

THROMBOCYTEST[®] plus

Test kit for the flow cytometric determination of reticulated platelets and for absolute counting of thrombocytes

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

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Reagents für 50 tests, 10 counting tubes (THROMBOCYTEST[®] plus)
Please read the instructions carefully before use!

SUMMARY and EXPLANATION

THROMBOCYTEST[®] plus allows the flow cytometric determination of reticulated platelets and the absolute count determination of thrombocytes during thrombocytopenia (number of thrombocytes < 20.000/μl). The test kit contains a RNA specific dye which stains the RNA from reticulated platelets, a monoclonal anti-thrombocyte antibody conjugated with the fluorescence dye phycoerythrin (PE) and fluorescent beads as counting standard.

The detailed instructions result from specific experience and precise validation assays. Critical steps and hints are in bold letters.

APPLICATIONS

Reticulated platelets are young platelets that contain increased levels of mRNA and rRNA compared to mature cells. When thrombocytes are released from the bone marrow into the peripheral circulation they contain residual RNA which is subsequently degraded as the cells circulate. These young reticulated platelets appear normally in the peripheral blood at low levels, up to 4.5% of total thrombocytes.

An increased proportion of reticulated platelets indicates increased thrombopoiesis. The ability to detect increased platelet production has proven to be useful clinically in patients with thrombocytopenia. If these patients have elevated levels of reticulated platelets it implies that they have a disease resulting in peripheral destruction of platelets. In contrast, if their levels of reticulated platelets are depressed, it implies that they have a disease which impairs the ability of the bone marrow to make new platelets.

The determination of reticulated platelets is useful for the evaluation of thrombopoietic disorders like thrombocytopenia, for monitoring course and treatment of Idiopathic Thrombocytopenic Purpura (ITP) (see Fig. 4), of bone marrow transplantation success und during chemotherapy. Platelet-associated immunoglobulin (PA-Ig) which has been correlated with ITP can be detected by the separate kit THROMBOCYTEST[®] immune.

Also, the effect of drugs on the rate of platelet production can be studied.

The Idiopathic Thrombocytopenic Purpura (ITP) often results in microparticles and fragmentocytes of erythrocytes. This makes it more difficult to count thrombocytes accurately by using haematology analysers. At low thrombocyte numbers immunological counting of cells is useful.

TEST PRINCIPLES

THROMBOCYTEST plus allows the flow cytometric determination of reticulated platelets as well as the determination of absolute counts of thrombocytes. The test kit contains a specific RNA dye which stains the RNA from reticulated platelets, a monoclonal antibody against a thrombocyte specific antigen conjugated with the fluorescence dye phycoerythrin (PE) and fluorescent beads as counting standard for absolute counting of thrombocytes.

The anti-thrombocyte antibody ("CD42b RPE Conjugate") is added into a test tube which contains counting beads („COUNTING TUBES“) or into a test tube without counting beads, if only the percentage of reticulated platelets has to be determined. Then, EDTA whole blood and a RNA specific dye („RNA/DNA STAINING SOLUTION“) is added. After an incubation period of 30 min at room temperature the cell suspension can be analyzed by flow cytometry.

MATERIAL and REAGENTS

The test kit contains:

1. 1 vial (1 ml) antibody reagent ("**CD42b RPE Conjugate**"), contains the monoclonal antibody anti-CD42b-PE.
2. 1 vial (0.5 ml) containing concentrated RNA specific dye („**RNA/DNA STAINING SOLUTION**“).
3. 1 vial (50 ml) containing **DILUTION BUFFER**.
4. 10 disposable test tubes containing freeze-dried fluorescent counting beads („**COUNTING TUBES**“), only THROMBOCYTEST plus.

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.

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. 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). **COUNTING TUBES** can be stored at room temperature. The diluted RNA solution has to be discarded after use. 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 Dilute the RNA stock solution with DILUTION BUFFER:
- „RNA/DNA STAINING SOLUTION“, 1 : 100 (e.g., 10 µl in 1000 µl, 1000 µl pro test)
- 1.2 Prepare ice bath.
- 1.3 Switch on and calibrate the flow cytometer.

