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PHAGOBURST™

Reagent kit for the quantification of the oxidative burst activity of monocytes and granulocytes in heparinized human whole blood

Reagent kit containing fluorogenic substrate, stimulants and reagents for 100 tests

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Key to symbols used

Symbol	Meaning	Symbol	Meaning
IVD	In Vitro Diagnostic Medical Device	Σ	Contains sufficient for <n> tests
CE	European Conformity		Temperature limitation
	Manufactured by	i	Consult instructions for use
REF	Catalogue number		Use by
LOT	Batch code	REAG	Reagent*
CONT	Contains		

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

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SUMMARY AND EXPLANATION

This test kit allows the quantitative determination of leukocyte oxidative burst in heparinized whole blood. It contains unlabeled opsonized bacteria (E.coli), phorbol 12-myristate 13-acetate (PMA) and the chemotactic peptide N-formyl-Met-Leu-Phe (fMLP) as stimulants, dihydrorhodamine (DHR) 123 as a fluorogenic substrate and necessary reagents. It determines the percentage of phagocytic cells which produce reactive oxidants (conversion of DHR 123 to R 123) and their enzymatic activity (amount of R 123 per cell).

The evaluation of oxidative burst activity 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

PHAGOBURST™ is intended to investigate the altered oxidative burst activity found in various disorders and to evaluate the effects of drugs.

Reduced or missing burst activity is observed in inborne defects like the chronic granulomatous disease (CGD). CGD is a heterogenous group of inherited disorders that usually manifests itself during the first two years of life (3, 4). The disease is characterized clinically by repeated and life-threatening infections caused by bacterial and fungal organisms. These infections typically consist of pneumonia, lymphadenitis, or abscesses that involve lymph nodes, lungs, and liver. The NADPH oxidase is the enzyme system responsible for producing superoxide anion, which is quickly converted to hydrogen peroxide and hydroxyl radicals. Abnormalities in the constituent peptides of the NADPH oxidase enzyme system lead to the dysfunctions characteristic of CGD. Neutrophils from CGD patients fail to produce a significant oxidative burst following stimulation. Different forms of CGD are described (classical X-linked CGD and autosomal recessive patterns). PHAGOBURST™ is a rapid and sensitive method for the diagnosis of CGD and for the detection of X-linked carriers.

Reduced oxidative burst activity was found in patients with AIDS (5), in elderly people (6), in patients with severe infections (7), in patients undergoing therapies with N-acetylcysteine (8) or in recipients of bone marrow and blood transplants (9).
The spontaneous and fMLP-induced neutrophil respiratory burst was shown to be increased in neonates without laboratory signs of infection (10).

Various immunomodulators (e.g., cytokines (GM-CSF, G-CSF, TNFα) or drugs) seem to have effects on the oxidative burst. By using fMLP as a low stimulant one can investigate additive or priming effects (11) of test substances.

The test kit is compatible with blood of mice, rats, rabbits, dogs, cattle and other species (12 – 14).

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 (oxidative burst) and oxygen-independent mechanisms (1, 2).

PHAGOBURST™ allows the quantitative determination of leukocyte oxidative burst. The PHAGOBURST™ kit contains unlabelled opsonized E.coli bacteria (reagent B) as particulate stimulus, the protein kinase C ligand phorbol 12-myristate 13-acetate (PMA, reagent D) as high stimulus and the chemotactic peptide N-formyl-MetLeuPhe (fMLP, reagent C) as low physiological stimulus, dihydrorhodamine (DHR) 123 (reagent E) as a fluorogenic substrate and necessary reagents. Heparinized whole blood is incubated with the various stimuli at 37 °C, a sample without stimulus serves as negative background control. Upon stimulation, granulocytes and monocytes produce reactive oxygen metabolites (superoxide anion, hydrogen peroxide, hypochlorous acid) which destroy bacteria inside the phagosome. Formation of the reactive oxidants during the oxidative burst can be monitored by the addition and oxidation of DHR 123. The reaction is stopped by addition of lysing solution (reagent F), which removes erythrocytes and results in a partial fixation of leukocytes. After one washing step with wash solution (reagent A), DNA staining solution (re-

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agent G) is added to exclude aggregation artifacts of bacteria or cells. The percentage of cells having produced reactive oxygen radicals are then analyzed as well as their mean fluorescence intensity (enzymatic activity).
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: MIGRATEST™ to measure chemotaxis, PHAGOTEST™ to measure ingestion of microbes, PHAGOBURST™ to measure oxidative burst.

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** (non-labelled) **E.coli suspension**, 1 x solution, ready to use, approx. 1-2 x 10^9 bacteria per ml.

