

SUMMARY and EXPLANATION

This test allows the quantitative flow cytometric determination of the chemotactic function of neutrophilic granulocytes. In analogy to the Boyden chamber technique, this kit contains multiwell plates and precoated cell culture inserts for chemotaxis studies, the chemotactic peptide N-formyl-Met-Leu-Phe (fMLP), an antibody reagent, counting beads and necessary reagents. It allows determination of the number of neutrophils which have migrated through cell culture inserts towards a concentration gradient of the chemoattractant fMLP. Also, the decrease of expression of the cell adhesion molecule L-selectin can be analysed together with the shape-change of the cells by analysing the changes of the forward scatter signals.

The detailed instructions result from specific experience and precise validation assays. Critical steps and hints are in bold letters.

APPLICATIONS

By MIGRATEST™ it is intended to investigate the altered chemotactic function of neutrophils found in various disorders and to evaluate the effects of drugs on this process.

Abnormal chemotaxis was observed and described with a variety of clinical disorders (3, 4). These defects are usually accompanied by recurrent infections of the skin or respiratory tract. Clinical syndromes with frequent infections are already observed in the infant. A functional chemotaxis defect diminishes the number of granulocytes which migrate from the circulation into inflamed tissues and therefore the amount of active cells at the sites of inflammation.

Known inborn defects are Leukocyte Adhesion Deficiency (5), Hyperimmunoglobulin E or Job's syndrome and Chédiak-Higashi syndrome (CHS). These disorders are associated with a reduced chemotactic activity of neutrophilic granulocytes (6, 7).

Acquired defects can be observed in renal failure, diabetes, hepatic cirrhosis (8) and Hodgkin's disease. Circulating immune complexes in rheumatoid arthritis also inhibit chemotaxis (10).

Various drugs and immunomodulators can enhance or decrease the chemotactic activity of granulocytes. These effects can be investigated in vitro or ex vivo by application of MIGRATEST™.

PRINCIPLES of the PROCEDURE

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

MIGRATEST™ allows the quantitative determination of neutrophil chemotaxis. The number of cells can be determined which have migrated through cell culture inserts with a pore size of 3.0 µm towards a concentration gradient of the chemoattractant fMLP. In parallel, the decrease of L-selectin (11) expression can be measured. Downregulation of this cell adhesion molecule correlates directly with the activation of neutrophils by exposing to chemotactic factors. The shape-change of the cells precedes the cell migration and can be measured by analysing the changes of the forward scatter signals using flow cytometry. The test kit contains the chemoattractant fMLP, cell culture inserts and necessary reagents. Leukocytes are isolated from heparinized whole blood by spontaneous sedimentation over reagent A (leukocyte separation medium) and placed into cell culture inserts. Chemotaxis is conducted at 37°C towards a gradient of fMLP in comparison to a control of reagent B (incubation buffer). The cells are then stained with reagent D (anti-L-selectin antibody reagent) which also contains counting beads. Just prior flow cytometric analysis a special vital DNA dye (reagent E) is added.

In summary, chemotaxis is the initiation of the multistep and multifactorial process of phagocytosis (1, 2). The whole cascade of events can be investigated individually under controlled conditions by separate assays: MIGRATEST™ to measure chemotaxis, PHAGOTEST™ to measure ingestion of microbes, BURSTTEST (PHAGO-BURST™) to measure oxidative burst.

MATERIAL and REAGENTS

The reagent kit contains:

SORB

24 specially treated **cell culture inserts** (3.0 µm pore size), preloaded in two 24-well dishes.

REAG A

12 vials (1 ml) containing leukocyte **separation medium** for isolation of leukocyte-rich plasma (LRP).

REAG B

1 vial (20 ml) containing **incubation buffer**.

REAG C

1 vial (100 µl) containing the **chemotactic peptide fMLP** (200 x stock solution, 10 µM). Dilute 5 µl in 1 ml of reagent B for use.

REAG D

1 bottle (0.9 ml) **antibody and counting reagent** contains the monoclonal antibody anti-L-selectin-FITC and counting beads.

REAG E

1 vial (100 µl) containing **vital DNA staining solution**. Dilute 5 µl in 250 µl of reagent B.

