

Detection of intracellular antigens by flow cytometry: comparison of two chemical methods and microwave heating

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Detection of intracellular antigens by flow cytometry requires effective fixation and permeabilization of the cell membrane. This study compares three fixation/permeabilization techniques: two commercial chemical reagents, the ORTHOPermeaFix™ (OPF) and the FIX&PERM Cell Permeabilization Kit® (F&P), and a novel method based on microwave heating (MWH). They have been applied to the detection of two nuclear (p53 and rb/p105) and two cytoplasmic (bcl-2 and mdr-1/gp-170) antigens, using positive- and negative-control cell lines and peripheral blood mononuclear cells. Western blotting was performed as a control of protein expression. For the four antigens assessed, cellular morphology, discrimination between intact cells and debris, percentage of positive cells, and mean fluorescence intensity were examined. For this last parameter, the assessment of the MWH technique was performed using SD and a graphical approach inspired by the concepts described by Bland and Altman (*Lancet* 1986;346:1085–7) as well as Petersen et al. (*Clin Chem* 1997;43:2039–46). The statistical analysis shows that MWH is comparable to the commercial methods and that its reproducibility is also equivalent to OPF and F&P. As assessed for some of the most clinically relevant intracytoplasmic and intranuclear antigens, the MWH method appears to be a valuable and inexpensive alternative. It is worth noting that, unlike commercial reagents, MWH altered surface antigens. Interestingly, this feature, which would prevent cell selection on the basis of combined membrane and intracellular epitopes,

is associated with a decrease of nonspecific background fluorescence.

Flow cytometry is a versatile method for the detection and quantification of cell surface antigens. Combined analysis of immunofluorescence labeling and scatter features such as size and granularity of the various cell types allows rapid automated investigation of a large number of cells and the delineation of distinct subpopulations. Regarding the detection of intracellular antigens, standardization of this procedure remains, however, a real challenge. Preliminary cell permeabilization and fixation, the two crucial steps that are mandatory for the detection and quantification of intracellular antigens, can indeed be performed in different ways. Saponin, Tween 20, or Triton X-100 are used to permeabilize the membrane. Cell fixation is often performed with alcohol or paraformaldehyde (PFA)³ (1–4). Although individual reports have mentioned satisfactory results for the detection of either cytoplasmic or nuclear antigens, no methodological consensus has yet been achieved among laboratories (4). Indeed, optimal conditions depend on the type of cell examined and the characteristics of the antigen to be detected. Moreover, limitations such as insufficient access to target antigen, cell losses, formation of small cellular aggregates, alteration of cell morphology, and light scatter patterns are commonly encountered during membrane permeabilization and can hamper the consistency and reproducibility of the results.

Two commercial reagents, ORTHOPermeaFix™ (OPF; Ortho) and FIX&PERM Cell Permeabilization Kit® (F&P; An Der Grub Bio Research GmbH, Imtec) have recently been claimed to avoid these limitations. They have been shown to be suitable for the detection of many cytoplas-

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³ Nonstandard abbreviations: PFA, paraformaldehyde; OPF, ORTHOPermeaFix; F&P, FIX&PERM Cell Permeabilization Kit; MWH, microwave heating; PCNA, proliferating cell nuclear antigen; CL, cell line; mAb, monoclonal antibody; PBS, phosphate-buffered saline; and MCF, mean cell fluorescence.

mic (CD22, CD3, and myeloperoxidase) or nuclear (terminal deoxynucleotidyl transferase) antigens and can be used for the simultaneous detection of membrane and intracellular antigens (4–7).

As an alternative, a very promising noncommercial method, based on microwave heating (MWH) treatment, has recently been reported to achieve the same performance with intracellular targets [CD68, proliferating cell nuclear antigen (PCNA), Ki-67] (2).

In this study, we compare OPF, F&P, and MWH, focusing on the detection of cytoplasmic (bcl-2 and gp-170) and nuclear antigens (p53 and rb/p105), all of which are proteins of interest in diagnostic and research areas: the bcl-2 gene product plays a key role in the inhibition of the programmed cell death or apoptosis; the glycoprotein gp-170, encoded by the *MDR-1* gene, is the marker of classical multidrug resistance, for which the importance of quality-control procedures and standardization of detection methods have been emphasized recently (8). The latter two proteins, p53 and rb/p105, are the products of the corresponding tumor suppressor genes and are meant to play a key role in the control of the cell cycle. These four antigens, which are detectable by flow cytometry (9–17), have all been involved, alone or in association, with the occurrence of drug resistance. This makes interesting the use of a simple and standardized method that allows the simultaneous detection of cytoplasmic and intracellular antigens. A panel of cell lines (CLs) constitutively

expressing or lacking expression of those antigens were used as controls.

Materials and Methods

CLs

Cells were cultured in RPMI-1640 cell culture medium (BioWhittaker) supplemented with 100 mL/L fetal calf serum (Life Technologies), glutamine, and antibiotics (penicillin and streptomycin) at 37 °C in a 5% CO₂ humidified incubator.

Positive and negative CLs (listed in Table 1) were chosen for their differential expression of the four antigens of interest.

p53. The presence of a wild-type or mutated *p53* gene was determined by sequence analysis of the whole open reading frame in IM9, DOHH2, and DG75. Mutated *p53* was found only in DG75 (J-L. Gala, unpublished results). RAJI and CCRF-CEM both express a mutated *p53* protein (18).

rb. rb/p105, the retinoblastoma gene product, is not expressed in the negative control lymphoblastoid line IM9 (19). 6M1 and 6M2, which are IM9 subclones stably transfected with the control plasmid pZipNeoSV(X), have been shown to lack expression of the protein and were also included as negative controls (17). Positive-control CLs expressing the *rb* gene included 3C3 and 5C3, which

Table 1. CLs used as negative and positive controls in regard to the antigens assessed.

