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Automated immunochemistry

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Abstract

A prototype immunostainer, featuring totally enclosed slide chambers, a reagent carousel and a microcomputer controlled fluid transfer system, was developed. The machine offers total flexibility in choice of primary and detection reagents for each slide and has been successfully used for the immunochemical demonstration of a variety of antigens in paraffin wax and blood smear preparations. It is considerably cheaper to use than manual immunochemistry and will be even cheaper in a production version currently under development.

Immunochemistry has become a widely used technique in diagnostic and research cellular pathology, and an increasing number of laboratory workers are required to use it. Staffing problems and the laborious nature of immunochemistry may limit its full application. Furthermore, as laboratory personnel may be required to process immunochemical runs comprising a complex variety of antibodies and to handle large numbers of slides technical errors can arise.

Given that immunochemistry is a repetitive technique consisting of a repeated cycle of buffer wash, reagent application, and buffer wash, it should be possible to automate the procedure. A specification for automation should include individual slide flexibility, so as to provide for reaction with several primary antibodies, low antisera volumes to match manual costs, and a rigorous quality assurance system to ensure that sections are treated only with correct reagents.

In this paper we describe the development and use of a prototype automated immunostainer based on an isolated slide chamber with a computer controlled micro fluid transfer system. Using this machine immunochemical preparations, equivalent to manual staining, have been achieved at about two thirds of the total cost of the bench technique.

Methods

The immunostainer is composed of three functional areas (fig 1).

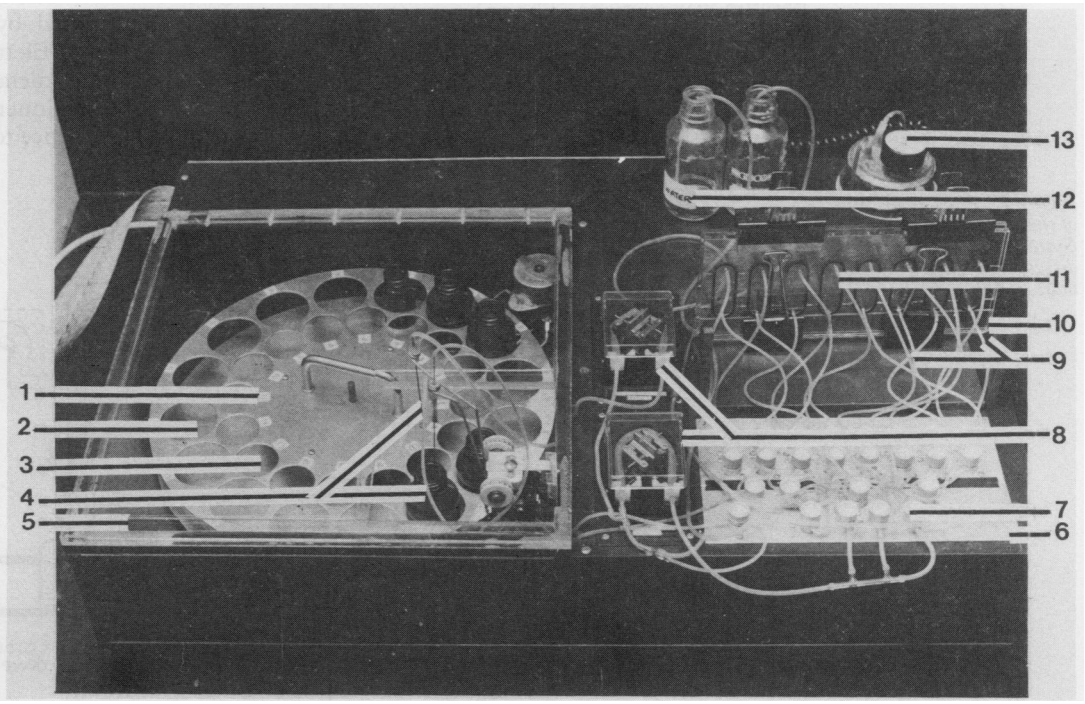

Figure 1 General arrangement of the immunostainer

KEY

1 Carousel
2 Outer circle of reagent containers
3 Inner circle of reagent containers
4 Dippers
5 Recessed box
6 Valve block
7 Solenoid pinch valve
8 Peristaltic motors
9 Silicon tubing
10 Slide chamber
11 "O" ring
12 Buffer and water containers
13 Waste vacuum container

