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A Procedure to Prepare Human Platelets for Fluorocytometric Analyses Using a Pipet Column Packed with Sepharose 2B

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Summary. **A procedure to prepare substrate platelets for fluorocytometry was developed using a plastic pipet column packed with Sepharose 2B and plastic microtubes. The gel fractionation proved to be reliable in collecting platelets free from plasma. Collected platelets were centrifuged with ACD, incubated with ammonium oxalate, centrifuged and resuspended in phosphate buffered saline as substrate platelets. Reactions between the substrate platelets obtained from blood group-0 healthy men and alloimmunized anti-platelet antibodies, i.e., anti-Yuk^a and anti-Yuk^b serum, were analyzed by a laser flow cytometer, Cytoron (Ortho Diagnostic Systems), with satisfying results. This preparation procedure seems to be useful for fluorocytometric analyses of platelets and has advantages of being able to reduce both overall assay time and blood volume.**

INTRODUCTION

Since platelets are highly susceptible to both gravity and low temperature, special care must be taken to minimize damage during the isolation procedure. Without an addition of 1/10 volume of acid-citrate-dextrose solution to platelet rich plasma prior to centrifugation, the sedimented platelets would fail to be resuspended. When the starting volume of PRP is more than 5 ml, platelets are easy to observe as sediments on each centrifugation. For platelet antigen and/or antibody detections, however, it is often difficult to prepare more than 5 ml of platelet rich plasma, especially from patients with thrombocytopenia. Platelet preparation through Sepharose 2B gel was described by Tangen et al., in 1972,[1] using 15 by 300 mm or larger columns. Gel filtration of platelet rich plasma makes it possible to eliminate the laborious washing of centrifugations before obtaining pure platelets and so conserve time.

This paper describes a procedure to prepare substrate platelets for fluorocytometric analyses using a 5ml plastic pipet column. Gel-filtrations of about 1 ml of platelet rich plasma were monitored both on platelet counts and protein concentrations. Collected platelet fractions were treated to hemolyse contaminated red cells, and reactions between these processed platelets and alloimmunized anti-platelet sera were analysed by a flow cytometer.

MATERIALS AND METHODS

Equipment

1. Plastic tubes (Shionogi, 4 ml, inner size; 10 to 7 by 70 mm)
2. Microtubes (Eppendorf, 1.5 ml, #3810)
3. Plastic squirts (Maruemu, PS-1)
4. Disposable plastic pipets (Corning, 5 ml, #7075-5)
5. Nylon net (Pharmacia, 40 μm, #71-1600-02)
6. Centrifuges (Beckman TJ-6 and Kubota KH-150)
7. Pipet column for gel-filtration

The out-let of a 5 ml disposable plastic pipet was covered with a sheet of nylon net (ca. 10 by 10 mm) which had been immersed in water, and then was inserted into the in-let portion of a scalp vein needle (Tarumo, 19G). The adjoining section was sealed with an instant glue (Sankyo, Aron Alfa A), to fix and prevent water leakage. The needle portion was cut off. The entire appearance of the pipet column is illustrated in Fig. 1.

