ThromboStep (2nd Generation) Platelet associated immunoglobulin detection kit (PAIg Kit)

REF

TBS-X-50T 50 test RUO

INTRODUCTION

A physiological platelet count typically ranges from 150,000 to 450,000 platelets per microliter (µL) of peripheral blood in healthy individuals. Thrombocytopenia is clinically defined as a significant reduction in the circulating platelet count. Because a decreased platelet count can arise from diverse pathophysiological processes, the uantification of platelet-associated immunoglobulin (PAIg) serves as a critical diagnostic tool to differentiate whether thrombocytopenia is driven by impaired platelet production or by accelerated peripheral destruction.

MATERIALS PROVIDED

The ThromboStep Kit comprises the following components sufficient for 50 determinations:

-Platelet Identification Reagent: 1 vial of anti-CD42a PE monoclonal antibody (clone validated for flow cytometry; recognizes a 17–22 kDa single-chain integral membrane glycoprotein, also known as GPIX, expressed on platelets and megakaryocytes).
-Immunoglobulin Detection Reagents:

- 1 vial of FITC-conjugated polyclonal antiboy to total human immunoglobulins (Anti-total Igs FITC).
- 1 vial of FITC-conjugated polyclonal antibody to human IgA.
- 1 vial of FITC-conjugated polyclonal antibody to human IgG.
- 1 vial of FITC-conjugated polyclonal antibody to human IgM.

-Control Reagent: 1 vial of FITC-conjugaed polyclonal antibody to total rabbit immunoglobulins (Goat anti-Ig Rabbit FITC).
-Buffer Components:

- 2 bottles of Tyrode's Solution (without sodium bicarbonate), 20X concentrated (2 x 50 mL).
- 2 bottles of Sodium Bicarbonate (powder format).
- 1 bottle of Ammonium Oxalate Solution (powder format).

MATERIALS REQUIRED BUT NOT PROVIDED

- Blood collection tubes containing EDTA as an anticoagulant.
- 12 x 75 mm polystyrene or polypropylene centrifuge tubes.
- Fixation solution: 1% paraformaldehyde, 10 mM EDTA and 0.5% BSA in PBS.
- Phosphate-Buffered Saline (PBS).
- Distilled or deionized water.
- Calibrated micropipettes and disposable tips.
- Vortex mixer.
- Centrifuge equipped with a swing-out rotor.
- Flow cytometer equipped with appropriate excitation lasers (488 nm) and fluorescence detection channels (FITC/FL1 and PE/FL2).

INTENDED USE / RECOMMENDED USAGE (RUO Version)

ThromboStep™ (Ref. TBS-X-50T) is a research use only reagent kit intended for the simultaneous detection and profiling of platelet-associated immunoglobulins (PAIg: IgG, IgA, and IgM) in human peripheral blood samples utilizing multicolor flow cytometry.

This assay is specifically developed to support research investigations regarding platelet immunophenotyping, facilitating the characterization of antibody-mediated platelet mechanisms versus alternative processes affecting platelet homeostasis. The reagent is optimized for direct immunofluorescence staining of human blood platelets and is intended for laboratory research applications by qualified technical personnel. For Research Use Only. Not for use in diagnostic or therapeutic procedures.

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 °C and 8 °C. DO NOT FREEZE. The antibody is stable until the expiry date stated on the vial label if kept at 2°C-8°C. Do not use after the date indicated. Once the vial is open, the product is stable for 90 days.

EVIDENCE OF DETERIORATION

Reagents must not be used if any visual or physical signs of deterioration or contamination are observed. The standard appearance of the liquid components is a clear, colorless, and odorless solution. The presence of any visual turbidity, microbial growth, discoloration, or precipitation indicates product compromise, and the affected reagent must be immediately discarded. In the event of suspected reagent degradation, suspend testing and contact Immunostep's Technical Support Department directly at: tech@immunostep.com.

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

AMMONIUM OXALATE 1X PREPARATION (500 ml)

1. Measure 450 mL of H_2Ousing a graduated cylinder.
2. Transfer the $H_2O$to a clean beaker.
3. Add the complete contents of the bottle of Ammonium Oxalate Solution (powder format).
4. Mix gently until completely dissolved and the solution is homogeneous.
5. Adjust the final volume to 500 mL with $H_2O$and mix thoroughly.
6. Filter through a 0.45 µm membrane into a suitable bottle and record the preparation date.

Note: Once prepared, the 1X Ammonium Oxalate working solution remains stable for up to 1 year when stored at room temperature (15–25 °C) in a tightly sealed container.

PREPARATION OF 1X COMPLETE TYRODE`S BUFFER WORKING SOLUTION

1. Measure 950 mL of distilled or deionized water utilizing agraduated cylinder.
2. Transfer the distilled water from the graduated cylinder into a clean, appropriately sized beaker.
3. Quantitatively add the 50 mL contents of one bottle of 20X concentrated Tyrode's solution to the beaker.
4. Incorporate the Sodium Bicarbonate from one dedicated vial into the mixture, and agitate or stir continuously until the powder is fully dissolved.
5. Filter the fully homogenized solution through a sterile 0.45 µm membrane filter directly into a clean 1 L storage container, and clearly record the preparation date on the bottle label.

Note: Preparation of 1X Complete Tyrode's Washing Buffer. And following the addition of sodium bicarbonate, the complete 1X Tyrode's buffer matrix is stable for up to 90 days when stored at 2–8 °C. Discard the solution if visual turbidity or microbial contamination develops.