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

1. Blood collection tubes containing **heparin anticoagulant**.
2. 1 or 2 ml disposable test tubes (e.g., Eppendorf, #0030 102.002 or Nunc, #341173).
3. 12 x 75 mm disposable test tubes (Falcon, Becton Dickinson No. #352052) and appropriate test tube racks.
4. Ice bath with cover.

Required apparatus:

1. Variable volume micropipettes 20 - 200 µl, 100 - 1000 µl and disposable tips.
2. Dispenser pipette and dispenser tips.
3. Waterbath.
4. Digital thermometer.
5. Vortex mixer.
6. 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 E contains sodium azide as preservative. Under acidic conditions, sodium azide yields hydrazoic acid, an extremely toxic and volatile compound. Solutions with azide should be diluted with tap-water before dis-

- posal to avoid deposits in plumbing, where explosive conditions may develop.
3. The reagent E contains ethanol.
Hazard statements: H225 Highly flammable liquid and vapour.
Precautionary statements: P210 Keep away from heat/sparks/open flames/hot surfaces. – No. smoking. H233 Keep container tightly closed.

STORAGE and STABILITY

Store the kit in the dark at 2 - 8° C (in refrigerator). **Cell culture inserts and multiwell plates** can be stored at **room temperature**. The reagent C and E working solutions have to be discarded after use. The reagents are supplied with a preservative that does not influence the chemotactic function of basophilic granulocytes. The reagents are stable for the period shown on the packaging label, when stored as described.

PROCEDURE**1. Preparations:**

- 1.1 Dilute the stock solutions with reagent B:
 - Reagent C, 1 : 200 (volume as needed, e.g., 5 µl in 1 ml, 350 µl per test).
 - Reagent E, 1 : 50 (e.g., 5 µl in 250 µl, 20 µl per test).
- 1.2 Prewarm water bath to 37°C (**precise temperature control!**).
- 1.3 Prepare ice bath.
- 1.4 Switch on and calibrate the flow cytometer.

2. Leukocyte isolation:

Collect 5 ml of **heparinized** whole blood from each patient and control person to be examined. Obtain blood with standard aseptic techniques. **DO NOT USE blood anticoagulated by EDTA or citric acid!**

- 2.1 Carefully overlay 1 ml of heparinized whole blood on top of reagent A.
- 2.2 The vial is left motionless at room temperature. After 40 min the following phases have formed: upper phase containing leukocytes and platelets (**leukocyte-rich plasma = LRP**) and lower phase containing erythrocytes. The sedimentation of erythrocytes takes a longer time when using older blood samples.
- 2.3 Remove approx. 500 µl of the leukocyte-rich plasma (upper phase), pipette the cell

suspension into one 1 ml or 2 ml test tube. The isolated cells should be used within 1 hour for the chemotaxis assay.

3. MIGRATEST™ set-up:

3.1 Prepare the following samples for each patient or control person by pipetting reagent B or reagent C working solution into two wells of a multiwell plate:

Negative control sample:

350 µl of reagent B

Positive control (test) sample:

350 µl of the reagent C working solution

3.2 Place cell culture inserts into both wells. Add **100 µl of leukocyte-rich plasma** into each cell culture insert.

3.3 Incubation:
The multiwell plate is incubated for **30 min at 37°C** in a **water bath** without shaking.

3.4 Precisely at the end of the incubation time remove manually the cell culture inserts from the multiwell plate. Add the cell suspensions from both wells of the multiwell plate to two separate 5 ml tubes (**tube 1** and **tube 2**) and place the tubes on ice. In addition, unstimulated cells from the leukocyte-rich plasma serve as **L-selectin control**. For this purpose, mix **20 µl of the cell suspension from the cell culture insert** of the **negative control sample** with **180 µl of reagent B** and transfer this mixture to a **third 5 ml tube (tube 3)**.

3.5 Labelling with reagent D:
Add **20 µl of reagent D to each tube (3 tubes per patient)**. Vortex and incubate the tubes for **10 min** in an **ice bath, covered to prevent exposure to light**.

3.6 Vital staining:
Add **20 µl of 1 x reagent E** per tube, mix and incubate **5 min on ice** (light protected in the ice bath).
Measure the cell suspension within 120 min.

