl-CoA racemase (AMACR), Prostate Specific Antigen (PSA)
24	Pituitary	Adrenocorticotropin (ACTH), Follicle Stimulating Hormone (FSH), Prolactin (PRL)
25	Skeletal muscle	Myoglobin
26	Skin	Ck 5/6, Ck Pan
27	Skin (Melanoma)	Melan-A
28	Small bowel (GIST)	Dog1
29	Testicle	Myeloperoxidase
30	Thyroid	Thyroglobulin, Thyroid transcription Factor-1(TTF1)
31	Tonsil	B Cell Specific Octamer Binding Protein1 (BOB1), Immunoglobulin A(IgA), Immunoglobulin D (IgD) Immunoglobulin G (IgG), Immunoglobulin M (IgM), Ki67, Lysozyme, Multiple myeloma oncogene 1 (Mum1)

Reducing (ref. S3022), Dako Denmark A/S, and incubated overnight at 2–8°C. Concentrations used in the manual routine are presented in **Table 2**, these data were used to compare concentrations of antibodies in the manual technique with those in the automated technique, and automation dilutions were also classified as lesser than, equal to, or greater than those used in the manual technique. The detection system used was Dako EnVision + Dual Link System-HRP (K4065) with Dab Chromogen, following manufacturer's instructions; the counterstaining step was performed with Harris' hematoxylin, followed by washing in 1% ammonium hydroxide. Washing buffer was phosphate-buffered saline, pH 7.0.

Automated IHC staining and evaluation

Seventy-eight concentrate antibodies were tested (**Table 2**), immunohistochemistry reactions were performed on VENTANA BenchMark XT (Ventana Medical Systems, Oro Valley, AZ, EUA) equipment, and the detection system used was UltraView Universal DAB Detection Kit (Ventana Medical Systems, Oro Valley, AZ, EUA, ref. 760-500). Endogenous peroxidase blockade was done for all antibodies in automation it is performed by the ultraView Universal DAB reagent Inhibitor contains 3% hydrogen peroxide solution, time is not provided by the manufacturer. The counterstain reagent was Hematoxylin II (Ventana Medical Systems, Oro Valley, AZ, EUA, ref. 790-2208),

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Table 2. Primary antibodies tested in automation.

Antibody	Clonality	Cellular localization	Lot	Supplier	Manual dilution
ACTH	02A3	Cytoplasmic	10044413	DAKO	1:1500
ALK Protein	ALK1	Cytoplasmic/nucleus	00056980	DAKO	1:300
Alpha-1- Fetoprotein	Polyclonal	Cytoplasmic	00058169	DAKO	1:500
AMACR	EPMU1	Cytoplasmic	6005488	NOVOCASTRA	1:500
Bcl 2	124	Cytoplasmic	00056477	DAKO	1:300
Bcl 6	PG-B6p	Nucleus	00053818	DAKO	1:200
BOB 1	TG14	Cytoplasmic/nucleus	6003030	NOVOCASTRA	1:200
Calcitonin	CL1948	Cytoplasmic	6003520	NOVOCASTRA	1:2000
CEA	Polyclonal	Cytoplasmic	00034436	DAKO	1:500
CD X2	AMT 28	Nucleus	6000213	NOVOCASTRA	1:700
CD 7	CBC.37	Membrane	00053089	DAKO	1:50
CD 8	C8/144B	Membrane	00056474	DAKO	1:300
CD10	NCL-L-CD10-270	Membrane	6019294	NOVOCASTRA	1:25
CD 15	BY87	Membrane	6015073	NOVOCASTRA	1:200
CD 20	L 26	Membrane	00050873	DAKO	1:600
CD 21	1 F 8	Membrane	00080760	DAKO	1:200
CD 23	MHM6	Membrane	00049836	DAKO	1:200
CD 30	Ber - H2	Membrane	00057005	DAKO	1:700
CD 31	JC70A	Cytoplasmic/membrane	00051102	DAKO	1:100
CD 34	QBEnd- 10	Membrane	00053768	DAKO	1:100
CD 38	SPC32	Membrane	6006807	NOVOCASTRA	1:100
CD 57	TB 01	Membrane	00061124	DAKO	1:200
CD 68	PGM1	Cytoplasmic	00058931	DAKO	1:500
HCG	Polyclonal	Cytoplasmic	00053071	DAKO	1:1500
Chromogranin A	DAK - A3	Cytoplasmic	00052696	DAKO	1:1000
Cytokeratin 5/6	D5/16B4	Cytoplasmic	00060846	DAKO	1:400
Cytokeratin 7	OV-TL 12/30	Cytoplasmic	00050559	DAKO	1:800
Cytokeratin 8/18	5D3	Cytoplasmic	L106751	NOVOCASTRA	1:700
Cytokeratin 20	Ks 20.8	Cytoplasmic	00057249	DAKO	1:700
CKPAN	MNF 116	Cytoplasmic	00048696	DAKO	1:200
CMV	CCH2+DDG9	Nucleus	00057285	DAKO	1:1000
Collagen IV	CIV	Membrane	00050818	DAKO	1:50
Desmin	D 33	Cytoplasmic	00052230	DAKO	1:300
DOG-1	K9	Cytoplasmic	6007637	NOVOCASTRA	1:100
EBV	CS.1-4	Cytoplasmic	00055677	DAKO	1:700
E-cadherin	NCH-38	Membrane	10042951	DAKO	1:400
EMA	E29	Cytoplasmic/membrane	00051982	DAKO	1:300
Estrogen Receptor	SP1	Nucleus	00050839	DAKO	1:400
FSH	C10	Cytoplasmic	10039598	DAKO	1:800
Granzime B	GrB-7	Cytoplasmic	00055619	DAKO	1:300
GFAP	6F2	Cytoplasmic	00062411	DAKO	1:400
Glucagon	Polyclonal	Cytoplasmic	10040085	DAKO	1:500
Her2	SP3	Membrane	R026	DBS	1:1500

