

THROMBOCYTEST™ immune

Reagent kit for the flow cytometric quantitation of platelet associated immunoglobulin










Antibodies and reagents for 25 tests

For Research Use Only.
Not for Use in Diagnostic and Therapeutic Procedures.



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Key to symbols used

	Manufactured by		Temperature limitation
	Catalogue number		Consult instructions for Use
	Batch code		Use by
	Contains		Reagent*
	Contains sufficient for <n> tests		

* See chapter MATERIALS AND REAGENTS for a full explanation of symbols used in reagent component naming.

SUMMARY and EXPLANATION

THROMBOCYTEST™ immune allows the flow cytometric quantitation of platelet associated immunoglobulin. The test kit contains various polyclonal antibodies labelled with the fluorescence dye phycoerythrin (PE) as follows: antibodies against total human immunoglobulins, against human IgA, IgM und IgG and polyclonal antibodies against rabbit immunoglobulin as a negative control. The test kit also contains a fluorescein isothiocyanate (FITC) conjugated monoclonal antibody which recognizes a platelet specific antigen and further reagents. The fluorescence 2 intensity values correspond to the amount of platelet associated immunoglobulin.

The quantitation of platelet associated immunoglobulin should be performed by flow cytometry. The detailed instructions result from specific experience and precise validation assays. Critical steps and hints are in bold letters.

APPLICATIONS

THROMBOCYTEST™ immune is useful for the evaluation of thrombocytopenia (low platelet count). The main effect of this reduced platelet count is an increased risk of bleeding. There is a high risk of spontaneous bleeding once the platelet count drops below 5,000 to 20,000 platelets per μl . The quantitation of platelet associated immunoglobulin allows the differential diagnosis to distinguish the cause of thrombocytopenia. Thrombocytopenia may result from a failure of platelet production (myelosuppression caused by cancer, drugs such as chemotherapy agents, alcohol or viral infections) or an increased rate of platelet destruction. Increased destruction of platelets can occur due to two different general mechanisms (immunologic and nonimmunologic thrombocytopenia).

Idiopathic thrombocytopenic purpura (Morbus Werlhof, ITP) is the most common acquired thrombocytopenia. ITP is an autoimmune disorder characterized by antibodies directed against platelets. These antibodies bind to various structural platelet antigens and can cause destruction and removal of platelets.

The determination of autoantibodies against thrombocytes helps differentiate immune from nonimmune thrombocytopenia. The direct flow cytometric test allows the quantitation of platelet associated total immunoglobulin as well as the determination of the class (IgA, IgM or IgG) of immunoglobulin.

THROMBOCYTEST™ immune may also be useful for the evaluation of functional defects of thrombocytes. In some cases, the binding of antibodies to platelets may induce platelet dysfunction.

TEST PRINCIPLES

THROMBOCYTEST™ immune allows the flow cytometric quantitation of platelet associated immunoglobulin. The test kit contains various polyclonal antibodies labelled with the fluorescence dye phycoerythrin (PE) as follows: antibodies against total human immunoglobulins, against human IgA, IgM und IgG and polyclonal antibodies against rabbit immunoglobulin as a negative control. The test kit also contains a fluorescein isothiocyanate (FITC) conjugated monoclonal antibody which recognizes a platelet specific antigen and further reagents.

Platelet rich plasma is isolated from EDTA whole blood by several washing steps using BUF/WASH (Wash Buffer) and incubated over night at 2-8°C. Addition of Reagent B (goat serum) prevents unspecific binding. The thrombocytes are stained with different PE conjugated polyclonal antibodies against human immunoglobulins, human IgA, IgM and IgG and with a FITC conjugated monoclonal anti-CD42a antibody. Polyclonal antibodies against total rabbit immunoglobulins serve as negative control. The samples are incubated for 20 min on ice, washed and analysed by flow cytometry.

