FagoFlowEx Kit

100 tests | Cat. No. **ED7042**

IVD CE

Instructions for Use (EN)

Version: ED7042_IFU_v8_EN

Date of Issue: 14-04-2023

Symbols used in the device labeling

IVD	In Vitro diagnostic medical device		Temperature limit
CE	CE marking of conformity		Keep away from sunlight
	Manufacturer		Keep Dry Keep away from rain
UDI	Unique Device Identifier	CONTENTS	Contents
	Consult instructions for use	UK CA	UKCA mark
	Contains sufficient for <n> tests		
REF	Catalogue number		
LOT	Batch code		
	Use by date		

1. Intended Purpose

FagoFlowEx Kit is intended for the determination of phagocytic activity of neutrophil granulocytes by measuring respiratory (oxidative) burst in whole blood by flow cytometry.

What is detected and/or measured

The device detects and measures two parameters using fluorogenic substrate Dihydrorhodamine 123:

- percentage of neutrophil granulocytes that produce reactive oxygen species (ROS) in response to ingestion of *E. coli* bacteria
- intracellular activity of ROS-producing enzymes.

Device function

The device is intended for screening/aid to diagnosis of congenital or acquired immunodeficiency.

Context of a physiological or pathological state

Inability of neutrophil granulocytes to catalyze production of reactive oxygen species causes Chronic Granulomatous Disease (CGD), a group of inherited disorders with a common phenotype of recurrent severe bacterial and fungal infections and tissue granuloma formation [1, 2, 3, 4]. Results consistent with CGD may also originate from MPO deficiency which is the most common phagocyte defect presenting usually as a normal phenotype without increased incidence of infections [5].

A decrease of phagocytic activity without the defect in ROS producing enzymes occurs in various other clinical conditions that are associated with immune suppression, either primary variable immune deficiencies and plasma opsonin deficiencies, or secondary immunodeficiencies [6, 7].

Type of assay

Not automated

Quantitative

Type of specimen required

Human heparin-anticoagulated whole blood

Testing population

A patient with suspected defect of granulocyte function

2. Intended user

The device is intended for professional laboratory use only. Not for near-patient testing or self-testing.

Requirements on qualification

Intended user shall have state-of-the-art expertise in flow cytometry analysis of human cells, standard laboratory techniques including pipetting skills, safe and proper handling of specimens derived from the human body.

Intended user shall be compliant with standard EN ISO 15189 or other national provisions, where applicable.

3. Test principle

The test is based on measuring the production of ROS in neutrophil granulocytes using a fluorogenic substrate Dihydrorhodamine 123 (DHR123).

During the test a sample of human blood is incubated with heat inactivated *E. coli* bacteria and with DHR123. The reaction mixture is brought to 37 °C to promote phagocytosis of *E. coli* by neutrophile granulocytes. During the incubation the bacteria are actively engulfed by the cells while non-fluorescent DHR123 passively enters the intracellular environment by its concentration gradient. Bacteria become entrapped inside the cellular phagosomes and trigger enzymatic reactions that result in production of ROS. ROS ions oxidize DHR123 to fluorescent rhodamine 123 (R123) that is excited by the laser beam from a flow cytometer during acquisition of a blood specimen. Subsequent emission of light from R123 corresponding to the intracellular activity of ROS-producing enzymes is collected and analyzed by flow cytometer.

Two other reactions are performed in parallel to the *E. coli* stimulation, the negative control reaction, which is the reaction without *E. coli*, and the positive control reaction, which is a reaction that uses Phorbol 12-myristate 13-acetate that activates ROS producing enzymes without phagocytosis.

The cells are considered actively phagocyting if their fluorescence exceeds the fluorescence of cells from the negative control reaction. The result is reported as percentage of phagocyting cells. Fluorescence intensity of phagocyting cells is directly proportional to the intracellular activity of ROS-producing enzymes.

4. Reagent(s) provided

Contents

The device FagoFlowEx Kit, sufficient for 100 tests, is provided with the following reagents:

E. coli (5 vials) containing lyophilized *E. coli* bacteria, 1 vial is sufficient for stimulation of 20 blood samples (ED7042-1).

DHR123 (5 vials) containing lyophilized Dihydrorhodamine 123, 1 vial is sufficient for staining of 60 blood samples (ED7042-2).

Stimulation Control (5 vials) containing lyophilized PMA (Phorbol 12-myristate

13-acetate), 1 vial is intended for 20 positive control tests (ED7042-3).

Lysing Solution (1 bottle) containing 15 ml of ready to use solution (ED7042-4).

