

# Specificity and sensitivity of immunocytochemistry for detecting P-glycoprotein in haematological malignancies

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**Abstract**

**Aims**—To determine the optimal working conditions of the alkaline phosphatase-antialkaline phosphatase (APAAP) method to establish a specific and sensitive assay for the detection of low numbers of MDR positive cells in patients with hematological malignancies.

**Methods**—Three monoclonal antibodies (C-219, JSB-1, MRK-16) were used for the detection of P-glycoprotein (P-gp) in cell lines and in samples from 43 patients with haematological malignancies. The results of the APAAP method were compared with western blotting for specificity and sensitivity.

**Results**—Excellent correlation was obtained between optimised APAAP and western blotting, except in the case of multiple myeloma. JSB-1 seemed to be the more useful monoclonal antibody for the APAAP which was more sensitive than western blotting in its ability to detect single P-gp positive cells.

**Conclusions**—Methods for P-gp detection, as defined by multidrug resistant (MDR) cell lines, are not necessarily optimal and specific for clinical samples and may lead to higher false positive and negative results, according to the conditions and the monoclonal antibodies used.

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Emergence of resistance is a major cause of treatment failure in cancers. The MDR phenotype is characterised by overexpression of a 170 kilodalton transmembrane protein, P-glycoprotein (P-gp),<sup>1-4</sup> and a decreased intracellular drug concentration.<sup>5-8</sup> Over expression of P-gp is the hallmark of classic MDR<sup>9-11</sup> and is associated with cross-resistance to a spectrum of structurally and functionally unrelated chemotherapeutic agents, such as anthracyclines, vinca alkaloids, and podophyllotoxins. Several monoclonal antibodies<sup>12-16</sup> and different methods, including protein blotting techniques,<sup>17-22</sup> flow cytometry,<sup>23-25</sup> and immunocytochemistry,<sup>25-30</sup> (Sparrow R L, *et al.* Abstract presented at Haematology Society of Australia Meeting, September 1991, Melbourne, Australia) have been used for its detection. Results vary between laboratories throughout the world, presumably because of methodological differences. Discrepancies have been observed in

results obtained from cases of chronic lymphocytic leukaemia (CLL)<sup>19 24 25</sup> and acute myeloid leukaemia (AML).<sup>21-23</sup> Such discrepancies make comparisons of data difficult. The prognostic value of P-gp detection by immunocytochemistry on human samples has been reported.<sup>30-33</sup> Very few reports give information on the degree of specificity and sensitivity of method used.<sup>19 34</sup>

The aim of this study was to determine the optimal working conditions of the alkaline phosphatase-antialkaline phosphatase (APAAP) method using the three widely reported monoclonal antibodies (C-219, JSB-1, MRK-16),<sup>13 14 17</sup> and to compare this with western blotting to establish a specific and sensitive APAAP method which would allow the detection of low numbers of MDR positive cells in patients with haematological malignancies.

**Methods**

**SAMPLE PREPARATION**

Bone marrow and peripheral blood samples from 43 patients with haematological malignancies, including drug resistant and relapsed cases, were examined. These comprised 15 cases of multiple myeloma (MM), 13 of acute myeloid leukaemia (AML), four of non-Hodgkin's lymphoma (NHL), and 11 of lymphoid leukaemia. Samples from patients with MM contained between 15% and 80% tumour cells. All specimens were collected into EDTA anticoagulant. Samples were washed in phosphate buffered saline (PBS) (pH 7.4) and the cells resuspended in TRIS-buffered saline (TBS) at a concentration of 10<sup>6</sup> cells/ml. Cytospin preparations were made, air-dried for 24 hours at room temperature, then wrapped unfixed in aluminium foil and stored at -80°C until required.

Part of the separated specimens were kept for western blot analysis. Normal controls comprised mononuclear cells from healthy bone marrow donors. Negative and positive MDR cell lines with known degrees of doxorubicin or vinblastine resistance were used as controls (table 1).

