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Article

Simultaneous de-waxing and standardisation of antigen retrieval in immunohistochemistry using commercially available equipment

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Summary
In this article we describe our experiences using a commercial piece of equipment to achieve antigen retrieval of tissue sections. The successful introduction of the LabVision PT Module has led to the attainment of a single antigen retrieval protocol to replace six original protocols. It has also led to standardisation of antigen retrieval within our Department which together with the use of automated staining has meant complete standardisation of all our immunohistochemistry procedures. The Department has also benefited from a significant decrease in the time spent on antigen retrieval thus freeing staff for other tasks.

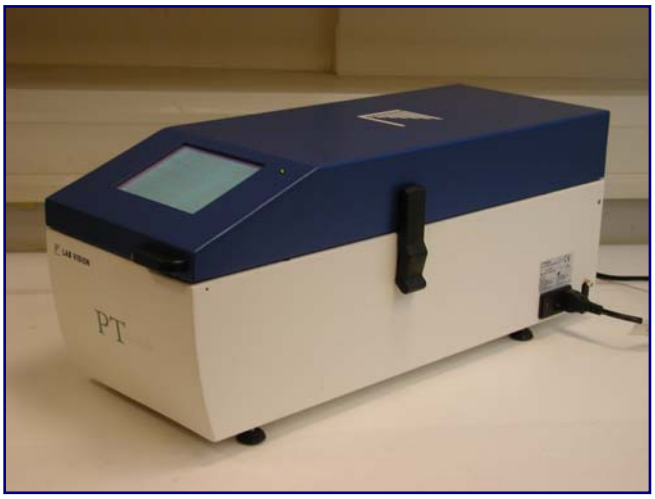
INTRODUCTION

In modern immunohistochemistry the key to optimum demonstration of many antigens is adequate antigen retrieval, which is necessary due to the masking of epitopes by tissue fixation. A plethora of antigen retrieval techniques are currently in use including various heat mediated methods and enzyme digestion.

We recently became aware of a commercial piece of equipment designed to achieve optimum antigen retrieval in a simple manner. The concept of simplicity pertaining to antigen retrieval is not new[1]. The equipment is known as a PT Module (pre-treatment module) and is marketed by LabVision (LabVision UK Ltd, The Pines, Fordham Road, Newmarket CD 8 7LG). The PT Module is illustrated in Figure 1.

After reviewing the company literature[2], we decided to try the pre-treatment module in our Department.

Figure 1. The LabVision PT Module.



MATERIALS AND METHODS

All tissues which were used had been fixed in 10% buffered formalin for 24-48 hours and processed to paraffin wax on an automated processor using an overnight schedule. Sections were then cut at 3µm, picked up on Superfrost plus slides (Thermo-Electron, 93-96 Chadwick Road, Astmoor, Runcorn, Cheshire WA7 1PR) and dried for 30 minutes at 60°C.

The slides were removed from the oven and inserted into Autostainer racks (Dako Ltd, Denmark House, Angel Drove, Ely, Cambridgeshire CB7 4ET) which were then placed into the PT Module. Each Tank contained 1.5 litres of Tris-EDTA buffer pH 8.0 preheated to 65°C. A warm-up cycle was then initiated and the slides incubated at 98°C for 25 minutes. After the pre treatment the racks were removed and rapidly cooled in de-ionised water. The racks were then loaded on a Dako Autostainer and stained by a standard method using Dako ChemMate reagents (Cat. No. K5001) and Peroxidase block (Cat. No. S2023); other secondary reagents can be used if desired (unpublished personal observation). All primary antibodies were diluted using Dako ChemMate antibody diluent (Cat. No. S2022).

Our standardised staining protocol employs the following incubation times:

- Primary antibody 30 minutes
- ChemMate Reagent A 30 minutes
- ChemMate Peroxidase Block 5 minutes
- ChemMate Reagent B 12 minutes
- ChemMate DAB+ 3 minutes (x2)

All buffer washes between reagents were carried out using Tris Buffered Saline pH 7.6. Counterstaining was carried out using Harris' Haematoxylin (R. A. Lamb, Units 4&5, Parkview Industrial Estate, Alder Close, Lottbridge Drove, Eastbourne, East Sussex BN23 6QE) for 1 minute.