5. Materials required but not provided

Round bottom test tubes (12 x 75 mm)

Deionized water (Reagent-grade)

6. Equipment required

Automatic pipette with disposable tips (10 – 1000 µl) for pipetting specimen and reagents

Vortex mixer

Thermostat (air incubator) or water bath able to incubate test tubes at 37 °C

Flow cytometer laser excitation source (488 nm), detectors for scatters, optical filters and emission detector appropriate to collect signal from fluorochrome provided in Table 1.

Table 1 Spectral characteristic of fluorochrome used in the device

Fluorochrome	Excitation [nm]	Emission [nm]
Rhodamine 123	488	525

NOTICE: The device was tested on flow cytometers BD FACSCanto™ II (BD Biosciences), BD FACSLyric™ (BD Biosciences), Navios EX (Beckman Coulter), DxFLEX (Beckman Coulter) and Sysmex™ XF-1600 (Sysmex Corporation).

7. Storage and handling

Store at 2-8 °C.

Avoid prolonged exposure to light.

Do not freeze.

See Section 10 Procedure (Reagent Preparation) for information about In-Use stability and shelf-life following the first opening, together with the storage conditions and stability of working solutions (where applicable).

8. Warnings, precautions and limitations of use

GHS Hazard Classification

WARNING: Lysing Solution (ED7042-4) contains formaldehyde (CAS No. 50-00-0) and methanol (CAS No. 67-56-1) in concentrations classified as hazardous.

Label elements	Signal word
 	**Danger**
H-phrases	H302 Harmful if swallowed. H315 Causes skin irritation. H317 May cause an allergic skin reaction. H319 Causes serious eye irritation. H335 May cause respiratory irritation. H341 Suspected of causing genetic defects. H350 May cause cancer.
P-phrases	P201 Obtain special instructions before use. P264 Wash hands and exposed parts of the body thoroughly after handling. P280 Wear protective gloves/protective clothing/eye protection. P301+P312 IF SWALLOWED: Call a doctor if you feel unwell. P302+P352 IF ON SKIN: Wash with plenty of water and soap. 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. P333+P313 If skin irritation or rash occurs: Get medical advice/attention. P362+P364 Take off contaminated clothing and wash it before reuse.

Consult Safety Data Sheet (SDS) available on the product page at www.exbio.cz for the full information on the risks posed by chemical substances and mixtures contained in the Product and how they should be handled and disposed.

Biological Hazard

Human biological samples and blood specimens and any materials coming into contact with them are always considered as infectious materials.

Use personal protective and safety equipment to avoid contact with skin, eyes and mucous membranes.

Follow all applicable laws, regulations and procedures for handling and disposing of infectious materials.

Evidence of deterioration

Normal appearance of the lyophilized reagents provided is a white powder (E. coli and Stimulation Control) or a solid lyophilized cake (DHR123). Do not use the reagent if you observe any change in appearance, for example a color change or liquefaction.

Normal appearance of the Lysing solution is a clear liquid. Do not use the reagent if you observe any change in appearance, for example turbidity or signs of precipitation.

Limitation of use

Do not use after the expiry date stated on the product labels.

9. Specimen

Use venous peripheral blood collected in specimen receptacle classified as a medical device, with the presence of heparin anticoagulant.

> **CAUTION:** Anticoagulants EDTA and citrate negatively affect results of the analysis.

Blood specimen in the collection tube must be stored at room temperature. Do not refrigerate.

Process the blood specimen no later than 24 hours after collection.

10. Procedure

Preparation of reagent(s) provided

E. coli

Reconstitute the content of *E. coli* vial in 250 µl deionized water. Prepare fresh each measuring day, store at 2-8 °C and use it within the next 8 hours. Alternatively, the reagent can be frozen at -20 °C to -80 °C and used within 7 days.

CAUTION: Avoid repeated freeze/thaw cycles.

DHR123

Reconstitute the content of DHR123 vial in 650 µl deionized water. Prepare fresh each measuring day, store at 2-8 °C and use it within the next 8 hours. Alternatively, the reagent can be frozen at -20 °C to -80 °C and used within 7 days.

NOTICE: Aliquoted solution will sustain up to 5 freeze/thaw cycles.

Stimulation Control

Reconstitute the content of Stimulation Control in 250 µl deionized water. Prepare fresh each measuring day, store at 2-8 °C and use it within the next 8 hours. Alternatively, the reagent can be frozen at -20 °C to -80 °C and used within 7 days.

NOTICE: Aliquoted solution will sustain up to 5 freeze/thaw cycles.

Lysing Solution

Reagent is ready to use.

NOTICE: Bring the reagent to room temperature prior to use.

Specimen staining

1. For the examination of one patient, label three 12 x 75 mm round bottom test tubes with the appropriate sample identification and marking for

   E. coli stimulated reaction,

   positive control reaction (PMA stimulation)

   and negative control reaction.