4. 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:

Acquire data by using fluorescence **triggering in the FL3 channel**, set a **first region 1** on **leukocytes** and **counting beads** (dot plot diagram SSC/FL3, see Fig. 1A, 1B).

After activating a gate on region 1, set a second region 2 around the **counting beads** (dot

plot diagram **SSC/FSC**, see Fig. 2A, 2B); **start acquisition and end data acquisition by count, acquire exactly 2,000 events** so that the amount of granulocytes in the control sample can be compared with the number of granulocytes in the positive control sample after stimulation with fMLP. **Set a third region 3** around the **population of granulocytes** (dot plot diagram SSC/FSC, see Fig. 2A, 2B).

Data evaluation:

After setting a logical gate on granulocytes in region 1 and 3, the following two parameters are analysed for each negative control and positive control (test) sample:

(1) Number of cells

(2) Mean value of the FSC signal (only for the test sample, the FSC control signal is obtained from the L-selectin control sample)

The evaluation of the **L-selectin expression** is done in a separate FL1 histogram. For that purpose, use the L-selectin control sample to set a marker for fluorescence-1 (FL1) so that less than approx. 15% of the cells are positive (see Fig. 3A). Then, determine the percentage of activated granulocytes showing a decreased expression of L-selectin in the test sample (see Fig. 3B). Also, the FSC control signal has to be analysed in the L-selectin control sample (dot plot diagram SSC/FSC). Please refer to the following summarizing table for data evaluation.

| Parameter | Tube No. 1 | Tube No. 2 | Tube No. 3 |
|-----------|-----------------|-------------------------------|-------------------------------|
| 1 | Number of cells | Number of cells | - |
| 2 | - | Mean value of FSC signal | Mean value of FSC signal |
| 3 | - | Percentage of activated cells | Percentage of activated cells |

FIGURES

Recommended gating during data acquisition, data are acquired by using fluorescence triggering in the FL3 channel (red fluorescence > 630 nm). Region 1 set on leukocytes and counting beads (see Figures 1A and 1B).

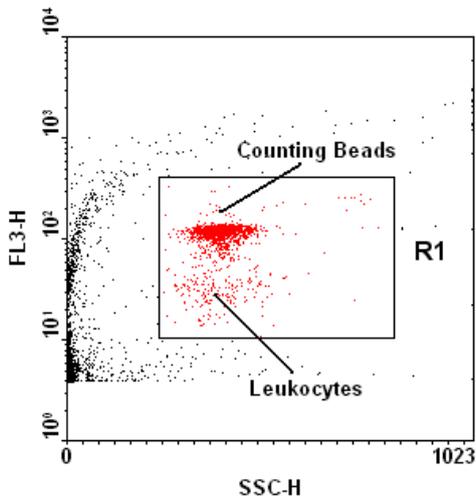


Figure 1A Region 1 set on leukocytes and counting beads, **negative control sample**

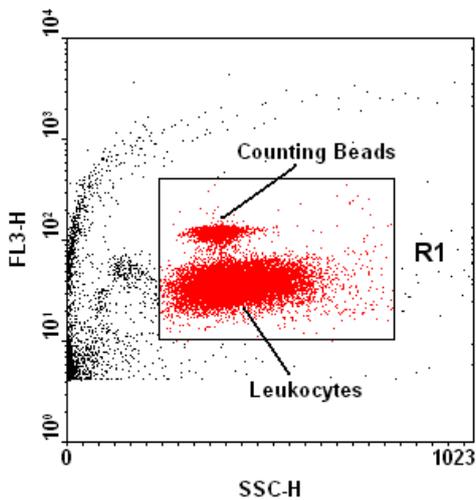


Figure 1B Region 1 set on leukocytes and counting beads, **positive control sample**

Typical dot plot (SSC/FSC) display during data acquisition and analysis, gate set on region 1 Region 2 set on counting beads, region 3 set on granulocytes (see Figures 2A and 2B).

