

## SUMMARY and EXPLANATION

This reagent kit allows the quantitative determination of leukocyte phagocytosis in heparinized whole blood. It contains fluorescein (FITC)-labelled opsonized E.coli bacteria 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 should be performed by flow cytometry. 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 (4, 5, 6). The defects can be associated with the neutrophil itself or with an immunoglobulin or complement defect. Known inborn defects are leukocyte integrin deficiencies, actin dysfunction, and complement components defects (7, 8, 9). 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, systemic infections and acute pancreatitis. Reduced phagocytosis was found in patients with recurrent bacterial skin and sinopulmonary infections (10), in patients with wound infections from burns (11), in patients with AIDS (12), in neonates (13), in elderly people (14) or in patients undergoing therapies with glucocorticoids (15) or anesthetics (16).

Various immunomodulators (cytokines such as interleukin-2 or interferon- $\gamma$ , 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 (17, 18).

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 (19-22).

## PRINCIPLES of the PROCEDURE

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, 3).

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 reagent B (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 reagent C (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 reagent A (wash solution) erythrocytes are then removed by addition of reagent D (lysing solution). The DNA staining solution (Reagent E), 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, 3). It is therefore investigated under controlled conditions by separate kits: MIGRATEST™ to measure chemotaxis, PHAGOTEST™ to measure ingestion of microbes, BURSTTEST (PHAGOBURST™) to measure oxidative burst. The critical factors are: degree of opsonization, temperature, time of incubation and ratio of bacteria to leukocytes.

## MATERIAL and REAGENTS

The reagent kit contains:

### REAG A

1 bottle of Instamed-Salts to be reconstituted in 1 L of double distilled water provides 1 L ready-to-use **1 x wash solution**.

### REAG B

1 bottle (2 ml) of stabilized and **opsonized FITC-labelled E.coli suspension**, 1 x solution, ready to use, approx.  $2 \times 10^9$  bacteria per ml.

### REAG C

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.

### REAG D

1 vial (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.

### REAG E

1 bottle (20 ml) of **DNA staining solution** for cytometric discrimination of bacteria during leukocyte analysis, pink reagent solution, 1 x solution.

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

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 wash solution (reagent A) and 1 x lysing solution (reagent D).

4. Ice bath with cover.
5. Double distilled water or water for injection for reconstitution of wash solution (reagent A) and for dilution of 10 x lysing solution (reagent D).

Required apparatus:

1. Variable volume micropipettes 20 - 200  $\mu$ l, 100 - 1000  $\mu$ l and disposable tips.
2. Dispenser pipette and dispenser tips.
3. Bottle-top dispensers for wash 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).

## WARNING

1. Blood samples must always be regarded as potentially infectious. Wear disposable gloves and protective clothing while handling blood samples.
2. The reagent A contains chloracetamide and ethylenediaminetetraacetic acid sodium salt. Hazard statements: H301 Toxic if swallowed, H302 Harmful if swallowed, H317 May cause an allergic skin reaction, H319 Causes serious eye irritation, H361f Suspected of damaging fertility, H412 Harmful to aquatic life with long lasting effects.  
Precautionary statements: P273 Avoid release to the environment, P280 Wear protective gloves/protective clothing/eye protection/face protection, P281 Use personal protective equipment as required, P301 + P310 IF SWALLOWED: Immediately call a POISON CENTER or doctor/physician, P302 + P352: IF ON SKIN: Wash with plenty of soap and water. P305 + P351 + P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. P337 + P313: If eye irritation persists: Get medical advice/attention. P405 Store locked up.
3. The reagent G contains diethylene glycol and formaldehyde.  
Hazard statements: H301 Toxic if swallowed, H302 Harmful if swallowed, H311 Toxic in contact with skin, H314 Causes severe skin burns and eye damage, H317 May cause an allergic skin reaction, H331 Toxic if inhaled, H351 Suspected of causing cancer.

Precautionary statements: P280 Wear protective gloves/protective clothing/eye protection/face protection. P301 + P310 IF SWALLOWED: Immediately call a POISON CENTER or doctor/physician, P303 + P361 + P353: IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower. P305 + P351 + P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. P308 + P313 IF exposed or concerned: Get medical advice/attention if you feel unwell. P501 Dispose of contents/container in accordance with local/regional/national/inter-national regulations.

4. 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.

