

PHAGOTEST®

TEST KIT FOR THE QUANTIFICATION OF PHAGOCYTTIC ACTIVITY OF MONOCYTES AND GRANULOCYTES IN HEPARINIZED WHOLE BLOOD

For *in vitro* diagnostic use.

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Opsionized *E.coli*-FITC and reagents for 100 tests.
Please read the instructions carefully before use!

SUMMARY AND EXPLANATION

This test kit allows the quantitative determination of leukocyte phagocytosis in heparinized whole blood. It contains fluorescein (FITC)-labelled opsonized bacteria (*E.coli*-FITC) and necessary reagents. It measures the overall percentage of monocytes and granulocytes showing phagocytosis in general (ingestion of one or more bacteria per cell) and the individual cellular phagocytic activity (number of bacteria per cell).

The investigation of phagocytosis can be performed either by flow cytometry or by fluorescence microscopy. Because of the quantitative analysis very accurate work is important, especially when day to day comparisons are required. The detailed instructions result from specific experience and precise validation assays. Critical steps are in bold letters. A graphic summary of the test is attached.

APPLICATIONS

PHAGOTEST® is intended to investigate the phagocytic activity found in various disorders and to evaluate the effects of drugs.

Abnormal phagocytosis can occur with a variety of clinical disorders (3, 4). The defects can be associated with the neutrophil itself or with an immunoglobulin or complement defect. Known inborn defects are actin dysfunction, tuftsin deficiency and complement receptor C3bi deficiency. These deficiencies can result in increased susceptibility to infection due to defective neutrophil phagocytosis. Acquired defects associated with altered phagocytic activity can be observed in trauma, diabetes, renal failure, and infection. Reduced phagocytosis was found in patients with recurrent bacterial skin and sinopulmonary infections (5), in patients with wound infections from burns (6), in patients with AIDS (7), in neonates or in elderly people.

Various immunomodulators (cytokines such as interleukin-2 or interferon- γ , lactic acid bacteria and plant extracts such as from Echinaceae Purpureae herba) can increase the phagocytic activity of neutrophils and monocytes. These effects can be investigated *in vitro* or *ex vivo* (8, 9).

PHAGOTEST® also allows the study of the phagocytic activity of HL-60 promyelocytic leukemia cells or of isolated monocytes or macrophages.

The test kit is compatible with blood of mice, rats, rabbits, dogs, cattle and other species.

TEST PRINCIPLES

Phagocytosis by polymorphonuclear neutrophils and monocytes constitutes an essential arm of host defense against bacterial or fungal infections. The phagocytic process can be separated into several major stages: chemotaxis (migration of phagocytes to inflammatory sites), attachment of particles to the cell surface of phagocytes, ingestion (phagocytosis) and intracellular killing by oxygen-dependent and oxygen-independent mechanisms (1, 2).

PHAGOTEST® allows the quantitative determination of leukocyte phagocytosis (ingestion of bacteria). It measures the percentage of phagocytes which have ingested bacteria and their activity (number of bacteria per cell). The phagocytosis test kit contains fluorescein-labelled opsonized *Escherichia coli* bacteria and other necessary reagents. Heparinized whole blood is incubated with the FITC-labelled *E.coli* bacteria at 37°C, a negative control sample remains on ice. The phagocytosis is stopped by placing the samples on ice and adding QUENCHING SOLUTION. This solution allows the discrimination between attachment and internalization of bacteria by quenching the FITC fluorescence of surface bound bacteria leaving the fluorescence of internalized particles unaltered. After two washing steps with WASHING SOLUTION erythrocytes are then removed by addition of LYSING SOLUTION. The DNA STAINING SOLUTION, which is added just prior flow cytometric analysis, excludes aggregation artifacts of bacteria or cells.

The *E.coli* bacteria are opsonized with immunoglobulin and complement of pooled sera. Cells of the phagocytic system (monocytes, polymorphonuclear neutrophils) have receptors for a complement component (C3b) and for the constant part of the immunoglobulin molecule (Fc) mediating the adhesion of the bacteria to the cell surface. By utilizing both opsonized and nonopsonized bacteria, which are also available, both opsonic capacity and phagocytosis can be measured at the same time. Thus, it can be determined whether abnormal phagocytosis is due to a failure in the opsonization process or to a defect in the ingestion capability of the phagocyte.