MATERIAL AND REAGENTS

The kit contains:

BUF	WASH
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2 vials (50 ml) containing 10 x WASH BUFFER

REAG	B
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1 vial (1.25 ml) containing Goat Serum

REAG	C
------	---

1 vial (0.5 ml) polyclonal antibody to rabbit immunoglobulins ("Polyclonal Anti-Human Rabbit, RPE Conjugate"), negative control

REAG	D
------	---

1 vial (0.5 ml) polyclonal antibody to total human immunoglobulins ("Polyclonal Anti-Human Immunoglobulins, RPE Conjugate")

REAG	E
------	---

1 vial (0.5 ml) polyclonal antibody to human IgA ("Polyclonal Anti-Human IgA, RPE Conjugate")

REAG	F
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1 vial (0.5 ml) polyclonal antibody to human IgG ("Polyclonal Anti-Human IgG, RPE Conjugate")

REAG	G
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1 vial (0.5 ml) polyclonal antibody to human IgM ("Polyclonal Anti-Human IgM, RPE Conjugate")

REAG	H
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1 vial (2.5 ml) anti-CD42a-FITC ("CD42a FITC Conjugate")

The test kit does not contain the following materials required for the assay:

1. Blood collection tubes containing **EDTA** anticoagulant.
2. 12 x 75 mm disposable test tubes (Falcon, BD Biosciences #352052) and appropriate test tube racks.
3. Ice bath with cover.
4. Double distilled water or water for injection for dilution of 10 x WASH BUFFER (BUF/WASH)

Required apparatus:

1. Variable volume micropipettes 10 - 200 µl, 100 – 1000 µl and disposable tips
2. Dispenser pipette and dispenser tips.
3. Vortex-mixer.
4. Refrigerated centrifuge with swinging buckets and 12 x 75 mm tube carriers.
5. Flow cytometer with 488 nm excitation wavelength (argon-ion laser).

WARNINGS

1. Blood samples must always be regarded as potentially infectious (hepatitis, HIV, etc.)! Wear disposable gloves and protective clothing while handling blood samples.
2. The reagents C, D, E, F, G and H contain sodium azide as preservative. Under acidic conditions, sodium azide yields hydrazoic acid, an extremely toxic and volatile compound. Solutions with azide should be diluted with tap-water before disposal to avoid deposits in plumbing, where explosive conditions may develop.

A **Material Safety Data Sheet (MSDS)** for THROMBOCYTEST™ immune is available on request.

STORAGE and STABILITY

Store the kit in the dark at 2-8°C (in refrigerator). The reagents are supplied sterile with a preservative that does not influence the performance of the assay. The reagents are stable for the period shown on the packaging label, when stored as described.

ASSAY PROCEDURE

1. Preparations:

- 1.1 Dilution of 10 x WASH BUFFER:
Dilute 10 x WASH BUFFER (BUF/WASH) 1:10 with Aqua bidest (e.g., 10 ml in 100 ml).
- 1.2 Prepare ice bath.
- 1.3 Switch on and calibrate the flow cytometer.

2. Isolation of thrombocytes

Collect 5 ml of **EDTA blood** from each patient or control person to be examined. Obtain blood with standard aseptic techniques. **DO NOT USE blood anticoagulated by heparin or citric acid!**

- 2.1 Centrifuge approx. **2 ml** of EDTA blood from each patient or control person at **100 x g** for **10 min** (without brake!) to obtain **platelet rich plasma (PRP)**. Remove the platelet rich plasma without any contamination by erythrocytes.
- 2.2 Centrifuge the platelet rich plasma at **700 x g** for **7 min** (with brake!). Aspirate and discard the supernatant. Resuspend the cell pellet with **3 ml** of **1 x BUF/WASH**.
- 2.3 Wash the platelets 3 times by centrifuging at 700 x g for 7 min, aspirating the supernatant and resuspending the cells in **3 ml** of **1 x WASH/BUF**.