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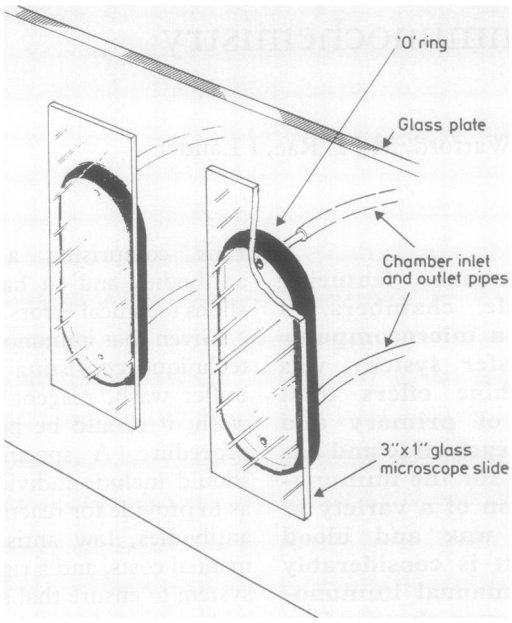

Figure 2 Schematic representation of the slide chamber. Note: slide backing plate is not shown.

THE SLIDE CHAMBER (fig 2)

A bank of eight slide chambers is fitted to the machine. Standard 25 × 75 mm microscope slides are placed on a glass backing plate over which a glass cover plate carrying eight "O" rings is secured by clips. Isolation of each slide from its neighbour and the formation of individual reagent chambers is achieved on closure as the "O" rings seal against the microscope slides. The area available for reaction on the microscope slide in the closed chamber is 822 mm^2, enough for two standard paraffin wax sections. The volume required to fill the closed chamber is 0·4 ml. Entry and exit of reagent into each slide chamber is through two 0·5 mm internal diameter stainless steel tubes fixed into the front plate.

THE REAGENT CAROUSEL

The carousel comprises two 450 mm diameter metal plates held apart by metal spacers. The top circle contains 16 50 mm in diameter outer and 16 40 mm in diameter inner numbered circular cut-outs for the positioning of reagent containers. Maximum reagent volumes for each position in the outer and inner rings are 50 ml and 20 ml, respectively. Use of the inserts, however, means that reagent volumes as low as 1 ml can be accommodated at any position. The carousel is isolated in a box with a perspex cover which is designed to allow for reagent refrigeration.

During operation reagents positioned in the inner ring are withdrawn to the outer ring for mixing purposes while those in the outer ring are used to fill the slide chambers. Separate dippers attached to the side of the carousel box are used to perform these functions, the carousel being automatically rotated to position the correct reagent container for withdrawal. To assist the mixing of reagents a magnetic stirring facility is also provided.

Bulk reagents, buffer, and water are held separately away from the carousel to allow for the larger volumes required in the washing and rinsing of chambers and silicon tubing.

FLUID TRANSFER SYSTEM (fig 3)

Reagents are transferred from the reagent containers to the slide chambers and then to waste in 0·5 mm internal diameter silicon coated tubing (Altec, Code 01-93-1404). The small diameter of the tubing ensures a maximum retention of 0·1 ml after the filling of a reagent chamber. The flow of reagents in the tubing is controlled by the use of solenoid pinch valves (Sirai Elettro Meccanica), which are secured to a metal block. Transfer of reagents from the inner to the outer ring of the carousel and hence to the slide chambers is