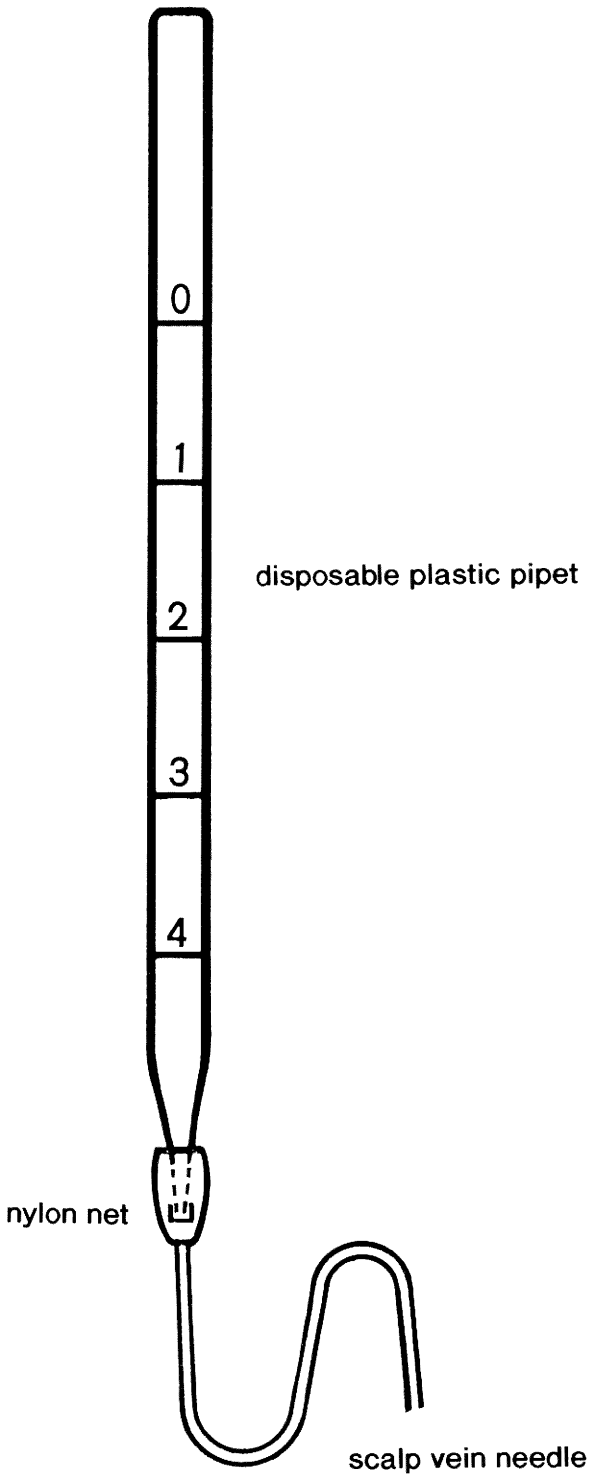

Fig. 1. Diagram showing the disposable pipet column for Sepharose 2B.

8. Automatic cell counter (Toa, Sysmex M-2000)
9. Tube mixer (Thermonics, TM-103)
10. Micropipettes (Eppendorf, 4710 and Rainin, EDP)
11. Flow cytometer (Ortho Diagnostics Systems, Cytoron)

Reagents

1. Phosphate buffered saline (PBS) 0.15 M, pH 7.4 containing 4.5 g $NaH_2PO_4 \cdot 2H_2O$, 32.25 g $Na_2HPO_4 \cdot 12H_2O$ and 8.5 g NaCl per liter of solution.
2. 1% Ammonium Oxalate containing 1.1 g $(NH_4)_2C_2O_4 \cdot H_2O$ per 100 ml distilled water.
3. Sepharose 2B (Pharmacia)

Prior to packing into the column it was necessary to wash Sepharose 2B with PBS more than ten times by decantation, and to deairate with a water vacuum pump for 30 min.

4. Platelet rich plasma (PRP)

Two ml of group-O blood containing one-eighth its volume of ACD-A solution was taken into the plastic tubes (blood height in the tube was about 38 mm) and centrifuged for 8 min at 800 rpm at room temperature using Beckman TJ-6 (r=196 mm). In the case of a crystallized EDTA-2K containing tube (Nipro, inner diameter: 11 mm), in which the height of blood in the tube was 22 mm, the centrifuging time was shortened from 8 min to 5 min. The supernatant PRP was transferred to another plastic tube using a disposable squirt.

5. ACD-A solution (Terumo)
6. Anti-platelet antibodies

Anti-Yuk^a and anti-Yuk^b serum[2,3] were kindly supplied from Dr. Yoh-ichi Shibata, Toranomon Hospital, Tokyo.

7. Tonein TP reagents (Ohtsuka Assay Laboratories)
8. FITC-labeled antihuman IgG goat IgG [$F(ab')_2$ fraction] (Tago, #4200)

Procedures

All procedures described below were carried out at room temperature.