Table 1 Cell lines

CCRF-CEM	Human P-gp negative leukaemic cell line
CEM/VLB8	P-gp positive subline resistant to VLB 8 ng/ml
CEM/VLB 100	P-gp positive subline resistant to VLB 100 ng/ml
8226/S human	P-gp negative multiple myeloma cell line
8226/DOX40	P-gp positive resistant to doxorubicin 40 ng/ml
K562	P-gp negative myeloid cell line
U937	Fc receptor positive P-gp negative histiocytic cell line

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Table 2 Anti-P-glycoprotein monoclonal antibodies

	Source	Type	Subclass	Recognition site
C219	Dr Ling, OCI Tronoto, Canada	Ascites	IgG2a	Internal cytoplasmic epitope
JSB-1	Monosan Sanbio, Holland	Ascites	IgG1	Internal cytoplasmic epitope
MRK 16	Dr Tsuruo, CCC Tokyo, Japan	Ascites	IgG2a	External cytoplasmic epitope

#### APAAP

The APAAP technique was performed, as described before,<sup>35,36</sup> with three anti-P-gp monoclonal antibodies (C219, JSB1, and MRK16) (table 2). Isotype matched monoclonal antibodies were included as controls. Slides were examined with a light microscope by two independent observers. Intensity of positive staining was graded from 1+ to 5+, based on the MDR negative and positive cell lines. In the experiments tumour samples were defined as positive when at least 30% of the malignant cells in the slide exhibited positive staining.

Seven cell lines (table 1) were used to analyse the following factors: monoclonal antibody concentration and incubation time, sample fixation, and temperature of fixation. By examining one variable after another, the optimal conditions required to obtain specific results—that is, results comparable with those of western blotting, were ascertained. A monocytic cell line, U937, which expresses CD32 (Fc receptor), was used as a control to test non-specific binding of the anti-P-gp and isotype matched monoclonal antibodies. This cell line also allowed the best method of blocking non-specific staining to be determined. VLB8 cell line, resistant to 8 ng vinblastine/ml and expressing a low level of P-gp, was used as a control to assess the sensitivity of the technique.

To avoid interassay variability, the assay was repeated at least three times for each parameter tested on P-gp positive and P-gp negative cell lines.

#### Fixation

To select the optimal fixative to preserve both antigen expression and cellular morphology, several different compounds were tested alone and in combination. Time of fixation was also tested as were the effects of three different fixation temperatures (table 3).

#### Monoclonal antibodies

For JSB-1, the concentration evaluated varied

from 1.5–2 to 6–8 µg/ml, for C219, from 5 to 10 µg/ml, and for MRK16, from 46 to 23 µg/ml. The optimal antibody concentration was initially determined by titration on all cell lines to obtain the best discrimination between P-gp positive and P-gp negative cell lines and adjusted to obtain the best correlation with the western blot results.

Isotype matched IgG1 and IgG2a control antibodies were used at the same concentration as the corresponding anti-P-gp monoclonal antibodies. For each anti-P-gp monoclonal antibody, overnight incubation at 4°C was compared with one hour of incubation time at room temperature.

#### Agents blocking non-specific binding

Different agents and dilutions were compared during the APAAP assay. Normal decomplexed human donor sera, Intragam (pure immunoglobulin preparation for intravenous use), or human group AB serum were diluted in TBS and each of them evaluated at the following dilutions: blocking agent: TBS 1 in 1, 1 in 2, 1 in 3, 1 in 4. Three different blocking strategies were also evaluated: (1) incubation of slides with blocking agent before starting APAAP; (2) incubation using blocking agent together with the primary monoclonal antibody; (3) incubation using blocking agent together with second monoclonal antibody (rabbit anti-mouse). A combination of strategies two and three was also tested.

The enhancing effect of repeat applications of the second and third layer antibodies was examined to improve assay sensitivity.