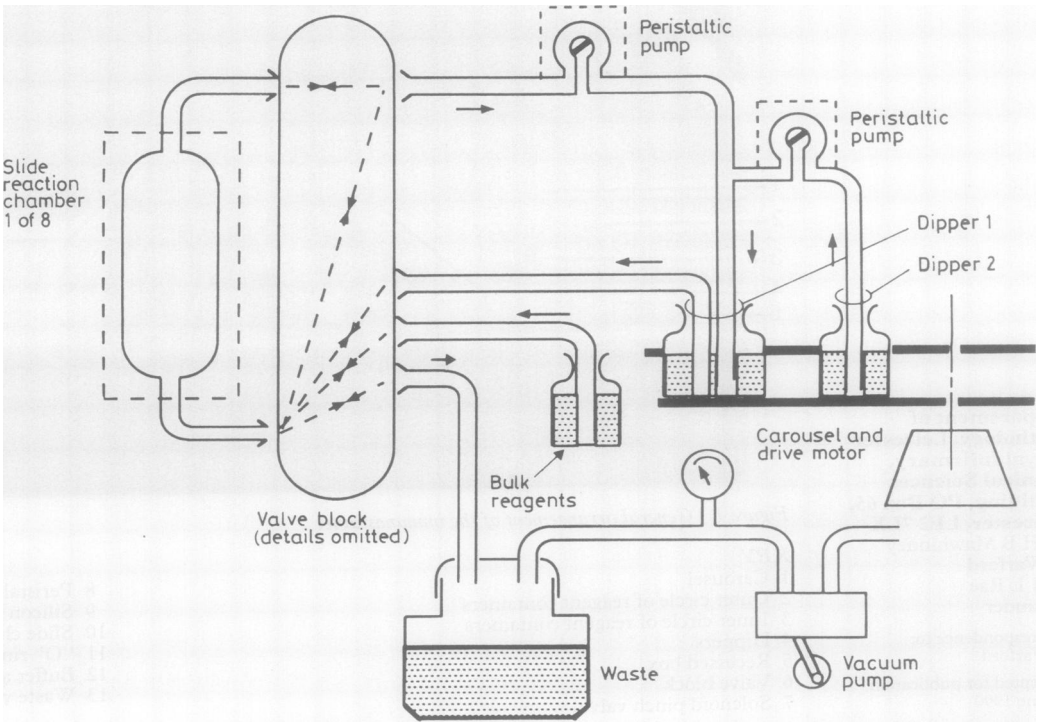

Figure 3 Flow diagram of the Reagent Transfer System. Possible flow direction of reagents is indicated by arrows.

achieved using separate peristaltic pumps and dippers. In contrast, a vacuum system is used to empty the slide chambers to waste. For buffer washing and rinse programmes a combination of the use of both peristaltic and vacuum systems is used.

Programming and control

All functions of the immunostainer are controlled by a BASIC program held on floppy disc and operated via a BBC Master 128 microcomputer. A standard dot matrix printer is attached to provide hard copies of techniques and a real time trace of immunochemical staining.

After performing an automatic start procedure a menu providing seven operating modes is displayed. These are construct technique, application, edit, add stages, test, run, rinse and wash.

Immunochemical technique programs are constructed using the first option. Each step is entered giving information on reagent, time, and, if required, mixing volumes. Two letter/number codes are used for the identification of each reagent supplemented, as required, by the entry of a third control code. Using a separate part of the program, reagent codes can be translated into full text, a feature that is useful when checking the completed technique.

Control codes for reagents include W, for washing the tubing and dippers before the transfer of a reagent so as to avoid cross contamination, and M for mixing reagents on the carousel. Control codes for time are S for seconds, M for minutes, and H for hours. For mixing, the volume required to be transferred from the inner to outer carousel position is entered in 1 ml increments.

Thus for methanol blocking for 20 minutes the entry is:
Reagent ME Time 20M.
For reaction with κ antisera, with washing before and after its addition, it is:
Reagent KPW Time 30M.
For the mixing of diaminobenzidene (DAB) with hydrogen peroxide, it is:
Reagent DBM Time 00M Mixing 05, and for the use of mixed DAB substrate, it is:
Reagent DB Time 10M.

Construction of a technique is terminated by entering 99H in the time code for the last reagent, after which the programme is displayed with translation of reagent codes for checking (fig 4), named by the operator and written to disc if satisfactory. Edit and add stages options are available at this point, providing a ready means of modifying techniques.

Using the edit option, small changes, such as substitution of an alternative primary antisera or deletion of a reagent, can be made to a technique. Extra reagents can be added at any point in an existing technique using the add stage option. Thus a single APAAP technique suitable for sections can be modified to a double APAAP method for the immunostaining of blood smear preparations.

Before starting immunostaining the mechanical operation of the machine may be checked using the test option. Using single key entries this allows the correct functioning of valves, motors, peristaltic pumps, vacuum and reagent transfer systems to be monitored.