RESULTS

Table 1 shows the range of antibodies with which the PT Module was used, while Figure 2 shows the results achieved with our range of primary antibodies. The overall quality of staining was excellent with minimum background. Staining was crisp, with no, or at worst, minimal patchiness when demonstrating antigens expressed as nuclear, cytoplasmic or membrane staining patterns.

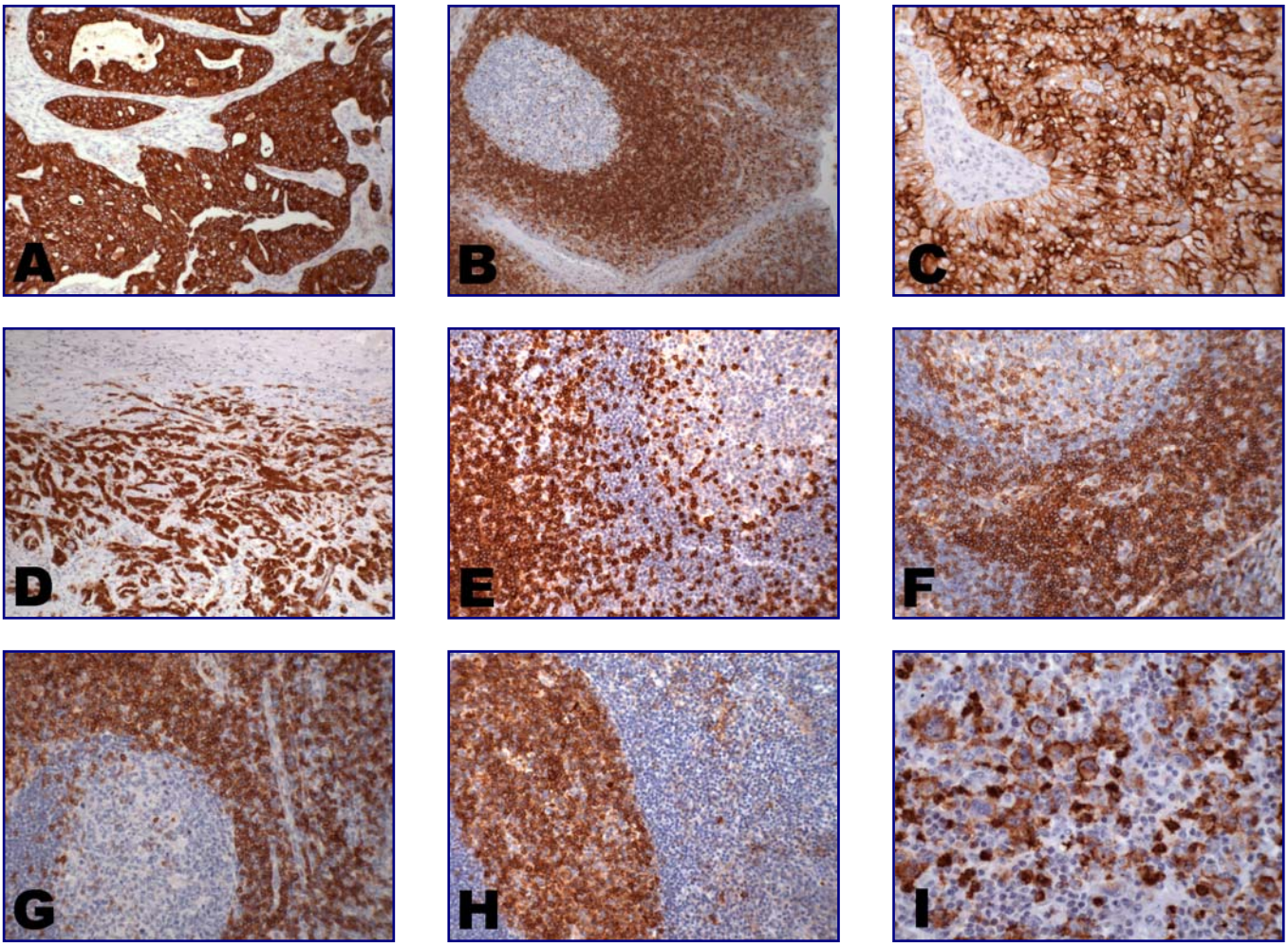
Primary Antibody / Antigen	Supplier	Clone	Dilution Used Previously	Dilution Used With PT Module	Comment
Bcl2	Dako	124	1:50	1:100	Increased dilution
Bcl6	Dako	BG-B6p	1:50	1:100	Increased dilution
Ca125	Dako	M11	1:100	1:100	Dilution unchanged
Calretinin	Dako	DAK-Calret 1	1:50	1:100	Increased dilution
CD1a	Dako	010	1:50	1:50	Dilution unchanged
CD2	Novocastra	AB75	1:100	1:100	Dilution unchanged
CD3	LabVision	SP7 (rabbit monoclonal)	1:50	1:200	Increased dilution
CD4	Novocastra	4B12	1:100	1:100	Dilution unchanged
CD5	Dako	CD5/54/F6	1:50	1:100	Increased dilution
