The EnVision™+ system: a new immunohistochemical method for diagnostics and research. Critical comparison with the APAAP, ChemMate™, CSA, LABC, and SABC techniques

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Abstract

***Aim*—To assess a newly developed immunohistochemical detection system, the EnVision™+.**

***Methods*—A large series of differently processed normal and pathological samples and 53 relevant monoclonal antibodies were chosen. A chessboard titration assay was used to compare the results provided by the EnVision™+ system with those of the APAAP, CSA, LSAB, SABC, and ChemMate™ methods, when applied either manually or in a TechMate 500 immunostainer.**

***Results*—With the vast majority of the antibodies, EnVision™+ allowed two- to fivefold higher dilutions than the APAAP, LSAB, SABC, and ChemMateTM techniques, the staining intensity and percentage of expected positive cells being the same. With some critical antibodies (such as the anti-CD5), it turned out to be superior in that it achieved consistently reproducible results with differently fixed or overfixed samples. Only the CSA method, which includes tyramide based enhancement, allowed the same dilutions as the EnVision™+ system, and in one instance (with the anti-cyclin D1 antibody) represented the gold standard.**

***Conclusions*—The EnVision™+ is an easy to use system, which avoids the possibility of disturbing endogenous biotin and lowers the cost per test by increasing the dilutions of the primary antibodies. Being a two step procedure, it reduces both the assay time and the workload.**

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Keywords: immunohistochemistry; detection method; EnVision™+; antigen retrieval

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Immunohistochemistry is nowadays an integral part of both the diagnostic process and research activity. However, there is a lack of standardisation of the techniques used and results can vary from laboratory to laboratory, making comparisons difficult. Many technical factors contribute to this situation. First, the type of tissue available for immunohistochemistry is not always adequate because of problems with sampling or processing. Second, most if not all fixatives produce a certain degree of antigen masking by modifying the protein structure of the tissue.[1 2] Hence, immunophenotyping has mainly been performed on fresh tissue, though the fixation problems can be partly overcome by treating routine sections with proteolytic enzymes.[3–5] At the beginning of the 1990s, new techniques for antigen retrieval from routine sections were proposed, based on the use of heat. These rapidly gained ground because they had the following advantages: (1) they preserve cytological detail, (2) they are easy to use, (3) they enhance the sensitivity of immunohistochemical methods, and (4) their effectiveness is almost independent of the fixative used.[6–22] Finally, the detection method chosen is crucial, since its sensitivity greatly influences the results.[23–39] This is particularly important when dealing with routine material where the target availability can be very slight—either because of antigen masking or because the antigen is only present in trace amounts. In assessing the performance of immunohistochemical methods, the feasibility of using high dilutions of the primary antibody needs to be examined, since high sensitivity contributes to the specificity of the results and undoubtedly reduces the cost per test.

In this paper, we report on the results of a clinical trial of the recently introduced EnVision™+ system.[23–31]

Methods

THE ENVISION™+ SYSTEM

EnVision™+ is a two step staining technique in which the primary antibody is followed by a polymeric conjugate in sequential steps.[23–31] The polymeric conjugate consists of a large number of peroxidase and secondary antibody molecules bound directly to an activated dextran backbone (fig 1). The polymeric conjugates hold up to 100 enzyme molecules and up to 20 antibody molecules per backbone.

TISSUE SELECTION

For this study, a large series of samples from normal and pathological tissues was selected. The former comprised hyperplastic bone marrow, reactive lymph nodes, and placenta at term; the latter included granulomatous lymphadenitis, lymphomas belonging to the main categories of the REAL classification,[32] non-lymphoid leukaemias grouped according to the

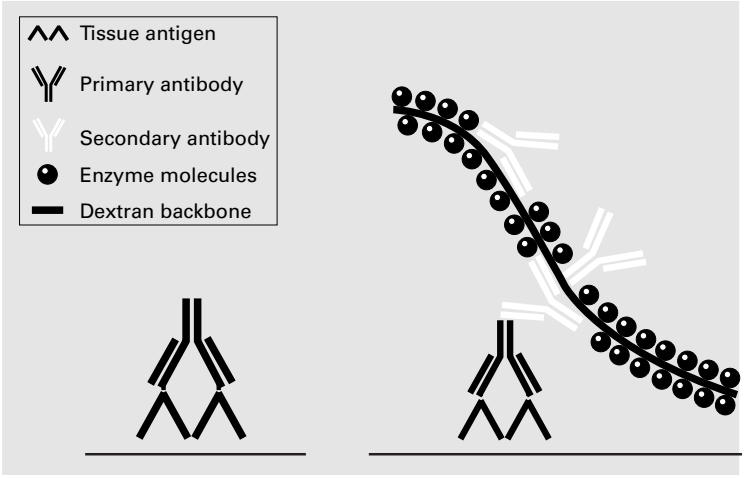


Figure 1 Schematic drawing of the EnVision™+ system.