Gel Filtration

A plastic pipet column was packed with Sepharose 2B to the zero line and 5 ml of PBS were passed through to wash the gel before filtration. About 1 ml of PRP was applied on top of the gel, allowed to run completely into the gel, and 2 ml of PBS was then added. The first 2 ml of eluate was discarded, and next 1 ml of eluate was collected in a microtube as

the platelet fraction.

Substrate Platelets for Fluoucytometric Analyses

To the collected platelet fraction, one tenth volume of ACD-A solution was added, and then centrifuged at 5,200 rpm for 2 min using a Kubota KH-150 (r=65 mm). The sediments were resuspended in 1 ml of 1% ammonium oxalate solution and left for 5 min to hemolyse contaminated red blood cells. After centrifugation at 5,200 rpm for 2 min, the sedimented platelets were resuspended in 500 μl of PBS. The suspension was adjusted to a platelet count of 200,000/μl by dilution with PBS.

Immunological Reaction

Using micropipettes, 2 μl of alloantibody (anti-Yuk^a serum or anti-Yuk^b serum) and 100 μl of platelet suspension described above were mixed in a microtube and incubated for 30 min at room temperature. A 900 μl of PBS was added, mixed end-over-end, and then centrifuged at 5,200 rpm for 2 min using a Kubota KH-150. The supernatant was discarded and another 900 μl of PBS was added and mixed for 3 sec using a tube mixer. The tube again was centrifuged, and the sediment was resuspended in 100 μl of PBS. Five μl of FITC-labeled anti-human IgG goat IgG $F(ab')_2$ fraction diluted 5 times with PBS was added. After 30 min of incubation at room temperature, the tube was washed twice with PBS also as described above. The sediment was resuspended in 500 μl of PBS and was subjected to laser fluorocytometry.

Fluorocytometry

All procedures were followed to the instructions described to the flow cytometer.

RESULTS

Platelet and Protein Profile of Sepharose 2B Gel-Filtration

Eight hundred μl or 1200 μl of PRP were applied on each top of the Sepharose 2B gel in a pipet column, respectively. Eluated fractions were collected every 0.5 ml. Protein concentrations and platelet counts were measured using Tonein TP reagents and an automated blood cell counter. Platelets were fractionated mainly in tubes 5, 6 and 7, whereas plasma protein appeared in tube 8 and were fractioned mainly in tubes 11, 12 and 13. These results suggested that the first 2 ml of eluates should be discarded and the next 1.0 or 1.5 ml should be collected as the platelet fraction (Fig. 2). In this study, 1.0 ml of eluate was used.

Fluorocytometric Analyses of Platelets

Platelets reacting with allo-antibodies were shown in Figs. 3 and 4. As seen in the figures, fluorescence curves clearly distinguish two platelet antigens. In the Yuk (a−b+) person, SS, the flurescence peak of platelets reacting with anti-Yuk^a counted less than 20, whereas in the Yuk(a+b+) person, SK, that was more than 30 (Fig. 3). The fluorescence peaks of platelets from SS and SK reacting with anti-Yuk^b were located essentially at the same places, counting more than 70 (Fig. 4).

DISCUSSION

Compared to red cells, studies on platelet antigens and the antibody system have not yet been established as a routine work because of the fragility of platelets. To obtain substrate platelets with minimum impairment is essential for such studies, the first key point being to collect enough platelet rich plasma from the blood. In the preparation of PRP, the centrifuging time must be determined in each laboratory using a constant speed, with the variables of duration of the centrifuging and the height of the blood in the tube. For instance, in the case of 8 ml or 4 ml of blood in a plastic tube (Eiken Kizai, inner diameter: 15 mm), tubes were centrifuged at 800 rpm for 15 min or 10 min with Beckman TJ-6. This means that the height of the blood from the bottom of the tube is the regulating factor in obtaining large amounts of PRP. Four ml of normal blood were enough to prepare 1 ml of PRP with a concentration of around 400,000/μl and the processed platelets were sufficient for several different antibody analyses. From thrombocytopenic patients, several columns were prepared to harvest platelets, applying 1,200 μl of PRP at most per column, and collecting 1.5 ml of the eluate.