   Pipette to the bottom of the test tubes

   - 10 µl of E. coli into the tube marked as E. coli stimulated reaction.
   - 10 µl of Stimulation Control into the tube marked as positive control reaction.
   - Do not pipette anything into tube marked as negative control reaction.

2. Pipette 50 µl of well-mixed blood specimen to the bottom of each of the test tubes and vortex gently.

   CAUTION: Avoid pipetting blood on the side of the test tube. If blood smear or droplet remains on the side of the tube, it may not be stained with the reagent or erythrocytes may not be lysed and the test result may not be valid.

3. Pipette 10 µl of DHR123 to the bottom of each of the test tubes and vortex gently.
4. Place the test tubes to 37 °C for 20 minutes in a water bath or for 30 minutes in an air incubator.
5. Add 50 µl of Lysing Solution to each of the test tubes. Vortex gently and incubate the test tubes for 5 minutes at room temperature in the dark.
6. Add 1 ml of deionized water into each of the test tubes, vortex gently, and incubate for 10 minutes at room temperature in the dark.
7. Acquire the stained sample immediately on the flow cytometer. If the stained sample will not be acquired immediately, cap the test tube, store at 2-8 °C in the dark and analyze within 2 hours.

   CAUTION: The fluorescence of Rhodamine 123, produced by oxidation of DHR123, is detected in FITC channel (525 nm). Since the Rhodamine 123 is quickly released from granulocytes as shown in Figure 8, samples need to be **measured as soon as possible** (no later than 2 hours after lysis) preferable **in a standardized narrow time window** (see page 18).

   CAUTION: Vortex the stained sample immediately before acquisition on the flow cytometer to avoid aggregates.

Flow cytometry analysis

The flow cytometer selected for use with the device FagoFlowEx Kit shall be calibrated on a routine basis using fluorescent microbeads to ensure stable sensitivity of detectors according to the cytometer manufacturer's instructions.

If not maintained properly the flow cytometer may produce false results.

Refer to the manufacturer's cytometer specifications for lasers and fluorescence detectors according to the excitation and emission characteristics of the fluorochromes in Section 6 Equipment required.

Set voltages on the fluorescence detectors of interest prior to stained specimen analysis. Voltage on a PMT detector should be set high enough, so that minimum of negatively stained events interfere with 0th channel on the fluorescence axis. Also, PMT detector voltage should not exceed values at which positive events are pressed to right axis.

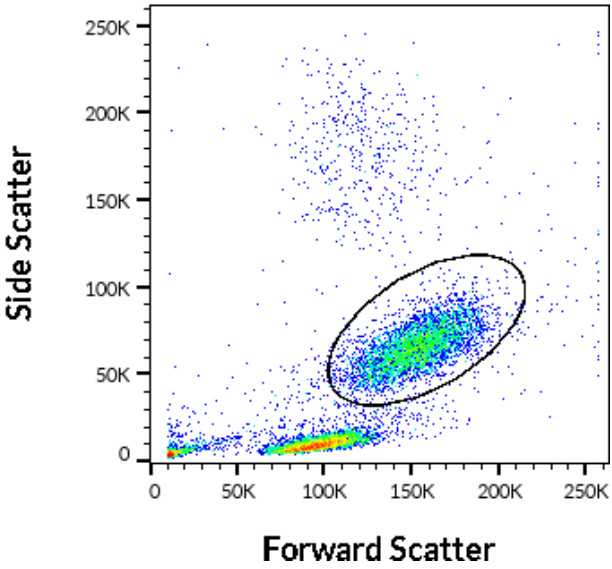
For measured data analysis, it is possible to use cytometer software developed by the manufacturer, or software dedicated for offline cytometry data analysis (for example FlowJo™, VenturiOne®, Infinicyt™).

Analysis of a patient's sample

Acquire at least 5.000-10.000 leukocyte events. Visualize acquired events in the side-scatter (SSC) versus forward-scatter (FSC) dot-plot. Set the gate around granulocytes as shown in Figure 1.

CAUTION: Ingestion of bacteria influences the position of granulocytes in the SSC-FSC dot-plot. Due to this, adjust the gate individually for each reaction.

Figure 1 Delineation of granulocyte population



Visualize gated granulocytes as histograms where the X-axis represents fluorescence intensity in FITC channel. Use the negative control reaction to set an appropriate gate to discriminate positive (actively phagocyting ROS producing cells) and negative (non-phagocyting non-ROS producing cells) granulocytes. Copy the gate to the E. coli stimulation reaction and to the positive control reaction (Figure 2a, 2b, 2c).