FAB classification,[33] primary or secondary carcinomas arising in the breast, small bowel, prostatic gland, endometrium, thyroid, and larynx, as well as retinoblastomas and rhabdomyosarcomas.

SAMPLE PROCESSING

The procedures differed depending on the type of sample. In particular, the bone marrow biopsies were fixed in B5 for 2.30 hours, washed in 70% alcohol for 30 minutes, and then decalcified for 2.30 hours in bisodic salts of ethilendiaminotetracetic acid (EDTA); the lymph nodes were partly fixed in B5 for 3.0 hours and then washed in 70% alcohol, and partly fixed in 10% buffered formalin for periods ranging from 24 hours to one week. The last procedure (10% buffered formalin for 24 hours to one week) was also used for the remaining samples.

Table 1 Comparison between the optimal dilutions of primary antibodies against haemo-lymphopoietic markers allowed by the EnVision™+ system and the CSA, SABC, LSAB, and APAAP methods

Antibody (clone if monoclonal/commercial source)	*SABC/LSAB/APAAP*	*EnVision/CSA*
CD1a (O10/Immunotech)	1:2	1:12
CD3 (polyclonal/Dako)	1:300	1:1200
CD5 (CLA229/Medac)	1:5 (inconsistent results)	1:20
CD8 (C8/144B/Dako)	1:5	1:20
CD15 (C3D-1/Dako)	1:5	1:40
CD20 (L26/Dako)	1:200	1:1000
CD21 (IF8/Dako)	1:10	1:40
CD23 (MHM6/Dako)	1:50	1:100
CD30 (Ber-H2/Prof. Stein)	1:10	1:40
CD34 (QBEND-10/Immunotech)	1:20	1:80
CD40 (MAB89/Dako)	1:100	1:400
CD43 (DF-T1/Dako)	1:200	1:800
CD45 (PD7/26+2B11/Dako)	1:500	1:1000
CD45R (UCHL-1/Dako)	1:120	1:480
CD45R (Ki-B3/Prof Parwaresh)	1:20	1:80
CD57 (Leu7/Becton Dickinson)	1:20	1:40
CD45RA (4KB5/Dako)	1:20	1:80
CD61 (Y2-51/Dako)	1:5	1:5
CD68 (KP1/Dako)	1:640	1:2560
CD68 (PG-M1/Prof Falini)	1:20	1:80
CD79a (JCB117/Prof Mason)	1:10	1:60
Glycophorin A (JC159/Dako)	1:320	1:1280
Neutrophilic elastase (NP57/Dako)	1:10	1:40
FVIIIRAg (F8/86/Dako)	1:6	1:24
Lysozyme (polyclonal/Dako)	1:800	1:3200
TdT (polyclonal/Dako)	1:30	1:80
Cyclin D1 (D1-GM/Novocastra)	Negative	Negative/1:40
κ light chain (polyclonal/Dako)	1:11 000	1:22 000
λ light chain (polyclonal/Dako)	1:13 000	1:26 000
MPO (polyclonal/Dako)	1:10 000	1:40 000
S-100 (polyclonal/Dako)	1:2000	1:8000

After fixation and possible decalcification, all specimens were washed in 70% alcohol and processed in a VIP 2000 machine (Miles Scientific, Bayer Divisione Diagnostica, Milan, Italy), with paraffin embedding (Merck Histologie® non-caking paraffin) at 56°C.

ROUTINE SECTION PRETREATMENT FOR ANTIGEN UNMASKING AND ANTIBODIES

For this trial, the antigen unmasking technique was defined according to the experience gained in the course of a previous study, where a series of antibodies had been applied at different dilutions on routine sections from tissue samples fixed in 10% buffered formalin or B5, and from B5 fixed, EDTA decalcified bone marrow biopsies,[34] by using the APAAP,[35] LSAB,[36] and SABC[37] methods.