## STORAGE and STABILITY

Store the kit in the dark at 2 - 8° C (in refrigerator). Before use, mix the bacteria thoroughly (vortex mixer) or disaggregate by a syringe with a narrow needle. The reagents are supplied with a preservative that does not influence phagocytosis. The reagents are stable for the period shown on the packaging label, when stored as described.

## PROCEDURE

### 1. Preparations:

- 1.1 Dissolve the salts for wash solution (reagent A) in 1 L of double distilled water to prepare the wash solution.
- 1.2 Dilute the stock solution of reagent D 1: 10 in double distilled water (volume as needed, 2 ml per test).
- 1.4 Prepare ice bath.
- 1.5 Prewarm water bath to 37°C (**precise temperature control!**).
- 1.6 Switch on and calibrate the flow cytometer.

### 2. PHAGOTEST™ set-up:

#### 2.1 Dispensing:

**Heparinized whole blood** is mixed gently (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 (Reagent B)** well (vortex mixer) and add 20 µl per test and control sample to the whole blood samples.

#### 2.3 Incubation:

All tubes are mixed once more. The **control sample remains on ice**. The **test sample is incubated for 10 min at 37.0°C in a water bath**. **Incubation time and temperature 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 are taken out of the water bath together on one rack simultaneously and placed on ice in order to stop phagocytosis. **100 µl of icecold Reagent C (quenching solution)** is added to each of the samples. Mix the samples (vortex mixer).

#### 2.5 Washing.

Add **3 ml of wash solution (Reagent A)** to the tubes. Centrifuge the tubes (5 min, 250 x g, 2-8°C). Discard the supernatant. The samples are washed with **3 ml of wash solution (Reagent A)** once again (5 min, 250 x g, 2-8°C). Discard the supernatant.

#### 2.6 Lysis and fixation:

The whole blood samples are lysed and fixed with **2 ml of prewarmed (room temperature) 1 x reagent D (lysing solution)**.

Vortex and incubate the samples for **20 min at room temperature**.

Spin down cells (5 min, 250 x g, 2-8°C). Discard the supernatant.

2.7 Washing:

Add **3 ml of wash solution (Reagent A)** to the tubes. Centrifuge the tubes (5 min, 250 x g, 2-8°C). Aspirate the supernatant.

2.8 DNA staining:

Add **200 µl of DNA staining solution (Reagent E)** to the tubes. 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).

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**.

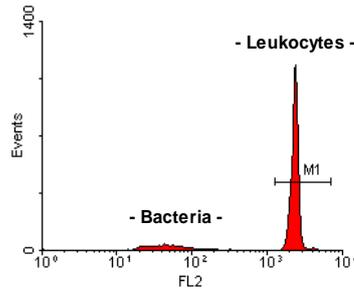
Data evaluation:

The percentages 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, see **Fig. 2A, 2C**) and its **green fluorescence histogram (FL1)** is analyzed (see **Fig. 2B, 2D**).

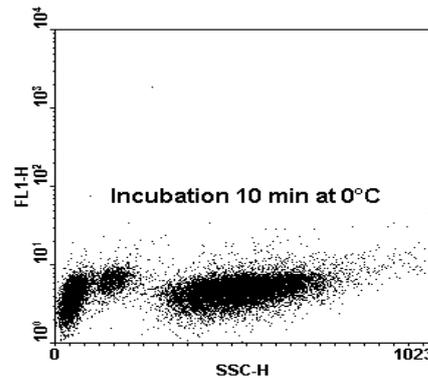
For that purpose, use the control sample to set a marker for fluorescence-1 (FL1) so that less than 1-3% of the events are positive. The percentage of phagocytosing cells in the test sample can then be determined by counting the number of events above this marker position. The mean fluorescence correlates with the number of bacteria per individual leukocyte.

**FIGURES**

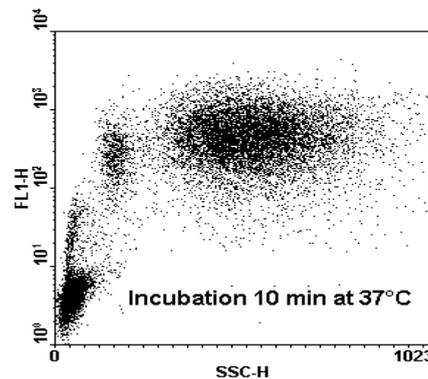
Recommended histogram/dot plot displays during data acquisition (see Figures 1A, 1B, 1C).