CL	Tumor	Intracellular antigens		Genetic status	Reference
		Co- ^a	Co+		
DOHH2	FL	p53	bcl-2	Wild-type t(14;18)	Personal data ^b (23)
IM9	Lb/MM	p53		Wild-type	Personal data (19)
6M1 (IM9 subclone)	Lb/MM	rb			(17)
6M2 (IM9 subclone)	Lb/MM	rb			(17)
KG1	AML		rb		(20)
3C3 (IM9 subclone)	Lb/MM		rb		(17)
5C3 (IM9 subclone)	Lb/MM		rb		(17)
RAJI	BL		p53	Mutated	(18)
			bcl-2	EBV positive	(21)
DG75	BL		p53	Mutated	Personal data (21)
		bcl-2		EBV negative	(21)
DG75 LMP	BL		p53	Mutated	Personal data (21)
			bcl-2	EBV (LMP) positive	(21)
DAUDI	BL	bcl-2		EBV negative	(21)
CCRF-CEM	T-ALL	gp-170		<i>MDR-1</i> unexpressed	(24)
			p53	Mutated	(18)
VLB8 (CEM subclone)	T-ALL		gp-170	<i>MDR-1</i> expressed	(24)
VLB100 (CEM subclone)	T-ALL		gp-170	<i>MDR-1</i> expressed	(24)

^a Co-, negative control; Co+, positive control; FL, follicular lymphoma; Lb/MM, lymphoblastoid cell line from a patient presenting with multiple myeloma; AML, acute myeloblastic leukemia; BL, Burkitt lymphoma; EBV, Epstein-Barr virus; LMP, latent membrane protein; T-ALL, T-cell acute lymphoblastic leukemia.

^b J-L. Gala, unpublished results.

are IM9 subclones transfected with pZipRb, a plasmid containing the rb gene-coding sequence, and have been shown to express the gene product (17). The rb-positive myeloid line KG1 was also included (20).

bcl-2. The Epstein-Barr virus-negative (group 1) Burkitt lymphoma CLs DAUDI and DG75 do not express *bcl-2* (21). *bcl-2* is expressed in the Epstein-Barr virus-positive CL RAJI and the subclone DG75 LMP, which is DG75 transfected with pSHISLMPd3, a plasmid containing the latent membrane protein-coding sequence (21, 22). *bcl-2* is also overexpressed in follicular lymphomas carrying the translocation t(14;18) and has been found in DOHH2, a CL derived from such a lymphoma (23).

gp-170. CCRF-CEM, derived from a T-cell acute lymphoblastic leukemia, does not express *gp-170*, the marker of *MDR-1*. VLB8 and VLB100, obtained by culturing CCRF-CEM in progressively increasing concentrations of vinblastine, express *gp-170*; however, expression is higher in VLB100 (24).

ANTIBODIES

Monoclonal antibodies (mAbs) recognizing intracellular antigens included the following: p53 (clone DO7; Dako, Prosan) at a final dilution of 1/100; *bcl-2* (clone 124; Dako, Prosan) at a final dilution of 1/70; rb (clone G3-245, Imtec) at a final dilution of 1/500; *gp-170* (clone JSB-1; Pharmingen, Imtec) at a final dilution of 1/60; and PCNA (clone PC10; Dako, Prosan) at a final dilution of 1/50.

mAbs mapping external epitopes were also used and included the *gp-170* monoclonal 4E3 (Imtec) and CD19 (clone HD37; Dako, Prosan) both used undiluted.

Two isotype negative-control antibodies, IgG1 (a gift from H. Bazin, Laboratory of Experimental Immunology, UCL, Brussels, Belgium) and IgG2a (clone DAK-GO5; Dako, Prosan) were used throughout the study.

PROCEDURES

Preliminary steps. For each CL, the presence or absence of the antigen assessed was confirmed by established methods, namely Western blot and an immuno-alkaline phosphatase method (APAAP) (17, 21, 24–27). Western blot and APAAP were performed with the same antibodies as for flow cytometry. The only exception was *gp-170* (*MDR-1*) detection by Western blotting where C219 (Dako, Prosan) at a final dilution of 1/400 was used in place of JSB-1, as described previously (24). Immunoblotting was obtained from 1×10^7 cells, and each blot was probed overnight at 4 °C with the respective mAb and incubated for 1 h at room temperature with either alkaline-phosphatase-conjugated anti-mouse antibody (DAKO, Prosan) for p53, *bcl-2*, and *gp-170*, or horseradish peroxidase-conjugated anti-mouse antibody (Amersham) for rb.

For p53 and *bcl-2*, the total cellular protein lysates were prepared by harvesting whole cells in hot Laemmli elec-

trophoresis sample buffer as described (21, 27, 28). After immunoblotting on nitrocellulose membrane (Bio-Rad), the specific binding was visualized with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma Chemical Co.).

For rb, whole cells extracts were separated by overnight electrophoresis in an 8% sodium dodecyl sulfate-polyacrylamide gel and transferred to an Immobilon poly(vinylidene difluoride) membrane (Millipore) as described (17, 26). The BM Chemiluminescence Western blotting kit (Boehringer Mannheim) was used for detection according to the manufacturer's instructions.

For *gp-170*, cells were resuspended in 0.5 mL of lysis buffer (0.01 mol/L Tris-HCl, pH 7.4) and run on sodium dodecyl sulfate-polyacrylamide gel as described (24). Immunoblotting and detection were identical to the procedure used for p53 and *bcl-2*.

The autofluorescence of each CL was measured before further evaluation. The three methods were performed according to the manufacturers' instructions or as described (2). For each antibody, experiments were repeated three times, and each trial with a particular CL was performed in triplicate.

Evaluation of the microwave oven performance. Although microwave ovens are simple apparatuses, they can differ by their total power capacity. To better characterize the performance of our system and to allow other laboratories to set up an identical procedure, we measured the time needed to boil 1000, 500, 250, 100, and 10 mL of distilled water in our microwave oven (Philips M610) at the maximum power setting (800 W).

The performance of our apparatus was as follows: 1000 mL of distilled water boiled at 12 min 9 s; 500 mL boiled at 6 min 22 s; 250 mL boiled at 3 min 35 s; 100 mL boiled at 1 min 52 s; and 10 mL boiled at 58 s.