2. **THROMBOCYTEST plus set-up:**

For each patient sample to be analysed a blood sample from a healthy control person should also be assayed. This procedure facilitates the setting of a gate in the SSC versus FL1 dot plot, see below.

If both the percentage of reticulated platelets and the absolute count of thrombocytes have to be determined, use a COUNTING TUBE for the patient sample. Use a test tube for the control sample (blood sample from a healthy control person).

2.1 Addition of CD42b RPE:

Add 20 µl of CD42b RPE conjugate into the bottom of the COUNTING TUBE or test tube.

2.2 Addition of EDTA blood:

Mix EDTA blood well. Pipette 10 µl of well-mixed anticoagulated blood into the bottom of the COUNTING TUBE or test tube. Perform **reverse pipetting** to ensure a higher precision. For reverse pipetting, the button is depressed to the second stop. When the button is released, excess sample is drawn up into the tip. A precise volume of sample is expelled by pressing the button to the first stop, leaving excess sample in the tip. Vortex gently and **incubate** the tubes for **10 min in the dark at room temperature**.

2.3 RNA staining:

Add 1 ml of 1 x RNA/DNA STAINING SOLUTION per tube, mix and **incubate for 30 min in the dark at room temperature. Measure the cell suspensions within 60 min!**

3. **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 contain nonlysed whole blood with a high number of erythrocytes.

Set a **first region 1** around the population of **CD42b positive thrombocytes** (dot plot diagram SSC/FL2, see **Fig. 1A, 1B**). If you also want to determine the absolute number of thrombocytes, set a **second region 2** around the counting **beads** (dot plot diagram SSC/FL2, see **Fig 1A, 1B**). In this case, start acquisition, end data acquisition by count and acquire exactly **500 events** in **region 2**. If **absolute counting** of thrombocytes is **not performed**, acquire **100,000 events**.

After activating a gate on region 1, set a **third region** in the **dot plot diagram SSC versus FL1** to determine the percentage of reticulated platelets (see **Fig. 2 A**, sample from healthy control person and **Fig 2B**, patient sample).

Data analysis

(1) Calculating absolute counts of thrombocytes:

After setting a logical gate on CD42b positive thrombocytes, determine the number of thrombocytes in region 1 and the number of counting beads in region 2.

Example for calculating absolute counts of thrombocytes in the sample:

Results:

# of events in region 1 containing thrombocytes	# of events in region 2 containing counting beads
2438	500

The calculation of the absolute number of thrombocytes (PLT/ μ L) is done according to the following formula:

$$\text{absolute count of thrombocytes (cells}/\mu\text{L)} = \frac{\text{\# of events per tube} * \text{\# of events in R2} * \text{\# of events in R1}}{\text{test volume}}$$

Results for the example:

$$\text{PLT}/\mu\text{L} = \frac{85,380/500 * 2,438}{10} = 41,630$$

* This value „# of events per tube“ (for this example 85,380) is found on the lot label for the COUNTING TUBES.

(2) Determining the percentage of reticulated platelets:

After setting a logical gate on CD42b positive thrombocytes, the percentage of reticulated platelets is determined in region 3 (dot plot diagram SSC/FL1). Fig 2 shows examples for a blood sample from a healthy control person (Fig. 2A) and from a patient sample (Fig. 2B).

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. Pipettes should be calibrated according to the frequency recommended by the manufacturer to obtain optimal results.

EXPECTED VALUES:

The following **normal range (central 95%)** of the **percentage of reticulated platelets** was determined using fresh EDTA blood samples from **56 normal individuals**. Results are shown in Table 1.

Table 1: Percentage of reticulated thrombocytes – normal range

	Percentage of reticulated platelets
95% Range	0.41 – 3.25
Mean	1.83
N	56

PRECISION of the METHOD:

The intra-assay precision of this assay was determined on ten replicate tests from each sample. Results are shown in Tables 2 and 3.

Table 2: Intra-assay precision for the determination of the percentage of reticulated platelets

Patient Sample	Average % of Reticulated Thrombocytes	Standard Deviation	Average CV (%)
1	2.46	0.50	20
2	12.41	1.20	10

Table 3: Intra-assay precision for the determination of the absolute number of thrombocytes

Patient Sample	Average Number of Thrombocytes	Standard Deviation	Average CV (%)
1	127,050	6,170	5
2	7,900	520	7

ABSOLUTE COUNTING versus COMPARATIVE METHOD

The flow cytometric absolute counting of thrombocytes was compared with a haematology analyser (Cell-Dyn® 3200, Abbott Diagnostics). Fig. 3 shows the correlation between the two methods.