Resuspend the washed platelets in **1.5 ml** of **1 x WASHING BUFFER** after the last centrifugation step and store the platelets **over night at 2-8°C**

3. THROMBOCYTEST™ immune set up:

The platelet rich plasma is washed once more by centrifugation after the over night incubation to reduce the amount of unspecific bound antibodies.

- 3.1 Centrifuge the platelet rich plasma at **700 x g** for **7 min**. Aspirate and discard the supernatant. Resuspend the cell pellet with **3 ml** of **1 x WASH/BUF**. Wash the platelets once more by centrifuging at **700 x g** for **7 min**. Aspirate and discard the supernatant. Resuspend the washed platelets in **1 ml** of **1 x WASH/BUF**.

The **labelling** of the **thrombocytes** with **polyclonal antibodies** must be done **on ice**. Prepare ice bath.

Count the platelets with the help of a counting chamber or with a hematology analyzer and resuspend the platelet suspension at approx. 20,000/ μ l.

Prepare and label 5 test tubes for each patient or healthy control person.

3.2 Pipette **100 μ l** of the **platelet suspension** into each tube. Add **10 μ l** of the **GOAT SERUM** to each tube. Vortex the tubes and incubate for at least **1 min** at **RT**.

3.3 Add **20 μ l** of the **polyclonal antibodies** to each tube („Polyclonal Anti-Rabbit Immunoglobulins“, „Polyclonal Anti-Human Immunoglobulins“, „Polyclonal Anti-Human IgA“, „Polyclonal Anti-Human IgG“, „Polyclonal Anti-Human IgM“). Vortex and incubate the tubes for **20 min** in an **ice bath**, covered to prevent exposure to light.

3.4 Add **2 ml** of **1 x WASHING BUFFER** to each tube. Mix the samples. Spin down cells (**7 min, 700 x g**). Discard the supernatant.

3.5 Pipette **20 μ l** of **CD42a FITC** („CD42a FITC Conjugate“) into each tube. Vortex and incubate the tubes for **20 min** in an **ice bath**, covered to prevent exposure to light.

3.6 Add **2 ml** of **1 x WAS/BUF** to each tube. Mix the samples. Spin down cells (**7 min, 700 x g**). Discard the supernatant.

Resuspend the cell pellet in 500 μ l 1 x WASH/BUF. Measure the cell suspension within 2 to 3 hours. If samples are not to be analyzed immediately after preparation, store them in the dark on ice.

4. Flow cytometric analysis:

Cells are analysed by flow cytometry using the blue-green excitation light (488 nm argon-ion laser, e.g., FACSCalibur™, CellQuest™ Software).

Measurement:

Set **FSC** and **SSC amplifier gains** to **log** mode and set **threshold** on **FSC** for data collection. The sample flow rate should be low, since the samples may contain a high number of erythrocytes.

Set a **region 1** around the population of **CD42a positive thrombocytes** (dot plot diagram **SSC/FL1**, see **Fig. 1A, 1B**). Acquire at least 5,000 thrombocytes in region 1.

Data analysis:

Activate a gate on region 1 (population of thrombocytes). Display **fluorescence 2 (FL2) histograms** of the gated data. Set a marker M1 to include the total peak

and analyse the respective **Median values** to quantify platelet associated.

FIGURES

Abb. 1: Recommended dot plot display **FSC/FL2** during data acquisition and analysis „Region“ R1 set on **CD42a positive thrombocytes**