FIXATION/PERMEABILIZATION METHODS

OPF. Cells (10^6) were washed in $1 \times$ phosphate-buffered saline (PBS; 1.44 g/L KH_2PO_4 –90 g/L NaCl–7.95 g/L Na_2HPO_4), resuspended in the OPF reagent, and then treated according to the manufacturer's instructions. Briefly, 10^6 cells were washed in PBS and fixed for 45 min in 1.5 mL of OPF reagent diluted twice, and then washed with PBS containing 15 g/L bovine serum albumin, 5 mg/L EDTA, and 50 mL/L fetal calf serum.

F&P. Cells (10^6) were washed in PBS and resuspended in 100 μL of reagent A (fixation) for 15 min, and then incubated in 100 μL of reagent B (permeabilization) and 20 μL of the primary mAb for 60 min. This prolonged permeabilization/incubation step was chosen after comparing a 15-min incubation as recommended by the manufacturer to 30 min and 60 min. In our hands, a 60-min permeabilization/incubation step allowed optimal immunolabeling without altered morphology or cell loss.

MWH treatment. Cells (10^6) were washed in PBS and fixed on ice for 30 min in 20 mL/L PFA in PBS. The supernatant was discarded after centrifugation (500g for 5 min at room temperature), and cells were resuspended in 10 mL of a 0.01 mol/L sodium citrate buffer, pH 6.0, containing 5 g/L bovine serum albumin in a 50-mL propylene tube. The cell suspension was heated in a microwave oven at the maximum power setting (800 W), reaching a temperature between 90 and 100 °C. Heating times of 30 and 60 s were compared for each marker. Cells were then chilled on ice for 10 min. After centrifugation (500g for 5 min at room temperature), the supernatant was discarded, and the cells were washed in PBS. The requirement for ice during fixation and after heating was assessed with the p53 monoclonal and corresponding control CLs. Results obtained with and without ice after PFA fixation and/or heating were compared.

IMMUNOLABELING

For each of the above techniques, incubation was performed with the mouse primary mAb (100 μ L) at the appropriate dilution for 60 min. This step was followed by incubation with a fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody (100 μ L) for 30 min in the dark. Cells were resuspended in 200 μ L of ISOTON II for analysis. Cells were washed in PBS before and after each incubation step. Incubation with the various antibodies was performed at room temperature.

COMPLEMENTARY ANALYSIS

Separate set of preliminary experiments. Recommended a few years ago for the detection of intracytoplasmic antigens (1, 29, 30), saponin was shown to detect the intranuclear antigen PCNA poorly (2). Accordingly, saponin (20 mL/L PFA for 30 min plus 1 g/L saponin for 5 min) was compared with OPF for the detection of both the intranuclear antigen p53 and the intracytoplasmic antigen bcl-2. For p53, RAJI and CEM were used as positive CLs, and IM9 served as negative control. For bcl-2, RAJI and DG75 LMP were used as positive CLs, and DAUDI served as negative control.

Additional controls. Peripheral blood samples collected from healthy blood donors were used as negative controls for all the intracellular antigens.

To assess the reproducibility of the study by Lan et al. (2), we repeated the published experiments using the PCNA mAb and the U937 monocytic CL.

Antigenic preservation of external epitopes after MWH was assessed on lymphocytes collected from patients presenting with a B-cell chronic lymphocytic leukemia as well as on the gp-170 VLB100 CL, using the CD19 and 4E3 antibodies, respectively.

The influence of delayed immunostaining on cellular morphology, immunoreactivity, and cell scatter criteria was evaluated for the three methods, using RAJI cells and p53 mAb. Cells fixed, permeabilized, and immunostained

immediately were compared with cells kept at 4 °C and immunostained 3 days and 1 week later, respectively.

FLOW CYTOMETRY

All samples were run on a flow cytometer (FACScan, Becton Dickinson) equipped with an argon ion laser (488 nm). Forward and right angle light scatter signals were collected for all samples, along with fluorescein isothiocyanate fluorescence measured through a 543 nm band-pass filter. The percentage of positive cells was measured from a cutoff set using an isotype-matched nonspecific control antibody. The mean channel fluorescence (MCF) was measured for a total of 5000 cells.

STATISTICAL ANALYSIS

Graphical representation. Bland and Altman (31, 32) and, more recently, Petersen et al. (33) emphasized the importance of graphical interpretation of analytical data when comparing a reference and a field method. They both advocated the use of difference plots. However, current data do not allow a strict application of these concepts. Data recorded with distinct intracytoplasmic and intranuclear antigens on distinct CLs cannot be pooled together. Moreover, an optimal cytometric method must be defined as the method giving the lowest MCF value with CLs defined as negative controls while giving the highest MCF value for CLs expressing the antigen of interest. The statistical agreement of the MWH technique was assessed using SD and a graphical approach adapted to the current situation. We considered the MCF values recorded for the negative-control CLs, the cytoplasmic antigens, and the intranuclear antigens separately.

Choice of the reference value. OPF and F&P have both been proposed as standard methods (3). There is no consensus yet on which method is the best; hence we have no way of considering a unique reference value. According to Bland and Altman (31), the mean of the two measurements is the best estimate we have when we do not know the true value. Considering the MCF differences existing between OPF and F&P, the MCF average of these two commercial techniques was therefore chosen as the reference value. To determine if the MWH method showed MCF values comparable with this chosen reference value, we took into account the Bland and Altman (31) statement that how far apart measurements can be without causing difficulties is a question of judgment. We considered a MCF difference (threshold) $\leq 15\%$ as good agreement. The arbitrary choice of 15% was considered to be conservative because a 15% difference is smaller than the average difference between OPF and F&P (which was 18%, with one-half of the values exceeding the 15% difference). The related questions focused on the agreement of the methods and on reproducibility.

Statistical analysis was performed on the triplicate results of one representative trial. Slight variations of intranuclear antigenic expression of rb and p53 were

Table 2. MCF and percentages of positive cells (%) obtained with OPF, F&P, and MWH on negative- and positive-control CLs for the four antigens assessed.

