

# BASOTEST™

Reagent kit for the quantitative determination of the degranulation  
of basophilic granulocytes in heparinized human whole blood

Reagent kit containing antibodies, allergens and reagents for 100 tests



Glycotope Biotechnology GmbH  
Czernyring 22  
69115 Heidelberg  
Germany  
Tel. +49 (0)62 21 91 05-0  
Fax +49 (0)62 21 91 05-10  
E-Mail: [info@glycotope-bt.com](mailto:info@glycotope-bt.com)  
[www.glycotope-bt.com](http://www.glycotope-bt.com)

## Key to symbols used

	In Vitro Diagnostic Medical Device		Contains sufficient for <n> tests
	European Conformity		Temperature limitation
	Manufactured by		Consult instructions for Use
	Catalogue number		Use by
	Batch code		Reagent*
	Contains		

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

## SUMMARY AND EXPLANATION

This reagent kit allows the quantitative determination of basophil degranulation in heparinized human whole blood. It contains the chemotactic peptide N-formyl-Met-Leu-Phe (fMLP) as positive control, important allergens, a two-colour antibody reagent for assessing the activation of human basophilic granulocytes and necessary reagents. It determines the percentage of basophilic granulocytes which have degranulated after incubation with allergen or fMLP.

The evaluation of basophil degranulation should be performed by flow cytometry. The detailed instructions result from specific experience and precise validation assays. Critical steps are in bold letters.

## APPLICATIONS

BASOTEST™ is intended for investigation of allergen induced activation of basophilic granulocytes.

Basophilic granulocytes are the least common circulating leukocyte in blood and account for only 0.5 - 1% of the total white blood cell population (1-3). Immediate-type hypersensitivity (1-3) is characterized by allergic reactions immediately following contact with innocuous foreign substances (allergens or antigens). Allergic reactions are predominantly due to the immunoglobulin E (IgE) class of antibodies (4) and develop when contact with an antigen triggers the formation of specific IgE antibodies by B cells with T cell help. The antigen-specific IgE antibodies bind to the very high-affinity  $Fc_\epsilon$  receptors I in the membrane of tissue mast cells and basophilic leukocytes (5). Renewed contact with the same antigen then leads to bridging: adjacent antibodies on the cell surface of sensitized cells are crosslinked by antigen molecules. This bridging of IgE molecules on the basophil cell surface by antigen or allergen activates the cell to secrete a number of pre-formed chemical mediators stored in secretory granules, such as histamine, heparin, neutral protease, and a number of acid hydrolases and chemotactic factors. Secondary mediators (leukotrienes and cytokines) are also generated as a result of cell activation. Allergic rhinitis or hay fever, asthma bronchiale, systemic anaphylaxis and urticaria are typical allergic diseases.

The immune response to environmental allergens is believed to depend on multiple factors including genetic, developmental, and environmental factors (6, 7, 8).

The flow cytometric method correlates well with histamine release assays. BASOTEST™ there-

fore allows the diagnosis of immediate-type hypersensitivity (type I reactions), especially in response to natural allergens. Furthermore, the success of immunotherapies (e.g., hyposensitisation) may be monitored. However, this test is not suited to detect any delayed type hypersensitivity (e.g., type IV reactions).

## PRINCIPLES OF THE PROCEDURE

BASOTEST™ allows the quantitative determination of human basophil degranulation (1-3).