In summary, phagocytosis and the subsequent digestion are a multistep and multifactorial process (1, 2). It is therefore investigated under controlled conditions by separate kits: PHAGOTEST® to measure ingestion of bacteria, BURSTTEST (PHAGOBURST®) to measure oxidative burst. The critical factors are: degree of opsonization, temperature, time of incubation and ratio of bacteria to leukocytes (see **Fig. 3, 4**).

MATERIAL and REAGENTS

The test kit contains:

1. 1 bottle (2 ml) of stabilized and **opsonized FITC-labelled *E.coli* suspension (E.COLI FITC OPSONIZED)**, 1 x solution, ready to use, 1×10^9 bacteria per ml.
2. 1 bottle (10 ml) of **QUENCHING SOLUTION** for suppressing fluorescence of the bacteria attached to the outside of the cell, blue reagent solution, 1 x solution.
3. 1 bottle (20 ml) of **DNA STAINING SOLUTION** for cytometric discrimination of bacteria during leukocyte analysis, pink reagent solution, 1 x solution.
4. 1 bottle (20 ml) of **LYSING SOLUTION** (10 x stock solution for storage), provides 200 ml of 1 x solution after 1 : 10 dilution with double distilled water for lysing erythrocytes and simultaneous fixing of leukocytes.
5. 1 bottle of Instamed-Salts as a **WASHING SOLUTION (SALTS f. WASHING SOLUTION)** to be reconstituted in 1000 ml aqua bidest, provides 1000 ml ready-to-use WASHING SOLUTION.

The test kit does not contain the following material:

1. Blood collection tubes containing **heparin anticoagulant**.
2. 12 x 75 mm disposable test tubes (Falcon, Becton Dickinson No. 352052) and appropriate test tube racks.
3. Flasks (500 ml and 1000 ml) for WASHING SOLUTION and 1 x LYSING SOLUTION.
4. Ice bath with cover.
5. Double distilled water or water for injection for reconstitution of WASHING SOLUTION and dilution of LYSING SOLUTION.

Required apparatus:

1. Variable volume micropipettes 20 - 200 µl and pipette tips.
2. Dispenser pipette and dispenser tips.
3. Bottle-top dispensers for WASHING SOLUTION and 1 x LYSING SOLUTION.
4. Waterbath.
5. Digital thermometer.
6. Vortex mixer.
7. Refrigerated centrifuge with swinging buckets and 12 x 75 mm tube carriers.
8. Flow cytometer with 488 nm excitation wavelength (argon-ion laser).
9. optional: fluorescence microscope with filter block for FITC-immunofluorescence and phase contrast.

STORAGE and STABILITY

Store the kit in the dark at 4° C (in refrigerator). **The bacteria have to be stored separately at below -20°C before use.** After thawing, the **bacteria** should be stored in **small aliquots at -20°C. Avoid repeated freezing and thawing.** Alternatively, **the bacteria** may be **stored at 4°C for up to 3 months.** Before use, the bacteria have to be mixed thoroughly (vortex mixer) or to be disaggregated by a syringe with a narrow needle. The reagents are supplied sterile with a preservative that does not influence phagocytosis.

ASSAY PROCEDURE**1. Preparations**

- 1.1 Dissolve the salts for WASHING SOLUTION in 1000 ml double distilled water. Dilute the 10 x LYSING SOLUTION 1 : 10 in double distilled water.
- 1.2 Prepare ice bath.
- 1.3 Cool bacteria and QUENCHING SOLUTION in the ice bath.
- 1.4 Prewarm water bath to 37°C (**precise temperature control!**).
- 1.5 Switch on and calibrate the flow cytometer or fluorescence microscope. The same amplifier adjustments as for immunofluorescence analysis with directly conjugated monoclonal antibodies are possible.

