

## **BURSTTEST (PHAGOBURST®)**

### **TEST KIT FOR THE QUANTIFICATION OF THE OXIDATIVE BURST ACTIVITY OF MONOCYTES AND GRANULOCYTES IN HEPARINIZED WHOLE BLOOD**

**For Research Use Only.**

**Not for use in diagnostic or therapeutic procedures.**

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Catalog No. 10-0200

Fluorogenic substrate, stimulants and reagents for 100 tests.

Please read the instructions carefully before use!

#### **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. The detailed instructions result from specific experience and validation assays. Critical steps are in bold letters. A graphic summary of the test is attached.

#### **APPLICATIONS**

BURSTTEST 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 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).

The oxidative burst of granulocytes is impaired in transplantation (6), AIDS (7), and in elderly people (8).

The spontaneous and fMLP-induced neutrophil respiratory burst was shown to be increased in neonates without laboratory signs of infection (9).

Various immunomodulators (e.g., cytokines (GM-CSF, G-CSF, TNF $\alpha$ ) or drugs) seem to have effects on the oxidative burst. By using fMLP as a low stimulant one can investigate additive or priming effects (10) of test substances.

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

BURSTTEST allows the quantitative determination of leukocyte oxidative burst. The BURSTTEST kit contains unlabelled opsonized *E. coli* bacteria as particulate stimulus, the protein kinase C ligand phorbol 12-myristate 13-acetate (PMA) as high stimulus and the chemotactic peptide N-formyl-MetLeuPhe (fMLP) as low physiological stimulus, dihydrorhodamine (DHR) 123 as a fluorogenic substrate (5) 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, which removes erythrocytes and results in a partial fixation of leukocytes. After one washing step with WASHING SOLUTION, DNA STAINING SOLUTION 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). The whole cascade of events can be investigated individually under controlled conditions by separate assays: MIGRATEST<sup>®</sup> to measure chemotaxis, PHAGOTEST<sup>®</sup> to measure ingestion of bacteria, BURSTTEST (PHAGOBURST<sup>®</sup>) to measure oxidative burst.

## **MATERIAL and REAGENTS**

The test kit contains:

1. 1 bottle (2 ml) of stabilized and **opsonized** (non-labelled) ***E. coli* suspension (*E. coli* OPSONIZED)**, 1 x solution, ready to use, ~ 1 x 10<sup>9</sup> bacteria per ml.
2. 1 vial (100 µl) containing the **chemotactic peptide fMLP** (200 x stock solution, 1 mM). Dilute 5 µl in 1 ml WASHING SOLUTION for use.
3. 1 vial (100 µl) containing **phorbol 12-myristate 13-acetate (PMA)** (200 x stock solution, 1.62 mM). Dilute 5 µl in 1 ml WASHING SOLUTION for use.
4. 12 vials each containing one **SUBSTRATE DISK** to be reconstituted by injection of 1 ml WASHING SOLUTION 30 - 45 min before use.
5. 1 bottle (20 ml) of **DNA STAINING SOLUTION** for cytometric discrimination of bacteria during leukocyte analysis, pink reagent solution, 1 x solution.
6. 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.
7. 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. Disposable 12 x 75 mm Falcon<sup>®</sup> polypropylene (#2053) or polystyrene (#2052) test tubes and appropriate test tube racks.
3. Flasks for WASHING SOLUTION (1000 ml) and 1 x LYSING SOLUTION (500 ml).
4. Ice bath with cover.
5. Reagent-grade (both distilled and deionized) water for reconstitution of WASHING SOLUTION and dilution of 10 x 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).

**STORAGE and STABILITY**

**Store the kit in the dark at 2-8° C.** Before use, the bacteria have to be mixed thoroughly (vortex mixer) or to be disaggregated by a syringe with a narrow needle. The fMLP and PMA working solutions have to be discarded after use. The reagents are supplied sterile with a preservative that does not influence phagocytosis and oxidative burst.