Granulocytes which undergo the oxidative burst exhibit bright fluorescence of Rhodamine 123. Calculate the mean fluorescence intensity of positive and negative granulocytes. The fluorescence intensity is directly proportional to the intracellular activity of ROS-producing enzymes.

Figure 2a Histogram of granulocyte fluorescence intensity from negative control reaction

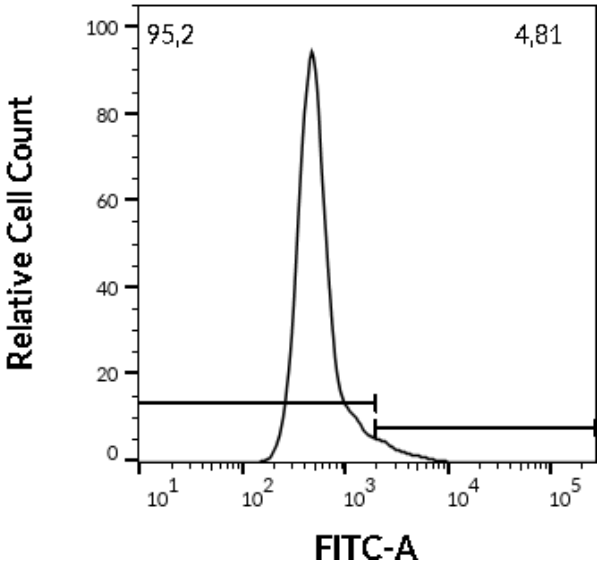


Figure 2b Histogram of granulocyte fluorescence intensity from E. coli stimulated reaction

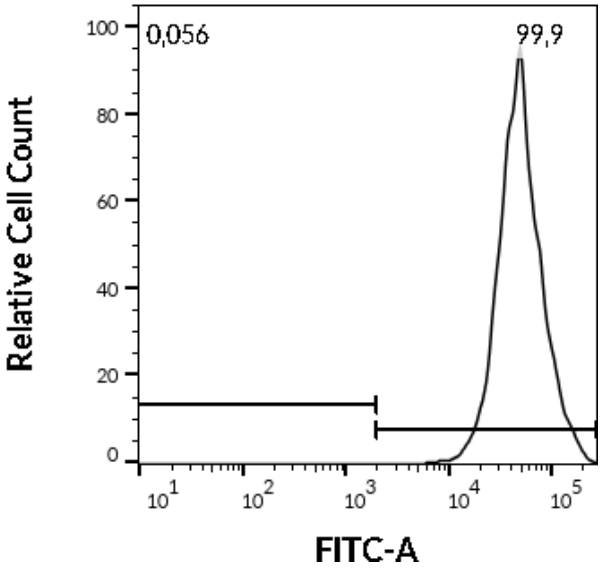
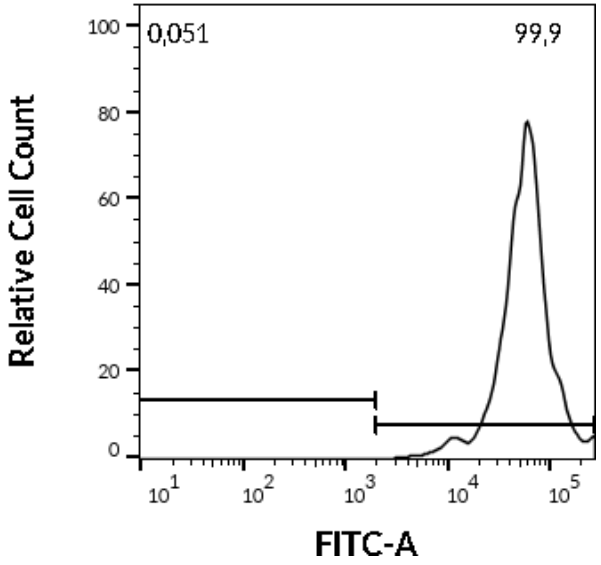


Figure 2c Histogram of granulocyte fluorescence intensity in positive control reaction



Calculation and interpretation of analytical results

Quantitative parameters

Two quantitative parameters are reported and interpreted in terms of whether there is any indication of a defect in phagocytic activity or a defect in ROS production:

a) Relative number of positive granulocytes which exhibited respiratory burst after the E. coli stimulation.

b) Stimulation index (SI) calculated as the mean fluorescence intensity (MFI) ratio of positive granulocytes of *E. coli* stimulated reaction and negative granulocytes of negative control reaction.