	OPF		F&P		MWH		
	MCF auto ^a	MCF (±SD)	% (±SD)	MCF (±SD)	% (±SD)	MCF (±SD)	% (±SD)
p53							
Co-: IM9	19	90 (14)	0.1 (0.2)	89 (13)	0.5 (0.2)	92 (10)	0.6 (0.1)
DOHH2	27	53 (4)	0.3 (0.2)	62 (11)	0.2 (0)	67 (7)	2 (1)
Co+: RAJI	19	373 (11)	87 (6.2)	340 (16)	79 (1)	350 (12)	52 (2)
CEM	15	238 (10)	66 (4)	267 (20)	56 (4.6)	308 (8)	64 (5.3)
LMP	12	155 (8)	75 (6)	109 (15)	50 (7.2)	170 (9)	67 (1.7)
DG75	17	229 (12)	85 (7.8)	195 (5)	61 (2.6)	277 (6)	79 (2.6)
rb							
Co-: IM9		63 (6.5)	1.2 (0.3)	50 (5.6)	0.2 (0.5)	76 (4)	1.6 (0.4)
6M1	11	87 (7)	1.8 (0.3)	68 (6)	0.5 (0.1)	48 (9)	2 (1)
6M2	14	61 (11)	6 (1)	49 (3)	1.8 (0.1)	52 (7)	1.5 (0.2)
Co+: KG1	13	395 (13)	93 (2.6)	221 (5)	90 (3.4)	277 (3)	82 (2.6)
3C3	18	130 (3)	50 (3.6)	168 (4)	66 (3.6)	166 (7)	88 (2.6)
5C3	16	132 (5)	54 (8)	171 (6)	69 (2.6)	159 (5)	90 (3.6)
bcl-2							
Co-: DAUDI	10	59 (7)	1.6 (0.1)	54 (8)	0.6 (0.1)	59 (5)	2.5 (0.2)
DG75		113 (5)	18 (3.6)	97 (6)	1 (0.5)	105 (4)	3 (1)
Co+: DOHH2		930 (16)	37 (2.6)	850 (5)	35 (1.7)	810 (13)	41 (3.6)
RAJI		320 (9)	35 (4.6)	283 (16)	30 (1.7)	268 (8)	39 (3)
LMP		203 (12)	28 (3)	162 (14)	25 (1.7)	171 (4)	21 (1)
gp-170							
Co-: CEM		67 (3.6)	1.2 (0.2)	50 (7.5)	1.3 (0.2)	57 (6)	1.1 (0.2)
Co+: VLB8	16	103 (7)	60 (2.6)	115 (6)	61 (1.7)	101 (9)	55 (3)
VLB100	16	165 (7)	64 (3)	164 (14)	69 (3)	242 (5)	94 (2.6)

^a MCF auto, MCF for cellular autofluorescence; Co-, negative control; Co+, positive control; LMP, DG75 LMP.

indeed observed over time with positive CLs, regardless of the method used, despite the log phase pattern of growth and despite comparable method reproducibility evidenced by SD. Such variations were therefore assumed to be cell cycle-related. Western blot analysis on rb-positive CLs confirmed this hypothesis and excluded a cytometric artifact (17). Because these variations were observed with any of the three methods performed at the same time on the same positive CLs, the trial giving the highest MCF value was chosen as representative.

Results

DETECTION OF ANTIGEN EXPRESSION

The preliminary set of experiments indicated that MCF values obtained with saponin for p53 and bcl-2 antigens on positive CLs were consistently weaker (by 37% and 47%) than the values recorded with OPF (data not shown). On this basis, experiments with saponin were not pursued.

Reproducibility of PCNA detection. Our findings confirmed those of Lan et al. (2) using MWH on the U937 CL and

Table 3. Background variations according to CLs and fixation/permeabilization method.

	OPF		MWH		Differences between the highest and lowest backgrounds ^a	
	MCF (±SD)	% (±SD)	MCF (±SD)	% ^b (±SD)	MCF (±SD)	% ^b (±SD)
Irrelevant (isotypic p53)						
IM9	86 (3)	13 (4)	54 (1)	12 (5)	32 (37%)	Nihil
RAJI	111 (5)	35 (3)	72 (2)	25 (3)	39 (35%)	10 (28%)
CEM	119 (2)	34 (5)	83 (2)	21 (6)	36 (30%)	13 (38%)
DG75	89 (3)	35 (4)	69 (4)	24 (2)	20 (22%)	11 (32%)
No primary antibody						
IM9	52 (8)	11 (4)	52 (4)	9 (2)	None	None
RAJI	107 (4)	23 (1)	67 (4)	17 (1)	40 (37%)	6 (26%)
CEM	96 (2)	27 (2)	80 (6)	12 (3)	16 (17%)	15 (55%)
DG75	119 (2)	24 (3)	77 (3)	20 (4)	42 (64%)	4 (17%)

^a The difference between the highest and lowest value in (%) was expressed as the percentage of decrease with respect to the highest value taken as 100%.

^b Percentage of positive cells.