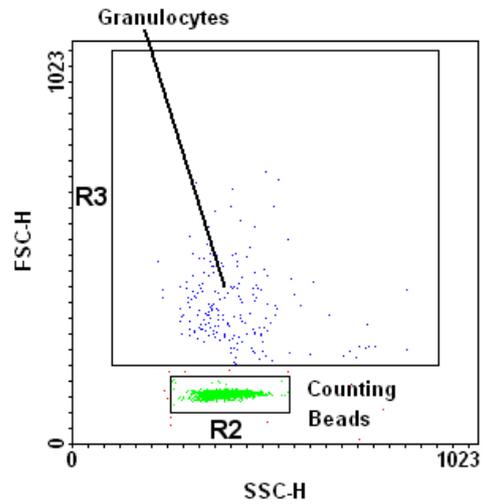


Figure 2A Region 2 set on counting beads, region 3 set on granulocytes, **negative control sample**

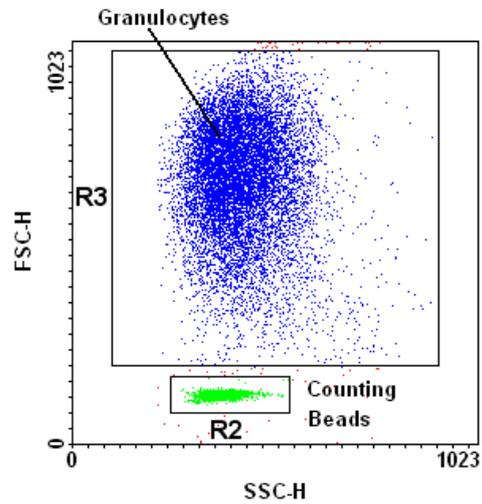


Figure 2B Region 2 set on counting beads, region 3 set on granulocytes, **positive control sample**
Note: Shape change of the granulocytes (FSC-H-signal)!"

FL1 histograms during data analysis, gate set on combination of region 1 and 3 (see Figures 3A and 3B).

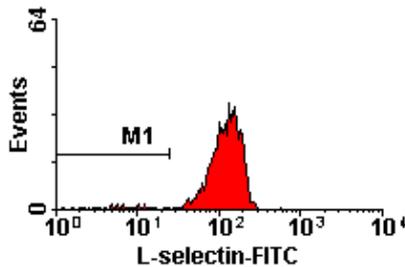


Figure 3A FL1 histogram of the granulocyte population in region 3, L-selectin control sample

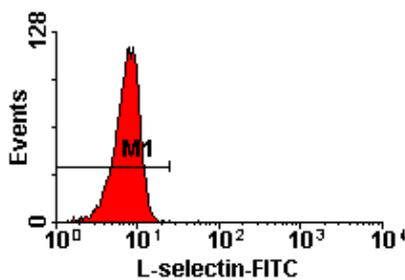


Figure 3B FL1 histogram of the granulocyte population in region 3, test sample

Note: Increase of the percentage of activated cells showing a reduced expression of L-selectin.

REMARKS

1. **Heparinized whole blood** should be processed **within 24 h of sampling**. **Blood samples** should remain at **room temperature** prior to processing.
2. Duplicate determinations are useful in establishing the assay.
3. The proposed test protocol allows to investigate the process of chemotaxis after an incubation time of 30 min. By extending the incubation time, higher values can be obtained for the parameter „number of cells migrated through cell culture inserts“.
4. When **testing the effects of drugs** on increasing or inhibiting the chemotactic function, it is useful to incubate at first the cell suspension with the drugs to be tested. Thereafter, the cells are stimulated by the chemoattractant fMLP.

PRECISION of the METHOD

The **intra-assay precision** of this assay was determined on triplicate test samples (stimulation with fMLP) from healthy individuals.

| | Number of cells after migration | Mean value of the FSC signal | % activated granulocytes |
|-----------------|---------------------------------|------------------------------|--------------------------|
| Range of values | 3,002 – 14,162 | 615 - 713 | 97.01 – 99.44 |
| Average CV (%) | 9.1 | 1.9 | 0.2 |
| n | 15 | 10 | 10 |

EXPECTED RESULTS

The **normal range** of the chemotactic activity of granulocytes was determined on **fresh blood samples** from **healthy subjects**.