#### WESTERN BLOT

Briefly,  $10 \times 10^6$  cells were washed twice in PBS and resuspended in 0.5 ml lysis buffer (0.01M TRIS-HCl, pH 7.4) and stored frozen at  $-20^\circ\text{C}$ . Cells were thawed and homogenised in a motor-driven Teflon glass homogeniser. Whole cells, nuclei, and debris were removed by centrifugation for 10 minutes at  $4000 \times g$ . The crude membrane fraction was collected from the supernatant

Table 3 Comparison of fixation conditions

Fixative	Fixation time	Fixation temperature
Acetone (A) Methanol (M) Ethanol (E) A/M (v/v) A/E (v/v)	Varied from 30 seconds to 10 minutes for fixatives not containing formalin	-20°C, -4°C, room temperature
Formalin (F)/TBS 0.5% 1.0% 2.0% 5.0%	Varied from 10 to 90 seconds for fixatives containing formalin	
A/M/F (19:19:2) A/M/F/ (19:19:1) A/E/F/ (19:19:2) A/E/F/ (19:19:1)		

Slides were incubated with the above fixatives for varying times, as indicated in column 2. The effect of temperature was determined after obtaining the optimal fixation time.

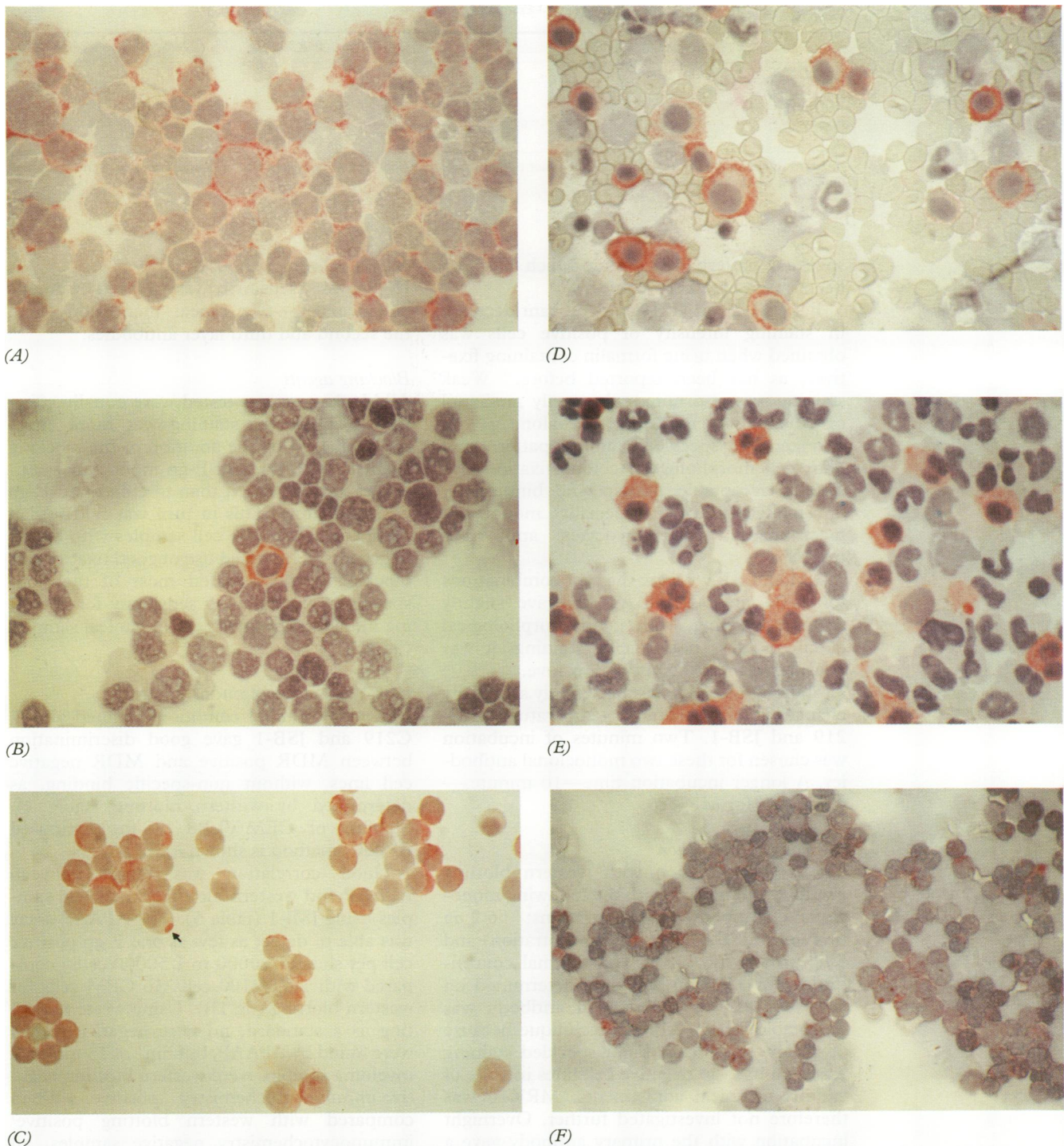


Figure 1 Immunocytochemical (APAAP) detection of P-glycoprotein overexpression using JSB-1: (A) CEM/VLB8; (B) acute myeloid leukaemia, single positive cell; (C) acute non-lymphoblastic leukaemia—presence of P-gp intracytoplasmic localisation (arrow) (D–E) multiple myeloma samples; (F) non-Hodgkin's lymphoma.

fluid by centrifuging at  $35000 \times g$  for 60 minutes. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed, as described before<sup>4,17</sup> in a Bio-Rad Mini Protean II and Mini Trans blot apparatus (Bio-rad Laboratories, New South Wales, Australia). Immunoblots were blocked in 5% skimmed milk in TBS-0.1% Tween for 60 minutes at room temperature and, after washing with TBS-0.1% Tween, were probed with C-219. The immunoblots were washed again in TBS-0.1% Tween, then overlaid with alkaline phosphatase conjugate anti-mouse immuno-