To undertake immunostaining the "run" option is selected. Initial prompts request the number of slides to be stained (one to eight) and technique names for each slide (eight different methods over eight slides). Using this information, the computer sorts a numbered carousel reagent container list for the first technique and then adds different reagents as required for subsequent methods. The operator then loads reagent containers on to the carousel and to secure the microscope slides on to the slide chamber. The integrity of the seal between the slides and the "O" rings is checked on the next display which

Figure 4 Example of a completed technique for the staining of κ antigen using a polyclonal PAP detection method

Stage	*Reagent code*	*Translation*	*Time*	*Mixing*
1	H2	Water	02M	—
2	ME	Methanol block	20M	—
3	H2	Water	02M	—
4	TS	TRIS buffered saline	02M	—
5	TR	Trypsin	30M	—
6	TS	TRIS buffered saline	02M	—
7	TS	TRIS buffered saline	02M	—
8	KPW	Rabbit anti-human κ	30M	—
9	TS	TRIS buffered saline	02M	—
10	TS	TRIS buffered saline	02M	—
11	SR	Swine anti-rabbit immunoglobulin	30M	—
12	TS	TRIS buffered saline	02M	—
13	TS	TRIS buffered saline	02M	—
14	PA	Rabbit PAP	30M	—
15	TS	TRIS buffered saline	02M	—
16	DBM	Diaminobenzidine (mix)	00M	05
17	TS	TRIS buffered saline	02M	—
18	DB	Diaminobenzidene (reaction)	10M	—
19	TS	TRIS buffered saline	02M	—
20	TS	TRIS buffered saline	99H	—

Notes (1) Trypsin step is at room temperature.
(2) To maximise reagent space on the carousel and to minimise deposition of dye in the transfer tubing programs do not usually include rehydration and counterstaining stages.

Table 1 Immunochemical techniques used on the immunostainer

Indirect—alkaline phosphatase —FITC immunofluorescence —immunoperoxidase	With polyclonal antisera
PAP	
Double APAAP	With monoclonal antisera
ABC—alkaline phosphatase —peroxidase	With polyclonal and monoclonal antisera

permits the filling of each slide chamber with buffer. The last display lists the techniques for final checking, asks whether a hard copy is required, and if a real time trace is needed. After responding to these enquiries the immunostainer starts the techniques using the computer's real time clock. No further operator intervention is required until immunostaining is completed. After this the slide chamber is disassembled and slides removed for counterstaining and mounting.

On completion of immunostaining, two options, rinse and wash, are available to clean thoroughly the fluid transfer system and slide chambers. Using the former the slide chambers are rinsed three times with buffer. In contrast, the latter allows the complete fluid transfer system to be washed with a cleaning agent positioned in the carousel.

Results

The immunostainer has been in regular use for two years and has proved mechanically reliable. In particular, no failures have been experienced with the solenoid pinch valves or "O" rings. Demonstrations[1] have confirmed that the operation of the computer program presents few difficulties.

Several immunochemical methods (table 1) in conjunction with monoclonal and polyclonal primary antisera have been used to evaluate the immunostainer. In the main, paraffin wax sections have been used but acetone fixed blood smears have been used with the double APAAP procedure. Trypsin digestion, 0·1% Difco 1/250 trypsin in 0·1% calcium chloride in water at pH 7·8, has been successfully used to unmask cytoplasmic antigens in formalin fixed paraffin wax sections. Due to the absence of any heating mechanism on the prototype, however, prolonged digestion times have been required.

The mixing facility has been used to prepare avidin-biotin enzyme complexes and DAB and 3-amino-9-ethyl carbazole peroxidase substrates. The preparation of fast red and naphthol phosphate substrates mixes for alkaline phosphatase demonstration has not, however, proved possible due to the decomposition of these solutions in the unmixed form during the immunochemical run. Thus manual visualisation of this enzyme has been used.

High quality immunostaining (fig 5) has been consistently achieved using the same conditions of antisera dilutions and incubation times as for manual staining. The automated procedure is also completed in the same overall time as an equivalent manual method. The possibility of antisera carry-over has been investigated by running slides programmed to omit a primary antisera adjacent to ones with a full program. In no instance has any immunostaining been noted in the control sections.

Discussion

Compared with the extent of automation of other pathology disciplines, cellular pathology lags far behind. Explanations for this include the receipt of non-standard samples and their analysis using a variety of methods which often have a high skill input. Exceptions are tissue processing and routine dye staining, for which the conditions that suit automation—the processing of large numbers of standardised samples by a repetitive technique—are found. These considerations are now driving the development of automated systems for immunochemistry of which several prototypes and commercial instruments have been developed.[2-4]

Common to all automated immunostainer concepts has been the recognition of certain essential features: low reagent volumes; the