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Continuation of Table 2.

Antibody	Clonality	Cellular localization	Lot	Supplier	Manual dilution
Hepatocyte	OCH1E5	Cytoplasmic	00061010	DAKO	1:400
IgA	N1CLA.	Cytoplasmic	6008931	NOVOCASTRA	1:10000
IgD	DRN1C	Cytoplasmic	6009435	NOVOCASTRA	1:10000
IgG	RWP49	Cytoplasmic	6009436	NOVOCASTRA	1:10000
IgM	8H6	Cytoplasmic	6007528	NOVOCASTRA	1:10000
KLC	CH15	Cytoplasmic	6006134	NOVOCASTRA	1:3000
Ki-67	MIB-1	Nucleus	00079939	DAKO	1:400
LLC	SHL53	Cytoplasmic	6005595	NOVOCASTRA	1:10000
LCA	2B11+PD7/26	Membrane	00055808	DAKO	1:600
Lysozyme	Polyclonal	Cytoplasmic	00051100	DAKO	1:1500
Melan-A	A 103	Cytoplasmic	00051087	DAKO	1:500
Muscle Actin	HHF35	Cytoplasmic	10042823	DAKO	1:500
Myeloperoxidase	59A5	Cytoplasmic	6000030	NOVOCASTRA	1:300
MUM 1	mum1 p	Nucleus/cytoplasmic	00060192	DAKO	1:200
Napsin A	01-0221	Cytoplasmic	60828	ARP	1:500
Myoglobin	MYO18	Cytoplasmic	6008887	NOVOCASTRA	1:500
NFP	2F11	Cytoplasmic	00085150	DAKO	1:200
NSE	BBS/NC/V I-H14	Cytoplasmic	00051206	DAKO	1:600
P53	DO-7	Nucleus	00050255	DAKO	1:800
P63	7JUL	Nucleus	6010555	NOVOCASTRA	1:200
PLAP	8A9	Cytoplasmic	00051152	DAKO	1:200
Polioma Vírus	PAb416	Nucleus	D00073537	CALBIOCHEM	1:200
Prolactin	Polyclonal	Cytoplasmic	10040256	DAKO	1:1500
PSA	ER-PR8	Cytoplasmic	00051198	DAKO	1:900
PSMA	3 E 6	Cytoplasmic	10042956	DAKO	1:900
Synaptophysin	sy-38	Cytoplasmic	00064754	DAKO	1:300
S100	Polyclonal	Nucleus/cytoplasmic	00048024	DAKO	1:3500
Smooth Muscle Actin	1A4	Cytoplasmic	00059322	DAKO	1:500
TDT	SEN28	Nucleus	6004211	NOVOCASTRA	1:100
Thyroglobulin	DAK-Tg6	Cytoplasmic	00051933	DAKO	1:1000
TTF1	SPT24	Nucleus	6011867	NOVOCASTRA	1:1100
VWF	F8-86	Cytoplasmic	00052965	DAKO	1:100
Villin	CWWB1	Cytoplasmic/membrane	6003038	NOVOCASTRA	1:2500
Vimentin	V9	Cytoplasmic	00051105	DAKO	1:3000
Wilms' Tumor 1	6F-H2	Nucleus	10042947	DAKO	1:400

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a modified Mayer's hematoxylin, time of counterstaining in hematoxylin II was defined according to the location of the antigen, with the objective of not masking nuclear antigens with Dab chromogen in the nucleus of the cells; for nuclear antigens, staining was done for 8 minutes, and for membrane antigens and cytoplasm, 12 minutes. All antibodies used were valid during the antibody testing period from January to December 2012.