**ASSAY PROCEDURE****1. Preparations**

- 1.1 Dissolve the salts for WASHING SOLUTION in 1000 ml of distilled water.
- 1.2 Dilute the stock solutions:
  - LYSING SOLUTION 1 : 10 in distilled water (volume as needed, 2 ml per test)
  - fMLP and PMA stock solutions 1 : 200 in WASHING SOLUTION (volume as needed, e.g., 5 µl in 1 ml, 20 µl is needed per blood sample).
- 1.3 Prepare SUBSTRATE SOLUTION: reconstitute 1 SUBSTRATE DISK per assay day by injecting 1 ml WASHING SOLUTION using a 1 ml syringe, **leave it for 30 - 45 min** before use and shake gently (**do not vortex!**). Unused SUBSTRATE SOLUTION may be stored frozen at -20°C for up to 2 weeks.
- 1.4 Prepare ice bath.
- 1.5 Cool bacteria in the ice bath.
- 1.6 Prewarm water bath to 37°C (**precise temperature control!**).
- 1.7 Switch on and calibrate the flow cytometer. The same amplifier and compensation adjustments as for immunofluorescence analysis with directly conjugated monoclonal antibodies are possible.

**2. Oxidative burst set-up**2.1 Dispensing:

**Heparinized whole blood** is mixed and pipetted into the bottom of 12 x 75 mm tubes, **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 5 to 10 min in order to cool them down to 0°C.

2.2 Activation:

Add **20 µl** of the **WASHING SOLUTION** to a test tube as a "**negative control**" (tube #1)

Mix the precooled ***E. coli*** bacteria well and **add 20 µl per test** to the whole blood (tube #2).

Add **20 µl** of the **fMLP working solution** to another test tube as a "**low control**" (tube #3)

Add **20 µl** of the **PMA working solution** 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**. The optimum of fMLP incubation time is different with each individual donor and might be shorter than 10 min (see **Fig. 3**).

**Incubation time and temperature must be monitored closely and the water bath must be closed and preheated.**

2.3 Oxidation:

After the 10 min incubation add **20 µl** of **SUBSTRATE SOLUTION** and **vortex the sample thoroughly.**

Incubate again for **10 min at 37.0 °C** in the water bath.

2.4 Stopping (lysis and fixation):

Precisely at the end of the incubation time all samples together on one rack simultaneously are taken out of the water bath.

The whole blood is lysed and fixed with **2 ml** prewarmed (room temperature, 20 to 25°C) **1 x LYSING SOLUTION**. Mix and incubate the samples for **20 min at room temperature.**

Spin down cells (5 min, 250 x g, 2-8°C). Aspirate the supernatant leaving approximately 100 µl.

2.5 Washing:

The samples are washed once with **3 ml of WASHING SOLUTION**. First, add 1 ml of WASHING SOLUTION to each tube, vortex gently. Finally, add 2 ml of WASHING SOLUTION. Centrifuge the tubes at 250 x g for 5 minutes at 2-8°C. Aspirate the supernatant leaving approximately 100 µl.

2.6 DNA staining:

Add **200 µl DNA STAINING SOLUTION**, vortex 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, e.g., 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 or platelet 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**).

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.

### REMARKS

1. Heparinized blood should be processed **within 24 h of sampling**. **Blood samples** should remain at **room temperature** (20 to 25°C) prior to processing.
2. The available **amount of substrate** is determined by the **incubation time** of the **SUBSTRATE DISK** in the washing solution (30 - 45 min are optimal).
3. **Eosinophils** (enhanced in allergies and parasitic infections) show an **increased autofluorescence**, which can be shown by the control assay.
4. Phagocytes incubated and stimulated at 37°C differ in size and granularity from cells in the negative 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. 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 to 240 min).

### EXPECTED VALUES

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	Mean Fluorescence Intensity (logarithmic gain, 4 decades 1-10,000)
Monocytes	<i>E. coli</i>	70 - 100	50 - 200
Granulozyten	<i>E. coli</i>	95 - 100	200 - 600
	fMLP	1 - 20	-
	PMA	99 - 100	500 - 1000

### PRECISION OF THE METHOD

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

	% Oxidizing Granulocytes	Mean Value FL1	% Oxidizing Monocytes	Mean Value FL1
Range of values	96.5 - 99.6	314 - 635	83.1 - 97.4	68 - 171
Average CV (%)	0.9	3.2	2.6	8.5
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 - burst positive cells in the control assay. Reasons for this phenomenon are platelet aggregates and dead cells with leaking DNA.
3. The ratio of bacteria ( $2 \times 10^7$  pro 20  $\mu\text{l}$ ) to leukocytes (in 100  $\mu\text{l}$  whole blood) is 25 : 1 assuming a white blood cell count of 8,000/ $\mu\text{l}$  and 40 : 1 at 5,000/ $\mu\text{l}$ . Samples with white blood cell counts differing from the normal range (4,000 – 10,000) require correction of the amount of bacteria added.
4. Samples ready for measurement without DNA STAINING SOLUTION are stable for 1 hour on ice, but they systematically loose fluorescence intensity.
5. The fluorogenic substrate DHR 123 is sensitive to oxidation and is therefore ampouled under inert gas.