CD7	Novocastra	CD7-272	1:100	1:100	Dilution unchanged
CD8	Novocastra	1A5	1:100	1:100	Dilution unchanged
CD10	Novocastra	56C6	1:40	1:40	Dilution unchanged
CD15	BD Biosciences	LeuM1	1:20	1:20	Dilution unchanged
CD20	Dako	L26	1:100	1:200	Increased dilution
CD21	LabVision	2G9	1:50	1:50	Dilution unchanged
CD23	LabVision	SP23 (rabbit monoclonal)	1:50	1:50	Dilution unchanged
CD30	Dako	BerH2	1:100	1:100	Dilution unchanged
CD31	Dako	JC/70A0	1:100	1:100	Dilution unchanged
CD34	Dako	QBEnd10	1:100	1:100	Dilution unchanged
CD45	Dako	2B11+PD7/26	1:100	1:100	Dilution unchanged
CD56	Novocastra	CD56-504	1:50	1:50	Dilution unchanged
CD68	LabVision	KP1	1:400	1:2000	Increased dilution
CD79a	Dako	JCB117	1:100	1:100	Dilution unchanged
CD138	Dako	M115	1:100	1:100	Dilution unchanged
CD246	Dako	ALK	1:50	1:50	Dilution unchanged
CK5/6	Dako	D5/16 D4	1:50	1:100	Increased dilution
CK7	Dako	OV-TL 12/30	1:50	1:100	Increased dilution
CK20	Dako	Ks20.8	1:50	1:100	Increased dilution