Pathologist analysis used the following criteria: (a) specific reaction with the antigen, classified as 0 (absent), 1 (weak), 2 (satisfactory), or 3 (strong); (b) technical quality, classified as 0 (unsatisfactory), 1 (regular), or 2 (satisfactory); and (c) background color, classified as 0 (absent), 1 (low), or 2 (present). During microscopic evaluation by the pathologist when the staining was weak, even after an increase in the antigenic recovery time and the concentration of the primary antibody, the Amplification Kit (Ventana Medical Systems, Oro Valley, AZ, EUA, ref. 760-080) was added to the protocol. The Amplification Kit (**Table 3**) may be used in conjunction with VENTANA detection kits to increase the signal intensity of weak-staining primary antibodies. After each run in the equipment the quality of the immunostaining was evaluated by the pathologist and this algorithm was followed for the decision making regarding changes in the protocols. When the result was considered unsatisfactory for any of the above reasons, the algorithm (**Figure 2**) for protocol change was followed. After defining the best protocol, each slide was digitalized using an Olympus BX51 microscope (Olympus Optical Co., Tokyo, Japan) connected to a digital color camera (Q-Color 5, Olympus); QCapture software was used for image capture.

Results

Protocols of 78 primary concentrated antibodies for automated use were standardized and are presented in **Table 3**. Immunostaining in automation photomicrographs are presented in **Figures 3-6**.

Regarding the types of antigenic retrieval, most of the antibodies benefited from an antigen retrieval in the Cell

Table 3. Protocols of concentrate antibodies for Ventana Benchmark XT- Automation.

Antibody	Antigen retrieval (min)	Incubation	Dilution	Antibody	Antigen retrieval (min)	Incubation	Dilution
ACTH	Not	32 min	1:1000	Granzime B	90 CC1	90 min	1:50
ALK Protein	60 CCl	32 min	1:250	GFAP	60 CC1	32 min	1:500
α-1- Fetoprotein	Not	32 min	1:50	Glucagon	Not	32 min	1:200
AMACR	60 CC1	32 min	1:100	Her2	60 CC1	32 min	1:500
Bcl 2	90 CC1	90 min	1:25*	Hepatocyte	60 CC1	32 min	1:300
Bcl 6	90 CC1	90 min	1:50*	IgA	60 CC1	32 min	1:200
BOB 1	90 CC1	90 min	1:25*	IgD	60 CC1	32 min	1:50
Calcitonin	4 P1	32 min	1:300	IgG	60 CC1	32 min	1:1000
CEA	60 CC1	32 min	1:1000	IgM	60 CC1	32 min	1:400
CDX2	90 CC1	90 min	1:25*	KLC	60 CC1	32 min	1:2000
CD 7	60 CC1	32 min	1:50	Ki-67	60 CC1	32 min	1:50
CD 8	60 CC1	32 min	1:1000	LLC	60 CC1	32 min	1:2000
CD10	60 CC1	32 min	1:20	LCA	60 CC1	32 min	1:500
CD 15	84 CC2	60 min	1:25	Lysozyme	Not	60 min	1:500
CD 20	60 CC1	32 min	1:500	Melan-A	60 CC1	32 min	1:100
CD 21	4P1	60 min	1:25*	Muscle Actin	60 CC1	32 min	1:300
CD 23	60 CC1	32 min	1:150	Myeloperoxidase	60 CC1	32 min	1:300
CD 30	90 CC1	60 min	1:30	MUM 1	60 CC1	32 min	1:100
CD 31	60 CC1	32 min	1:100	Napsin A	60 CC1	32 min	1:500
CD 34	90 CC1	32 min	1:100	Myoglobin	60 CC1	60 min	1:400
CD 38	60 CC1	32 min	1:400	NFP	60 CC1	32 min	1:50
CD 57	60 CC1	32 min	1:100	NSE	60 CC1	32 min	1:500
CD 68	60 CC1	32 min	1:300	P53	60 CC1	32 min	1:200
HCG	60 CC1	32 min	1:4000	P63	60 CC1	32 min	1:50
Chromogranin A	60 CC1	32 min	1:250	PLAP	60 CC1	32 min	1:100

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Continuation of Table 3.