Example of Stimulation Index calculation

Table 2 Negative control reaction: MFI of negative and positive granulocytes

Population	Number (%)	Mean FITC-A
negative	95.2	550
positive	4.81	3995

Table 3 *E. coli* stimulated reaction: MFI of negative and positive granulocytes

Population	Number (%)	Mean FITC-A
negative	0.056	1224
positive	99.9	53836

Ratio of MFI value of positive granulocytes from *E. coli* stimulated reaction (in Table 3) is divided by MFI value of negative granulocytes from negative control reaction (in Table 2).

$$\frac{\text{MFI value of positive granulocytes from } E.\,coli \text{ stimulated reaction}}{\text{MFI value of negative granulocytes from negative control reaction}} =$$

$$\frac{53836}{550} = \text{SI (Stimulation Index)} = 98$$

Qualitative parameters

Qualitative data interpretation incorporates histogram overlays to evaluate the signal distribution and to identify individual peaks that, in the case of occurrence of multiple granulocyte populations, need to be analyzed separately.

In case of **respiratory burst defects** (lack of oxidation of the DHR123) the resulting granulocyte histograms will show signal distribution agreement between *E. coli* stimulation reaction and positive control reaction (Figure 4, 5, 6).

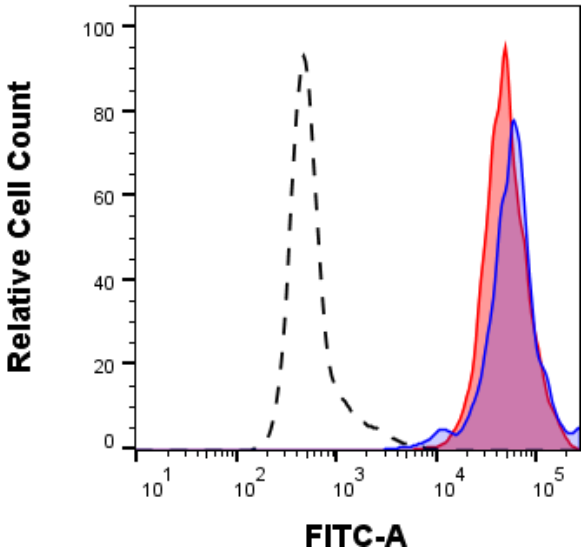
In case of **phagocytic activity defects** (decreased particle engulfment) the resulting granulocyte histograms will show signal distribution discrepancy between *E. coli* stimulation reaction and positive control reaction. *E. coli* stimulated reaction will have the granulocyte population divided into multiple peaks of different fluorescence intensities, positive control reaction will have a single peak (Figure 7).

> **NOTICE:** Detection of unusual results only indicates the suspicion of disease which needs to be confirmed by other tests.

Normal result of healthy donor

Granulocytes exhibit high respiratory burst after stimulation with **both** *E. coli* and positive control reaction (Figure 3).

Figure 3 Histogram overlay: Healthy donor without defect of respiratory burst, (SI = 98, relative number of positive granulocytes 99.9 %). Signal distribution of E. coli stimulated granulocytes (red-filled), granulocytes from negative control reaction (black-dashed) and granulocytes from positive control reaction (blue-filled)n in FITC detector.



Results indicating respiratory burst defect

1) A single peak with low signal intensity

If granulocytes exhibit low respiratory burst after stimulation with **both** *E. coli* and positive control reaction, it indicates either **myeloperoxidase (MPO) deficiency** (Figure 4) or less common **chronic granulomatous disease (CGD)** (Figure 5). The intensity of the respiratory burst in CGD depends on the mutation in NADPH oxidase enzyme complex. There are five autosomal recessive types (1-5) and one X-linked recessive type of the disease.

CAUTION: The assay cannot differentiate between CGD and MPO deficiency.

Figure 4 Histogram overlay: Patient with MPO deficiency, (SI = 11, relative number of positive granulocytes 89.7 %). Signal distribution of E. coli stimulated granulocytes (red-filled), granulocytes from negative control reaction (black-dashed) and granulocytes from positive control reaction (blue-filled) in FITC detector.