Fig. 1A **FSC/SSC** dot plot display, blood sample from a healthy control person

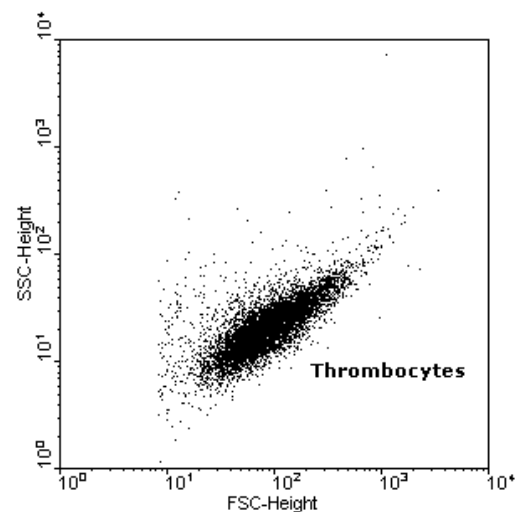


Fig. 1B **SSC/FL1** dot plot display, blood sample from a healthy control person

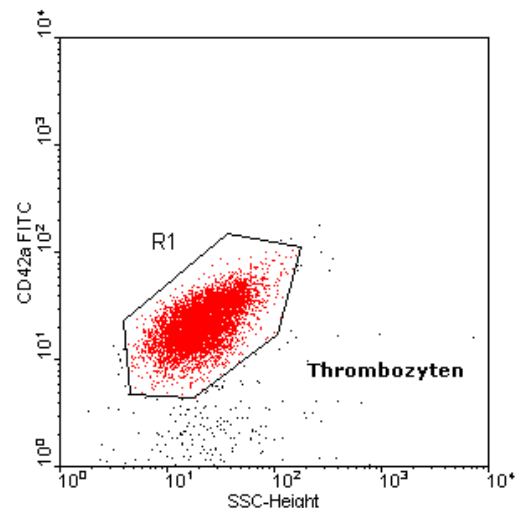


Fig. 2: **FL2 histograms** during data acquisition and analysis „Region“ R1 set on **CD42a positive thrombocytes**, healthy control person

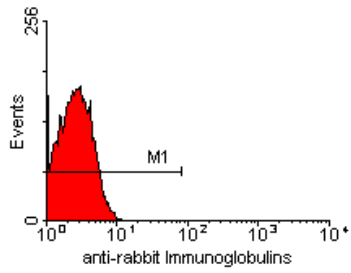


Fig. 2A anti-rabbit immunoglobulin

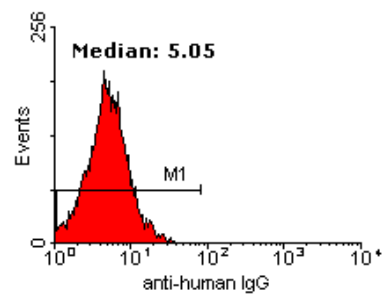


Fig. 2D anti-human IgG

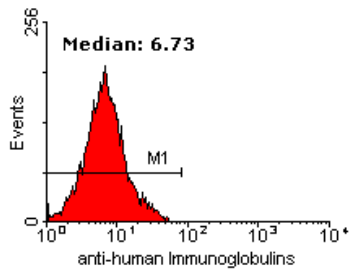


Fig. 2B anti-human immunoglobulin

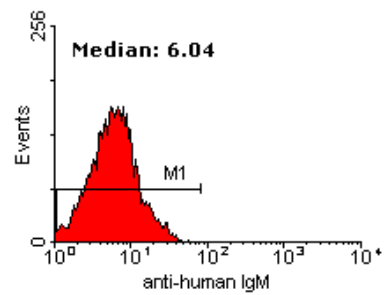


Fig. 2E anti-human IgM

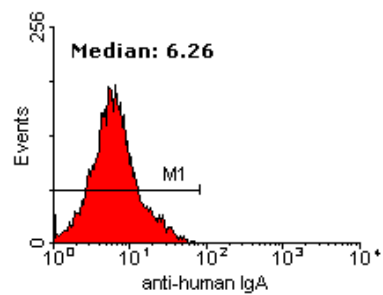


Fig. 2C anti-human IgA

Fig. 3: FL2 histograms during data acquisition and analysis „Region“ R1 set on CD42a positive thrombocytes, patient positive for IgG