**Figure 1A** Live gate on leukocyte DNA (FL2 histogram)



**Figure 1B** Dot plot lin SSC / log FL1 of control sample (0°C)



**Figure 1C** Dot plot lin SSC / log FL1 of test sample (37°C)

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 (see Figures 2A, 2B, 2C, 2D).

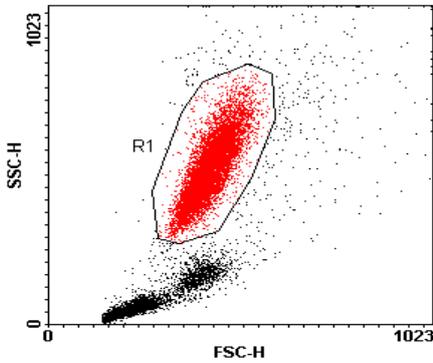


Figure 2A Typical dot plot FSC/SSC, gate set on granulocytes

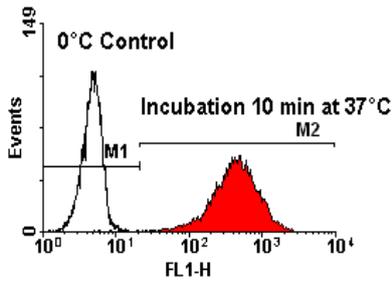


Figure 2B Typical FL1 histogram, gate set on granulocytes

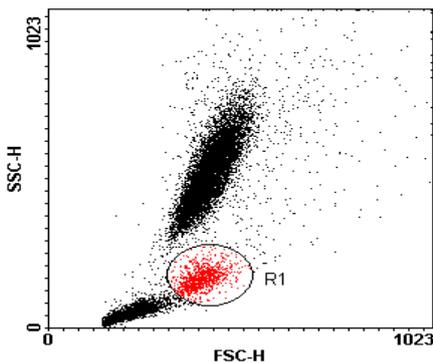


Figure 2C Typical dot plot FSC/SSC, gate set on monocytes

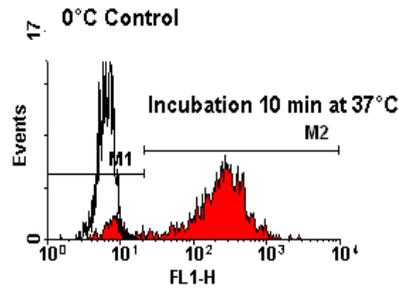


Figure 2D Typical FL1 histogram, gate set on monocytes

## 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 auto-fluorescence**, 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. Duplicates or 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 bacteria (reagent B) for 2.5 or 5 min) and **dilution of bacteria** (1:4 or 1:8).

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 FITC-labelled E.coli bacteria are also available as reagent F.** They can be used as stimulants in order to **test the opsonizing capacity of the patient's serum.**

**PRECISION of the METHOD**

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

	Range of values	Average CV (%)	n
% Phagocytizing Granulocytes	96.80 – 99.59	0.2	6
GeoMean FL1	264.30 – 616.60	1.5	6
% Phagocytizing Monocytes	78.4 – 95.5	1.1	6
GeoMean FL1	161.90 – 310.30	3.6	6

**EXPECTED RESULTS**

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*	GeoMean FL1*
Granulocytes	95 - 99	250 – 600
Monocytes	65 - 95	150 – 350

\* Results obtained using a shaking water bath, the GeoMan values are reduced by a factor of 20 to 30% when using a water bath without shaking.

**LIMITATIONS**

- Laboratories should establish their own normal reference ranges using their own test conditions.
- The samples should contain more than 95% viable cells and should be completely anticoagulated. Cells from older and incompletely anticoagulated blood samples may stain nonspecifically. Reasons for this phenomenon are platelet aggregates and dead cells with leaking DNA.
- The ratio of bacteria (approx.  $4 \times 10^7$  per 20 µl) to leukocytes (in 100 µl whole blood) is 50 : 1 assuming a white blood cell count of 8000/µl and 80 : 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.
- Samples ready for measurement without addition of reagent E (DNA staining solution) are stable for 24 hours on ice, but they systematically lose fluorescence intensity.

**IMPORTANT INSTRUCTIONS for QUANTITATIVE ANALYSIS**

- 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.
- Reproducible and standardized working is important. Therefore, please stick to these instructions and your own modifications thereof.