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 hours on ice after addition of the RNA/DNA STAINING SOLUTION.

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 antibody reagent ("CD42b RPE Conjugate") contains 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® plus is available on request.

REFERENCES:

- (1) Ault, K.A., Rinder, H.M., Mitchell, J., Carmody, M.B., Vary, C.P. & R.S. Hillman. 1992. The significance of platelets with increased RNA content (reticulated platelets). A measure of the rate of thrombopoiesis. *Am J Clin Pathol* 98: 637-646.
- (2) Ault, K.A. 1993. Flow cytometric measurement of platelet function and reticulated platelets. *Ann N.Y. Acad Sci* 677: 293-308.
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- (4) Richards, E. P. Baglin. 1995. Quantitation of reticulated platelets: Methodology and clinical application. *Br J Hem* 91: 445-51.
- (5) Himmelfarb, J., Holbrook, D., McMonagle E. & K.A. Ault. 1997. Increased reticulated platelets in dialysis patients. *Kidney Int.* 51: 834-839.
- (6) Ault, K.A. 1997. Reticulated Platelets: The birth of a new test for thrombopoiesis. *Clin Immunol Newsletter* 17(1): 1-7.
- (7) Ogata H. 1998. Measurement of reticulated platelets in thrombocytopenia. *Kurume Med* 45: 165-170.

FIGURES

Fig. 1: Recommended dot plot display FSC/FL2 during data acquisition and analysis

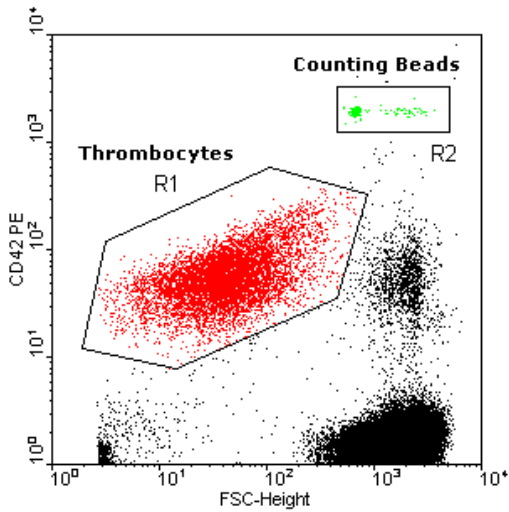
„Region“ R1 set on CD42b positive thrombocytes