## **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 DNA STAINING SOLUTION contains propidium iodide. Propidium iodide is highly toxic and a suspected mutagen. Avoid contact with skin and eyes. Wear suitable protective clothing, gloves and eye/face protection. Any materials in contact with this stain should be treated as hazardous waste and disposed of appropriately.
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. Phorbol 12-myristate 13-acetate might be carcinogenic!
5. The LYSING SOLUTION contains diethylene glycol and formaldehyde. Formaldehyde is harmful by inhalation, in contact with skin, and if swallowed. It is irritating to eyes and skin. Exposure can cause cancer. Possible risks of irreversible effects. May cause sensitization by skin contact. Keep locked up and out of the reach of children. Keep away from food, drink and animal feedingstuff. Wear suitable protective clothing and gloves. Even small amounts of diethylene glycol can be fatal. If swallowed, seek medical advice immediately and show this container or label. Dispose of according to federal, state, and local regulations.
6. *E. coli* OPSONIZED and DNA STAINING SOLUTION contain Thimerosal as a preservative. Thimerosal is a mercury compound. Exposure can cause reproductive toxicity. Harmful by inhalation, in contact with skin and if swallowed. Danger of cumulative effects. Keep away from food, drink and animal feedingstuff. After contact with skin, wash immediately with plenty of water. Wear suitable protective clothing. In case of accident or if you feel unwell, seek medical advice immediately. Dispose of according to federal, state, and local regulations.