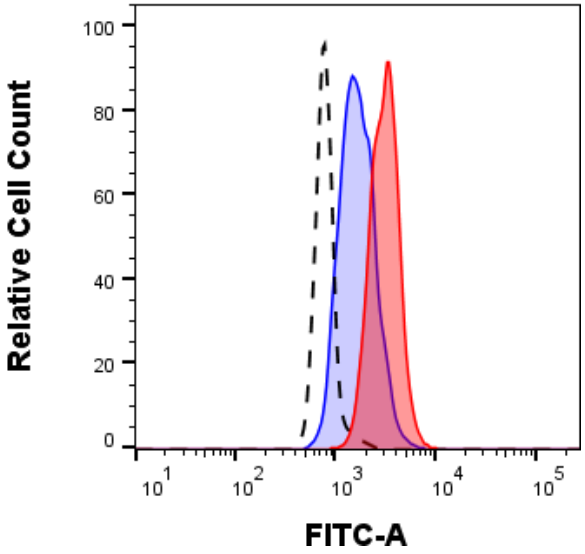
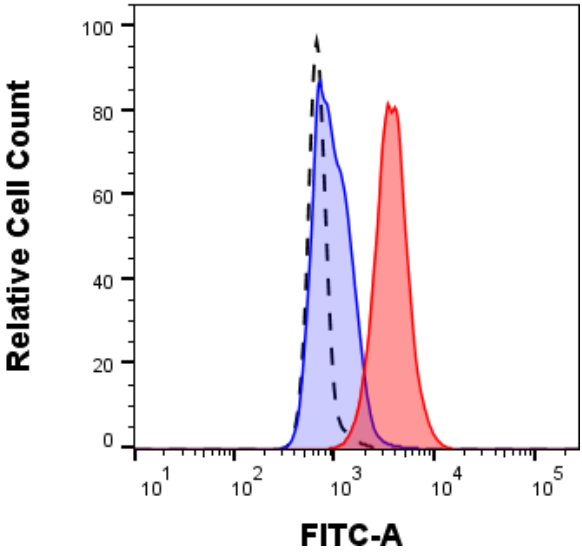


Figure 5 Histogram overlay: Male patient with X-linked CGD, (SI = 16, relative number of positive granulocytes 99 %). Signal distribution of E. coli stimulated granulocytes (red-filled), granulocytes from negative control reaction (black-dashed) and granulocytes from positive control reaction (blue-filled) in FITC detector.

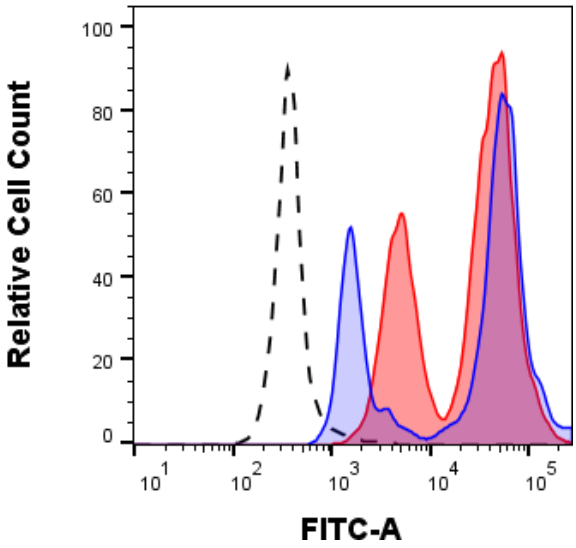


2) Two peaks with different signal intensity

If granulocytes of a female patient **exhibit two subpopulations** differing in intensity of respiratory burst after stimulation with both *E. coli* and positive control reaction (Figure 6), it indicates that the patient is a carrier of X-linked CGD.

> **CAUTION:** Three and more peaks in histogram indicate a contamination of granulocyte population gate in SSC vs. FSC dot-plot (Figure 1) with monocytes or with dead non-phagocyting cells.

Figure 6 Histogram overlay: Female carrying X-linked mutation of the NADPH oxidase gene. Two granulocyte subpopulations differ in respiratory burst intensity, (low MFI peak SI = 14, relative number of granulocytes 35 %, high MFI peak SI = 125, relative number of granulocytes 65 %). Signal distribution of *E. coli* stimulated granulocytes (red-filled), granulocytes from negative control reaction (black-dashed) and granulocytes from positive control reaction (blue-filled) in FITC detector.

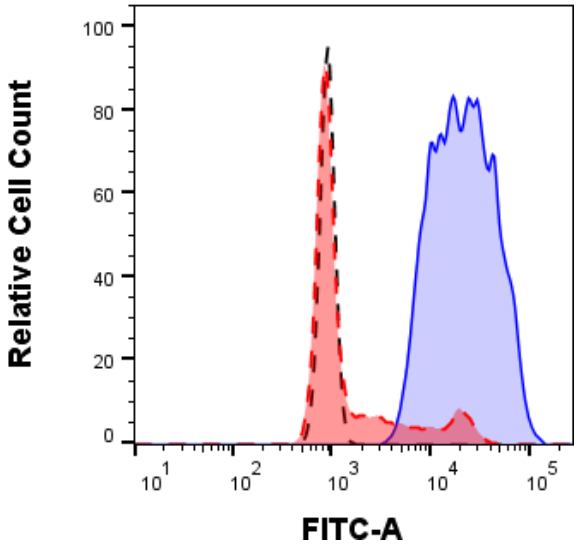


Results indicating phagocytic activity defect

Different peak pattern between the positive control reaction and the E. coli stimulated reaction

If granulocytes stimulated with *E. coli* exhibit low respiratory burst and granulocytes stimulated with positive control reaction show high respiratory burst (Figure 7), it indicates a defect in granulocyte phagocytic activity, alternatively the blood sample contains anticoagulant EDTA or citrate or the sample was old or improperly stored.