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 4° 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 apears in the botton of the tube after decant in 5 ml of 1X 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 mlof 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 adjust the platelet concentration to ≤100,000 platelets/µL.
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 2-8° 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 (RI) to select the platelets population. Set the RI around the CD42a PE positive population (trhombocytes). Acquire at least 10.000 events in region 1 (RI).

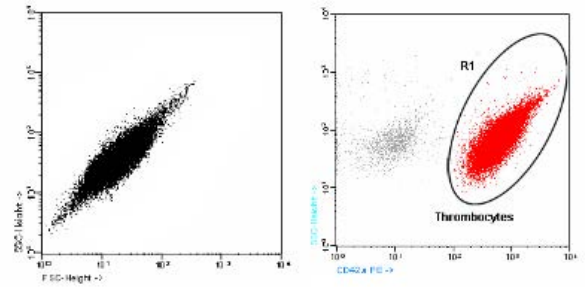

Figure 1. Control Thrombocytes (red events) are cells within CD42a gate (RI). Cells were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometer.

A

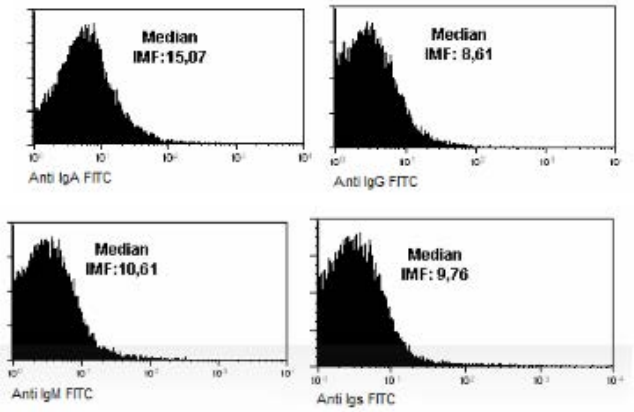

Figure 2. Immune Thrombocytopenia. The histograms represent comparison of a healthy control sample (A) and immune thrombocytopenia sample (B). Cells were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometer.

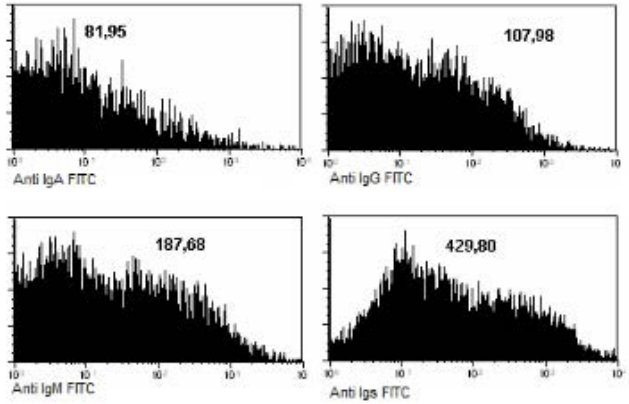

Figure 3. Immune Thrombocytopenia. The histograms represent an immune thrombocytopenia sample. Cells were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometer.

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 X106/µL
- Platelets: 150 - 450 X103/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.

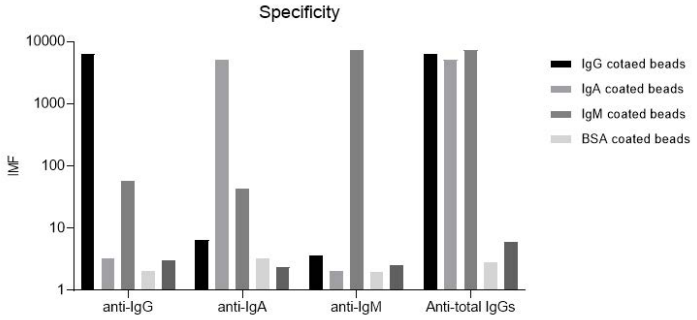

Figure 4: human immunoglobulins IgA, IgG and IgM and BSA (control) coated polystyrene beads were analyzed by FACSAria II (Becton Dickinson, San Jose, CA) flow cytometer.

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

1. Clinical and Laboratory Standards Institute (CLSI). Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture; Approved Standard - Fifth Edition (2003). CLSI Document H3-A5. Wayne, PA.
2. Kotylo PK, et al. Reference ranges for lymphocyte subsets in pediatric patients. Am J Clin Pathol. 100:111-5 (1993).
3. Reichert T, et al. Lymphocyte subset reference ranges in adult Caucasians. Clin Immunol Immunopathol. 60:190-208 (1991).
4. Schlossman SF, Boumsell L, Gilks W, et al., eds. Leukocyte Typing V: White Cell Differentiation Antigens. New York: Oxford University Press; 1995.

EXPLANATION OF SYMBOLS

	Form
REF	Catalog reference
Σ	Contains sufficient for > test
	Quantity per test
	Regulatory Status
RUO	Research Use Only
	Manufacturer

MANUFACTURED BY:

Address: Avda. Universidad de Coimbra, s/n
Cancer Research Center (C.I.C)
Campus de Unamuno
37007 Salamanca (Spain)
Telf./fax: (+34) 923 294 827
E-mail: info@immunostep.com
www.immunostep.com