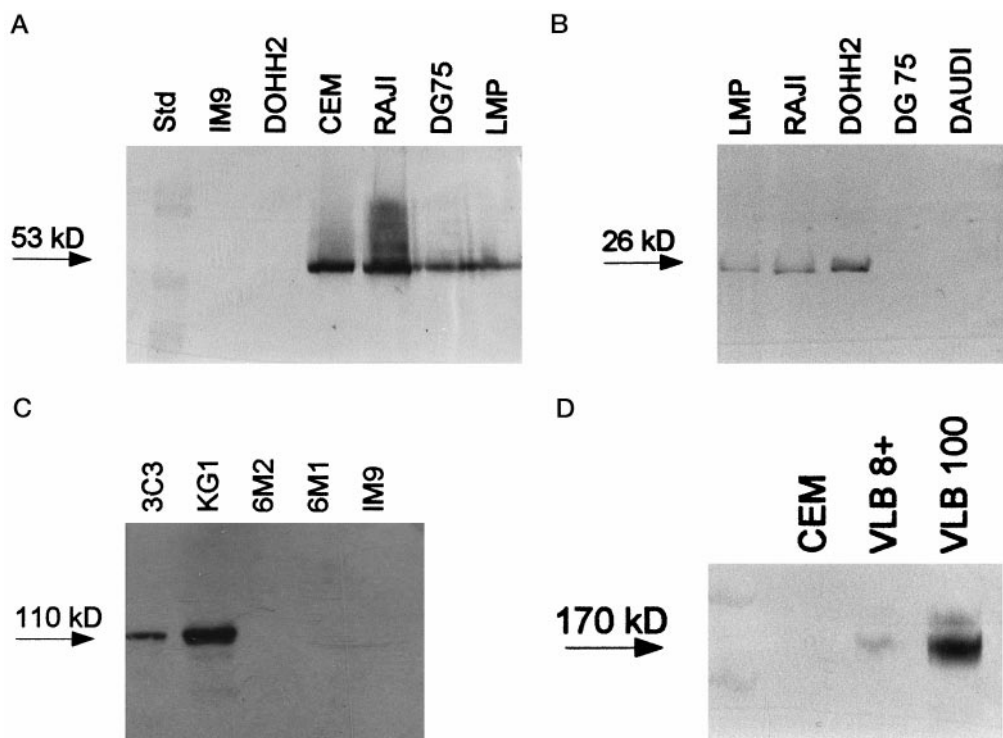


Fig. 1. Western blot results for positive- and negative-control CLs probed for the proteins p53 (A), bcl-2 (B), rb (C), and gp-170 (D).

(A) There was no protein expression in the negative controls *IM9* and *DOHH2*, whereas a specific band was found with the p53 mutated CLs. Band intensities corresponding to low (*LMP* and *DG75*), intermediate (*CEM*), and high (*RAJI*) expression were in agreement with flow cytometry data. (B) No protein expression was found in the negative controls *DAUDI* and *DG75*. On the other hand, a specific but progressively fainter band was observed with *DOHH2*, *RAJI*, and *LMP*, respectively. (C) No protein expression was observed in the negative controls *IM9*, *6M1*, and *6M2*. A stronger band was found in the positive control *KG1* compared with *3C3*, in accordance with flow cytometry data. [The clone *5C3* was not tested; however, APAAP staining was comparable with *3C3* (17).] (D) The negative-control CL *CEM* did not show any protein expression. According to APAAP staining (24) and flow cytometry data, the specific band for p-glycoprotein was much stronger in *VLB100* than in *VLB8*.

peripheral blood mononuclear cells with the marker PCNA. PCNA was detected in the majority of U937 cells (70% positive cells; MCF = 238), whereas peripheral blood mononuclear cells were negative (1.2% positive cells; MCF = 75). These results allowed us to proceed further and compare the three fixatives with other antibodies.

Results of the quantification for p53, bcl-2, rb, and gp-170. Results for the four antigens in the three methods are given in Table 2. Each commercial method was effective in distinguishing negative and positive CLs for expression of intracytoplasmic and intranuclear antigens. Variability of antigen expression was noticed, however, among the three different methods according to the cell type and the antigen assessed. Altogether, variability between the respective methods in this study, calculated as the difference between the highest and lowest MCF values, was between 9% and 36%. The MWH method compared favorably with OPF and F&P, especially with intranuclear antigens, where it ranked consistently first or second in the MCF intensity. In 5 of 12 antigen detection tests, MWH gave the highest MCF value. In the remaining experiments, the difference between the MWH method and the best MCF value was between 6% and 16% (the only exception being the 30% difference for rb detection in

KG1). The MCF with MWH was also associated with a consistent lower background observed with isotypic antibodies or when no primary antibody was added. When compared with OPF, the background for the CLs *IM9*, *RAJI*, *CEM*, and *DG75* was decreased between 17% and 64% with MWH (Table 3). Variation of antigen expression was also observed between positive CLs for one particular antibody, especially with the intranuclear antigens p53 and rb. Nonetheless, the same profile of expression, i.e., high, low, or intermediate constitutive expression, was consistently found in the positive CLs, irrespective of the method used. For each marker, Western blot and APAAP results confirmed the expected positivity and negativity of the control CLs. Although not designed for precise quantification of protein expression, Western blots were in total agreement with the antigenic profile (negative vs either low, intermediate, or high MCF value) of the various CLs (Fig. 1).

Technical factors potentially influencing MWH results. Extending the heating time from 30 s to 60 s did not improve the results with the MWH method. However, the loss of liquid and cells observed after heating for 60 s did not occur after 30 s. Performing the MWH method with or without ice during fixation and/or after heating yielded

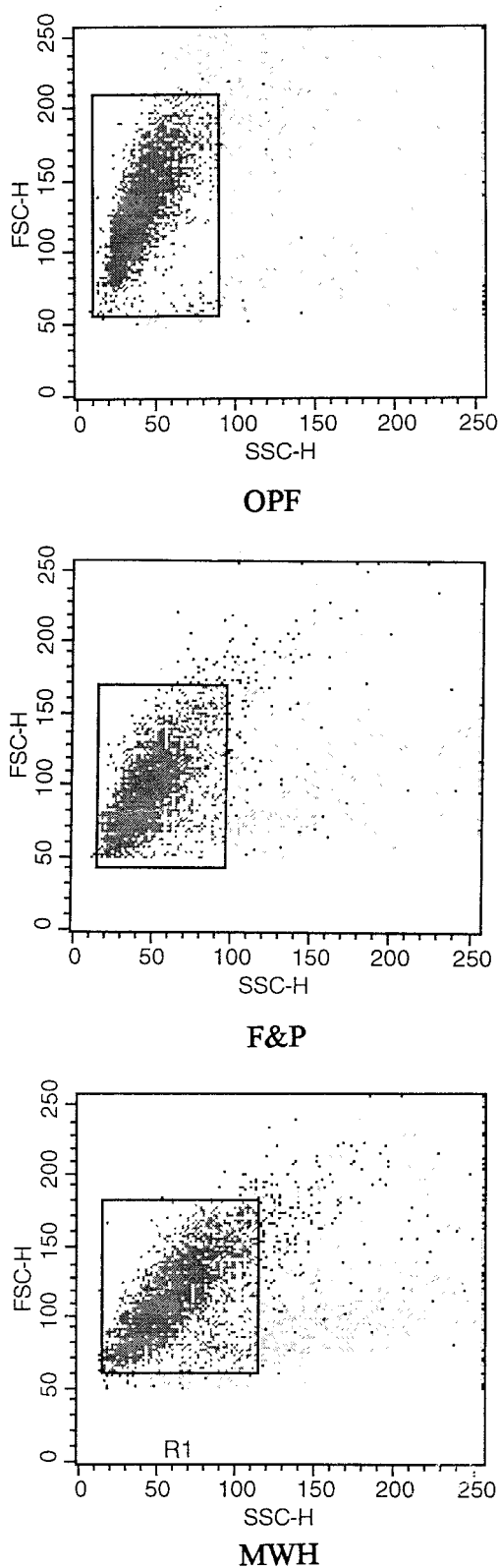


Fig. 2. Effects of each fixation/permeabilization method on cell morphology.