## **IMPORTANT INSTRUCTIONS FOR QUANTITATIVE ANALYSIS**

1. The **phagocytosis** and **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 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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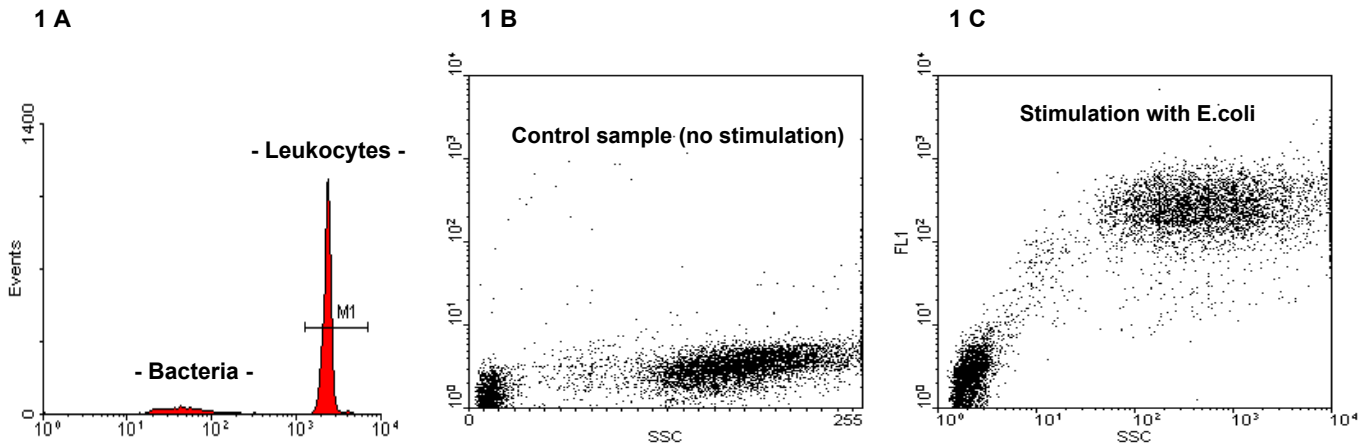
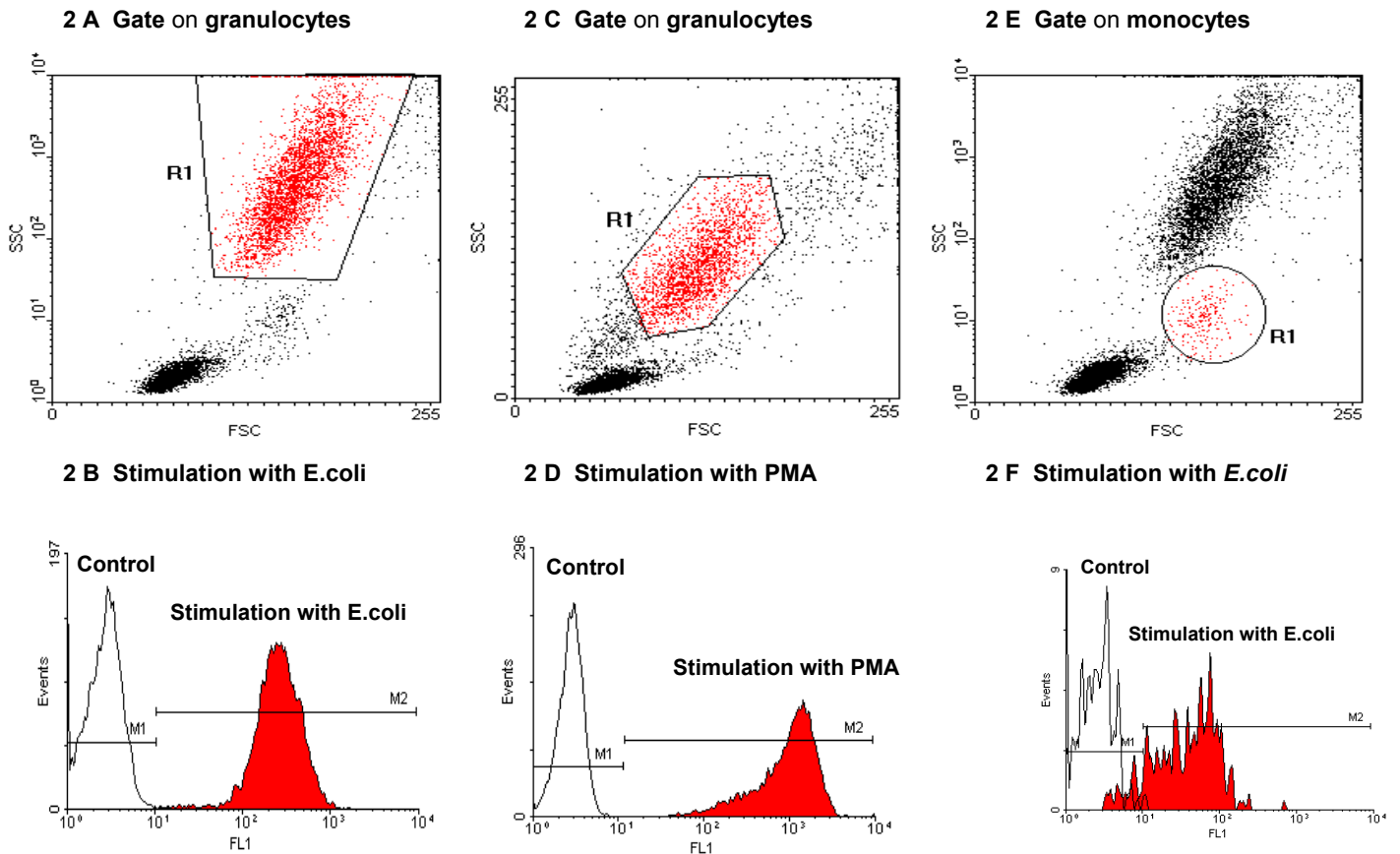
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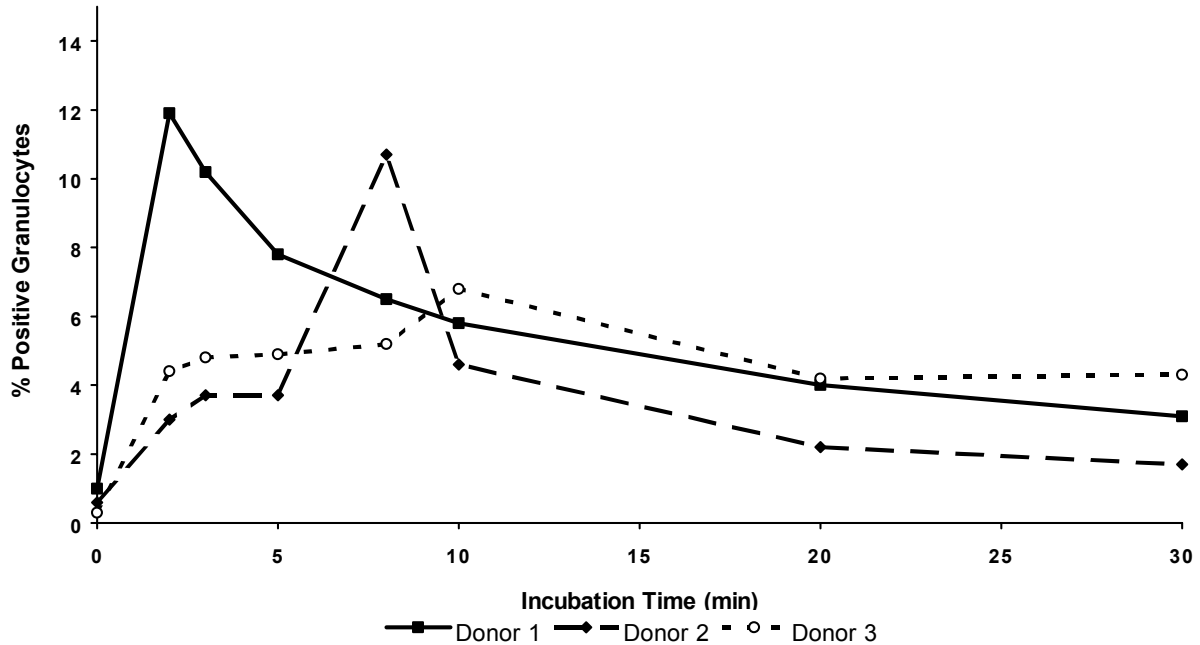
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**FIGURES****Fig. 1: Recommended histogram/dot plot displays during data acquisition**

