ThromboStep (2nd Generation)

Platelet associated immunoglobulin detection kit (PAIg Kit)

Reference	Test
TBS-50T	50 test

INTRODUCTION

A normal platelet count ranges from 150,000 to 450,000 platelets per microliter of blood in human. Thrombocytopenia is the presence of relatively few platelets in blood. Decreased platelet counts can be due to a number of disease processes, the quantification of platelet associated immunoglobulin allow to the cause of thrombocytopenia is decreased ratio in platelet production or an increase in the ratio of destruction.

MATERIALS PROVIDED

The kit includes:

- 1 vial monoclonal antibody anti CD42a PE. Recommended for use in flow cytometry for identification of Platelets and Megakaryocytes Reacts with a 17-22kDa single chain integral membrane glycoprotein, also known as GPIX.
- 1 vial FITC polyclonal antibody to total human immunoglobulin (Anti-total Igs FITC).
- 1 vial FITC polyclonal antibody to human IgA.
- 1 vial FITC polyclonal antibody to human IgG.
- 1 vial FITC polyclonal antibody to human IgM.
- 1 vial FITC conjugated polyclonal antibody to total rabbit immunoglobulins (Goat anti-Ig Rabbit FITC)
- 2 bottles of Tyrode's Solution without sodium bicarbonate 20X (50 ml).
- 2 bottles of Sodium bicarbonate

MATERIALS REQUIRED BUT NOT PROVIDED

- Blood collection tubes containing EDTA as anticoagulant.
- 12 x 75 mm polypropylene centrifuge tubes
- 1% of paraformaldehyde, 10 mM EDTA and 0,5% BSA
- Micropipette capable of dispensing 5 µl, 20 µl, 100 µl, and 500 µl volumes
- Serofuge or equivalent centrifuge
- Vortex mixer
- Flow Cytometer with 488 nm excitation wavelength (argon-ion laser)
- Ammonium oxalate (1 L): 1% Amonium Oxalate (Ej. Merk 101192; Sigma 09898 or 221716).

RECOMMENDED USAGE

"Thrombostep" from Immunostep is designed for use in flow cytometry detection and quantitation of platelet associated immunoglobulin.

CLINICAL RELEVANCE

Immune thrombocytopenic purpura (ITP) is an autoimmune disorder characterized by a low platelet count and mucocutaneuos bleeding. The autoantibodies are directed primarily to the platelet-specific receptors CD41a (GPIIb/IIIa) and CD42b (GPIb). As a result, the sensitized platelets are rapidly cleared by the monocyte-macrophage cell systems.

The determination of autoantibodies against thrombocytes allows differentiate immune from nonimmune thrombocytopenia.

APPROPRIATE STORAGE AND HANDLING CONDITIONS

Store in the dark, refrigerated between 2 ºC and 8 ºC. DO NOT FREEZE. The antibody is stable until the expiry date stated on the vial label if kept at 2ºC-8ºC. Do not use after the date indicated.

Once the vial is open, the product is stable for 90 days.

EVIDENCE OF DETERIORATION

Reagents should not be used if any evidence of deterioration is observed. For more information, please contact our technical service: tech@immunostep.com

The product's normal appearance is a semi-transparent, colourless liquid. It should not be used if liquid medium is cloudy or contains precipitate. It should be odourless.

RECOMMENDATIONS AND WARNINGS

a) The reagents contain sodium azide. In acid conditions, it is transformed into hydrazoic acid, a highly toxic compound. Azide compounds must be diluted in running water before being discarded. These conditions are recommended so as to avoid deposits in plumbing, where explosive conditions could develop. The safety data sheet (SDS) is available online at *www.immunostep.com*

b) Avoid microbial contamination of the reagent.

c) Protect from light. Use dim light during handling, incubation with cells and prior to analysis.

d) Never mouth pipette.

e) In the case of contact with skin, wash in plenty of water.

f) The samples should be handled in the same way as those capable of transmitting infection. Appropriate handling procedures should be guaranteed.

g) Do not use after the expiry date indicated on the vial.

h) Deviations from the recommended procedure could invalidate the analysis results.

i) FOR *IN VITRO* DIAGNOSTIC USE.

j) For professional use only.

k) Before acquiring the samples, it is necessary to make sure that the flow cytometer is calibrated and compensated.