Antibody	Antigen retrieval (min)	Incubation	Dilution	Antibody	Antigen retrieval (min)	Incubation	Dilution
Cytokeratin 5/6	60 CC1	32 min	1:200	Polioma Vírus	60 CC1	32 min	1:500
Cytokeratin 7	60 CC1	32 min	1:400	Prolactin	Not	32 min	1:1250
Cytokeratin 8/18	60 CC1	32 min	1:350	PSA	Not	32 min	1:100
Cytokeratin 20	60 CC1	32 min	1:350	PSMA	90 CC1	90 min	1:200
CKPAN	60 CC1	32 min	1:100	Synaptophysin	90 CC1	90 min	1:50*
CMV	4P1	32 min	1:800	S100	60 CC1	32 min	1:2000
Collagen IV	90 CC1	32 min	1:100	Smooth Muscle Actin	60 CC1	32 min	1:500
Desmin	60 CC1	32 min	1:150	TDT	60 CC1	32 min	1:200
DOG-1	90 CC1	32 min	1:100	Thyroglobulin	Not	32 min	1:500
EBV	60 CC1	32 min	1:600	TTF-1	60 CC1	32 min	1:600
E-cadherin	60 CC1	32 min	1:150	VWF	4P1	32 min	1:50
EMA	60 CC1	32 min	1:100	Villin	60 CC1	32 min	1:300
Estrogen Receptor	90 CC1	32 min	1:50	Vimentin	60 CC1	32 min	1:2000
FSH	4P1	32 min	1:100	Wilms' Tumor 1	8P1	90 min	1:50

Abbreviations: Ck: Cytokeratin; HMV: High Molecular Weight; AR: Antigen retrieval; CC1: Cell Conditioning 1; P1: Protease I; CC2: Cell Conditioning 2 * Use amplification kit.

Conditioning 1 buffer (**Table 4**). The most commonly used protocol was with antigen retrieval for 60 minutes and incubation time in the primary antibody for 32 minutes at 42°C. All the algorithms and figures presented here were developed by the authors.

Incubation times with the primary antibody were 32 minutes (83%), 60 minutes (6.4%), and 90 minutes (10.2%). The dilutions of the primary antibodies ranged from 1:20 to 1:4000, while in the manual IHC method, dilutions ranged from 1:25 to 1:10.000. We observed that most antibodies (83%) were used at lower (more concentrated) dilutions, while only 16% of antibodies could be used at the same or larger (less concentrated) dilutions than in the manual technique (**Figure 7**).

Table 4. Antigen Retrieval type.

Antigen retrieval	Antibodies	%
HIER*- Cell Conditioning 1	64	82.1
HIER*- Cell Conditioning 2	1	1.2
Enzymatic epitope retrieval	6	7.7
Not required	7	9

*HIER- Heat induced epitope retrieval

Discussion

All antibodies showed positive immunostaining, which indicates the feasibility of using the laboratory's in-stock concentrates when automation is implemented. The use of these reagents (concentrated antibodies) acquired and in stock represents savings for the laboratory, since their disposal, due to the mandatory purchase of antibodies ready for use from the Ventana, would represent an even greater expense as a result of the introduction of automation. In general, the antibodies in the automation platform needed to be more concentrated relative to the dilutions that were used in the manual routine. This may be due to the incubation time of the antibodies, which in the manual technique is at least 14 hours (2–8°C) and in the automated, at most 90 minutes. In the study by Gedda *et al*. (2010), using real-time immunohistochemistry, the authors propose a direct and quantitative antigen–antibody interaction analysis, indicating that when using incubations as recommended by many manufacturers for 30 minutes, the primary antibody had been linked to less than 10% of available sites. In addition, the authors point out that slight variations in incubation time and antibody concentration strongly affect the amount of bound antibody [**12**]. In our results, we observed that for more than 90% of the antibodies it was necessary to use some method of antigenic recovery, which is expected, since formalin (fixator commonly used in pathological anatomy) is a cross-link fixative, which can lead to loss of immunoreactivity, which can be restored by using the antigenic recovery technique [**13**]. Formalin´s ability to cross-link is potentially deleterious to antigenic strutucture, however there are some epitopes that are not significantly affected. Despite the fixation of all samples used as positive controls in formalin, for 7 antibodies, it was not necessary to use the antigen retrieval. Perhaps this preservation of reactivity may be related to the fact that some epitopes are irreversibly modified by formalin while others are not [**14**]. Regarding the sensitivity of epitopes to formalin they can be classified into three main groups: formalin-resistant epitopes, highly formalin-sensitive epitopes and epitopes with a time-dependent sensitivity to formalin fixation [**15**].