The plastic pipet column had advantages because the platelets were easy to observe in the eluate as a milky white color, while the plasma in the pipet column was yellow. Although the column and Sepharose were expensive, they afforded a stable and clear separation without losing many platelets. It took only 20 min at most for the gel-filtration.

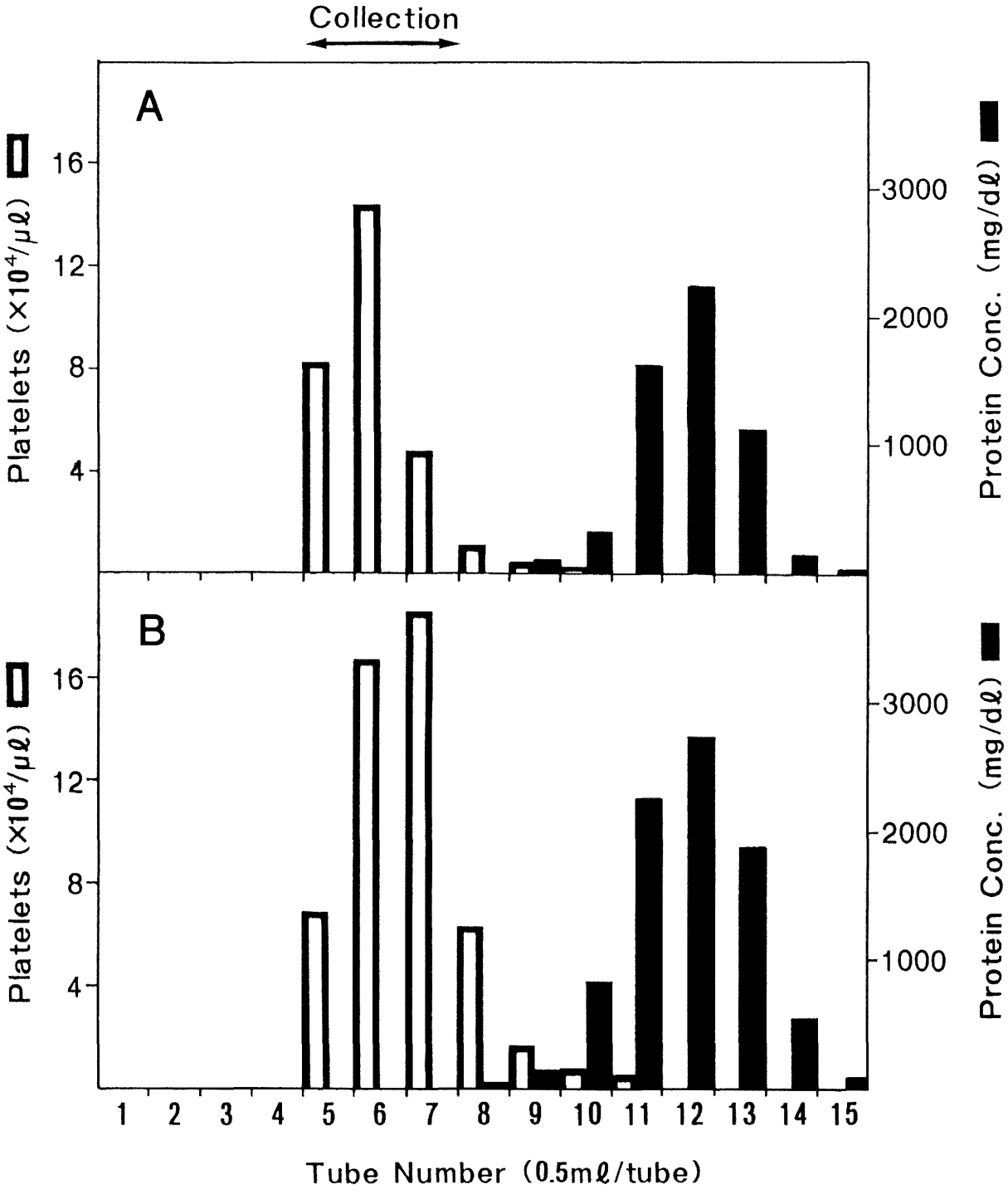

Fig. 2. Elution profile of PRP through the Sepharose 2B pipet column. Platelet rich plasma of 800 μl (A) and 1200 μl (B) was applied on top of the gel and eluates were collected every 0.5 ml. Platelet counts (open bar) and protein concentration (closed bar) are shown.