2. Phagocytosis set-up

- 2.1 Dispensing:
Heparinized whole blood is mixed (vortex mixer) and aliquoted on the bottom of a 5 ml tube, **100 µl per test**. As in immunofluorescence analyses, no blood should remain on the side wall of the tubes. **Do not use blood anticoagulated by EDTA or Citric acid!**
Before adding the bacteria, the blood samples should incubate in an ice bath for 10 min, in order to cool them down to 0°C.
- 2.2 Activation:
Mix the **precooled E.coli bacteria** well (vortex mixer) and add **20 µl per test** to the whole blood.
- 2.3 Incubation:
All tubes are mixed once more. The **control samples remain on ice**. The **test samples** are incubated for **10 min at 37.0°C** in a **water bath**.
Incubation time and temperatures must be monitored closely and the water bath must be closed and preheated.

- 2.4 **Quenching:**
Precisely at the end of the incubation time all samples together on one rack simultaneously are taken out of the water bath and placed on ice in order to stop phagocytosis. **100 µl** of icecold **QUENCHING SOLUTION** is added to each of the samples. Mix the samples (vortex mixer).
- 2.5 **Washing:**
Add **3 ml** of **WASHING SOLUTION** per tube. Mix the samples. Spin down cells (5 min, 250 x g, 4°C). Discard the supernatant.
- 2.6 The samples are washed with **3 ml** of **WASHING SOLUTION** once again (5 min, 250 x g, 4°C).
- 2.7 **Lysis and fixation:**
The whole blood is lysed and fixed with **2 ml** of prewarmed (room temperature) **1 x LYSING SOLUTION**. Mix and incubate the samples for **20 min at room temperature**. Spin down cells (5 min, 250 x g, 4°C). Discard the supernatant.
- 2.8 **Washing:**
The samples are washed once more with **3 ml** of **WASHING SOLUTION** (5 min, 250 x g, 4°C).
- 2.9 **DNA staining:**
Add **200 µl** of **DNA STAINING SOLUTION**, mix and incubate **10 min on ice** (light protected in the ice bath).
Measure the cell suspension 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., FACScan™, LYSIS™ II software or FACSCalibur™, CellQuest™ software).

Measurement:

During data acquisition a "**live**" gate is set in the **red fluorescence histogram** on those events which have at least the same DNA content as a human diploid cell (i.e. exclusion of bacteria aggregates having the same scatter light properties as leukocytes. See **Fig. 1A**). Alternatively, bacteria can be excluded by using **fluorescence triggering** in the **FL2** or **FL3 channel**.

Collect **10,000 - 15,000 leukocytes per sample**.

Data evaluation:

The percentage of cells having performed phagocytosis (granulocytes and monocytes) are analyzed as well as their mean fluorescence intensity (number of ingested bacteria). For that purpose the **relevant leukocyte cluster** is **gated** in the software program in the scatter diagram (lin FSC vs lin SSC) and its **green fluorescence histogram (FL1)** is **analyzed** (see **Fig. 2A, 2B**).

For that purpose, use the control sample to set a marker for fluorescence-1 (FL1) so that less than 1% of the events are positive. The percentage of phagocytosing cells in the test sample can then be determined by counting the number of events above this marker position. The mean fluorescence correlates with the number of bacteria per individual leukocyte.

REMARKS

1. **Heparinized blood** should be processed **within 24 h of sampling**. **Blood samples** should **remain at room temperature** prior to processing.
2. **Eosinophils** (enhanced in allergies and parasitic infections) show an **increased autofluorescence**, which can be shown by the control assay at 0°C.
3. Phagocytes incubated at 37°C differ in size and granularity from cells in the 0°C control sample. This has to be kept in mind when setting regions of interest (gates or bitmaps) in the scatter diagram. In addition, an increasing loss of cells can be observed because of adherence to the plastic surface at 37°C and autolysis.
4. Triplicates are useful in establishing the assay.