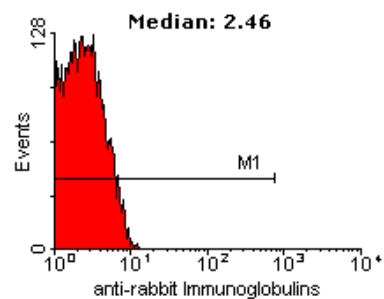


Fig. 3A anti-rabbit immunoglobulin

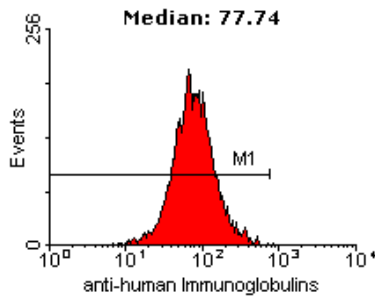


Fig. 3B anti-hHuman Immunglobulin

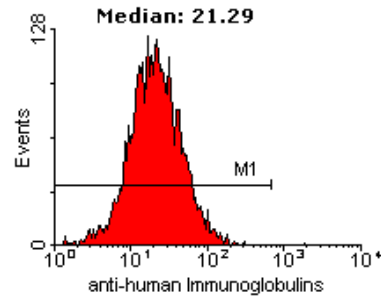


Fig. 4B anti-human Immunglobulin

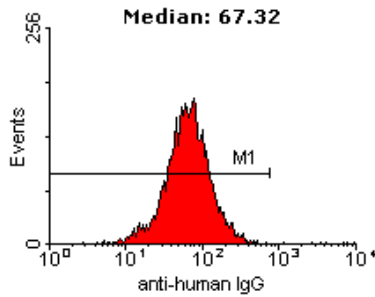


Fig. 3C anti-hHuman IgG

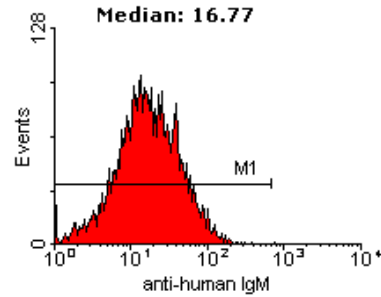


Fig. 4C anti-human IgM

Fig. 4: FL2 histograms during data acquisition and analysis „Region“ R1 set on CD42a positive thrombocytes, patient positive for IgM

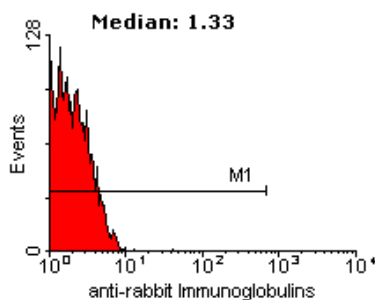


Fig. 4A anti-rabbit immunglobulin

REMARKS

1. **EDTA whole blood** should be processed **within 24 h after venipuncture**. **Blood samples** should remain at **room temperature** prior to processing.
2. Duplicate or triplicate determinations are useful in establishing the assay.
3. Thrombocytes have Fc receptors (CD32) which can cause background fluorescence. Incubation of the platelet suspension over night at 2-8°C reduces the amount of unspecific bound antibodies.

EXPECTED VALUES

The following **normal range (central 95%)** of the **Median values of the labelled thrombocytes** was determined using fresh EDTA blood samples from **normal individuals**. Results are shown in Table 1.