The test kit contains the chemotactic peptide N-formyl-Met-Leu-Phe (fMLP) as positive control (8), important allergens, a two-colour antibody reagent for detection of human basophilic granulocytes and determination of basophil activation and necessary reagents. Heparinized whole blood is incubated first with reagent B (stimulation buffer) for 10 min at 37°C, then with allergen at various concentrations for 20 min at 37°C. Reagent E contains the chemotactic peptide N-Formyl-Met-Leu-Phe (fMLP) which is used as a positive control, addition of reagent A (wash solution) serves as negative background control. Activation of basophilic granulocytes induces fusion of cytoplasmic granules with the plasma membrane and the successive release of inflammatory mediators. The degranulation process is stopped by incubating the whole blood samples on ice. The cells are then labelled reagent F which is a two-colour antibody reagent consisting of two different murine monoclonal antibodies conjugated with various fluorochromes. The monoclonal antibody anti-IgE-PE is conjugated with the fluorescent dye Phycoerythrin, reacts with human IgE and therefore detects basophilic granulocytes. The monoclonal antibody anti-CD63-FITC is conjugated with the fluorochrome fluorescein and recognizes a glycoprotein (gp53) expressed on activated basophils. After staining of basophils with this antibody reagent, erythrocytes are removed by addition of reagent G (lysing solution). After one washing step with reagent A (wash solution), the percentage of activated basophilic granulocytes is determined by flow cytometry.

## MATERIAL AND REAGENTS

The reagent kit contains:

### REAG A

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

### REAG B

1 vial containing lyophilized **stimulation buffer** to be reconstituted by addition of 2 ml of ultrapure, apyrogenic water. Store in aliquots at -20°C after reconstitution.

### REAG C

1 vial (200 µl) containing the allergenic extract **Mite mix**, concentrated stock solution. Dilute 10 µl in 1 ml of WASHING SOLUTION for use.

### REAG D

1 vial (200 µl) containing the allergenic extract **7 grass mix**, concentrated stock solution. Dilute 10 µl in 1 ml of WASHING SOLUTION for use.

### REAG E

1 vial (200 µl) containing the chemotactic **peptide fMLP** (200 x stock solution, 0.4 mM). Dilute 10 µl in 2 ml of 1 x wash solution (Reagent A).

### REAG F

1 bottle (2 ml) two-colour antibody reagent, contains two monoclonal antibodies: **anti-IgE-PE** and **anti-CD63-FITC**

### REAG G

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.

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 G).

4. Ice bath with cover.
5. Ultrapure, apyrogenic water or water for injection for reconstitution of wash solution (reagent A).
6. Double distilled water for dilution of 10 x lysing solution (reagent G).
7. Various other allergens or allergen mixtures.

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 ethylenediaminetetraacetic 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). Ethylenediaminetetraacetic acid sodium salt is irritating to eyes (R36) and may cause long-term adverse effects in the aquatic environment (R 52/53). Avoid release to the environment. Refer to special instructions/Safety data sheets (S61).
3. The reagent E contains dimethylsulfoxide (DMSO). Dimethylsulfoxide 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 F 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.

5. The reagent G 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 (R32). 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). Diethylene glycol is harmful if swallowed (R22). If swallowed, seek medical advice immediately and show this container or label (S46).

## STORAGE AND STABILITY

Store the kit in the dark at 2 - 8° C (in refrigerator). The fMLP and allergen working solutions have to be discarded after use. **Reagent B (stimulation buffer)** has to be stored in aliquots at <-15°C after reconstitution. The reagents are supplied sterile with a preservative that does not influence activation of basophilic granulocytes. 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 **ultrapure, apyrogenic** water to prepare the **wash solution**.
- 1.2 Reconstitute lyophilized reagent B by addition of 2 ml of ultrapure, apyrogenic water or thaw one aliquot of frozen reagent B.
- 1.3 Dilute the stock solutions:
  - 10 x reagent G: 1 : 10 in double distilled water (volume as needed, 2 ml per test)
  - reagent E stock solution: 1 : 200 in wash solution (volume as needed, e.g., 10 µl in 2 ml, 100 µl per blood sample).
  - allergen stock solutions (reagents C and D) 1 : 100 in wash solution (volume as needed, e.g., 10 µl in 1 ml, 100 µl per blood sample), further "ten-fold" serial dilutions are recommended.Dilute other allergens according to the instructions of the supplier.
- 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. BASOTEST™ 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!**

#### 2.2 Degranulation:

##### A)

Add **20 µl** of **reagent B** to the whole blood samples and vortex gently. Incubate the samples for **10 min** at **37°C** in a **water bath**.

##### B)

Add **100 µl** of **allergen** per test to the whole blood (**test sample**, tube #1).