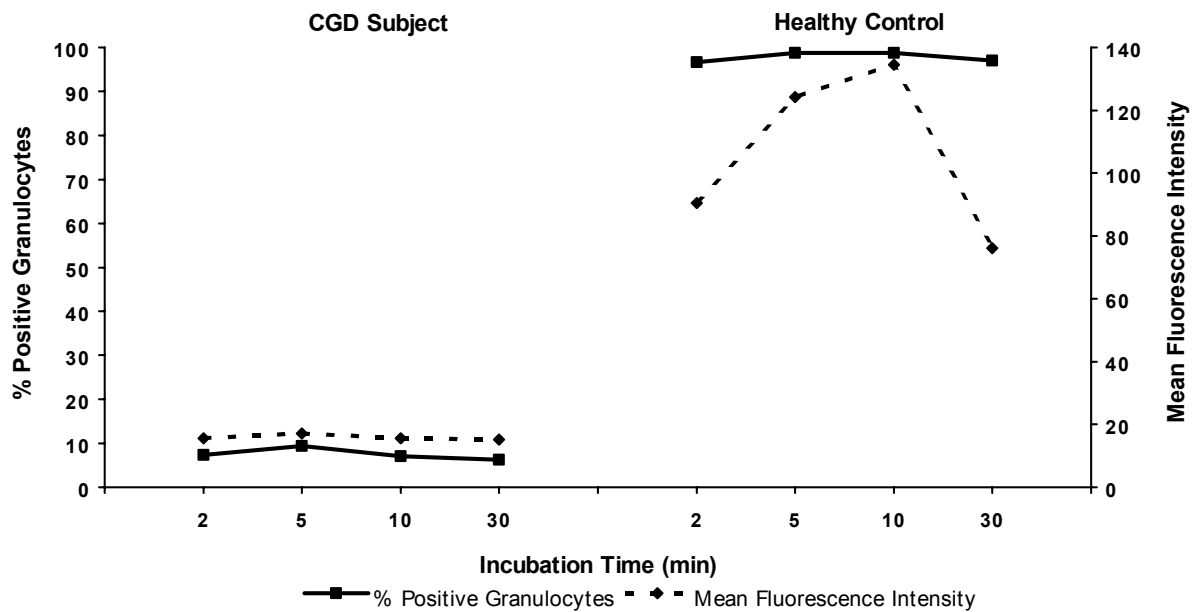
- A) Live gate on leukocyte DNA (FL2 histogram)  
 B) Dot plot lin SSC / log FL1 of control sample (no stimulation)  
 C) Dot plot lin SSC / log FL1 of test sample (stimulation with *E. coli*, 10 min, 37°C)

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

**Fig. 3: Kinetics of oxidative burst** by human granulocytes stimulated with the chemotactic peptide fMLP (incubation time with DHR 123 = 10 min). At different time intervals the **percentage of oxidizing granulocytes** was determined.