5. The proposed test protocol investigates the **phagocytosis process** under **optimal conditions** (opsonized bacteria, no isolation steps etc.). Therefore, on testing drugs in healthy persons only a limited increase in phagocytic activity *ex vivo* or *in vitro* can be expected. **Testing drug effects *in vitro***, it might be useful to **run kinetics for time dependence** (incubation with *E.coli*-FITC for 2.5 or 5 min) and **dilution of bacteria** (1 : 4 or 1 : 8). See **Fig. 3, 4**.
6. The bacteria are already opsonized, however an additional effect is achieved by the serum in the whole blood. This has to be kept in mind when working with other samples than whole blood.
The phagocytic activity of **HL-60 promyelocytic leukemia cells** or **isolated monocytes or macrophages** can be studied by incubating the cells with the FITC-labelled *E.coli* bacteria in culture medium containing **5 - 20 % fetal calf** or **human serum**. It might also be necessary to **extend the incubation time** (120 min - 240 min).
7. **Nonopsonized bacteria are also available**. They can be used as stimulants in order to **test the opsonizing capacity of the patient's serum**.

EXPECTED VALUES

The **normal range** of the phagocytic activity of granulocytes and monocytes was determined on **fresh heparinized whole blood samples** from healthy subjects.

Cell Type	% Phagocytizing Cells	Mean Fluorescence Intensity (4 decades, 1025 channels, log scale)
Monocytes	65 - 95	300 - 900
Granulocytes	95 - 99	800 - 1800

PRECISION OF THE METHOD

The intra-assay precision of this assay was determined on triplicate whole blood samples from healthy subjects.

	% Phagocytizing Granulocytes	Mean Value FL1	% Phagocytizing Monocytes	Mean Value FL1
Range of values	96.1 - 99.3	933 - 1816	62.5 - 91.8	435 - 806
Average CV (%)	0.8	5.1	5.0	6.6
n	6	6	6	6

LIMITATIONS OF THE METHOD

1. Every laboratory should establish its own range of normal values using its own test conditions.
2. The samples should contain more than 95% viable cells and should be completely anticoagulated. Older and incompletely anticoagulated blood samples can simulate - erroneously - phagocytosis positive cells in the control assay at 0°C. Reasons for this phenomenon are platelet aggregates and dead cells with leaking DNA, which include bacteria unspecifically and prevent quenching.
3. The ratio of bacteria (2×10^7 per 20 µl) to leukocytes (in 100 µl whole blood) is 25 : 1 assuming a white blood cell count of 8000/µl and 40 : 1 at 5000/µl. Samples with white blood cell counts differing from the normal range (4000 - 10000) require correction of the amount of bacteria added (see **Fig. 3, 4**).
4. Samples ready for measurement without DNA STAINING SOLUTION are stable for 24 hours on ice, but they systematically lose fluorescence intensity.

WARNINGS

1. Blood samples must always be regarded as potentially infectious (hepatitis, HIV etc.)! Wear suitable gloves and protective clothing. The bacteria are inactivated and stabilized.
2. The dye of the DNA STAINING SOLUTION might be carcinogenic!

3. The DNA-dye contaminates pipettes and the sample delivery system of the flow cytometer and might disturb future immunofluorescence analyses esp. in the case of phycoerythrin labelled antibodies. Diluted sodium hypochlorite (0.5 - 1.5 %) eliminates the DNA-dye contamination.
4. The LYSING SOLUTION contains formaldehyde, which is listed as a potential carcinogen.

A **Material Safety Data Sheet** for PHAGOTEST® is available on request.

IMPORTANT INSTRUCTIONS FOR QUANTITATIVE ANALYSIS

1. The **phagocytosis process** greatly **depends on temperature**. During the entire preparation of the samples **temperature** and **incubation time** must be **strictly observed**. The thermometer ought to give readings to the first decimal point.
2. Reproducible and standardized working is important. Therefore, please stick to the Operators Manual and your own modifications thereof.
3. Any changes at the flow cytometer must be taken into consideration, which influence the sensitivity of the fluorescence measurement and therefore the "mean" value. The use of a benchtop standard (fluorescent microbeads) is required for daily calibration.

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Further reference list on request.

PHAGOTEST® is a registered trademark of ORPEGEN Pharma, European Patent 0435226.