REAG	C

1 vial (100 µl) containing the **chemotactic petide fMLP** (200 x stock solution, 1 mM). Dilute 5 µl in 1 ml wash solution.

REAG	D

1 vial (100 µl) containing **phorbol 12-myristate 13-acetate (PMA)** (200 x stock solution, 1.62 mM). Dilute 5 µl in 1 ml wash solution.

REAG	E

12 vials each containing one **substrate disk** containing dihydrorhodamine 123 to be reconstituted by injection of 1 ml wash solution 20-30 min before use, provides 1 ml **substrate solution**.

REAG	F

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	G

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 µl, 100 - 1000 µl 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 ethylendiaminetetraacetic acid sodium salt. Chloracetamide is harmful by inhalation and contact with skin (R22) and may cause sensitization by skin contact (R43). Possible risk of impaired fertility (R62). Do not breathe dust (S22). Wear suitable protective clothing, gloves and eye/face protection (S36/37/39). Ethylendiaminetetraacetic acid sodium salt is irritating to eyes (R36) and may cause long-term adverse effects in the aquatic environment (R52/53). Avoid release to the environment. Refer to special instructions/Safety data sheets (S61).
3. The reagents C and D contain dimethyl sulfoxide. Dimethyl sulfoxide is irritating to eyes, respiratory system and skin (R36/37/38). Avoid contact with skin and eyes (S24/25). In case of

contact with eyes, rinse immediately with plenty of water and seek medical advice (S26).
4. The reagent D contains Phorbol-12-myristat-13-acetate (PMA). PMA is very toxic by inhalation, in contact with skin and if swallowed (R26/27/28). It is irritating to eyes, respiratory system and skin (R36/37/38). Possible risks of irreversible effects (R40). In case of accident or if you feel unwell, seek medical advixe immediately (S45), in case of contact with eyes, rinse immediately with plenty of water and seek medical advice (S26). Take off immediately all contaminated clothing (S27). Wear suitable protective clothing, gloves and eye/face protection (S36/37/39).
5. The reagent F contains diethylene glycol and formaldehyde. Formaldehyde is harmful by inhalation, in contact with skin and if swallowed (R20/21/22). It is irritating to eyes, respiratory system and skin (R36/37/38). Possible risks of irreversible effects (R40). May cause sensitization by skin contact (R43). In case of contact with eyes, rinse immediately with plenty of water and seek medical advice (S26). Wear suitable protective clothing and gloves (S36/37). In case of accident or if you feel unwell, seek medical advice immediately (S45). Use only in well-ventilated areas (S51). Diethylen glycol is harmful if swallowed (R22). If swallowed, seek medical advice immediately and show this container or label (S46).
6. 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 fMLP and PMA working solutions have to be discarded after usage. The reagents are supplied with a preservative that does not influence oxidative burst activity. 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 solutions of reagent C (fMLP stock solution) and reagent D (PMA stock solution) 1 : 200 in wash solution (volume as needed, e.g., 5 µl in 1 ml, 20 µl is needed per blood sample).

1.3 Dilute the stock solution of reagent F 1 : 10 in double distilled water (volume as needed, 2 ml per test).

1.4 Prepare substrate solution: reconstitute reagent E per assay day by injecting 1 ml wash solution using a 1 ml syringe, **leave it for 20 - 30 min** before use and shake gently (**do not vortex!**). Unused substrate solution may be stored frozen at < -15 °C for up to 2 weeks.

1.5 Prepare ice bath.

1.6 Prewarm water bath to 37 °C (**precise temperature control!**).

1.7 Switch on and calibrate the flow cytometer.

2. Oxidative burst 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:

(1) Add **20 µl** of **wash solution (reagent A)** to a test tube as a "**negative control**" (tube #1)
(2) Mix the precooled **E.coli bacteria (reagent B)** well and **add 20 µl per test to the whole blood** (tube #2).
(3) Add **20 µl** of the **fMLP working solution (reagent C)** to another test tube as a "**low control**" (tube #3)
(4) Add **20 µl** of the **PMA working solution (reagent D)** to another test tube as a "**high control**" (tube #4)
All tubes are mixed once more. The burst assay samples are 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.

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2.3 Oxidation:
After the 10 min incubation add **20 µl** of **substrate solution (reagent E)** and **vortex the sample thoroughly.**
All tubes are incubated for another **10 min at 37.0 °C** in the water bath.

2.4 Lysis and fixation:
The whole blood samples are lysed and fixed with **2 ml** of prewarmed (room temperature) **1 x reagent F (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.

2.5 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.