Figure 7 Histogram overlay: Sample anticoagulated with EDTA, (low MFI peak SI = 1, relative number of granulocytes 73 %, high MFI peak SI = 25, relative number of 9 %). Signal distribution of E. coli stimulated granulocytes (red-filled), granulocytes from negative control reaction (black-dashed) and granulocytes from positive control reaction (blue-filled) in FITC detector.



11. Analytical performance

Precision (repeatability and reproducibility)

Reproducibility of the assay was determined from data obtained by five operators analyzing six blood samples of healthy blood donors on the same day under the same experimental conditions.

The following parameters were calculated:

a) For the determination of relative number of positive granulocytes

CV = 2 %

b) For the determination of Stimulation index

CV = 11 %

Repeatability of the assay was not determined. Due the dynamic of MFI changes associated with R123 release from cells (Figure 8, 9, 10), the repeatability values would depend on the time that passed between the end of the sample processing (fixation/RBC lysis) and FACS analysis. It is recommended to perform analysis of small sample series and analyze them within the standardized narrow time window. Alternatively larger series can be analyzed later, e.g. after a larger time gap (40 minutes) to minimize the MFI variability.

Figure 8 Granulocyte mean fluorescence intensity (MFI) development in time after red blood cell lysis, one blood specimen as an example (healthy donor).

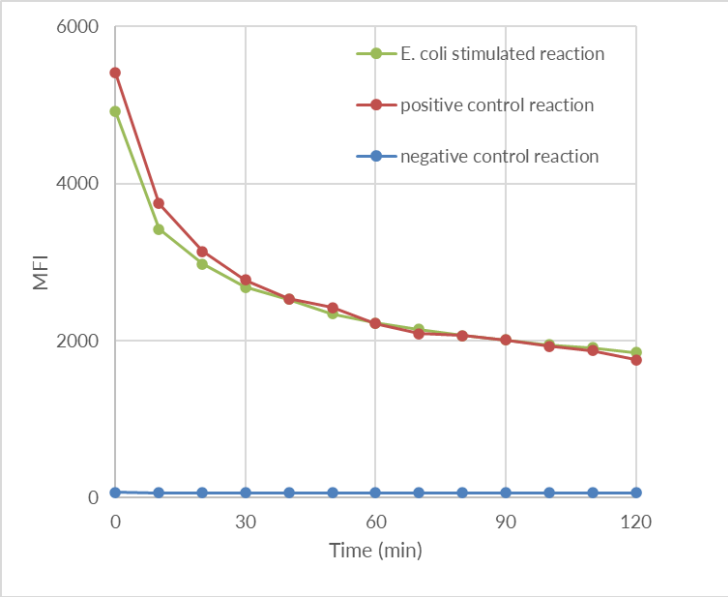


Figure 9 Granulocyte phagocytic activity (%) development in time after red blood cell lysis, 3 different blood specimens (healthy donors).