Cellular morphology was analyzed using light scatter histograms of the DG75 CL with p53. A clear discrimination between intact cells and debris is seen with each method.

comparable results with p53 on positive- and negative-control CLs (data not shown).

Preservation of external epitopes by MWH. It is worth noting that no CD19 and 4E3 labeling was observed with the MWH method when testing B-cell chronic lymphocytic cells and gp-170 subclones, respectively.

EFFECT ON CELL MORPHOLOGY AND IMMUNOREACTIVITY

The effect on cell morphology was measured by light scatter signals. The three methods preserved good cellular morphology and allowed easy discrimination between intact cells and debris, as illustrated with the p53 mAb and DG75 in Fig. 2.

Cell immunoreactivity, cell scatter properties, and excellent cellular morphology were obtained with the OPF and F&P techniques for a time interval as long as 1 week after fixation. However, immunolabeling had to be performed within 2 to 3 days after fixation with the MWH method. A 1-week delay caused a decrease in antigen immunodetection and an alteration of the cellular morphology (Fig. 3).

STATISTICAL ANALYSIS

Negative-control CLs. For five of eight MCF measurements, the difference between MWH and the MCF mean calculated on OPF and F&P results was <15% (Fig. 4A). However, regarding rb antigenic expression on 6M1 and IM9, we noted a higher difference: -38% and +35%, respectively. In both cases, the difference between OPF and F&P was also >15% (23% and 21%, respectively). Nonetheless, the graphical representation indicates that the SD of the three methods were comparable.

Nuclear antigens rb and p53. For four of seven measurements, the results were highly similar to the mean OPF and F&P (-10%, -2%, +5%, and +11%; Fig. 4B). In the other three measurements, analysis of p53 expression in LMP, DG75, and CEM cells showed a noticeably greater MCF with MWH (29%, 30%, and 22%, respectively). Altogether, MCF values tended to be higher (but not significantly) for MWH. SD were comparable for the three methods.

Cytoplasmic antigens bcl-2 and gp-170. For four of five measurements, results were comparable (variations between 0% and 11%; Fig. 4C). There was a slight trend towards lower MCF values with MWH. However, the MCF value of the CL VLB100 with MWH was much greater than the MCF mean of the other two methods (47%). Reproducibility of the three methods was comparable, as shown by the SD.

Discussion

Immunodetection of intracellular antigens requires that large molecules, such as antibodies, have access to the