globulin (Dakopatts). The filter was washed again and developed in a solution containing BCIP (5-bromo, 4-chloro, 3-indolylphosphate) and NBT (nitro blue-tetrazolium). This technique was sensitive to 1 P-gp positive CEM/VLB100 cells in 256 CCRF-CEM cells, as determined by the limiting dilutions technique.

## Results

### OPTIMAL CONDITIONS

#### Sample fixation

For MRK-16, PBS-formalin (3.5%) was the best fixative.<sup>34</sup> This antibody produced a



Table 4 Results of staining cell lines

	CEM	VLB8	VLB100	U937	K562	8226/S	8226/DOX40
C219	-	+	3	+	-	-	3+
JSB1	-	+	3	+	-	-	3+
MRK16*	+/-	+	1-2	+	+/-	+/-	1-2+
IgG2a	-	-	-	-	-	-	-
IgG1	-	-	-	-	-	-	-

\*Pattern of staining differs clearly from the other two monoclonal antibodies: presence of fine or coarse granules in the cytoplasm depending on the level of drug resistance.

speckled pattern of staining which has also been found by other workers.<sup>25 37</sup>

For JSB-1 and C219, a significant decrease in staining intensity of positive cells was obtained when using formalin containing fixatives, as has been reported before.<sup>14</sup> Weak staining was also noted when only methanol or ethanol was used. Acetone alone gave a stronger but speckled staining pattern and poor cell morphology. Acetone fixation also gave unacceptable non-specific binding by non-malignant cells especially monocytes, macrophages, megakaryocytes and neutrophils.

AM and AE v/v were the best combinations for JSB1 and C219. As AE gave slightly stronger staining, with good morphological detail and less non-specific staining, it was considered to be the optimal fixative.

Staining intensity was not greatly altered by changes in fixation time or temperature for C-219 and JSB-1. Two minutes of incubation was chosen for these two monoclonal antibodies. A longer incubation time—10 minutes—was better for MRK16.