„Region“ R2 set on counting beads for absolute counting of thrombocytes

A) Blood sample from a healthy control person

B) Blood sample from a patient with reduced number of thrombocytes

1 A



1 B

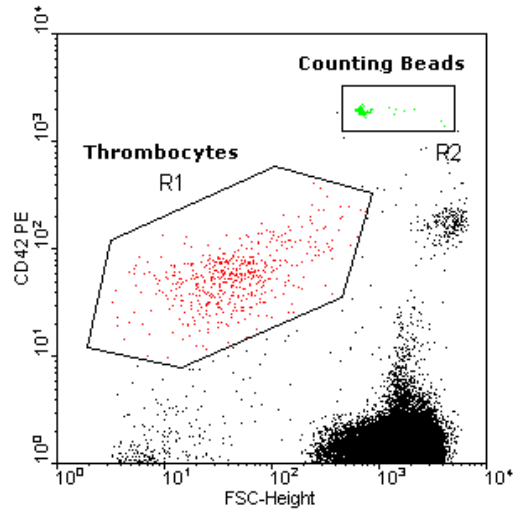


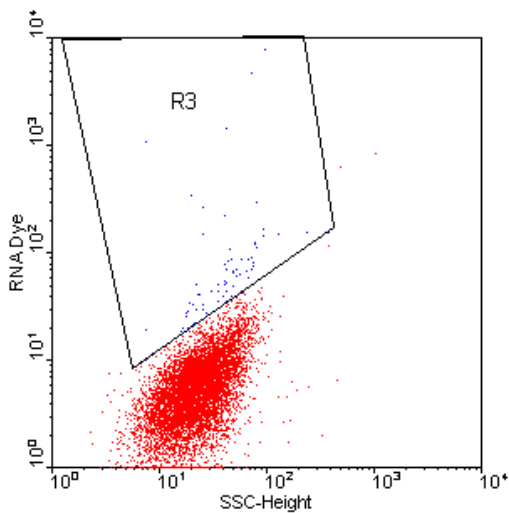
Fig. 2: SSC vs FL1 dot plot during data acquisition and analysis, gate set on region 1

Region R3 set on reticulated thrombocytes

A) Region 3 set on reticulated thrombocytes (1.1%), blood sample from a healthy volunteer

B) Region 3 set on reticulated thrombocytes (19.8), blood sample from a patient

2 A



2 B

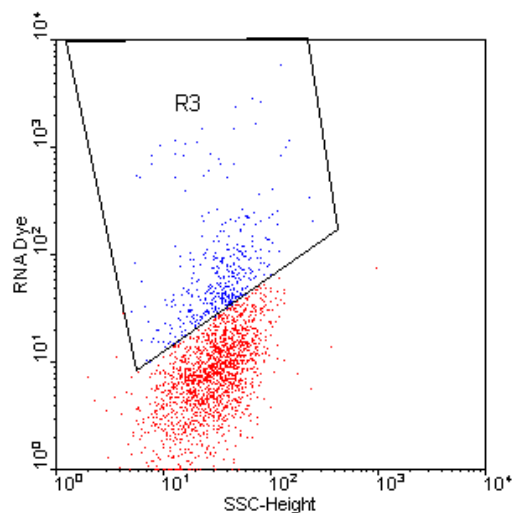


Fig. 3: Comparison of the absolute number of thrombocytes determined by a haematology analyser (Cell-Dyn® 3200) and with COUNTING TUBES (flow cytometric method)