Some issues became evident during the process of transi-

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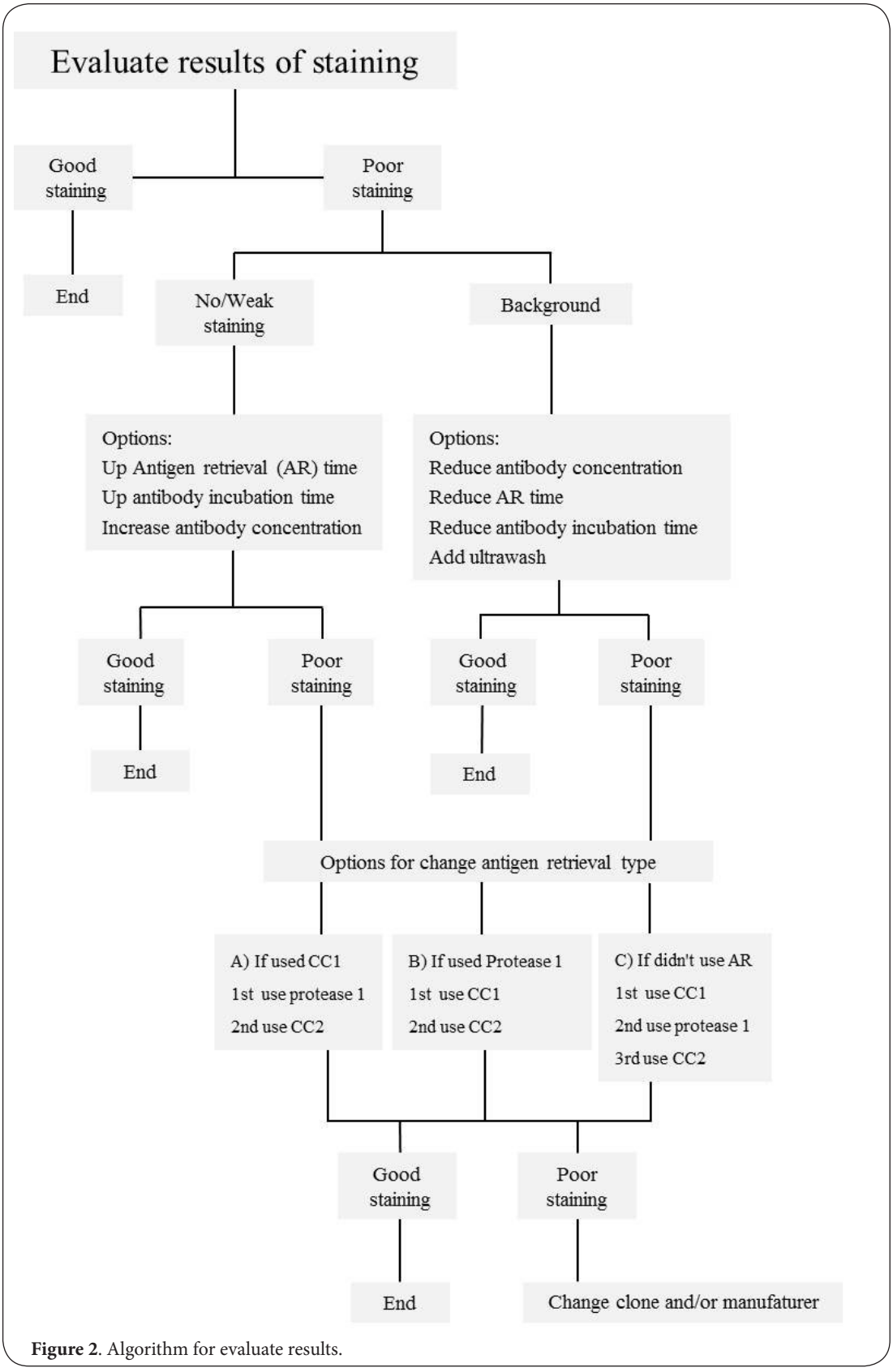

Figure 2. Algorithm for evaluate results.

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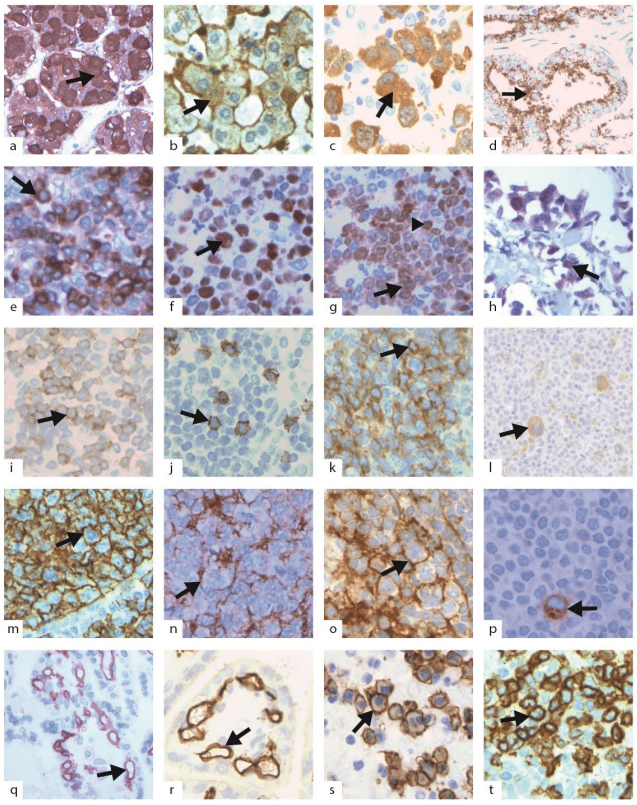