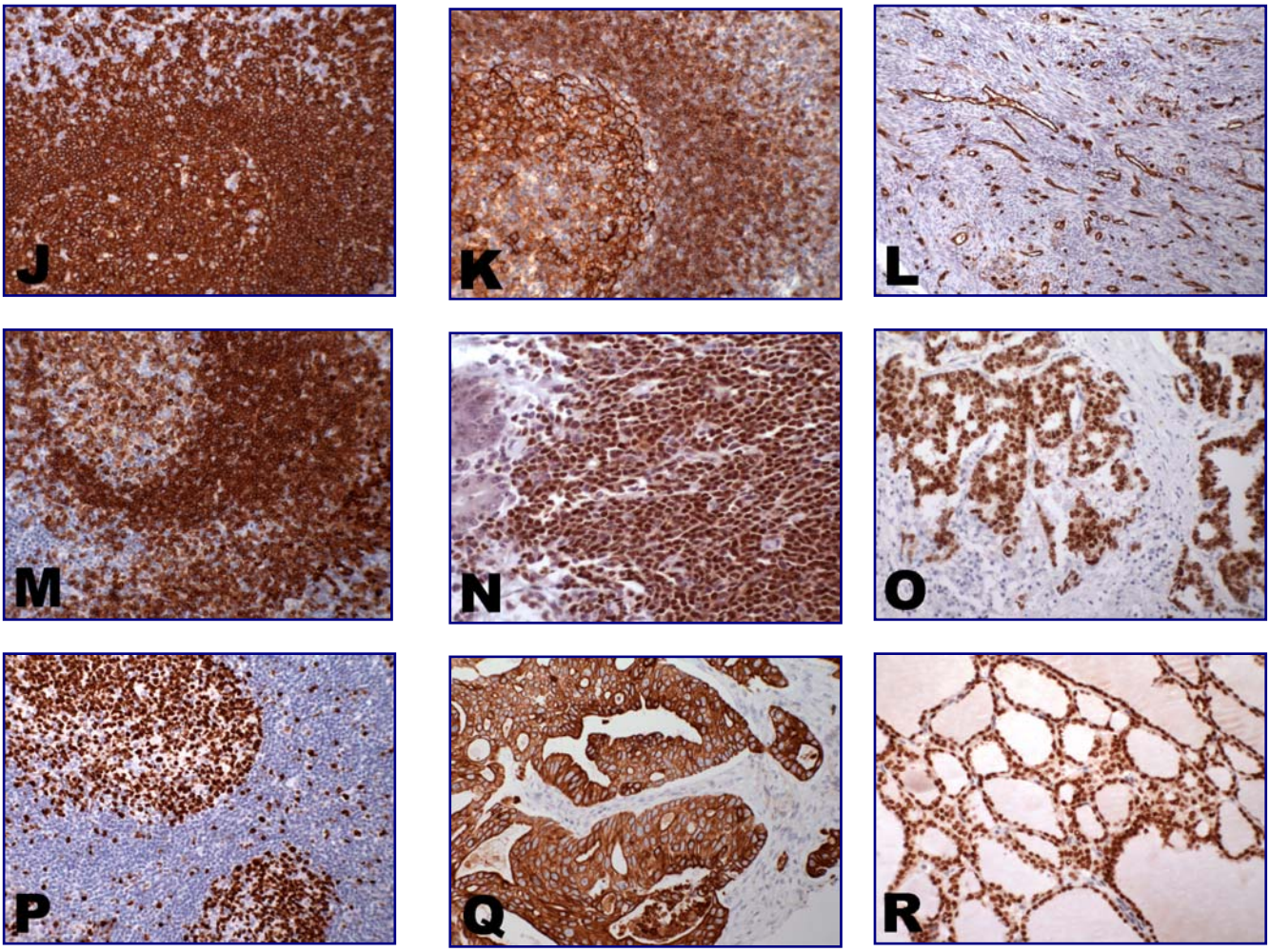
Table 1 continues on following page

Primary Antibody / Antigen	Supplier	Clone	Dilution Used Previously	Dilution Used With PT Module	Comment
Cyclin D1	LabVision	SP4 (rabbit monoclonal)	1:50	1:50	Dilution unchanged
Cytokeratins	Dako	AE1/AE3	1:100	1:100	Dilution unchanged
Cytokeratins	BD Biosciences	CAM 5.2	Neat	Neat	Dilution unchanged
Cytokeratins	Dako	MNF116	1:50	1:100	Increased dilution
Desmin	Dako	DE-R-11	1:100	1:100	Dilution unchanged
E-Cadherin	Dako	NCH-38	1:50	1:50	Dilution unchanged
EMA	Dako	E29	1:200	1:200	Dilution unchanged
Epithelial Antigen	Dako	BerEP4	1:100	1:100	Dilution unchanged
ER	LabVision	SP1 (rabbit monoclonal)	1:50	1:50	Dilution unchanged
Melanosome	Dako	HMB 45	1:100	1:100	Dilution unchanged
Inhibin	Oxford BioInnovation	MCA951S	1:100	1:100	Dilution unchanged
Kappa light chains	Dako	Rabbit polyclonal	1:5000	1:5000	Dilution unchanged
Lambda light chains	Dako	Rabbit polyclonal	1:5000	1:5000	Dilution unchanged
Ki67	Dako	MIB-1	1:100	1:100	Dilution unchanged
Thyroglobulin	Dako	DAK-Tg6	1:1000	1:1000	Dilution unchanged
TTF1	Dako	8G7G3/1	1:100	1:100	Dilution unchanged
Vimentin	Dako	V9	Neat	Neat	Dilution unchanged