**Fig. 4: Time-course of the increasing percentage of fluorescence-positive granulocytes in a normal healthy control a X-linked CGD subject on stimulation with *E. coli* bacteria.**



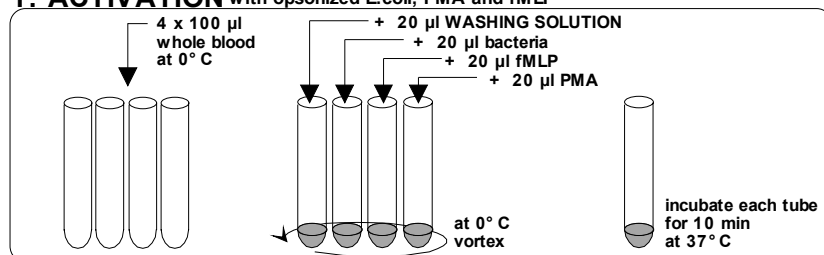
## Summary of Assay Procedure

### Preparations

1. Dissolve the salts for WASHING SOLUTION in 1000 ml deionized distilled water. Dilute the stock solutions (fMLP, PMA and 10 x LYSING SOLUTION).
2. Prepare ice bath (ice in water).
3. Prepare SUBSTRATE SOLUTION by reconstituting 1 SUBSTRATE DISK with 1 ml of WASHING SOLUTION.
4. Prewarm water bath to 37°C (**precise temperature control!**).
5. Switch on and calibrate flow cytometer.

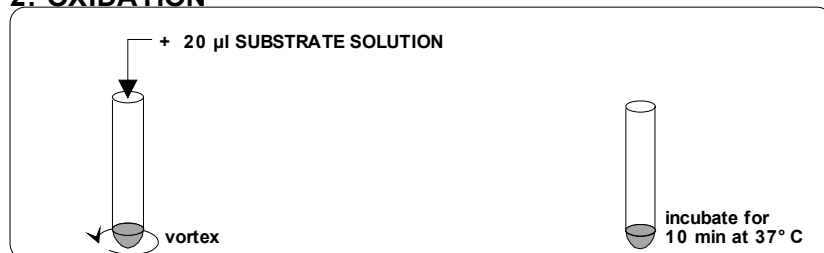
### BURSTTEST set up

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



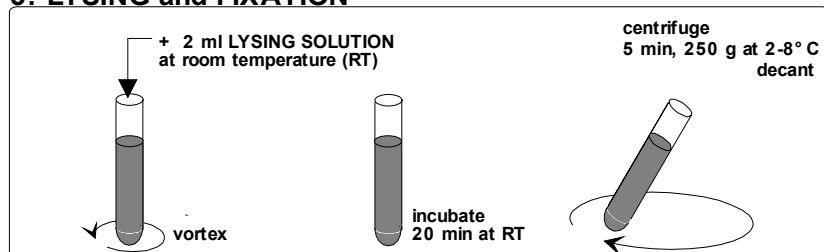
Use only **heparinized** whole blood. Incubate the blood samples for 5 to 10 min on ice in order to cool them down. **Avoid smearing blood down the side of the tube.** After addition of bacteria, PMA, fMLP or WASHING SOLUTION, vortex at low speed for 2 - 3 seconds.

#### 2. OXIDATION



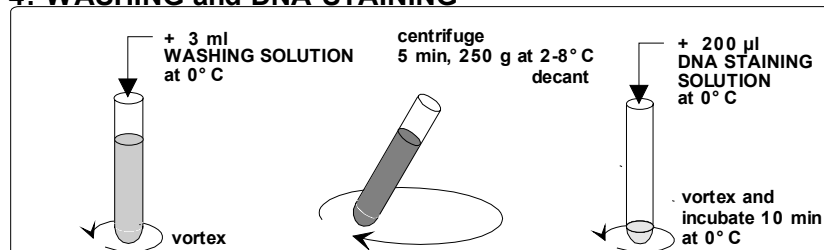
Add 20 µl of SUBSTRATE SOLUTION per sample, vortex.

#### 3. LYSING and FIXATION



Add **prewarmed** (room temperature, 20 to 25°C) **1 x LYSING SOLUTION**. Centrifuge the samples (250 g, 5 min, 2-8°C)

#### 4. WASHING and DNA STAINING



After addition of the DNA STAINING SOLUTION; measure samples ready for FACS analysis within 30 minutes.

#### 5. SAMPLES READY FOR FACS ANALYSIS

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

\* 1200 rpm for 16 cm rotor