Add **100 µl** of the **wash solution (reagent A)** to a further test tube as a **negative control** (tube #2)

Add **100 µl** of the **fMLP (reagent E) working solution** to a further test tube as a **positive control** (tube #3)

All tubes are mixed once more.

The samples are incubated for **20 min** at **37°C** in a **water bath**.

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

#### 2.3 Labelling with reagent F:

**Stop degranulation by incubating** the samples **on ice for 5 min**.

**Add 20 µl** of **reagent F** to each tube. Vortex and incubate the tubes for 20 min in an ice bath, covered to prevent exposure to light.

#### 2.4 Lysis and fixation:

The whole blood samples are lysed and fixed with **2 ml** of prewarmed (room temperature)

**1 x reagent G (lysing solution)**.

Vortex and incubate for **10 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. Add 200 µl of wash solution to the cell pellet, vortex. Incubate the tubes in a covered ice bath until analysis.

**Measure the cell suspension within 2 h!**

### 3. Flow cytometric analysis

Cells are analysed by flow cytometry using the blue-green excitation light (488 nm argon-ion laser).

#### Measurement:

Acquire data by using fluorescence **triggering in the FL2 channel (PE)** to **gate on basophilic granulocytes expressing high amounts of IgE** (see **Fig. 1A**). This live gating reduces the amount of data and saves memory capacity.

#### **Acquire at least 1,000 basophils per sample.**

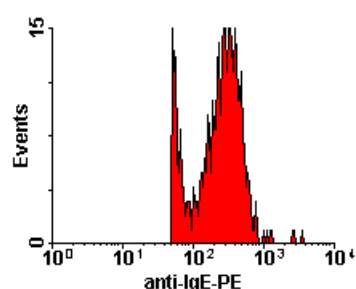
Alternatively, the **trigger threshold** may be set on the **Forward Scatter** signal. Then, **50,000 - 100,000 leukocytes** have to be **acquired per sample** (see **Fig. 1C**).

#### Data evaluation:

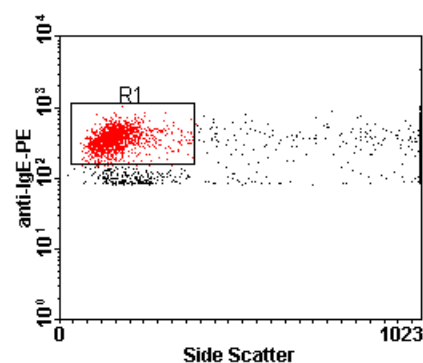
The **percentage of activated basophilic granulocytes** is analysed. For that purpose an **analysis gate** is set in the **linSSC/logFL2 dot plot** around **cells exhibiting high levels of IgE** (= basophilic granulocytes, see **Fig. 1B, C**). The **percentage of basophils expressing the activation antigen CD63** is analysed. For that purpose, use the control sample to set markers for FL1. The percentage of positive cells can then be determined by using the same marker positions (see examples in **Fig. 2A - C**).

## FIGURES

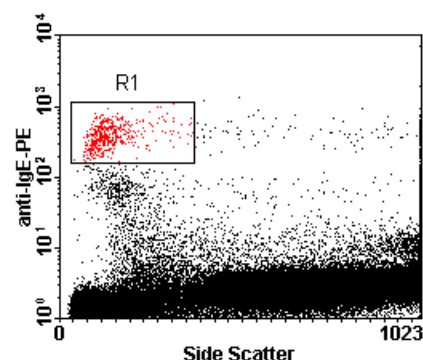
**Recommended gating during data acquisition and analysis (see Figures 1A, 1B, 1C).**



**Figure 1 A** Fluorescence **triggering in the FL2 channel (PE)** to gate on basophilic granulocytes expressing high amounts of IgE

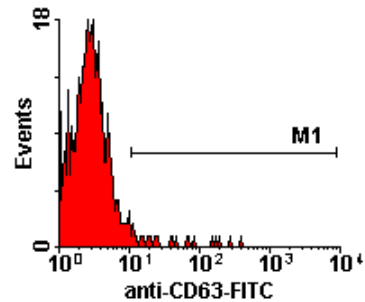


**Figure 1B** **Analysis gate (lin SSC / log FL2 dot plot)** on basophilic granulocytes during data analysis; the **trigger threshold** was set on **FL2**