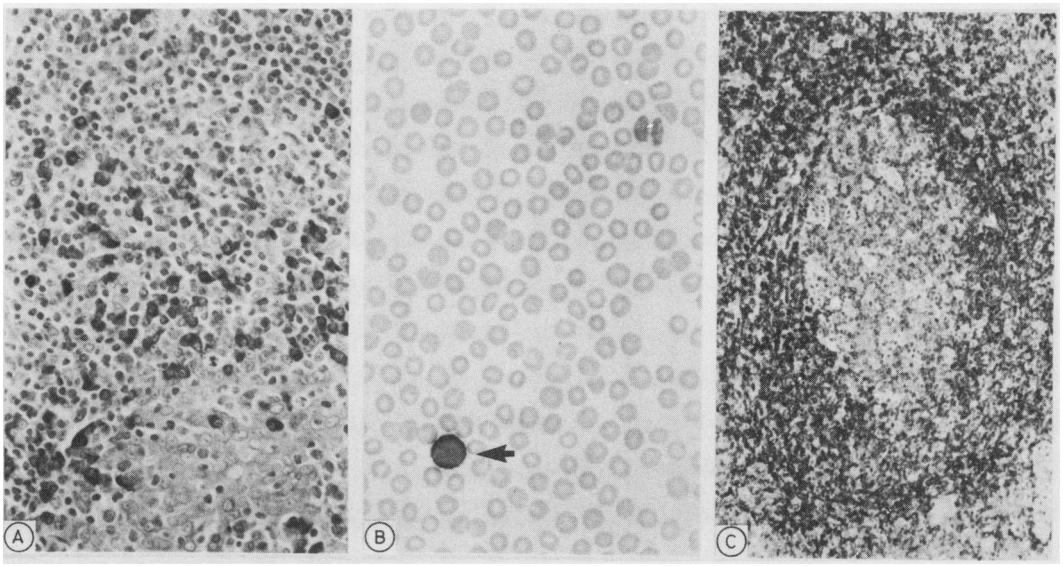

Figure 5 Examples of immunostaining achieved using the prototype immunostainer.
(A) Kappa light chains visualised cIg positive cells in formol sublimate fixed and paraffin wax embedded tonsil tissue using an ABC-HRP detection method.
(B) CD19 antigen in a B cell (arrow) shown in an acetone fixed normal blood smear using a double APAAP detection method.
(C) MB1 antigen shown in B cells surrounding a germinal centre in formol saline fixed and paraffin wax embedded tonsil tissue using an ABC-AP detection method.

necessity for precise time control of the process; and the provision of a rigorous quality control mechanism. The designs developed to meet these requirements however, have shown a great divergence.

In the simplest approach Stross *et al* modified a standard tissue processor to automate the technique from detection of the primary antibody and elected to undertake chromogen demonstration manually.[2] Economy is achieved by reusing reagents and man hours are reduced considerably compared with those for a manual technique. When one detection method is predominantly used and surplus processors are available for modification this approach may be useful. The variety of detection systems available for primary antibodies and the goal of reducing worker interaction time to a minimum, however, has led other groups to develop instruments capable of automating the entire technique.

The Code-On Immunology System developed by Brigati *et al*[3] in conjunction with Fischer Scientific was the first commercial immunostainer. The instrument is based on a modification of an automated dye stainer and provides heating for enzyme predigestion and (if desired) remaining stages, the mixing of solutions, and a 60 slide capacity. The IBM PC driven program, however, comprises 77 steps for an indirect method and the reagent blocks (isolon chambers) must be precisely charged to match the position of slides if correct section/antibody combinations are to be achieved. Furthermore, sections must be mounted of special slides, which makes the processing of referral sections impossible.

Another commercial product is that recently marketed by Shandon Southern Scientific Ltd. Like the Fischer machine it permits complete immunostaining, but it is a totally new development. Up to 20 slides can be processed in any run, each being held in a disposable coverplate which regulates the reaction of antibody with the sections. The machine is simply programmed but does not have any heating or mixing functions. Another novel concept has been reported by Stark *et al*.[4] This instrument is based on the controlled application of antisera from syringes and centrifugal buffer washes. The authors provide no indication of possible commercial development.

The prototype immunostainer described in this paper was developed from an earlier patented machine designed to standardise histological "special" staining. In its original form the instrument was controlled by punch tape. Although reliable, it offered little economic advantage over manual staining methods. Development was reactivated in 1986 when it was recognised that a computer controlled adaptation might offer considerable economies in immunostaining. The concept of a fluid transfer system through small bore tubing and regulated by pinch valves was retained but a new slide chamber with "O" ring sealing and reagent carrier was designed. In its present form the automated immunostainer offers many of the features of the instruments described above (table 2) and is unique in providing a totally enclosed reagent transfer system.