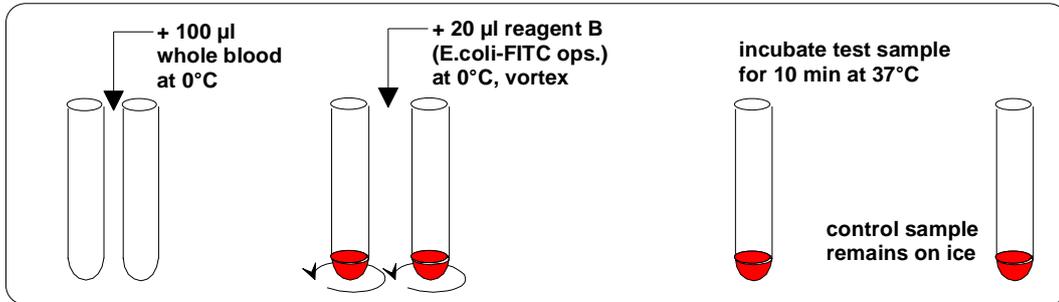
3. Any changes at the flow cytometer must be taken into consideration, which influence the sensitivity of the fluorescence measurement and therefore the "GeoMean" value. The use of a benchtop standard (fluorescent microbeads) is required for daily calibration.

## REFERENCES

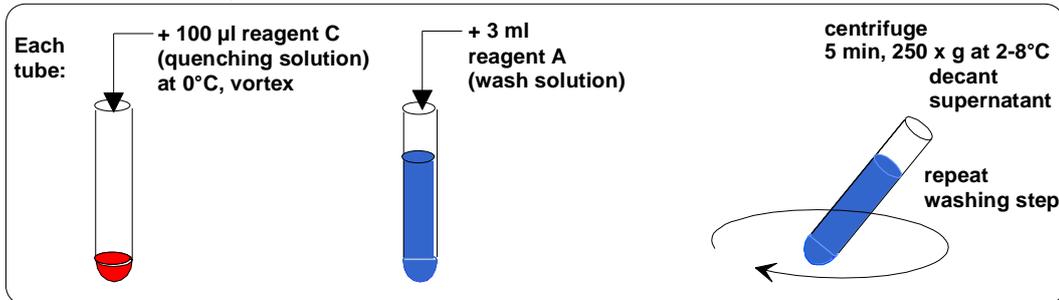
1. Male, D., Brostoff, J., Roth, D. & Roitt, I. 2006. Immunology, 7<sup>th</sup> ed. Elsevier Books, Oxford.
2. Sawyer, D.W., Donowitz, G.R. & G.L. Mandell. 1989. Polymorphonuclear neutrophils: An effective antimicrobial force. *Rev. Infect. Dis.* 11: S1532-S1544.
3. Janeway, C.A., Travers, P. & M. Walport. 2005. Immunobiology. 6<sup>th</sup> ed. Garland Publishing.
4. Rotrosen, D. & J.I. Gallin. 1987. Disorders of phagocyte function. *Ann. Rev. Immunol.* 5: 127-150.
5. Donadebian, H. D. 1989. Congenital and acquired neutrophil abnormalities. In: Klempner, M.S. et al. (eds) *Phagocytes and Disease*. Kluwer, Dordrecht Boston New York, pp 103-118.
6. Robinson, J.P. & W.O. Carter. 1993. Flow cytometric analysis of granulocytes. In: Bauer, K.D. et al. (eds) *Clinical Flow Cytometry, Principles and Applications*. Williams & Wilkins, Baltimore, pp 405-433.
7. Colten, H.R. & F.S. Rosen. 1993. Complement deficiencies. *Ann. Rev. Immunol.* 10: 809-824.
8. Fischer, A., Lisowska-Grospierre, B., Anderson, D.C. & T.A. Springer. 1988. Leukocyte adhesion deficiency: Molecular basis and functional consequences. *Immunodef. Rev.* 1: 39-54.
9. Malech H.L. & D.D. Hickstein. 2007. Genetics, biology and clinical management of myeloid cell primary immune deficiencies: chronic granulomatous disease and leukocyte adhesion deficiency. *Curr. Opin. Hematol.* 14: 29-36.
10. Basso, C.-F. 1984. Flow cytometric studies on phagocyte function in bacterial infections. *Acta path. microbiol. immunol. scand. Sect. C*, 92: 167 - 171.
11. Crogan, J.B. 1976. Altered neutrophil phagocytic function in burn patients. *J. Trauma* 16: 734-738.
12. Dobbmeyer, T.S., Raffel, B. Dobbmeyer, J.M., Findhammer, S., Klein, S.A., Kabelitz, D., Hoelzer, D., Helm, E.B. & Rossol. 1995. Decreased function of monocytes and granulocytes during HIV-1 infection correlates with CD4 cell counts. *Eur. J. Med. Res.* 1: 9-15.