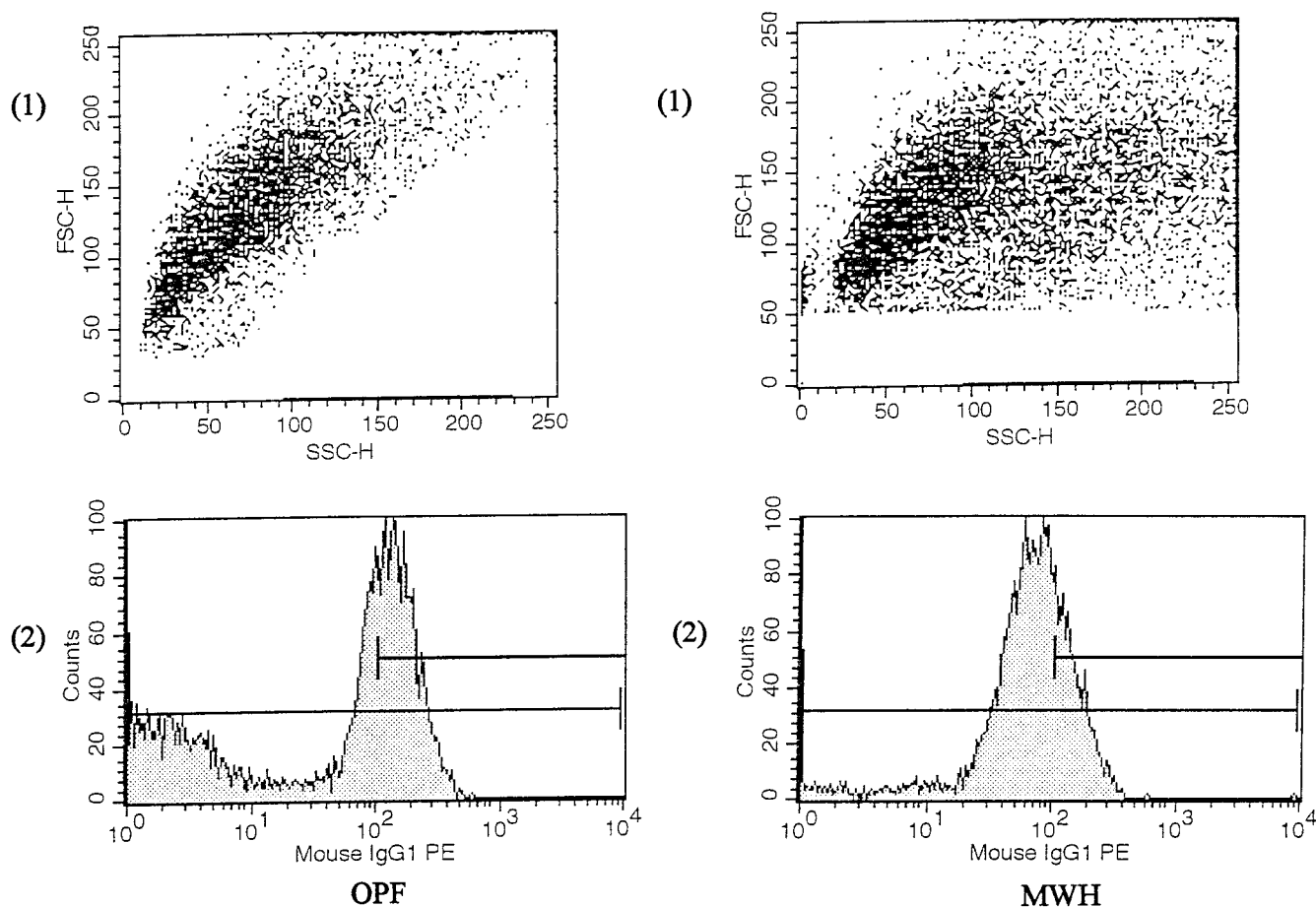


Fig. 3. Compared effects of delayed immunostaining (1 week after fixation with OPF and MWH) on cellular morphology, using light scatter histograms (1), and immunoreactivity, using fluorescence intensity histograms (2).

The immunostaining was performed on RAJI cells using the p53 antibody (*Mouse IgG1*). The positive cutoff for fluorescence, as defined by the vertical bars, was determined by using both an irrelevant isotopic antibody and no primary antibody on the positive CL. Results expressed in MCF and percentage of positive cells were identical for both negative controls. In *OPF*, the majority of cells are positive, whereas they are negative in the *MWH* histogram.

targeted antigen and an optimal antibody-ligand interaction. This depends on the type of fixation and permeabilization. Three fixation/permeabilization methods used in flow cytometry for the detection of cytoplasmic and nuclear antigens were compared in this study. *OPF* and *F&P* are two commercial reagents commonly used for that purpose (3–7), whereas *MWH* is a new promising non-commercial method (2). The four gene products (i.e., *bcl-2*, *p53*, *rb*, and *gp-170*) chosen for this comparison were not evaluated in the original report (2) nor in other studies using commercial reagents (3–7). They are good probes for flow cytometric analysis because their overexpression has been proven to be a valuable prognostic factor, especially in hematological malignancies (25–28, 34–36). Antibodies to these four markers are used widely in the clinical setting, in either staging or follow-up studies, and in research areas.

In a diagnostic laboratory, the choice of a particular technique is increasingly influenced by considerations such as versatility, rapidity, involvement of potentially harmful reagents, cost, and application to a wide range of

antigens. In this context, *OPF*, *F&P*, and *MWH* are easy to perform, require approximately the same amount of time to complete (~3 h 30 min) and have been shown to be suitable for a wide range of antibodies. None of them are particularly harmful. Because their computer-aided design improved optimization and standardization of the labeling procedure, *OPF* and *F&P* have recently been proposed as standards for the detection of intracellular antigens (3). The cost of *OPF* and *F&P*, however, may be prohibitive for routine diagnostic use. As an alternative, *MWH* requires only PFA and a citrate buffer, making it a much cheaper method. Chilling cells on ice during fixation and after heating, which is a somewhat cumbersome step described in the initial protocol (2), does not appear necessary. A microwave oven, required for *MWH*, is a very common and inexpensive piece of equipment.

Regarding the economic aspects, we also ran experiments with PFA and saponin, an inexpensive method recommended for detecting intracytoplasmic antigens when commercial fixatives were not yet available (1, 29, 30). However, MCF values recorded with this

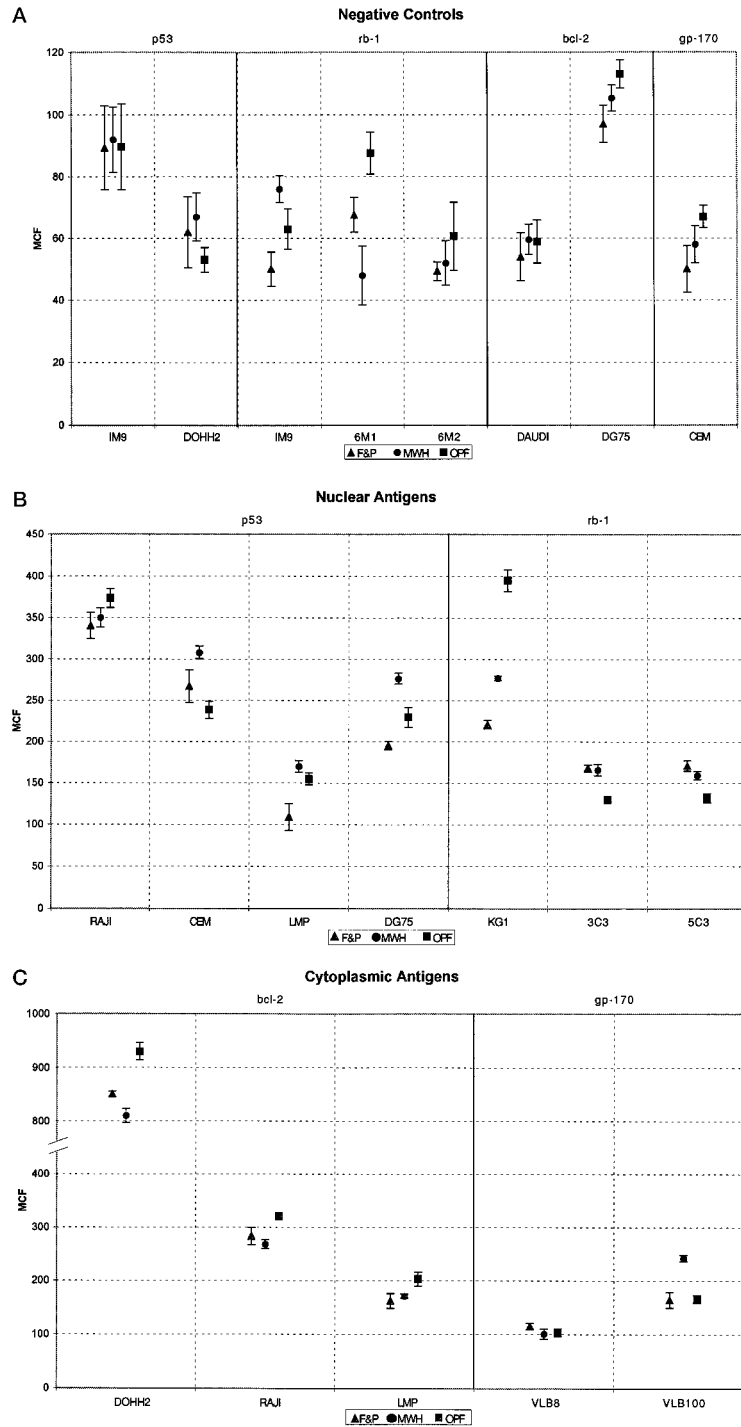


Fig. 4. Graphical representations of the SD for MWH (●), F&P (▲), and OPF (■).