PROCEDURE

WASHING BUFFER (50 ml) PREPARATION

Add one of the sodium bicarbonate content in one of the tyrode´s buffer bottle and close the stopper. Shake the bottle until the complete dissolution of the sodium bicarbonate. Dilute with H_20 the volume that will be used).

SAMPLE COLLECTION

1. Collect 10 ml of EDTA peripheral blood from each patient or individual control. Each sample, for both patients and control, are divided into two tubes and centrifuged without brake at low speed, 200xg for 10 minutes to obtain a separation between the rich plasma platelets and the fraction of blood cell[1,2]

2. After centrifugation is collected platelet-rich plasma with a pipette pasteur avoiding collect red blood cells and the platelet-rich plasma is centrifuged with brake at high speed at 900xg for 10 minutes. Decant the tube by collecting the plasma which was stored at 4º C.

The plasma will be used to confirm the result obtained in case it is necessary, using platelets from healthy individuals.

SAMPLE PREPARATION

1. Resuspend the button of platelets that appears in the botton of the tube after decant in 5 ml of 1% ammonium oxalate incubating 5 minutes at room temperature. The ammonium oxalate is a hypotonic solution to lyse red blood cells attached to platelets.

2. After incubation, cells are centrifuged at 900 x g for 10 minutes and the supernatant is decanted and resuspend the button of platelets in 3 ml of Washing Buffer.

3. Centrifuge the platelets at 900 x g for 10 minutes and decanting the supernatant. Wash the platelet one more time at 900 x g for 10 minutes.

4. Resuspend the washed platelets in 3 ml of 1% of paraformaldehyde, 10 mM EDTA and 0,5% BSA. Incubate 5 minutes at room temperature.

5. Wash cells twice with 3 ml of Washing Buffer. Centrifuge the platelets at 900 x g for 10 minutes and decant the supernatant.

6. Resuspend the cells in 1 ml of Washing buffer, counting the concentration of platelets on a haematology analyzer or with a counting chamber to match 100.000 / µl or less.

7. Finally the tubes are stored for at least 2 hours or a week at 4 °C to reduce the amount of unspecific bound antibodies.

SAMPLE LABELING METHOD

1. Five-tube are labelled for each patient or control (Polyclonal Anti-Rabbit Immunoglobulins; Polyclonal Anti-Human Immunoglobulins; Polyclonal Anti-Human IgA; Polyclonal Anti-Human IgG; Polyclonal Anti-Human IgM). It is recommended to do the labelling of the thrombocytes with antibodies on ice. Prepare ice bath.

2. Add 50 µl of platelets sample or control with anti-human Igs for each tube.

   - **Polyclonal Anti-Rabbit tube**: Add 20µl of the FITC Polyclonal Anti-Rabbit Igs + 20 µl of CD42a PE
   - **Polyclonal Anti-Human Igs tube**: Add 20µl of the FITC Polyclonal Anti-Human Igs + 20 µl of CD42a PE
   - **Polyclonal Anti-Human IgA tube**: Add 20µl of the FITC Polyclonal Anti-Human IgA + 20 µl of CD42a PE
   - **Polyclonal Anti-Human IgG tube**: Add 20µl of the FITC Polyclonal Anti-Human IgG + 20 µl of CD42a PE
   - **Polyclonal Anti-Human IgM tube**: Add 20µl of the FITC Polyclonal Anti-Human IgM + 20 µl of CD42a PE

3. Vortex and incubated the samples for 30 minutes in the dark ice bath.

4. Add 3 ml of Washing buffer to each tube, mix the samples and centrifuge the platelets at 900 x g for 10 minutes. Repeat the washing one more time.