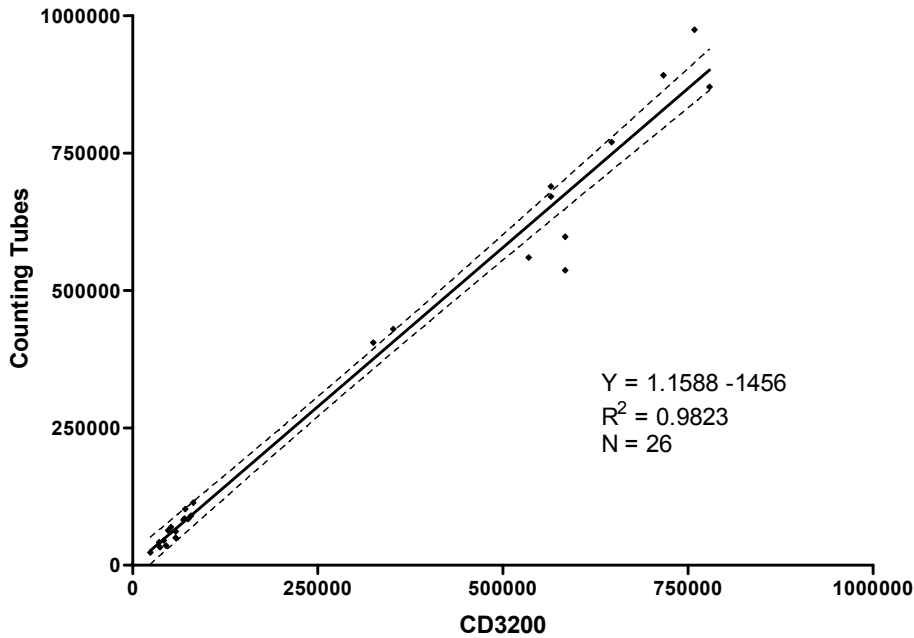
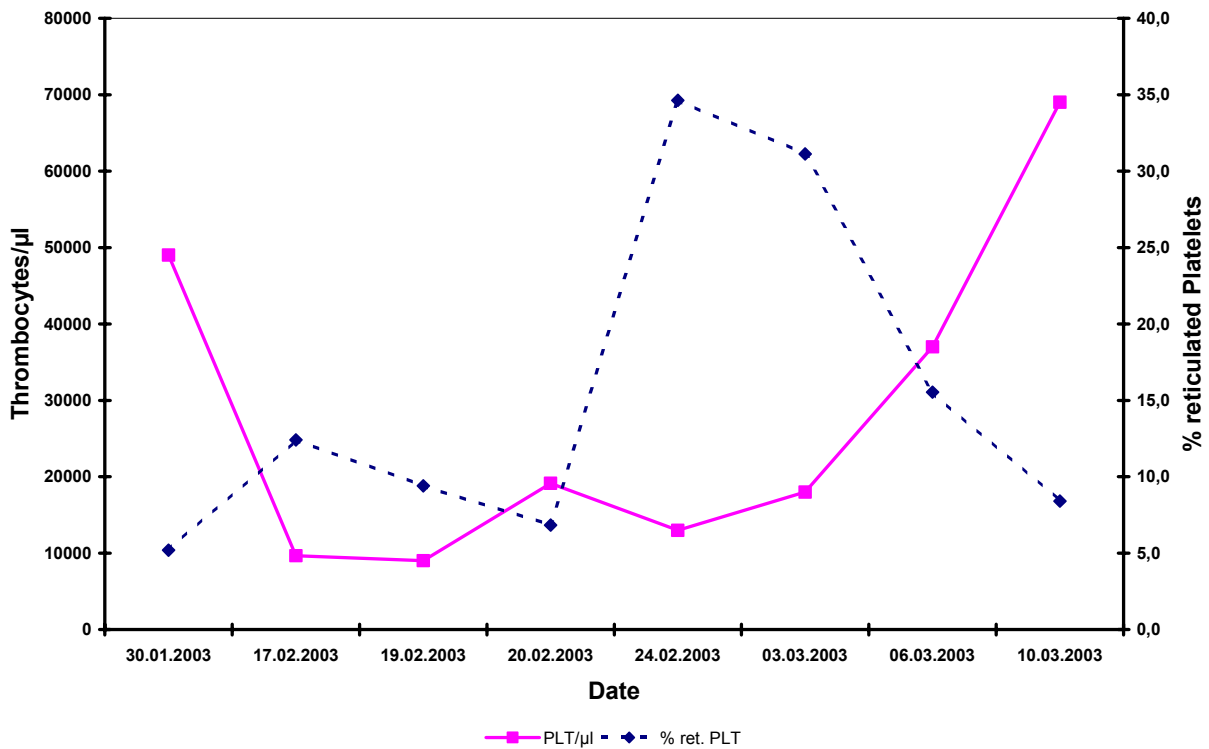




Fig. 4: Changes in the % reticulated platelets and the absolute number of thrombocytes, typical data from one patient


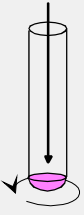
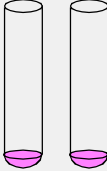


THROMBOCYTEST® Plus - Sample Preparation Procedure


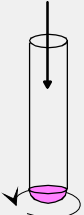
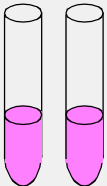
1. Addition of CD42b-PE

<p>Patient: + 20 µl CD42b-PE</p>  <p>"COUNTING TUBE"</p>	<p>Healthy control person: + 20 µl CD42b-PE</p>  <p>test tube</p>	<p>The lyophilisate of the counting beads is dissolved by addition of the antibody.</p>
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2. Addition of EDTA-Blut

<p>Patient: 10 µl EDTA whole blood</p> 	<p>Healthy control person: 10 µl EDTA whole blood</p> <p>Reverse pipetting: Depress the button to the second stop. When the button is released, excess sample is drawn up into the tip. A precise volume of sample is expelled by pressing the button to the first stop, leaving excess sample in the tip.</p> 	 <p>Incubate the samples for 10 min in the dark at RT.</p>
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3. RNA Staining

<p>Patient: + 1 ml RNA STAINING SOLUTION</p> 	<p>Healthy control person: + 1 ml RNA STAINING SOLUTION</p> 	 <p>Incubate the samples for 20 min in the dark at RT.</p>
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