Antiserum	Red fluorescence (95% range)	Red fluorescence (Median)	N
Anti-rabbit Ig`s	< 4.5	1.8	59
Anti-human Ig`s	< 16.6	11.1	59
Anti-human IgA	< 6.9	4.4	49
Anti-human IgM	< 14.3	6.7	59
Anti-human IgG	< 14.4	6.7	59

LIMITATIONS of the METHOD

1. Every laboratory should establish its own range of normal values (% reticulated platelets) using its own test conditions.
2. The samples should contain more than 95% viable cells and should be completely anticoagulated.
3. Samples ready for measurement are stable for 2 to 3 hours on ice.
4. This test is a suitable screening method. If results are positive, a further differentiation can be done with the help of other tests, e.g., MAIPA („Monoclonal Antibody Immobilization of Platelet Antigens“) test according to Kiefel.
5. Patients with very low numbers of platelets have so called microparticles („platelet dust“) caused by destruction of platelets (lysis by binding of autoantibodies and complement). These microparticles can be detected by staining of thrombocytes with anti-CD42a FITC.
6. This test does not allow the specific determination of heparin-induced thrombocytopenia (HIT type II).
7. The test has been developed for the analysis of thrombocytes in peripheral EDTA blood, but not from bone marrow.
8. Pseudothrombocytopenia induced by EDTA agglutination of platelets has to be excluded before performing the THROMBOCYTE Immune assay by examining a blood smear. It may occur only in EDTA blood due to EDTA-dependent platelet ag-

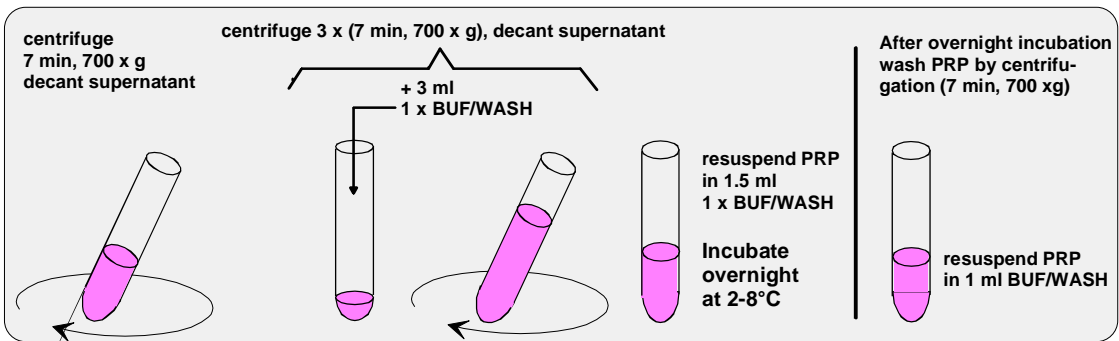
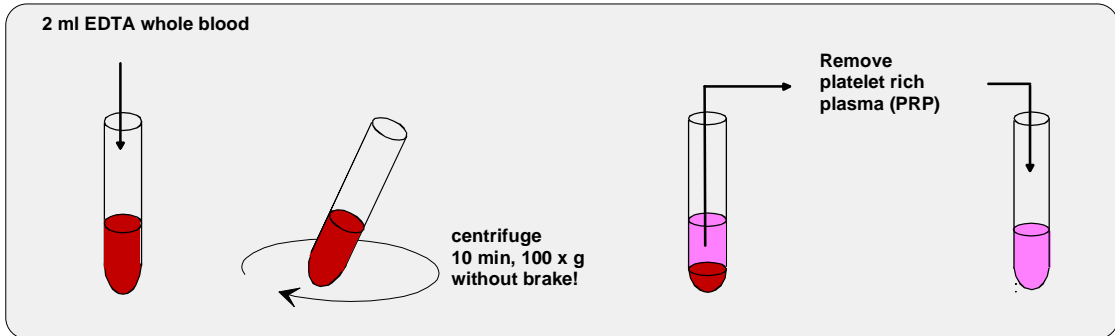
glutinins. Platelet satellitism also induced by EDTA is a phenomenon where platelets bind to neutrophils.

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THROMBOCYTEST™ immune - Sample Preparation Procedure

1. Isolation of platelet rich plasma (PRP)



2. Cell Labelling