5. Decanted the supernatant and resuspend the cell pellet in 500 ul of Washing buffer.

6. Analyzed by flow cytometry. If the samples are not to be analyzed immediately, store them in the dark at 2-8º C.

FLOW CYTOMETRY ANALYSIS

Verify that the cytometer is aligned and standardized to light scattering (FSC / SSC in logarithmic scale) and intensity of fluorescence (FL1, FL2 in logarithmic scale).

It is necessary to set a region (R1) to select the platelets population. Set the R1 around the CD42a PE positive population (trhombocytes). Acquire at least 10.000 events in region 1 (R1).

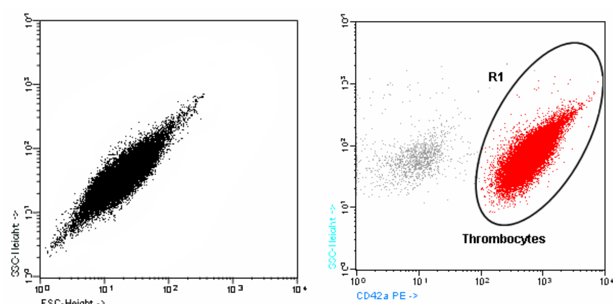
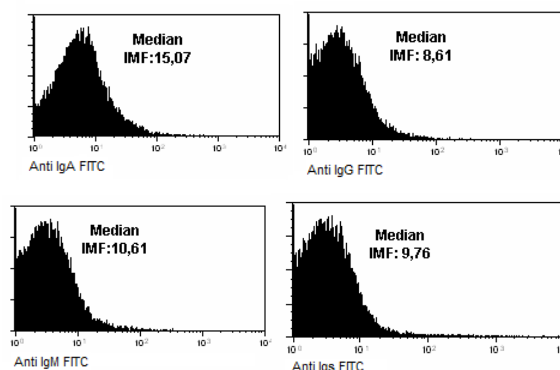


Figure 1. Control Thrombocytes (red events) are cells within CD42a gate (R1). Cells were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometer.

A



B

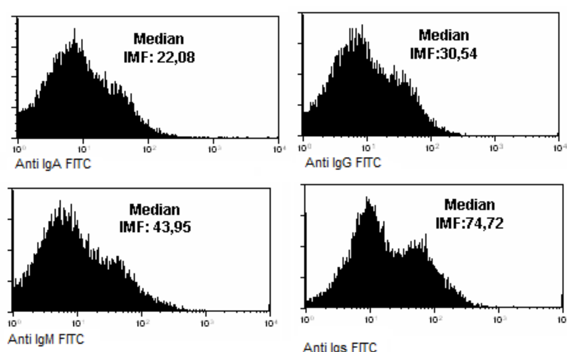


Figure 2. Immune Thrombocytopenia. The histograms represent comparison of a healthy control sample (A) and immune thrombocytopenia sample (B). Cells were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometer.

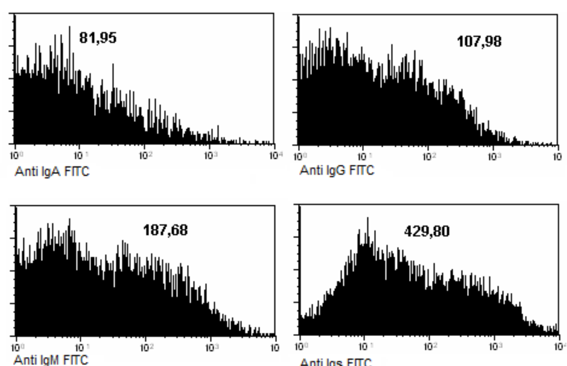


Figure 3. Immune Thrombocytopenia. The histograms represent an immune thrombocytopenia sample. Cells were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometer.

