Reagent kit for the flow cytometric quantitation of platelet associated immunoglobulin

Antibodies and reagents for 25 tests

Glycotope Biotechnology GmbH
Czernyring 22
69115 Heidelberg
Germany
Tel. +49 (0)62 21 91 05-0
Fax +49 (0)62 21 91 05-10
E-Mail: info@glycotope-bt.com
www.glycotope-bt.com

Key to symbols used

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>IVD</td>
<td>In Vitro Diagnostic Medical Device</td>
</tr>
<tr>
<td>CE</td>
<td>European Conformity</td>
</tr>
<tr>
<td></td>
<td>Contains sufficient for &lt;n&gt; tests</td>
</tr>
<tr>
<td></td>
<td>Temperature limitation</td>
</tr>
<tr>
<td></td>
<td>Consult instructions for Use</td>
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<td></td>
<td>Use by</td>
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<tr>
<td>REAG</td>
<td>Reagent*</td>
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<tr>
<td>CONT</td>
<td>Batch code</td>
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<td>Contains</td>
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* See chapter MATERIALS AND REAGENTS for a full explanation of symbols used in reagent component naming.
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 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.

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.

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The kit contains:

- 2 bottles (50 ml) containing 10 x WASH BUFFER
- 1 vial (1.25 ml) containing Goat Serum
- 1 vial (0.5 ml) polyclonal antibody to rabbit immunoglobulins ("Polyclonal Anti-Human Rabbit, RPE Conjugate"), negative control
- 1 vial (0.5 ml) polyclonal antibody to total human immunoglobulins ("Polyclonal Anti-Human Immunoglobulins, RPE Conjugate")
1 vial (0.5 ml) polyclonal antibody to human IgA ("Polyclonal Anti-Human IgA, RPE Conjugate")

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

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

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

The test kit does not contain the following materials required for the assay:
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.
4. Refrigerated centrifuge with swinging buckets and 12 x 75 mm tube carriers.
5. Flow cytometer with 488 nm excitation wavelength (argon-ion laser).

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.

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 overnight at 2-8°C

3. THROMBOCYTEST™ immune set up:

The platelet rich plasma is washed once more by centrifugation after the overnight 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.

A Material Safety Data Sheet (MSDS) for THROMBOCYTEST™ immune is available on request.
Count the platelets with the help of a counting chamber or with a hematology analyzer and resuspend the platelet suspension at approx. 20,000/µl.

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

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

3.3 Add 20 µl 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 µl 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 µl 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. 2B anti-human immunoglobulin

Fig. 2C anti-human IgA

Fig. 2D anti-human IgG

Fig. 2E anti-human IgM

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
**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 RESULTS

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.

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Red fluorescence (95% range)</th>
<th>Red fluorescence (Median)</th>
<th>N</th>
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<tbody>
<tr>
<td>Anti-rabbit Ig’s</td>
<td>&lt; 4.5</td>
<td>1.8</td>
<td>59</td>
</tr>
<tr>
<td>Anti-human Ig’s</td>
<td>&lt; 16.6</td>
<td>11.1</td>
<td>59</td>
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<tr>
<td>Anti-human IgA</td>
<td>&lt; 6.9</td>
<td>4.4</td>
<td>49</td>
</tr>
<tr>
<td>Anti-human IgM</td>
<td>&lt; 14.3</td>
<td>6.7</td>
<td>59</td>
</tr>
<tr>
<td>Anti-human IgG</td>
<td>&lt; 14.4</td>
<td>6.7</td>
<td>59</td>
</tr>
</tbody>
</table>

LIMITATIONS

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

REFERENCES

THROMBOCYTEST™ immune - Sample Preparation Procedure

1. Isolation of platelet rich plasma (PRP)

1. Isolation of platelet rich plasma (PRP)
2 ml EDTA whole blood

- Remove platelet rich plasma (PRP)
- Centrifuge 10 min, 100 x g without brake!

2. Centrifuge 7 min, 700 x g, decant supernatant

- + 3 ml 1 x BUF/WASH
- Resuspend PRP in 1.5 ml 1 x BUF/WASH

After overnight incubation wash PRP by centrifugation (7 min, 700 xg)

2. Cell Labelling

Cell Labelling:

- Mix samples
- Incubate for 20 min on ice

2. Cell Labelling:

- 5 x 100 µl à 2 x 10^6 PLT
- PRP suspension

- + 10 µl Reagent B per tube

- + 20 µl of different polyclonal antibodies

Cell Counting:

- Adjust concentration of thrombocytes to 20,000/µl

- Mix samples
- Incubate for 20 min on ice

2. Centrifuge samples 7 min, 700 x g, decant supernatant

- + 2 ml 1 x BUF/WASH

- Mix samples
- Incubate for 20 min on ice

2. Cell Labelling:

- + 2 ml 1 x BUF/WASH

- Mix samples
- Incubate for 20 min on ice

2. Centrifuge samples 7 min, 700 x g, decant supernatant

- + 0.5 ml 1 x BUF/WASH