13. Wilson, C.B. Development immunology and role of host defenses in neonatal infant. In: Remington, J.S. & J.O. Klein, eds. 1990. *Infectious diseases of the fetus and newborn infant*. Philadelphia, WB Saunders: 17:67.
14. Esparza, B., Sanchez H., Rulz M., Barranquero M., Sabino, E. & F. Merino. 1996. Neutrophil function in elderly persons assessed by flow cytometry. *Immunol. Invest.* 25: 185-190.
15. Goulding, N.J., Euzger, H.S., Butt, S.K. & M. Perretti. 1998. Novel pathways for glucocorticoid effects on neutrophils in chronic inflammation. *Inflammation Research.* 47: 158-165
16. Heller, A., Heller S., Blecken R. Urbaschek, R. & T. Koch. 1998. Effects of intravenous anesthetics on bacterial elimination in human blood in vitro. *Acta Anaesthesiol Scand* 42: 518-526.
17. Schiffrin, E.J., Rochat, F., Link-Amster, H., Aeschlimann, J.M. & A. Donnet-Hughes. 1995. Immunomodulation of human blood cells following ingestion of lactic acid bacteria. *J. Dairy Sci (USA)* 78: 491-497.
18. Brosche, T. & D. Platt. 1995. Nutritional factors and age-associated changes in cellular immunity and phagocytosis: A mini review. *Aging Immunol Infect Dis* 6: 31-42.
19. Menge, Ch. Neufeld, B., Hirt, W., Schmeer, N., Bauerfeind, R., Baljer, G. & L.H. Wieler. 1998. Compensation of preliminary blood phagocyte immaturity in the newborn calf. *Vet. Immunol. Immunopathol.* 62: 309-321.
20. Fachinger, V., Schlapp, T. Strube, W., Schmeer, N. & A. Saalmüller. 2000. Poxvirus-induced immunostimulating effects on porcine leukocytes. *J. Virol.* 74: 7943-7951.
21. Hoffmann-Jagielska, M., Winnicka, A., Jagielski, D. & R. Lechowski. 2003. Influence of some cellular aspects of the immune system in cats. *Veterinary Research Communications* 27: 643-652.
22. Kampen, A., Tollersrud, T., Larsen, S., Roth, J.A., Frank, D.E. & A. Lund. 2004. Repeatability of flow cytometric and classical measurement of phagocytosis and respiratory burst in bovine polymorphonuclear leukocytes. *Veterinary Immunology and Immunopathology* 97: 105-114.

## PHAGOTEST™ - Sample Preparation Procedure

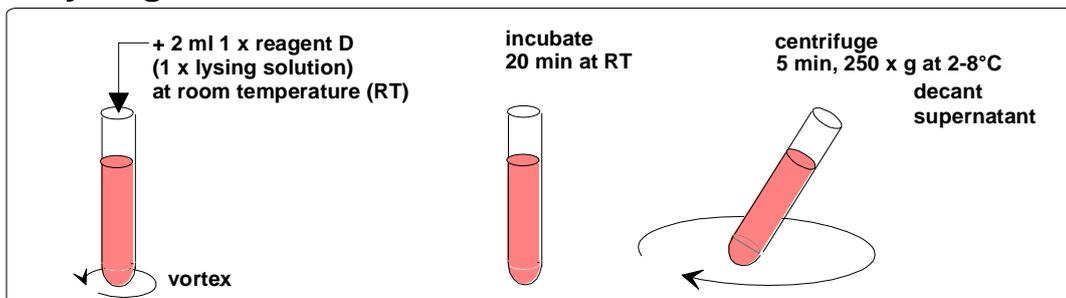
### 1. Labelling



### 2. Quenching



### 3. Lysing and Fixation



### 4. Washing and DNA Staining