Clearly the reagent/slide volumes on the prototype are very much in excess of the other concepts, but two paraffin wax sections can be comfortably accommodated or several frozen sections in each chamber. Furthermore, redesign of the chamber to provide an equivalent staining area with reagent volumes of under 150 μl is currently being undertaken with a United Kingdom based commercial company (Anglia Scientific Ltd). Nevertheless, under present conditions sections may be immunostained at one quarter of the non-antibody reagent costs and about two thirds of the total cost including antisera, other reagents, and staff time (Appendix).

Projection from prototype running costs to those of a production machine (Appendix) indicate that reagent expenditure should be similar to that of manual immunostaining. For a production immunostainer total costs must also include purchase price and maintenance expenditure. In making a decision to purchase an automated immunostainer the benefits of substantial savings in staff time and the facility to run the machine out of normal working hours would also have to be considered.

Other features which are currently being addressed are the number of slides that can be accommodated in a run, the application of heat, and the development of dedicated software, obviating the need for separate computer control.

It is currently envisaged that to accommodate the numerically divergent needs of general diagnostic laboratories and large research institutions a central control unit will simultaneously operate up to four, 24 slide chamber slave units, each with its own reagent carousel.

Table 2 Comparison of automated immunostainers

Instrument feature	*Prototype immunostainer*	*Production immunostainer*	*Fischer*	*Shandon*	*Stark et al*	*Stross et al*
Volume of reagent/slide (μl)	500	150	150*	100	80	230†
Individual time control for slides	Yes	Yes	No	Yes		No
Programming	Simple	Simple	Complex	Simple		Simple
Enclosed processing	Yes	Yes	No	Partial	No	Partial
Temperature control	No	Yes	Yes	No	No	No
Specialised slides/adaptor	No	No	Yes	Yes	Yes	Yes
Mixing facility	Yes	Yes	Yes	No	No	No
Primary antigen processing	Yes	Yes	Yes	Yes	Yes	No
Slide numbers	8	96	60*	20	20	

*Slides are positioned in pairs; therefore, 150 μl is required to cover one pair and up to 30 different primary antibodies can be used.
†Based on reuse of 400 ml of antisera over 17 weeks.

Heating of slide chambers is important for optimal enzyme predigestion of samples and the provision of a rapid staining schedule. This is being addressed by the construction of a metal backed slide chamber which will be electrically heated and air cooled.

Perhaps the overriding criterion in automated immunostaining is quality assurance. Although manual methods are prone to technical error, visual quality control is provided at every step in the technique. The prototype immunostainer is fitted with a trace facility which identifies the correct withdrawal of reagent from the carousel. To close the quality assurance cycle, however, a detector will be fitted to each slide chamber to check reagent filling.

Appendix

A cost comparison of manual and automated immunostaining of eight paraffin wax sections for κ light chains by PAP using the prototype immunostainer.

(a) *Antibodies*

Type	*Dilution*	*Source*	*Manual cost (p) 100 µl × 8*	*Automated cost (p) 500 µl × 8*
Kappa	1/1000	DAKO A191	2	12
Link	1/50	DAKO Z196	32	160
PAP	1/100	DAKO Z113	32	160
		Totals	£0·66	£3·32

(b) *Reagents*

	Manual		*Automated*	
	Volume (ml)	*Cost (p)*	*Volume (ml)*	*Cost (p)*
Methanol blocking	60	14	10	2·3
Trypsin	60	5	10	0·8
DAB	10	2	10	2·0
TRIS buffer	380	17	100	4·5
	Totals	38p		9·6p

(c) *Combined consumable costs*

	Manual (£)	*Automated (£)*
Antibodies	0·66	3·32
Reagents	0·38	0·10
Totals	£1·04	£3·42

(d) *Time comparison*

	Manual (minutes)	*Automated (minutes)*
Reagent preparation	35	35
Handling of slides	85	15
Totals	120	50

(e) *Employee costs*

MLSO 1+++, including national insurance and superannuation £5·90/hour

	Manual	*Automated*
Costs	£11·80	£4·92

(f) *Total costs*

	Manual (£)	*Automated (£)*
Reagents	1·04	3·42
Staff	11·80	4·92
Totals	£12·84	£8·34

(g) *Equivalent staining using the production immunostainer with a slide chamber volume of 150 µl*

	£
Antibodies	1·00
Reagents	0·10
Staff	4·92
Total	£6·02

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Notes