2.6 DNA staining:
Add **200 µl of DNA staining solution (reagent G)** to the tubes. Mix and incubate **10 min on ice** (light protected in the ice bath).
Measure the cell suspension within 30 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 **percentage of cells** having produced reactive oxygen metabolites (**recruitment**) are analyzed as well as their **mean fluorescence intensity** (amount of cleaved substrate, **activity**). 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 - 2F**).

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 positive cells in the test samples can then be determined by counting the number of events above this marker position. The mean fluorescence correlates with oxidation quantity per individual leukocyte.

FIGURES

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

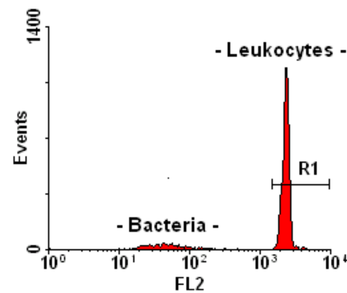

Figure 1A Live gate on leukocyte DNA (FL2 histogram)

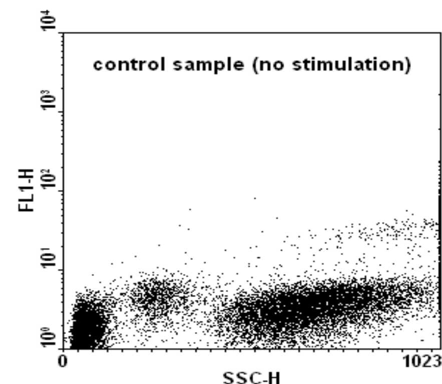

Figure 1B Dot plot lin SSC / log FL1 of control sample (no stimulation)

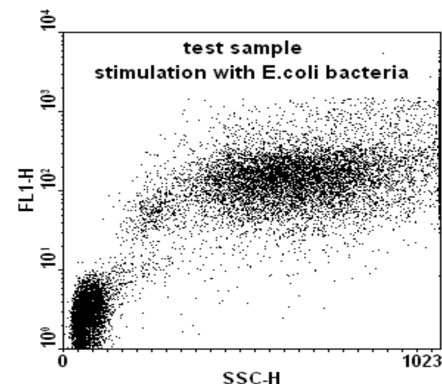

Figure 1C Dot plot lin SSC / log FL1 of test sample (stimulation with E.coli bacteria)

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Typical dot plots FSC/SSC and **FL1 histograms** of the oxidative burst assay (incubation time of 10 + 10 min at 37 °C). Histograms for the control samples a re presented on the left **(see Figures 2A – 2F)**.

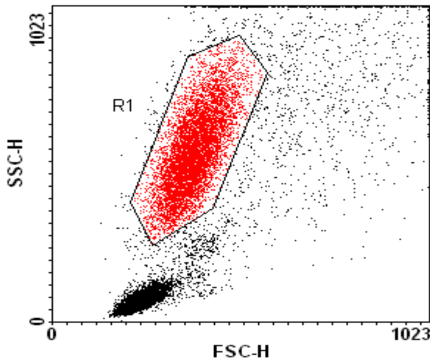

Figure 2A Typical dot plot FSC/SSC, gate set on granulocytes, stimulation with E.coli bacteria

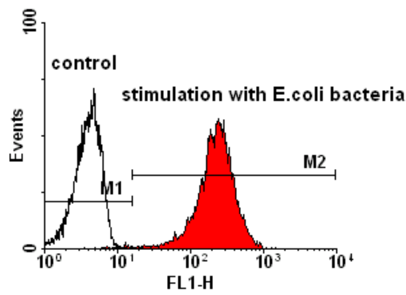

Figure 2B Typical FL1 histogram, gate set on granulocytes, stimulation with E.coli bacteria

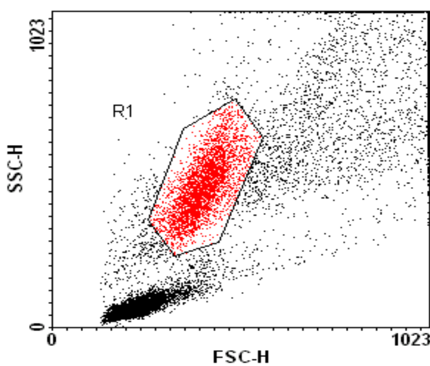

Figure 2C Typical dot plot FSC/SSC, gate set on granulocytes, stimulation with PMA