FIGURES

Fig. 1: Recommended histogram/dot plot displays during data acquisition

- A) Live gate on leukocyte DNA (FL2 histogram)
- B) Dot plot lin SSC / log FL1 of control sample (0°C)
- C) Dot plot lin SSC / log FL1 of test sample (37°C)

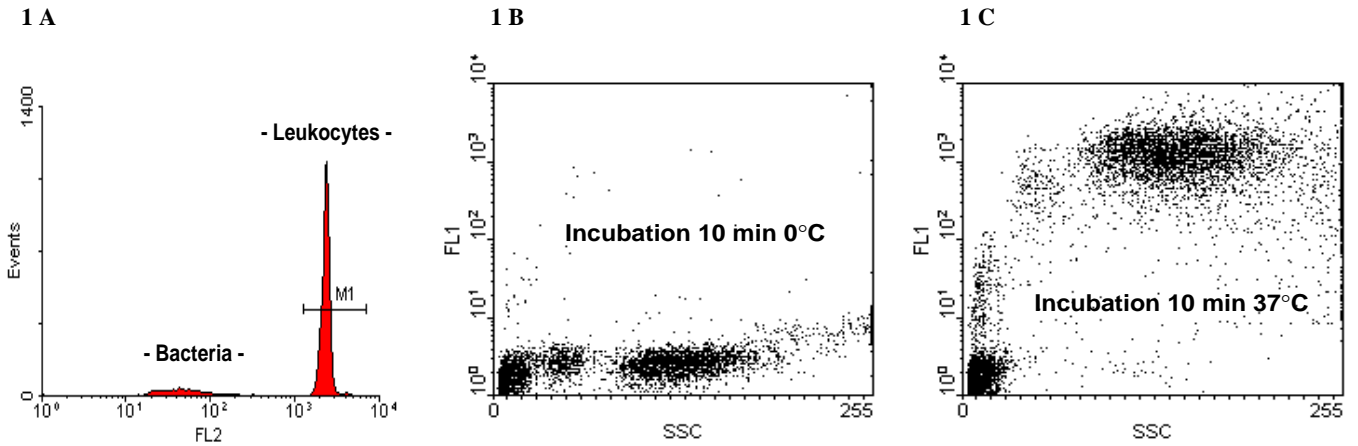
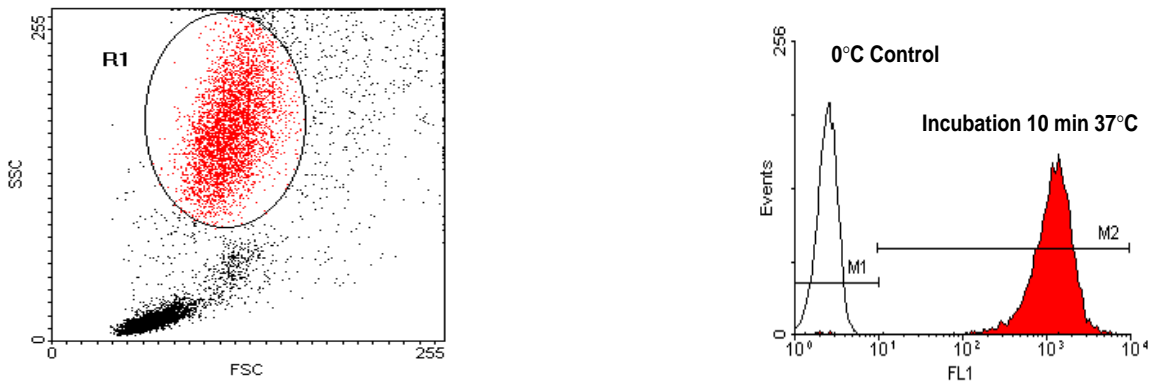


Fig. 2: Typical dot plots FSC/SSC and FL1 histograms of the phagocytosis test (incubation time of 10 min at 37°C). Histograms for the 0°C control samples are presented on the left.