Figure 3. Immunostaining, magnification 400x for all panels. **a**) ACTH, cytoplasmic (arrow); **b**) Alpha-1- Fetoprotein, cytoplasmic (arrow); **c**) Alk, cytoplasmic (arrow); **d**) AMACR, cytoplasmic (arrow); **e**) Bcl 2, cytoplasmic (arrow); **f**) Bcl6, nucleus (arrow); **g**) BOB 1, cytoplasmic (arrow) and nuclear (arrow head▲); **h**) Calcitonin, cytoplasmic (arrow); **i**) Cd7, membrane (arrow); **j**) Cd8, membrane (arrow); **k**) Cd10, membrane (arrow); **l**) Cd15, membrane (arrow); **m**) Cd20, membrane (arrow); **n**) Cd21, membrane (arrow); **o**) Cd 23 membrane (arrow); **p**) Cd30, membrane (arrow); **q**) Cd31, membrane (arrow); **r**) Cd34 membrane (arrow); **s**) Cd38, membrane (arrow); **t**) Cd57, membrane (arrow).

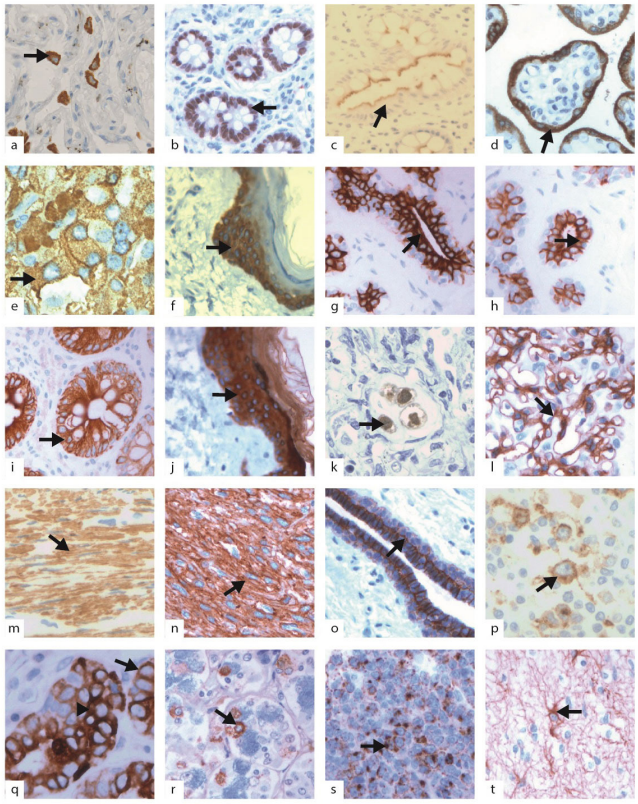

Figure 4. Immunostaining, magnification 400x for all panels. **a**) Cd68, cytoplasmic (arrow); **b**) Cdx2, nuclear (arrow); **c**) CEA, cytoplasmic (arrow); **d**) HCG, cytoplasmic (arrow); **e**) Chromogranin A, cytoplasmic (arrow); **f**) Ck 5/6, cytoplasmic (arrow); **g**) Ck 7, cytoplasmic (arrow); **h**) Ck8/18, cytoplasmic (arrow); **i**) Ck 20, cytoplasmic (arrow); **j**) CKPAN, cytoplasmic (arrow); **k**) CMV, nuclear (arrow); **l**) Collagen IV, membrane (arrow); **m**) Desmin, cytoplasmic (arrow); **n**) DOG-1, cytoplasmic (arrow); **o**) E-cadherin, membrane (arrow); **p**) EBV, cytoplasmic (arrow); **q**) EMA, membrane (arrow) and cytoplasmic (▲arrow head); **r**) FSH, cytoplasmic (arrow); **s**) Granzyme B, cytoplasmic (arrow); **t**) GFAP, cytoplasmic (arrow). Abbreviation: Ck (Cytokeratin).

tion from manual to automated IHC: first, it is imperative that at least one professional in the technical area be in charge of capturing, along with the experienced pathologist, the inadequacies in the marking obtained and making the appropriate change in the protocol. Second, knowing the demand of each antibody is critical in order to avoid previous dilution for an extended length of time, and on the platform tested, it is possible to keep 5 mL of diluted antibody. At the time of refilling of the diluted antibody, it is necessary to discard any dead volume present in the vial to avoid changing the antibody concentration determined by titration. Third, in centers where acquisition is complex or time-consuming, the management and purchase of reagents should be carefully planned given the importance of expiration dates of certain essential reagents (e.g., detection kits)—the equipment does not perform IHC until reagents with acceptable validity are recorded. Last, the use of purified water without contaminants is essential for the dilution of bulky fluids such as antigen retrieval buffers. While the use of purification stations for distilled water supply is an alternative, it requires periodic microbiological control.