| | Number of cells after migration | Mean value of the FSC signal | % activated cells |
|--------------------|---------------------------------|------------------------------|-------------------|
| L-selectin control | - | 250 – 450 | < 10% |
| Control sample | 100 – 1,500 | - | - |
| Test sample | 2,500 – 15,000 | 500 -750 | > 90% |

LIMITATIONS

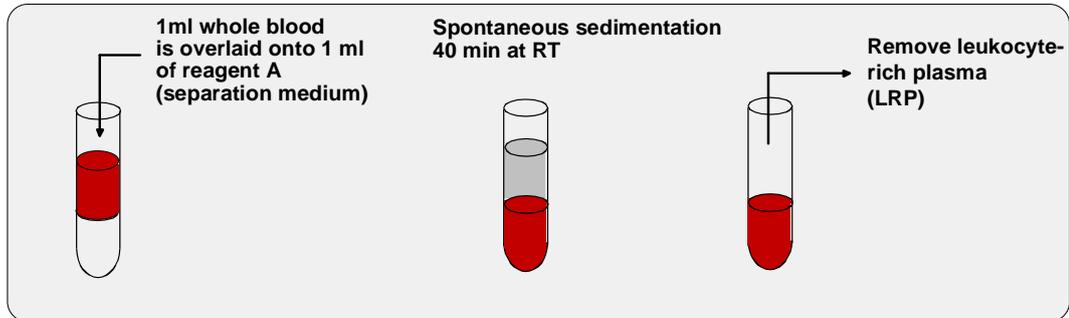
1. Laboratories should establish their own normal reference ranges using their own test conditions.
2. The samples should contain more than 95% viable cells and should be completely anticoagulated.
3. Samples ready for measurement are stable for 2 hours on ice after addition of the reagent E (vital DNA staining solution).

REFERENCES

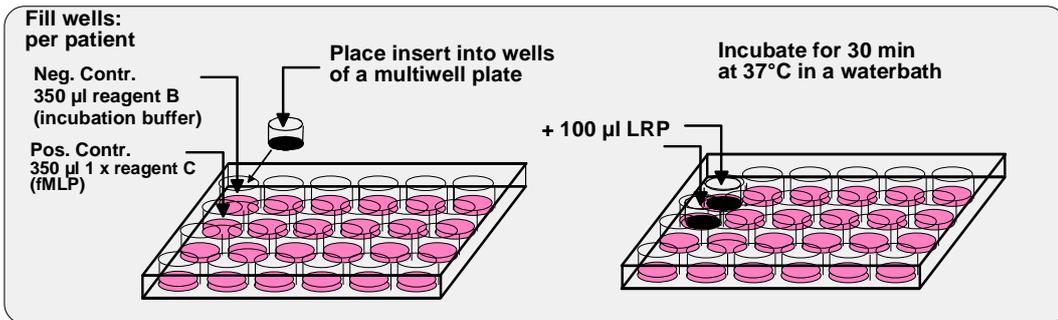
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MIGRATEST™ - Sample Preparation Procedure

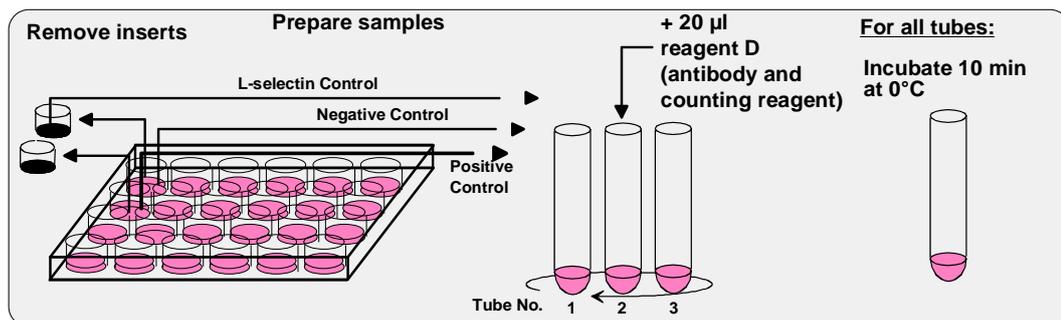
1. Leukocyte Isolation



2. Stimulation and Chemotaxis



3. Cell Labelling



4. DNA Staining, Measurement