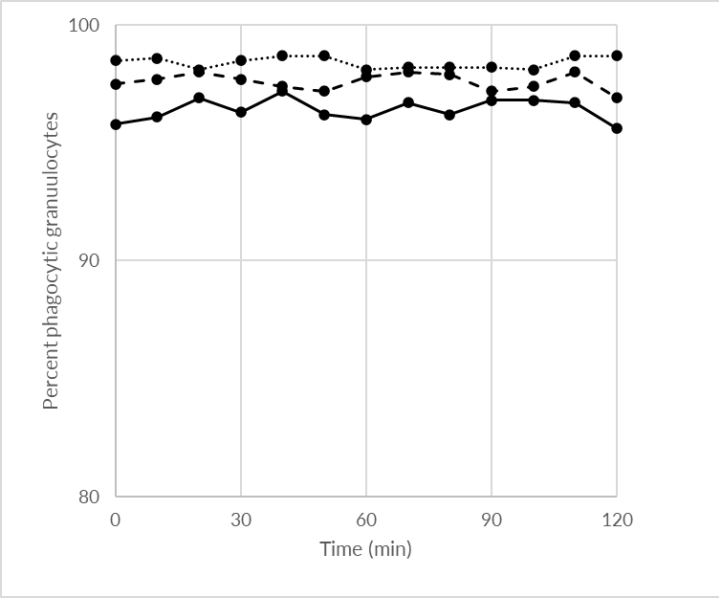
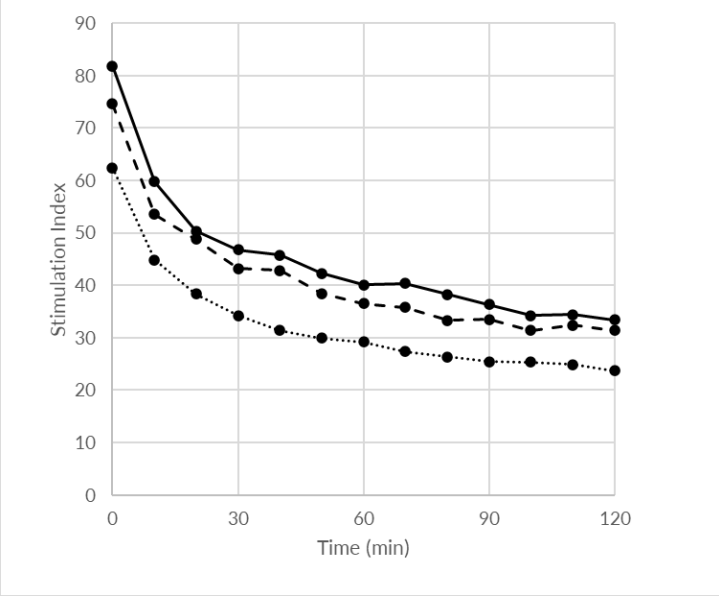


Figure 10 Stimulation Index development in time after red blood cell lysis, 3 different blood specimens (healthy donors).



12. Clinical performance

The assay was evaluated by comparative measurements to PhagoBurst (Orpegen Pharma GmbH) using specimen from total of 47 patients (Table 4). Both kits were able to detect: a) failure of particle ingestion (low phagocytic activity) and b) disorders of oxidative burst (MPO deficiency, CGD) with 100% sensitivity and 100% specificity.

Table 4 Patient characteristics in the performance evaluation study

Patient characteristic	n
Healthy donor (unrelated immunological disorder)	40
CGD (2 diseased and one CGD carrier)	3
MPO deficiency	2
Low phagocytic activity (disease model - EDTA anticoagulant)	2
Old samples (repeated measurements of healthy donors in 48 hours)	4

13. Expected values

Normal range of respiratory burst activity of granulocytes has been determined in 40 peripheral blood samples of healthy adults.

- Granulocytes with respiratory burst activity
  90-100 %
- Stimulation index of granulocytes > 30
  3^{rd} percentile = 31
  Median = 56
  97^{th} percentile = 97

Since the stimulation index may vary in different laboratories and instruments, each laboratory MUST establish the normal range using its own test conditions on samples from the local population of normal donors.

14. Interfering substances and limitations

Anticoagulants EDTA and citrate negatively affect results of the analysis.

15. References

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2) de Oliveira-Junior EB, et al. The human NADPH oxidase: primary and secondary defects impairing the respiratory burst function and the microbicidal ability of phagocytes. Scand J Immunol. 2011 May;73(5):420-7. doi: 10.1111/j.1365-3083.2010.02501.x. PMID: 21204900.
3) Song E, et al. Chronic granulomatous disease: a review of the infectious and inflammatory complications. Clin Mol Allergy. 2011 May 31;9(1):10. doi: 10.1186/1476-7961-9-10. PMID: 21624140; PMCID: PMC3128843.
4) Kuijpers T, et al. Inflammation and repeated infections in CGD: two sides of a coin. Cell Mol Life Sci. 2012 Jan;69(1):7-15. doi: 10.1007/s00018-011-0834-z. Epub 2011 Nov 15. PMID: 22083605; PMCID: PMC3249194.
5) Klebanoff SJ. Myeloperoxidase: friend and foe. J Leukoc Biol. 2005 May;77(5):598-625. doi: 10.1189/jlb.1204697. Epub 2005 Feb 2. PMID: 15689384.
6) Rotrosen D, et al. Disorders of phagocyte function. Annu Rev Immunol. 1987;5:127-50. doi: 10.1146/annurev.iy.05.040187.001015. PMID: 3297103.
7) Donabedian, H. (1989). Congenital and Acquired Neutrophil Abnormalities. In: Klempner, M.S., Styrt, B., Ho, J. (eds) Phagocytes and Disease. Immunology And Medicine Series, vol 11. Springer, Dordrecht. https://doi.org/10.1007/978-94-009-1279-3_6

16. Trademarks

17. Revision History

Version 8, ED7042_IFU_v8

IFU layout changed.

18. Manufacturer

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NOTICE: Any serious incident that has occured in relation to the device shall be reported to the manufacturer and the local competent authority.