Fifty three antibodies (listed in tables 1 and 2) were tested with the EnVision™+ system. They were diluted according to a chessboard titration method, starting from the highest dilution reached in the previous study.[22]

The EnVision™+ system was used both manually and automatically, with horseradish peroxidase and diaminobenzedene hydrochloride (DAB) being the enzyme and chromogen employed. In the manual mode, the results were compared with those from the APAAP,[35] CSA,[38] LSAB,[36] and SABC[27] techniques performed at room temperature. With the automatic approach, the EnVision™+ system was used in a TechMate 500 (Biotech Solutions, Santa Barbara, California, USA), the results being compared to those obtained with the APAAP, LSAB, SABC, and ChemMate™ methods. When DAB was the chromogen, endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 15 minutes at room temperature, in keeping with the characteristics of the ready-to-use solution included in the EnVision™+ package and with our previous experience, which had shown this approach to be efficient when using critical samples such as bone marrow.[39]

COST OF THE DETECTION METHODS EMPLOYED

All the detection systems used for the present study were purchased from Dako A/S (Glostrup, Denmark). According to prices on the Italian market, the cost/test of the EnVision™+ system[23 31] was 20% higher than that of the corresponding reagents/kits for the APAAP, CSA, SAB, and SABC methods, while it was 40% cheaper then the ChemMate™ package.

RESULT EVALUATION

The following indices were considered in evaluating the results and comparing the EnVision™+ system with the other methods: (1) the type of immunoreactivity (staining intensity and specificity), and (2) the highest/optimal dilution reachable with each antibody. The results were scored from − to ++++ according to the percentage of cells expected to be positive and the staining intensity.

Results

When manually applied and compared with the APAAP, LSAB, and SABC methods, the

Table 2 Comparison between the optimal dilutions of primary antibodies against non-haemo-lymphopoietic markers allowed by the EnVision™+ system and the CSA, SABC, LSAB, and APAAP methods

Antibody (clone if monoclonal/commercial source)	*SABC/LSAB/APAAP*	*EnVision/CSA*
Kinetic markers, oncogene and infective agent associated antigens		
bcl-2 (124/Dako)	1:20	1:80
p53 (DO-7/Dako)	1:40	1:160
Ki-67 (MIB-1/Prof Gerdes)	1:4	1:16
LMP (CS1-4/Dako)	1:7	1:28
CMV (CCH2+DDG9/Dako)	1:35	1:140
HP (polyclonal/Dako)	1:20	1:80
Intermediate filaments		
Cytokeratin (MNF116/Dako)	1:70	1:300
CK-HMW (Dako)	1:50	1:200
CK-LMW (Dako)	1:50	1:200
VIM (V9/Dako)	1:200	1:800
DESM (D33/Dako)	1:200	1:800
NF (2F11/Dako)	1:20	1:80
Other markers		
Actin (HHF35/Dako)	1:200	1:800
EMA (E29/Dako)	1:5	1:40
PSA (ER-PR8/Dako)	1:30	1:120
HMB45 (Dako)	1:50	1:200
Thyroglobin (polyclonal/Dako)	1:4000	1:16 000
α_1 fetoprotein (polyclonal/Dako)	1:2000	1:4000
Chromogranin A (polyclonal/Dako)	1:8000	1:32 000
NSE (BBS/NC/VI-H14/Dako)	1:150	1:1200
ER (1D5/Dako)	1:100	1:400
PRG (polyclonal/Dako)	1:200	1:400

EnVision™+ system provided superior results with all the antibodies employed, irrespective of the fixative used, as shown in tables 1 and 2, where the optimal working dilution of each reagent is matched with the immunohistochemical technique employed. In particular, the EnVision™+ system usually allowed two- to fivefold greater dilutions than the other techniques for the same staining intensity and percentage of expected positive cells (for example, figs 2 and 3). Remarkably, these results were also obtained with some antibodies which are known to give uncertain responses in formalin fixed material. Thus when applying the anti-CD5 monoclonal antibody to samples fixed for 24 hours, the EnVision™+ system provided clear cut staining of the neoplastic cells both in B cell chronic lymphocytic leukaemia and mantle cell lymphoma (six and five cases tested, respectively) (fig 4), while the APAAP, LSAB, and SABC techniques gave rise to inconstant results since the positivity of the lymphomatous elements ranged from weak to null while that of reactive T lymphocytes remained distinct in all cases. However, it should be noted that also the EnVision™+ technique showed progressive fading of the CD5 staining when the fixation was longer than 24 hours. Moreover, with the EnVision™+ system the Ber-H2/CD30—which unpredictably works in overfixed material[5 22]—continued to produce vivid staining even in samples soaked in formalin for one week (fig 5). In all instances, the detected antigens showed their proper location (at the membrane or cytoplasmic level) and the staining appeared stable with time when the sections were exposed to light for three months. Neither background staining nor endogenous peroxidase activity were observed when optimal dilutions of the primary antibodies and the peroxidase inhibition system of the EnVision™+ package were used (figs 2–12).