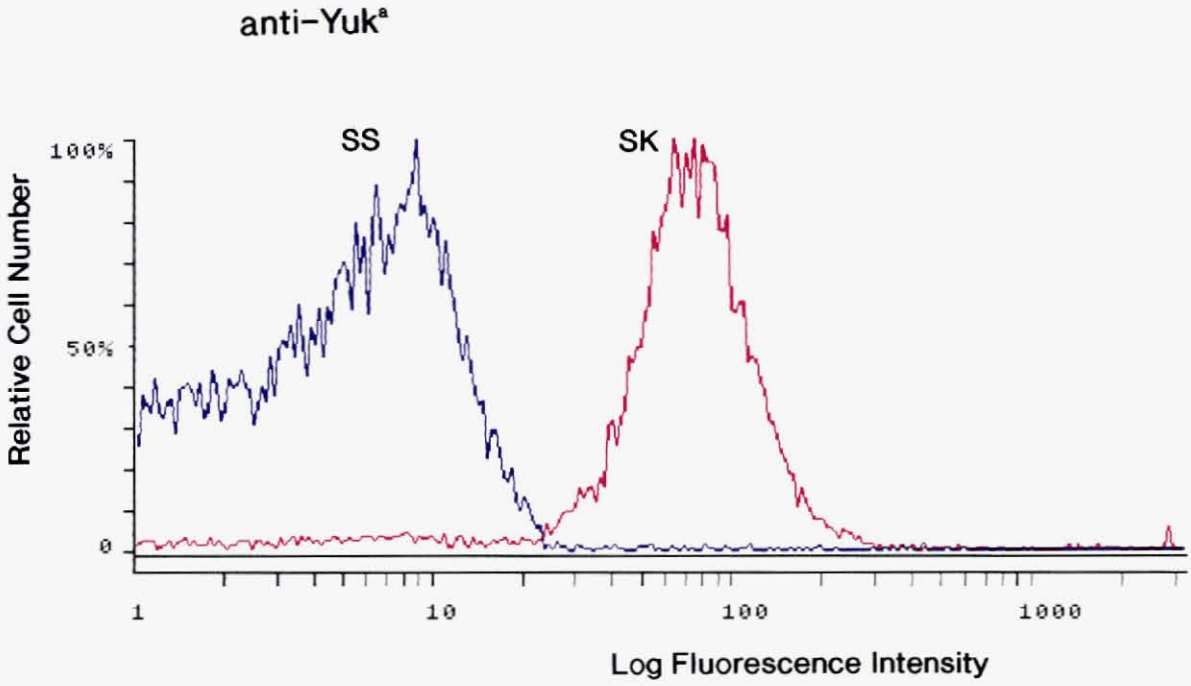

Fig. 3. Fluorocytometric patterns of platelets reacting with anti-Yuk^a serum. Platelets were incubated sequentially with anti-Yuk^a serum and FITC labeled anti-human IgG goat IgG F(ab')$_2$. Cells were analysed by flow cytometry using log amplification of the fluorescence intensity. SS (blue line) is the person whose platelet is Yuk (a−b+) and SK (red line) is Yuk (a+b+).