(A) SD for the four antigens on their respective negative-control CLs: *IM9* and *DOHH2* for *p53*; *IM9*, *6M1*, and *6M2* for *rb*; *DAUDI* and *DG75* for *bcl-2*; and *CEM* for *gp-170*. In most cases, the difference between MWH (●) and the MCF mean calculated on OPF (■) and F&P (▲) results is <15%. The SD (bars) of the three methods are also comparable (except for *rb* on *IM9* and *6M1*). (B) SD for the nuclear antigens *p53* and *rb* on the following CLs: *RAJI*, *CEM*, *LMP*, and *DG75* for *p53*; and *KG1*, *3C3*, and *5C3* for *rb*. Altogether, MCF values appear to be higher for MWH (●), whereas the SD (bars) are comparable for the three methods. (C) SD for the cytoplasmic antigens *bcl-2* and *gp-170* on the following CLs: *DOHH2*, *RAJI*, and *LMP* for *bcl-2*; and *VLB8* and *VLB100* for *gp-170*. Weak variations are observed between MWH (●) and the other two methods (except for the *VLB100* CL). SD (bars) are comparable for the three methods.

method were, in our hands, substantially lower (>30% decrease) than with OPF, an observation that is in accordance with the comparison between Tween 20 and OPF (6) and with the poor detection of PCNA with saponin (2). Such lower performance associated with a lack of consensus on optimal PFA and saponin concentrations, a lack of consensus on the optimal time for fixation and permeabi-

lization, and finally, the reported lack of detection of some intracytoplasmic antigens with saponin (37, 38) prompted us not to pursue the comparison between saponin and other fixatives further.

Regarding versatility, cell morphology preservation and antigenic retention were carefully examined. Morphology is a critical point directly affecting the quality of

the results. This is a necessary feature to ensure accurate flow cytometric measurements. Whereas permeabilization methods using detergents have been reported to cause cellular damage (1–3), morphological characteristics of the cells appeared to be equally preserved with OPF, F&P, or MWH. These methods fixed and permeabilized the cell membrane without major damage to the cell. The light scatter patterns provided clear discrimination between entire cells and cellular debris. Antigenic retention clearly distinguished OPF and F&P from MWH. As already experienced by Lan et al. (2) with MHC class I antigen, we confirmed that the other membrane antigens do not withstand microwave treatment, making MWH unsuitable for such detection. This may, in part, explain the consistent lower background obtained with this method when assessing isotypic antibodies. Each of the three methods appeared effective for the detection of the four antigens on the respective CLs. Flow cytometry results confirmed the preliminary assessment by immunocytochemistry and Western blot analyses of the expected phenotypes of the cells. For each antibody, the method comparison showed a good agreement between MCF values. Likewise, analysis of SD indicated that MWH reproducibility is comparable to OPF and F&P. The SD tended to be smaller for the MWH method than for OPF and F&P (SD = 7.2, 8.5, and 9.5, respectively), but this was not significant. Slight variations of MCF values recorded for each antibody and CL according to the method used might reflect a difference in accessibility or preservation of the antigens. Variations between OPF and F&P methods have also been reported with other antigens (5, 7), with both studies concluding that F&P was more specific and sensitive. Current data show that MWH is as effective as OPF and F&P for detecting intracellular proteins. In several instances, MWH was one of the best methods for the detection of nuclear antigens. MWH therefore appears particularly appropriate for studying variations of nuclear protein content related to cell cycle regulation, a problem that has an increasing clinical impact. The mechanism by which MWH permits intracellular antigen detection while preserving good cellular morphology is not well understood. It has been shown that heat denaturation of native protein secondary structure can be prevented by the cross-linking fixative formaldehyde (2). This could also be related to the combined effects of PFA and microwaves. It seems that successful detection with MWH depends on the nature and localization of antigens.

As reported for OPF (4), fixation and permeabilization may permit a delay before the immunostaining and cytometric analysis. The length of this delay would be of particular interest to small laboratories lacking facilities for flow cytometry. This would allow them to accumulate and send patients samples to reference centers. In the current study, it was found that immunostaining and cytometric analysis could be delayed for up to 1 week following fixation with OPF or F&P and at least 3 days for

MWH without adverse effects. Although this delay is shorter for MWH, it would allow routine analysis in common daily practice. It means also that samples that arrived on Friday afternoon could be analyzed on the next Monday.

In conclusion, our data indicate that OPF, F&P, and MWH methods are suitable for the analysis of intracellular antigen expression by flow cytometry. Although slight differences were observed according to cell type and the antigen assessed, each method provides effective fixation and membrane permeabilization, allowing immunologic interaction while preserving cellular morphology. They offer good reproducibility and can be used in routine flow cytometric protocols. In several instances, especially with intranuclear antigens, MWH compared favorably with the commercial methods while being considerably cheaper. This can be of importance in clinical laboratories as well as research settings. Loss of external epitopes appears to be a genuine limitation of the MWH assay, but this seems interestingly associated with lower background staining. Besides nuclear protein analysis, MWH could be used reliably in a timesaving manner to establish appropriate conditions for quantification of intracellular proteins before performing other time-consuming and/or expensive assays (39). According to the present results, the relevance of the MWH method in the clinical setting deserves additional study.

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