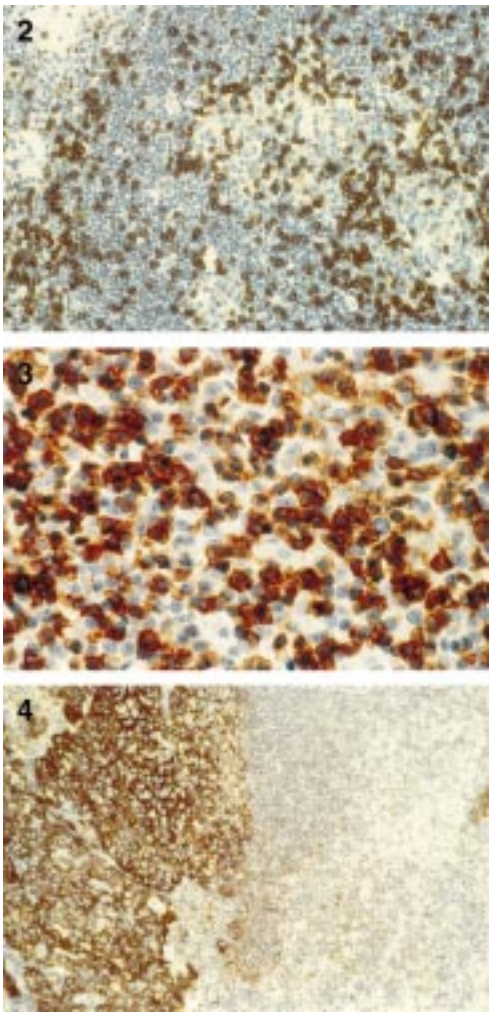
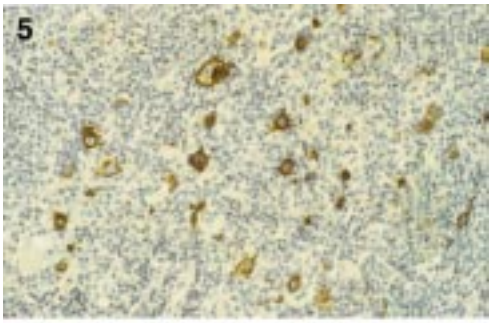
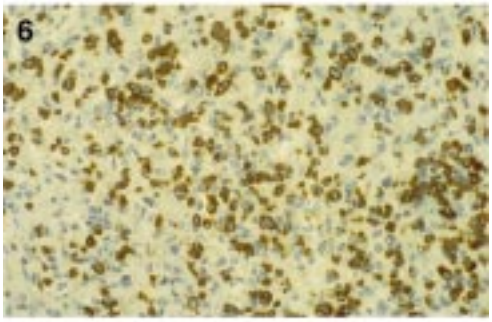


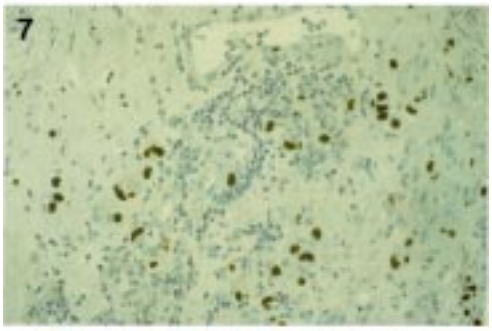
Figure 2 Follicular hyperplasia: staining of T lymphocytes within the mantle zone and germinal centre with the UCHL1/CD45R0 monoclonal antibody (EnVision™+ system; Gill's haematoxylin nuclear counterstaining, ×160).
Figure 3 Follicular hyperplasia: staining of follicular dendritic cells with the MHM6/CD23 monoclonal antibody (EnVision™+ system; Gill's haematoxylin nuclear counterstaining, ×400).
Figure 4 Peripheral mantle B cell lymphoma: neoplastic cells are strongly stained by the anti-CD5 monoclonal antibody (EnVision™+ system; Gill's haematoxylin nuclear counterstaining, ×320).