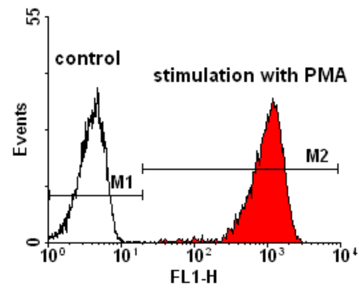

Figure 2D Typical FL1 histogram, gate set on granulocytes, stimulation with PMA

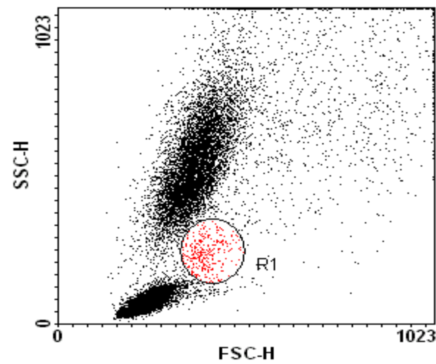

Figure 2E Typical dot plot FSC/SSC, gate set on monocytes, stimulation with E.coli bacteria

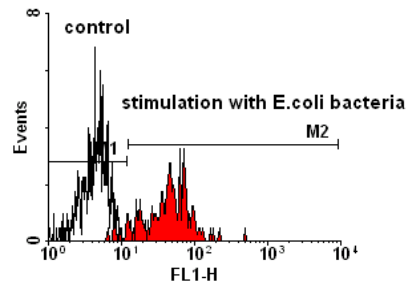

Figure 2F Typical FL1 histogram, gate set on monocytes, stimulation with E.coli bacteria

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REMARKS

1. Heparinized blood should be processed **within 24 h of sampling**. **Blood samples** should remain at **room temperature** prior to processing.
2. The available **amount of substrate** is determined by the **incubation time** of the **substrate disk** in the wash solution **(20 - 30 min are optimal)**.
3. **Eosinophils** (enhanced in allergies and parasitic infections) show an **increased autofluorescence**, which can be shown by the control assay at 0 °C.
4. Phagocytes incubated at 37 °C differ in size and granularity from cells in the 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.
5. Duplicates or triplicates are useful in establishing the assay.
6. The proposed test protocol with bacteria allows investigation of burst processes under **optimal conditions** (whole blood, opsonized bacteria, no isolation steps etc.). Therefore, **on testing drugs** in healthy persons only a limited increase in burst activity *ex vivo* or *in vitro* can be expected. **Testing drug effects in vitro**, it might be appropriate to use the weak stimulant **fMLP**, or to run **kinetics for time dependence** (incubation with E.coli bacteria for 2.5 or 5 min) and **dilution of bacteria** (1 : 4 or 1 : 8, ratio of bacteria per leukocyte).
7. 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 oxidative burst activity of **isolated monocytes/macrophages** or **cell lines** can be studied by incubating the cells with E.coli bacteria in culture medium containing **5 - 20% fetal calf** or **human serum**. It might also be necessary to extend the incubation time (60 min - 240 min).

PRECISION OF THE METHOD

The **intra-assay precision** of this assay was determined on triplicate whole blood samples from healthy subjects (stimulation with E. coli bacteria).

	Range of values	Average CV (%)	n
% Oxidizing Granulocytes	**99.60 – 99.95**	**0.1**	**6**
GeoMean FL1	**154.50 – 395.75**	**4.8**	**6**
% Oxidizing Monocytes	**81.80 – 96.67**	**1.1**	**6**
GeoMean FL1	**49.60 – 88.65**	**6.5**	**6**

EXPECTED RESULTS

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

Cell Type	Stimulus	% Oxidizing Cells*	GeoMean FL1*
Granulocytes	**E.coli**	**97 -100**	**150 – 500**
	fMLP	**1 -10**	**-**
	PMA	**98 - 100**	**300 - 1000**
Monocytes	**E.coli**	**70 - 100**	**50 - 100**

* Results obtained using a shaking water bath, the GeoMan values are reduced by a factor of 10 to 20% when using a water bath without shaking.

LIMITATIONS

1. Laboratories should establish their own normal reference ranges using their own test conditions.
2. The samples should contain more than 95% viable cells and should be completely anticoagulated. Cells from older and incompletely anticoagulated blood samples may stain non-

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specifically. Reasons for this phenomenon are platelet aggregates and dead cells with leaking DNA.

3. Samples ready for measurement without addition of reagent G (DNA staining solution) are stable for 1 hours on ice, but they systematically loose fluorescence intensity.
4. The fluorogenic substrate DHR 123 is sensitive to oxidation and is therefore ampuled under inert gas.

IMPORTANT INSTRUCTIONS FOR QUANTITATIVE ANALYSIS

1. The **phagocytosis** and **oxidative burst 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 this 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.