The benefits of automation with IHC seem to meet the requirements of higher reproducibility, quality, and standardization concomitant with increased demand. Automation has been applied in many branches of the medical sciences, arriving definitively in pathology laboratories [16]. Among the innovations that are changing the practice in laboratories of pathological anatomy are genetic tests, telepathology, and adherence to automation [17]. Another form of innovation is the implementation of laboratory and bar code information

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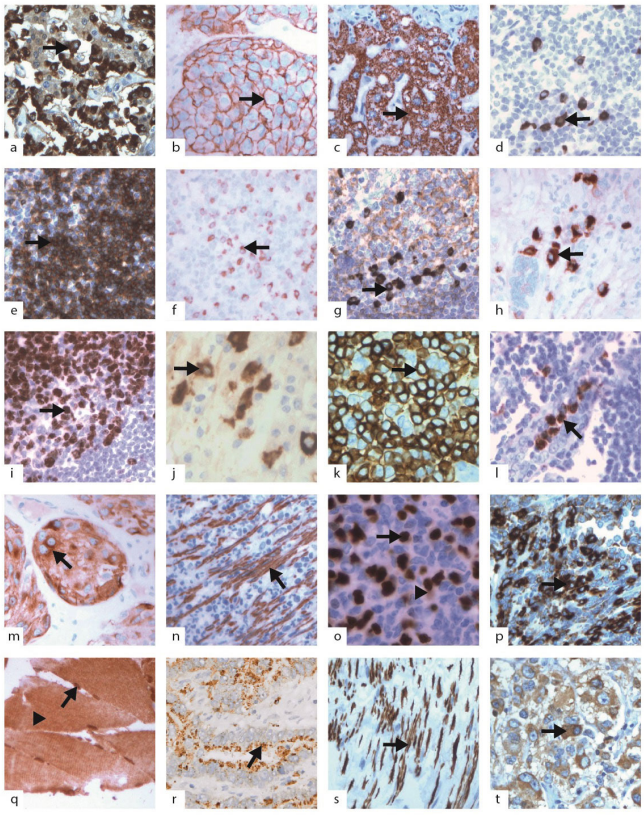

Figure 5. Immunostaining, magnification 400x for all panels. **a**) Glucagon, cytoplasmic (arrow); **b**) Her2, membrane (arrow); **c**) Hepatocyte, cytoplasmic (arrow); **d**) IgA, cytoplasmic (arrow); **e**) IgD, cytoplasmic (arrow); **f**) IgG, cytoplasmic (arrow); **g**) IgM, cytoplasmic (arrow); **h**) Kappa Light Chains, cytoplasmic (arrow); **i**) Ki-67, nuclear (arrow); **j**) LLC, cytoplasmic (arrow); **k**) LCA, membrane (arrow); **l**) Lysozyme, cytoplasmic (arrow); **m**) Melan-A, cytoplasmic (arrow); **n**) Muscle Actin, cytoplasmic (arrow); **o**) MUM 1, nuclear (arrow) and cytoplasmic (▲arrow head); **p**) Myeloperoxidase cytoplasmic (arrow); **q**) Myoglobin, nuclear (arrow) and cytoplasmic (▲arrow head); **r**) Napsin A, cytoplasmic (arrow); **s**) NFP, cytoplasmic (arrow); **t**) NSE, cytoplasmic (arrow).