As shown in tables 1 and 2, no significant differences were observed in terms of sensitivity between the EnVision™+ and CSA methods. Only with the antibody against cyclin D1 did the latter indeed appear superior, producing a clear cut positivity which was never detected with the EnVision™+ system. When the immunohistochemical procedures were carried out automatically by the TechMate 500 machine, the results remained superior to those provided by the APAAP, LSAB, and SABC methods, as well as those of the specifically developed ChemMate™ technique.

On economic grounds, although the Envision™+ system was 20% more expensive than the APAAP , LSAB, and SABC detection methods, the final cost per test was 35–60% less, thanks to the much higher dilution of the primary antibody, which allowed two to five more preparations to be stained with the same amount of reagent. Comparison with the







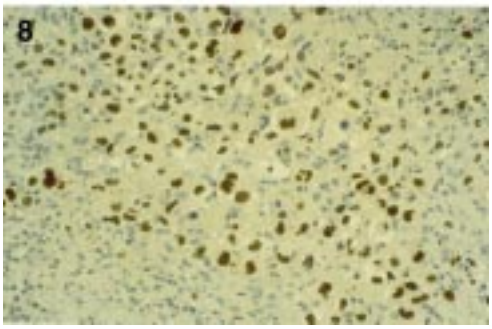
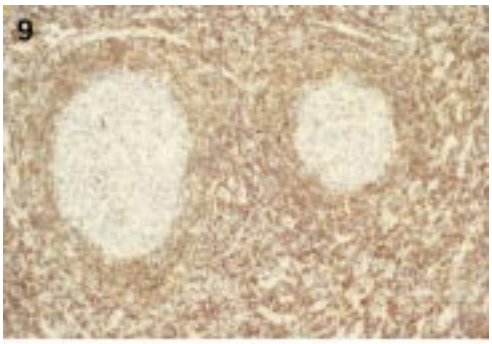
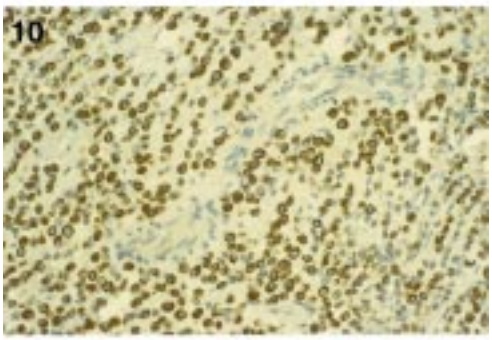
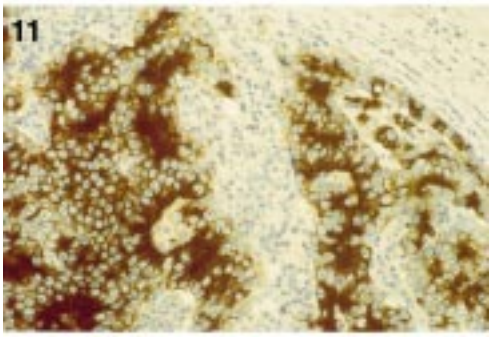


Figure 5 Hodgkin's disease: strong Ber-H2/CD30 positivity of Hodgkin and Reed-Sternberg cells in a sample, fixed in formalin for one week (EnVision™+ system; Gill's haematoxylin nuclear counterstaining, ×240).
Figure 6 Myeloid metaplasia of the spleen: positivity of myeloid elements with the polyclonal antibody anti-myeloperoxidase (EnVision™+ system; Gill's haematoxylin nuclear counterstaining, ×320).
Figure 7 Ulcerative colitis in an HIV positive patient: the specific monoclonal antibody shows the presence of cytomegalovirus (EnVision™+ system; Gill's haematoxylin nuclear counterstaining, ×240).
Figure 8 Anaplastic large cell lymphoma: most neoplastic cells react with the anti-p53 monoclonal antibody DO-7 (EnVision™+ system; Gill's haematoxylin nuclear counterstaining, ×80).