Table 1. Details of primary antibodies used in this study. As a result of initial pilot studies we found that we could increase the dilution of a number of primary antibodies while retaining excellent quality of staining.

Figure 2. Photomicrographs illustrative of the immunostaining results achieved are shown below, and on the following page. The legend for this figure is to be found at the foot of the following page.





A) AE1AE3 staining of cytokeratins in breast carcinoma (x10). **B**) Bcl2 staining in a reactive tonsil (x10). **C**) CA125 staining of ovarian carcinoma (x20). **D**) Calretinin staining in malignant mesothelioma (x10). **E**) CD3 staining in a reactive tonsil (x20). **F**) CD4 staining in a reactive tonsil (x20). **G**) CD5 staining in a reactive tonsil (x20). **H**) CD10 staining in a reactive tonsil (x20). **I**) CD15 staining of Hodgkin lymphoma (x40). **J**) CD20 staining in a reactive tonsil (x20). **K**) CD23 staining in a reactive tonsil (x20). **L**) CD34 staining of endothelial cells in uterine stroma (x20). **M**) CD79a staining in a reactive tonsil (x20). **N**) Cyclin D1 staining of a mantle cell lymphoma in stomach (x40). **O**) SP1 staining of oestrogen receptors in breast carcinoma (x40). **P**) Ki67 staining in a reactive tonsil (x40). **Q**) MNF116 staining of cytokeratins in breast carcinoma (x20). **R**) TTF1 staining in a thyroid (x20). Figures in closed brackets represent the original objective magnification.

DISCUSSION

The PT module consists of two tanks each 1.5 litre capacity to hold buffer. These tanks insert into the body of the machine and can be independently heated to 100°C.

The lid of the machine incorporates a touch screen for programming and controlling the unit. The unit is designed for use with an Autostainer (Dako or Lab Vision) and slides can be loaded in the tanks using Autostainer racks. The unit will cope with 48 slides i.e. 4 Autostainer racks each holding 12 slides.

The unit is supplied with commercial buffers covering the pH range 6.0 – 10.0. These buffers are also designed to achieve section de-waxing as well as antigen retrieval and are supplied as x100 concentrates. The buffers currently available are:

- Citrate buffer pH 6.0 (Cat. No. TA050-PM1X)
- Tris-EDTA buffer pH 8.0 (Cat. No. TA050-PM2X)
- Tris-EDTA buffer pH 9.0 (Cat. No. TA050-PM4X)
- Tris HCL buffer pH 10.0 (Cat. No. TA050-PM3X)

We began using the unit in March 2005. Initially we selected Tris-EDTA pH 8.0 buffer since our protocols in use for pre-treatment at that time spanned the range pH 6.0 to pH 9.9. They were as follows:

- Microwaving in Citrate buffer pH 6.0 for 12 minutes
- Microwaving in Citrate buffer pH 6.0 for 10 minutes, followed by incubation in α-chymotrypsin for 30 seconds
- Microwaving in Citrate buffer pH 6.0 for 30 minutes
- Incubation in α-trypsin pH 7.8 for 15 minutes
- Microwaving in Target Retrieval Solution (Dako, Cat. No. S1699) for 12 minutes
- Microwaving in Target Retrieval Solution High pH 9.9 (Dako, Cat. No. S3307) for 15 minutes.

It seemed reasonable to select a buffer in the mid point of this range for initial studies. All results discussed in this article were obtained using Tris-EDTA buffer pH 8.0.