LIMITATIONS OF THE PROCEDURE

1. Anti-IgA and anti-IgM can produce nonspecific labeling on IgG. In case the sample is positive for IgG, IgA and IgM consider only the first
2. Incubation of antibody with cells for other than the recommended procedures may result in a reduction or loss of antigenic determinants from the cell surface.
3. The values obtained from normal individuals may vary from laboratory to laboratory; it is therefore suggested that each laboratory should establish its own normal reference range.
4. Abnormal cells or cell lines may show a higher antigen density than normal cells. In some cases, this could require the use of a greater quantity of monoclonal antibody than is indicated in the procedures for sample preparation.
5. In whole blood samples, red blood cells found in abnormal samples, as well as nucleated red cells (from both normal and abnormal specimens) may be resistant to lysis. Longer periods of red blood cell lysing may be needed in order to avoid the inclusion of unlysed cells in the lymphocyte gated region.
6. Blood samples should not be refrigerated for an extensive period (more than 24 hours), since the number of viable cells will gradually decrease, and this may have an effect on the analysis. In order to obtain the best values, they should be kept at room temperature immediately prior to incubation with the monoclonal antibody.
7. Accurate results with flow cytometric procedures depend on correct alignment and calibration of the lasers, as well as correct gate settings.

REFERENCE VALUES

Abnormal results in the percentage of cells expressing the antigen or in its levels of expression may be due to pathological conditions. It is advisable to know the normal antigen expression patterns in order to ensure a proper interpretation of the results[4,5,6].

Percentage in Peripheral Blood of a Normal Patient

Red Blood Count : 3,8 - 5,6 $X10^6$/µL
Platelets: 150 - 450 $X10^3$/µL

The values obtained from healthy individuals may vary from laboratory to laboratory; it is therefore suggested that each laboratory should establish its own normal reference range.

CHARACTERISTICS

Values of the green fluorescence of stained thrombocytes from normal individuals. Cells were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometer.

Antibody	Green Fluorescence (Median)	Maximum Value	Minimum Value	N
Anti-human IgA	**29,05**	99,74	5,45	17
Anti-human IgG	**11,72**	72,34	4,43	17
Anti-human IgM	**13,89**	68,11	5,25	17
Anti-human immunoglobulin	**12,37**	65,91	3,98	17

Values of the green fluorescence of stained thrombocytes from pathological individual sample.

Antibody	Green Fluorescence (Median)	Maximum Value	Minimum Value	N
Anti-human IgA	**144.63**	446,30	23,53	15
Anti-human IgG	**62,58**	457,07	13,83	15
Anti-human IgM	**83,54**	258,44	20,85	15
Anti-human immunoglobulin	**91,49**	272,37	17,38	14

SPECIFICITY

For kit specificity assay anti-human Igs were incubated respectively with human immunoglobulins IgA, IgG and IgM and BSA (control) coated polystyrene beads.

Anti-human Igs specificity analysis show a very low cross-reactivity (>7%) for all of them (Fig 4), making easy the correct identification of non-pathological samples.

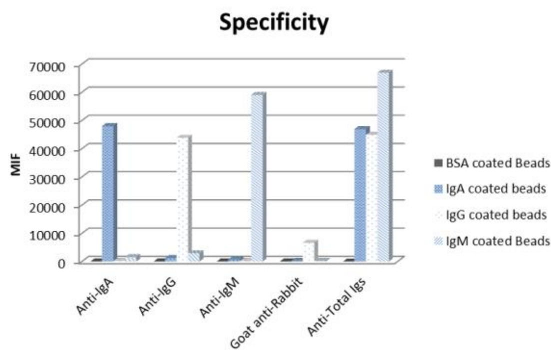


Figure 4: human immunoglobulins IgA, IgG and IgM and BSA (control) coated polystyrene beads were analyzed by FACSAria II (Becton Dickinson, San Jose, CA) flow cytometer.

WARRANTY

Garranted only to conform to the quantity and contents stated on the label or in the product labelling at the time of delivery to the customer. Immunostep disclaims hereby other warranties. Immunostep's sole liability is limited to either the replacement of the products or refund of the purchase price.

REFERENCES

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