A) Gate set on granulocytes



B) Gate set on monocytes

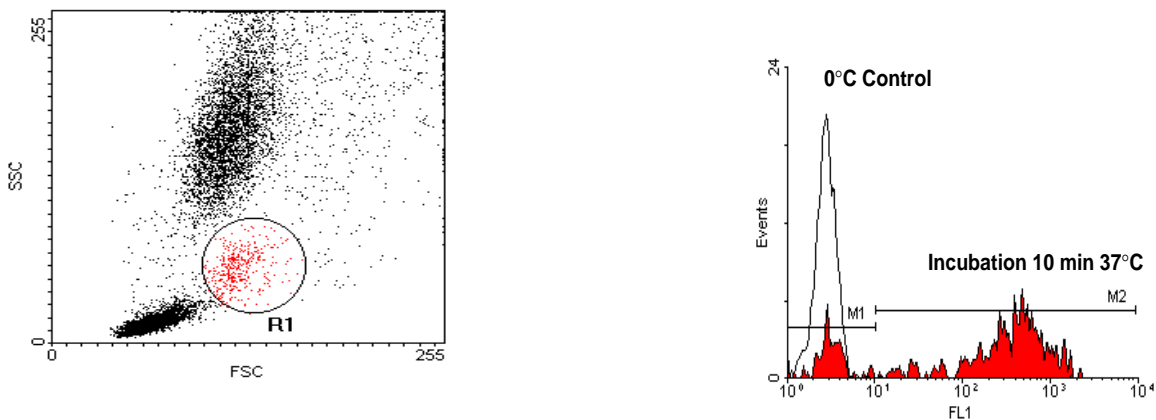


Fig. 3: Kinetics of phagocytosis by human granulocytes at various bacteria-to-cell ratios. At different time intervals the percentage of phagocytosing granulocytes was determined.

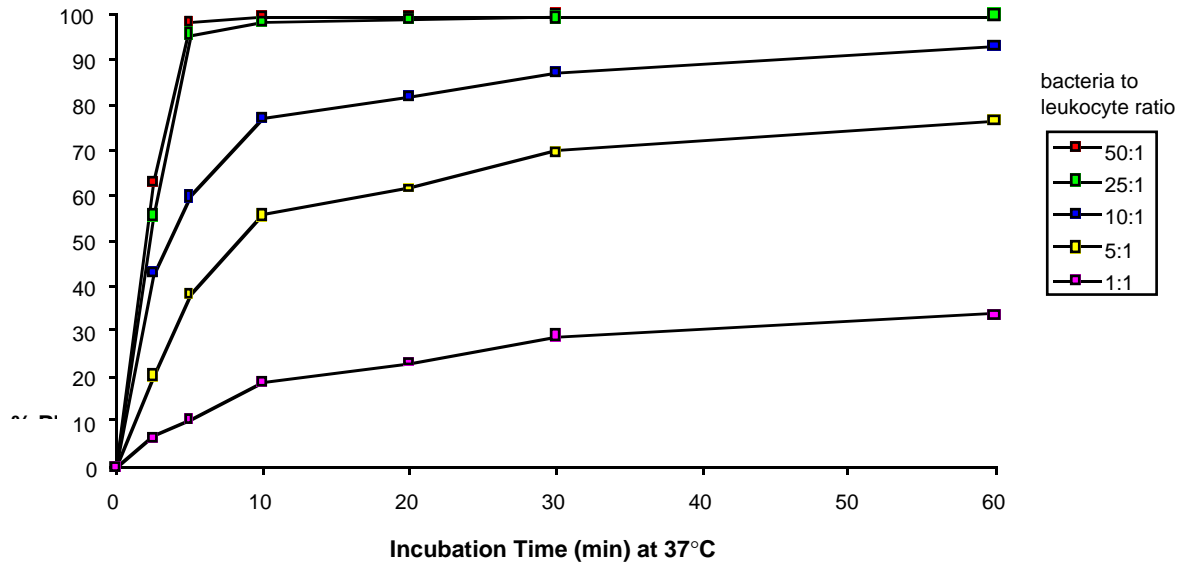


Fig. 4: Kinetics of phagocytosis by human granulocytes at various bacteria-to-cell ratios. The mean fluorescence of phagocytosing granulocytes is graphed as a function of time.