The PT module operates on two cycles (via the touch screen). These are the pre-heat cycle and the warm-up cycle. During the pre-heat cycle the buffer tanks will heat to a pre-set temperature of 65°C. The pre-heat cycle can be set to continuous, one time pre-heat or disable pre-heat. On continuous pre heat the tanks will heat to 65°C and hold at this temperature until a warm-up cycle is started. When a run is finished the tanks will cool to 65°C and hold at this temperature until a new run is started. On one time pre-heat each tank will pre-heat to 65°C until warm up cycle is started. When the run is finished the tanks will cool to 65°C. On disable pre-heat no pre-heating will take place before a run. If a pre-heat cycle is being used when the buffer has reached the required temperature (65°C) the slides can be loaded. The unit will then hold the slides at this temperature until prompted by the operator. Using the touch screen the operator can then select the warm-up cycle. The slides are then heated up to the temperature selected by the user and incubated for a time period, again selected by the user.

We routinely use a temperature at 98˚C and an incubation time of 25 minutes. The unit takes approximately 10 – 15 minutes to reach pre-heat temperature and approximately 10 – 15 minutes to reach warm-up temperature. This means a total time for antigen retrieval of around 55 – 60 minutes. On completion of an incubation period each tank has a cooling fan which switches on automatically to return the temperature to pre-heat value or ambient, if pre-heat is not being used. When the run is finished slides can be stained using standard protocols.

Initially we used the module with a small number of antibodies and we achieved very good results in terms of quality of staining. These included CK (MNF116), CD3, CD20 (L26), CD5, CD10, and CD45 (LCA). The results with these antibodies encouraged us to extend the range of antibodies tested, and we now use the PT module routinely for all antigens in our repertoire, which require antigen retrieval (Table 1).

We have now been using the PT Module in routine practice for 10 months and we have found it to be reliable and to produce consistent results. The benefits of this are:

1. Reduced time spent on antigen retrieval.
2. Simultaneous de-waxing of sections.
3. Slides can be transferred to Autostainer directly after retrieval.
4. Complete standardisation of immunohisto-chemistry.
5. Increased dilution of a number of antibodies.
6. Reduction of antigen retrieval protocols from six to one.
7. Less harsh treatment of delicate tissues such as breast and lymph node together with improved section adherence and better nuclear morphology on counterstaining.
8. The PT Module is user friendly and simple to operate.

As a result of using a single buffer with the PT Module for section de-waxing and antigen retrieval together with automated staining we have achieved total standardisation of immunohistochemistry in our Department. This is a desirable goal as evidenced by the writings of several experts in this field[3,4,5]. The commercial buffers should not be re-used as we have found a reduction in staining quality if they are used more than once, however from a costing perspective this method of antigen retrieval compares favourably with our former protocols in terms of reagent costs.

Overall the benefits obtained by using the PT Module have meant we are now in a position where we can meet the demands of a sustained increase in requests for immunohistochemistry with static staffing levels and minimally increasing budgets.

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REFERENCES

1. **Taylor CR, Shi SR, Gu J**. The antigen retrieval technique for Immunohistochemistry: "Think Simple". In: Antigen Retrieval Techniques: Immunohistochemistry and Molecular Morphology (1st edition). Shi SR, Gu J, Taylor CR (editors). Eaton, MA 2000; 1-5
2. **http://www.labvision.com**
3. **Taylor CR, Shi SR**. Practical Issues: Fixation, Processing and Antigen Retrieval. In: Immunomicroscopy: A Diagnostic

tool for the Surgical Pathologist (3rd edition). Taylor CR, Cote RJ (editors). Saunders Elsevier, Philadelphia 2006; 47-74
4. **O'Leary T**. Standardization in Immunohistochemistry. *Appl Immunohistochem Mol Morphol* 2001; **9**: 3-8
5. **Shi SR, Cote RJ, Taylor CR**. Antigen Retrieval Immunohistochemistry and Molecular Morphology in the year 2001. *Appl Immunohistochem Mol Morphol* 2001; **9**: 107-11