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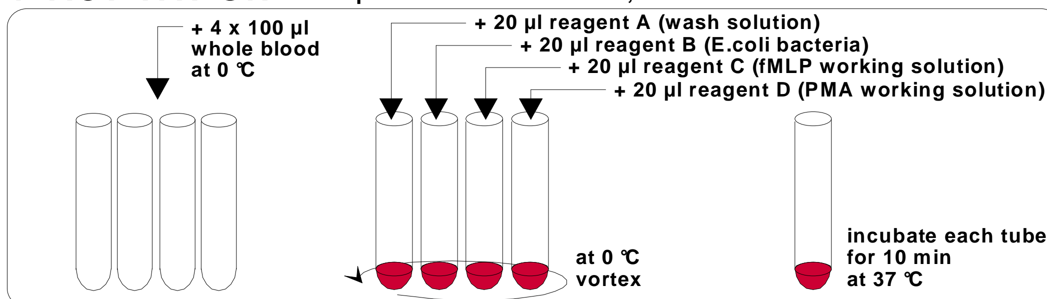
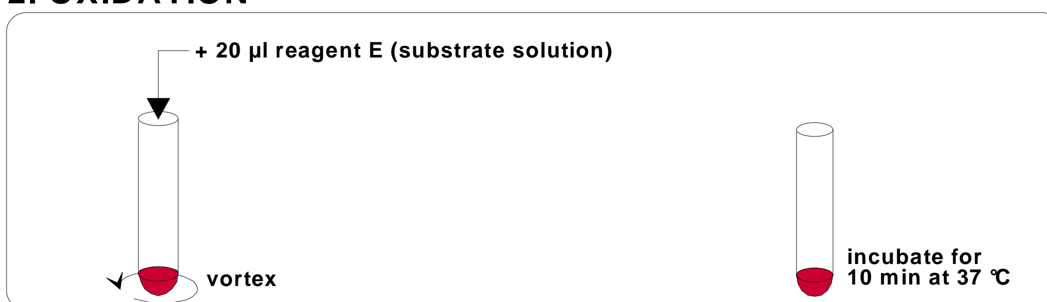
PHAGOBURST™ - Sample Preparation Procedure

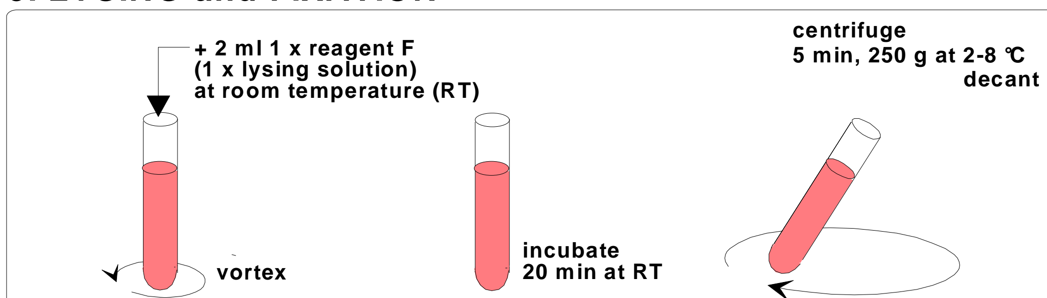
1. ACTIVATION with opsonized E.coli bacteria, PMA and fMLP



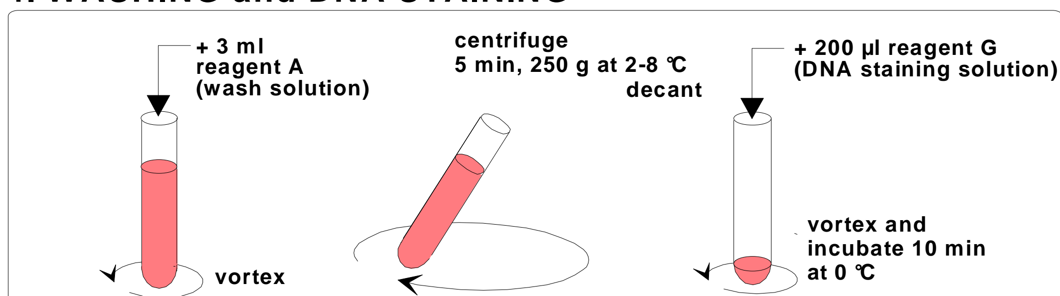
2. OXIDATION



3. LYSING and FIXATION



4. WASHING and DNA STAINING



5. SAMPLES READY FOR FACS ANALYSIS

store at 0 °C, protected from light!   Measure within 30 min!