#### Antibody concentration

The best correlation with western blotting results was obtained with the following monoclonal antibody concentrations: 6.2 µg protein/ml JSB-1 (average concentration) and 5 µg protein/ml C219. The optimal concentration for MRK16 was only determined on cell lines as this monoclonal antibody was unsuitable for the APAAP technique because of its low specificity. Faint speckled staining was seen in P-gp negative cell lines in spite of various technical adjustments. MRK16 was therefore not investigated further. Overnight incubation with the primary antibody gave a stronger staining pattern than one hour of incubation.

Non-specific staining of plasma cells, monocytes, and granulocytes, as demonstrated by the isotype matched control antibodies, was seen in clinical samples. These cells showed more non-specific staining than tumour cells which reflects the variability of APAAP specificity according to cell type.

#### Enhancement

Higher assay sensitivity was achieved by double enhancement—two repeat incubations with the second and third layer antibodies.

#### Blocking agents

All blocking agents tested were equally effective. Non-specific staining was more effectively blocked by the addition of the blocking agent to both the anti-P-gp monoclonal antibodies and the RAM than to either anti-P-gp or RAM alone. This in turn was better than preincubation of the cell samples with blocking agents. Combined use of good fixative and blocking agents avoided most of the non-specificity and retained sensitivity for detecting individual positive cells in clinical samples (fig 1B).

#### CELL LINES AND CLINICAL SAMPLES

Using the optimal conditions defined above, C219 and JSB-1 gave good discrimination between MDR positive and MDR negative cell lines, without non-specific binding, as determined by western blotting (table 4). Staining of CEM/VLB8 by the optimised APAAP method is shown in fig 1A.

Good correlation was found between APAAP and western blotting on clinical samples using JSB-1 (table 5). The APAAP assay was able to detect as few as one P-gp positive cell per slide (1/10000 to 1/50000 cells) compared with 1 VLB100 cell/256 CEM cells for western blotting (fig 1B). Using western blotting as a standard, no false negative results were found by APAAP, but nine of 15 multiple myeloma samples were western blotting negative/immunocytochemistry positive. When compared with western blotting positive/immunocytochemistry negative samples, all these nine contained a smaller number of positive plasma cells (about 30%) or showed weaker staining intensity (1+ to 2+). Examples of western blotting positive/immunocytochemistry positive leukaemia (c), myeloma (d, e), and lymphoma (f) are, respectively, shown in fig 1 C, D-E and F (APAAP positive staining) and fig 2 (positive western blot).

Table 5 Comparison of immunocytochemistry and western blotting results for 43 clinical samples

	MM	AML	ALL	NHL
Western blotting + /immunocytochemistry +	6	6	5	1
Western blotting - /immunocytochemistry -	-	7	6	3
Western blotting - /immunocytochemistry +	9	-	-	-

Immunocytochemistry results were obtained with JSB-1 and C219. Western blotting results were obtained using C219.