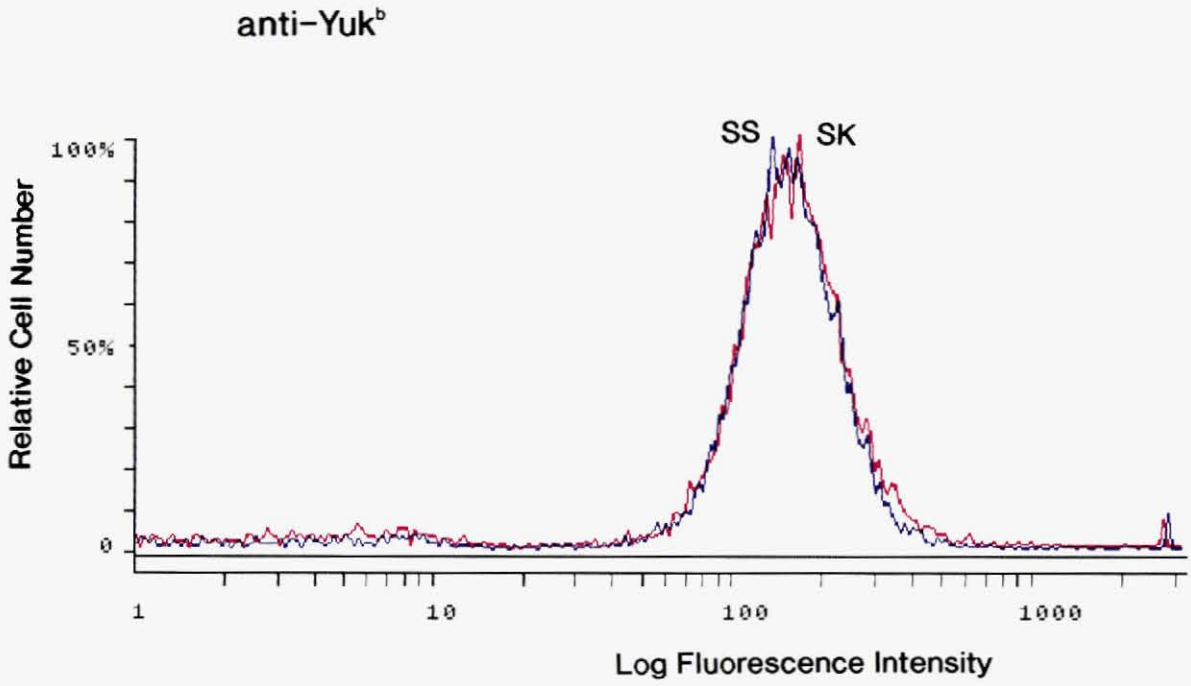

Fig. 4. Fluorocytometric patterns of platelets reacting with anti-Yuk^b serum. Reaction procedure is the same as that described in Fig. 3.

Microtubes shortened the centrifugation time to 2 min to harvest platelets at the bottom of the tube. It took about 10 min for the preparation of substrate platelets from the filtrated fraction. In immunoreactions, washing procedures were also easily performed in a short time by centrifuging each for 2 min.

Phosphate buffered saline (PBS) instead of Tyrode buffer[1] was used only because the former has been more routinely used in red cell compatibility tests.

The immunofluorescence test using a fluoromicroscope[4] and the mixed passive agglutination using a microplate[5] have been established as the standards for the identification and for the screening of platelet antibodies, respectively. The former method required the labor of a highly specialized person to obtain convincing results; the latter sometimes gave inconclusive results. The flow cytometer is now becoming a useful tool for investigating antigen and antibody reaction on platelets, especially in their identification. As far as is known, however, only a few applications on platelet analyses have previously been described.[6,7]

The peak positions of Yuk^a and Yuk^b were distinctly different; the former shifted weakly and the latter strongly. These results might be due to the epitope numbers or to the titer of the serum used. More detailed studies should be performed.

In order to obtain satisfactory results in fluorocytometric analyses, the cell to be tested must be a single particle separated from others. Using another flow cytometer, a wider distribution of fluorescence was obtained with substrate PRP and allo-antibodies.[8] To represent a clear and narrow peak of the fluorescence of platelets, gel-filtrated platelets seem most advantageous. Microtubes are also recommended for washing platelets because they conserve both time and labor.

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