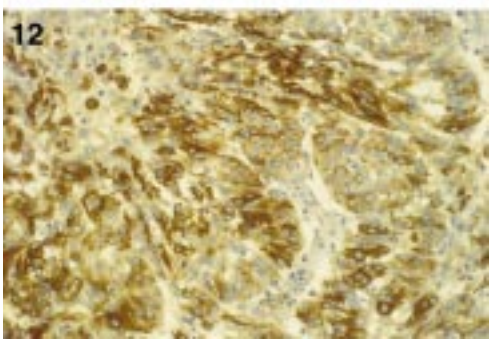


Figure 9 Follicular hyperplasia: paracortical T lymphocytes and mantle B cells express the bcl-2 product, while germinal centre cells are negative (EnVision™+ system; Gill's haematoxylin nuclear counterstaining, ×320).
Figure 10 Lymph node metastatic involvement by rhabdomyosarcoma: neoplastic cells are strongly stained by the D33 monoclonal antibody raised against the intermediate filament desmin (EnVision™+ system; Gill's haematoxylin nuclear counterstaining, ×320).
Figure 11 Lymph node metastatic involvement by prostatic carcinoma: the ER-PR8 monoclonal antibody anti-prostate specific antigen reacts with most tumour elements (EnVision™+ system; Gill's haematoxylin nuclear counterstaining, ×240).
Figure 12 Lymph node metastatic involvement by malignant melanoma: the HMB45 monoclonal antibody stains the vast majority of neoplastic cells (EnVision™+ system; Gill's haematoxylin nuclear counterstaining, ×320).

ChemMate™ was even more favourable, the final cost per test with the Envision™+ system being 45–68% less depending on the dilution of the primary antibody. Only the CSA method, which allowed the same dilutions of the primaries, appeared cheaper than the Envision™+ system; however, the time required to immunostain the slides with the CSA method was longer than with the Envision™+ system

(five hours *v* two hours). Thus when the investigation was carried out manually the former incurred a significant extra cost owing to the prolonged involvement of the technical staff.

Discussion

Our results show that the EnVision™+ system is a very sensitive method that usually allows high dilutions of the primary antibodies while maintaining the specificity of the reaction. These advantages become particularly evident when the system is applied manually and its results are compared with those of the most commonly applied methods (the APAAP, LSAB, and SABC techniques). The EnVision™+ system is, however, also readily applicable to immunostainers, with which it gives optimal stains, equivalent to the specifically developed detection systems (for example, the ChemMate™). Only the CSA technique,[38] which includes the tyramide based enhancement, appeared to be as effective as the EnVision™+ system; indeed with the anti-cyclin D1 monoclonal antibody it represented the gold standard.

Besides allowing cheaper immunohistochemical assays—as shown by comparative analysis of the cost per test with the other methods employed—the high dilutions of the primary antibody achieved by the EnVision™+ system also enable more reliable results to be obtained, since very high dilutions prevent background staining, formation of electrostatic or other non-immunological non-specific bonds, or unexpected cross reactivities (especially with polyclonal antibodies).[4]

In addition, the immunostains obtained with the EnVision™+ system do not seem to be influenced either by the fixation employed or by its accuracy—in our hands, analogous findings were observed in samples soaked both in formalin (for various lengths of time) and B5, as well as in B5 fixed, EDTA decalcified bone marrow biopsies. This positive effect should probably be interpreted as a combination of two factors—the system sensitivity on the one hand, and the type of antigen exposure performed[22] on the other.

It is important to emphasise that the use of the EnVision™+ system does not influence or have a negative effect on previously developed and optimised antigen retrieval techniques[23 24]: on the contrary it allows even higher dilutions to be achieved.

The EnVision™+ system also helps in overcoming the problems frequently encountered with some critical antibodies (for example, anti-CD5, Ber-H2/CD30),[5 22] where reactivity on routine sections depends both on the amount of the target molecule present in the tissue and on antigen preservation; the latter in turn is largely determined by how the sample has been treated.

Finally, the EnVision™+ system provides some additional advantages which are related to its intrinsic structural characteristics, such as the lack of endogenous biotin activity, and to the reduction of the staff workload and assay time.

In the light of these results, we think that the EnVision™+ method represents a powerful tool both for daily routine and for research work, which can contribute to the standardisation of immunohistochemical techniques.

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