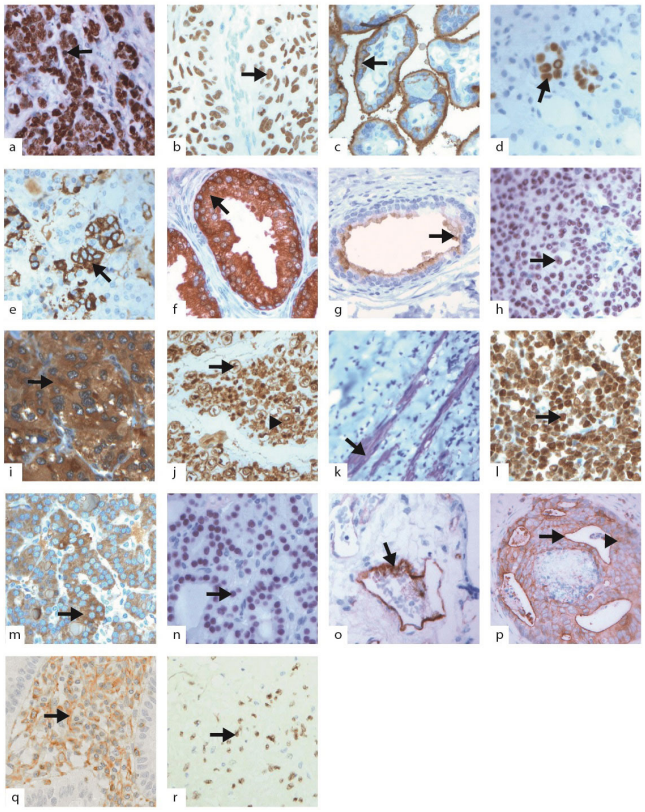

Figure 6. Immunostaining, magnification 400x for all panels. **a**) P53, nuclear (arrow); **b**) P63, nuclear (arrow); **c**) PLAP, cytoplasmic (arrow); **d**) Polioma Vírus, nuclear (arrow); **e**) Prolactin, cytoplasmic (arrow); **f**) PSA, cytoplasmic (arrow); **g**) PSMA, cytoplasmic (arrow); **h**) Estrogen Receptor, nuclear (arrow); **i**) Synaptophysin, cytoplasmic (arrow); **j**) S100, nuclear (arrow) and cytoplasmic (▲arrow head); **k**) Smooth Muscle Actin, cytoplasmic (arrow); **l**) TDT, nuclear (arrow); **m**) Thyroglobulin, cytoplasmic (arrow); **n**) TTF1, nuclear (arrow); **o**) Von Willebrand Factor, cytoplasmic (arrow); **p**) Villin, membrane (arrow) and cytoplasmic (▲arrow head); **q**) Vimentin, cytoplasmic (arrow); **r**) WT1, nuclear (arrow).

systems [18]. Promoting innovation in pathology is desirable; however, increasing costs with equipment and reagents are challenging and need to be overcome, the literature on the challenges in this area is scarce, and information on the subject of standardization, integration, and innovation in the laboratory often comes of other areas [17].

There are a few limitations of the study. First, there was an inability to compare immunostaining of concentrated antibodies with their ready-to-use counterparts. Because the main motivation of the study was the existence of antibody stocks in the laboratory, the laboratory did not acquire the equivalent ready-to-use stocks marketed by the manufacturer. The option to test automation in this laboratory was conditioned to the possibility of using the portfolio of pre-existing concentrated antibodies, which represented invested money. Second, no negative controls were used in the standardization, and the objective of the use of negative controls is to demonstrate that the reaction visualized is due to the interaction of the target protein epitope and the antibody parotope [19]. Last, a reaction cost analysis for manual and automated techniques was not performed.

Many factors make it impossible to establish a universal standard protocol for IHC, and it is up to each laboratory to choose and validate the protocol for each marker, whether the technique is automated or not. The standardization of an antibody that will be used in automation is part of the transition to automated IHC in open systems that allow the use of concentrated antibodies. In this case, the manufacturer's

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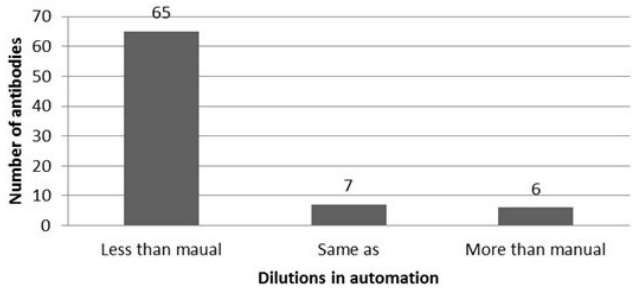

Figure 7. Antibody concentration used in automation in relation to manual technique.

recommendations are a good starting point [7,20].

Conclusion

If one is opting for an automation platform to perform immunohistochemistry according to the results presented here, it is possible to use concentrated antibodies that may already be in the laboratory, either for economic reasons or because a manufacturer does not have the antibody that is needed. For other equipment or antibodies, the standardization must be performed again. The process of standardization in large centers that have many antibodies can become an arduous task. However, with the possibility of migration to this incremental innovation, standardization should serve as a subsidy for a conscious migration in all areas of the laboratory, especially among pathologists, technicians, and administrators.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	EFSP	CGZ
Research concept and design	✓	✓
Collection and/or assembly of data	✓	--
Data analysis and interpretation	✓	✓
Writing the article	✓	--
Critical revision of the article	✓	✓
Final approval of article	✓	✓
Statistical analysis	✓	--

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