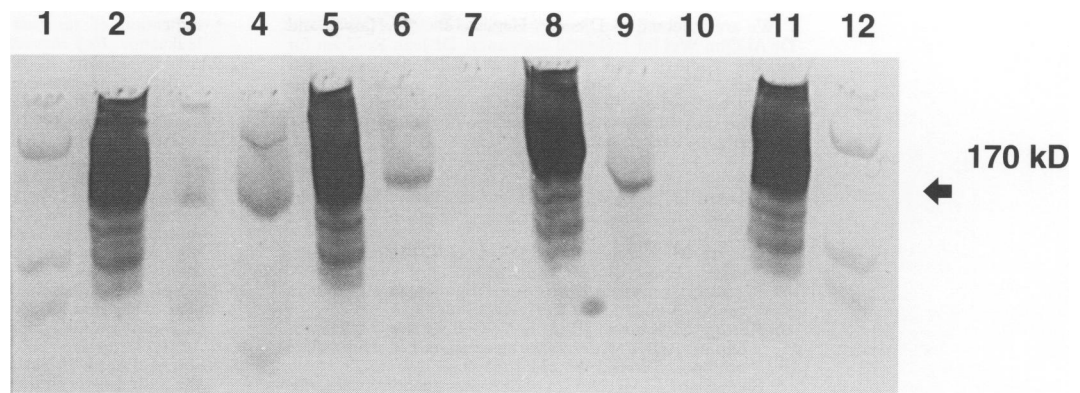


Figure 2 Western blot analysis of plasma membrane extracts using C219 as a probe: lanes 1–12, molecular weight; lanes 2, 5, 8 and 11, CEMVLB-100; lane 3, P-gp positive acute lymphoblastic leukaemia; lanes 4, 6, P-gp positive multiple myeloma; lane 9, P-gp positive non-Hodgkin's lymphoma; lane 7, CCRF-CEM; lane 10, P-gp negative multiple myeloma.

The results for C219 paralleled those of JSB-1 for blast cells, but staining of some non-neoplastic cells such as monocytes and neutrophils was observed in most clinical samples. These findings did not correlate with the western blotting results and were interpreted as non-specific staining. Lower concentrations of C219 reduced the sensitivity of the assay.

#### Discussion

Few clinical studies of MDR in haematological tumours have provided a comparison of immunocytochemical results with those of other methods.<sup>34–37</sup> The optimal conditions for the detection of P-gp are often determined using cell lines only.<sup>27–32</sup> Because the APAAP method avoids the problem of endogenous peroxidase inherent in the immunoperoxidase technique,<sup>27–29–33–39</sup> and provides excellent morphology,<sup>25–30–38</sup> we optimised the assay by examining the influence of technical conditions on its specificity and sensitivity.

Non-specific labelling, defined as the association of a weakly positive APAAP result on most malignant and non-malignant cells of a sample and a negative western blot result on the same sample, was found to be a major problem with anti-P-gp antibodies in this study. According to other data,<sup>38</sup> false positive staining was observed especially with monocytes, neutrophils, and megakaryocytes in the clinical samples. Positive staining of these cells was likely to be due to Fc receptor binding.

Manipulation of blocking agents, fixation, and antibody concentration largely resolved this technical problem and enabled us to obtain an excellent correlation with immunoblotting for cell lines, peripheral blood, and bone marrow samples.

Compared with all the other fixatives, the combined use of acetone-ethanol was a good compromise between staining intensity and prevention of non-specific binding. This was effective in preventing Fc receptor binding in both clinical samples and on the FcR positive, P-gp negative cell lines U937.

Increasing the JSB-1 and C219 concentrations above their optimal range decreased the assay's specificity as shown by the staining of non-neoplastic cells.

The anti-P-gp monoclonal antibody used also affected the specificity of the staining. The three monoclonal antibodies used in this study have been compared before,<sup>34–37</sup> but not with a panel of human haematopoietic tumours. With MRK16, we could not make a distinction between positive and negative results on cell lines. In this respect, it has been suggested that MRK16 might cross-react with molecules other than P-gp.<sup>37</sup> C219 gave comparable results with those of JSB-1 on blast cells, but it was less specific because it gave more positive staining of monocytes and neutrophils. JSB-1 seemed to be the most suitable monoclonal antibody with APAAP method.

In this study the only major discrepancy found between western blotting and APAAP results was with some multiple myeloma samples (table 5). It occurred with weakly positive samples (1+ to 2+) and indicates that cautious interpretation is required for P-gp expression by different cells types (notably plasma cells), especially in the case of weak staining. APAAP, however, showed a greater sensitivity compared with western blotting: it could detect single positive malignant cells in a multicellular sample (fig 1B).

In summary, immunocytochemistry using APAAP offers many advantages compared with other methods for the analysis of P-gp expression in clinical samples. APAAP can potentially detect very small numbers of positive cells, as demonstrated by this study. This, combined with the advantage of morphological identification of the positive cells, makes it a versatile method that is particularly suitable for hypocellular samples. It is also a simple, inexpensive, and quick assay. P-gp seems to be an important prognostic factor in the prediction of clinical outcome in many tumours. It would also be useful to study the clinical relevance of low numbers of MDR positive cells in malignant as well as normal haematopoietic tissues using the APAAP method.

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