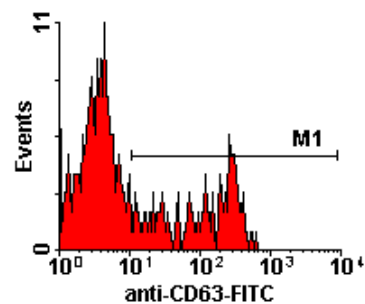


**Figure 1C** Analysis gate (lin SSC / log FL2 dot plot) on basophilic granulocytes during data analysis; the trigger threshold was set on FSC

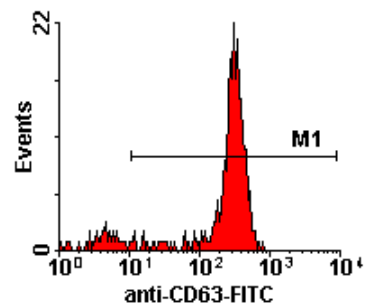
Typical FL1 histograms during data evaluation, representative experiment with cells from a patient allergic to grass pollens, incubation time of 10 + 20 min at 37°C (see Figures 2A, 2B, 2C)



**Figure 2A** Negative control (Incubation with wash solution, reagent A): 1.9% activated basophils



**Figure 2B** Positive control (incubation with fMLP, reagent E): 28.4% activated basophils



**Figure 2C** Test sample (Incubation with grass pollen mix, reagent D): 94.3% activated basophils

### REMARKS

1. Heparinized blood should be processed **within 24 h of sampling**. **Blood samples** should remain at **room temperature** prior to processing.  
The **blood collection systems** have to be **pyrogen free** (heparin from natural sources is often contaminated with pyrogen). In case of trouble pharmaceutical heparin should be used (200 IE per ml of blood).
2. When using **natural (unmodified) allergens**, the **optimum allergen concentration** has to be determined as follows: Dilute the allergen with wash solution to a concentration of **1 µg/ml, 100 ng/ml, 10 ng/ml and 1 ng/ml**. Determine the optimum allergen concentration with whole blood from patients allergic for the respective allergen.
3. Duplicate or triplicate determinations are useful in establishing the assay.
4. The use of **ultrapure, apyrogenic water for reconstituting wash solution** is **essential** because pyrogens may lead - erroneously - to degranulation of basophils. It is essential to use wash solution as negative control and also for dilution of fMLP and allergen stock solutions (reagents C, D and E). Therefore, only tissue culture grade water or water for injection should be used for this purpose. **Reagent G (10 x lysing solution)** may be diluted with **deionized double distilled water**.
5. **Contamination of blood samples with aeroallergens** should be **avoided** during basophil stimulation (all steps in sections 1 and 2 of the assay procedure). Potential sources of aeroallergens are dust mites, pollinating plants and open windows in the laboratory. Therefore, it is recommended to protect blood samples accordingly.
6. Activated basophils show a higher level of orthogonal light scattering than basophils in the negative control sample. This has to be kept in mind when setting regions of interest (gates or bitmaps) in the FL2/SSC dot plot diagram (see **Fig. 1B, 1C**).
7. Blood samples from **patients with high IgE level** (> 100 IU/ml) should be washed once with 3 ml of wash solution (250 x g, 5 min, 2-8°C) prior to the addition of reagent F.
8. The problem of a high amount of **aggregated platelets** in some blood samples may be circumvented by triggering on leukocytes using an **anti-CD45 antibody** labelled with PerCP or PE-Cy5.

### PRECISION OF THE METHOD

The **intra-assay precision** of this assay was determined on triplicate whole blood samples from allergic and non-allergic individuals. The values are presented as percentage of activated basophilic granulocytes.

	<b>Negative Control (incubation with reagent A)</b>	<b>Positive Control (incubation with reagent E)</b>
<b>Range of values</b>	<b>1.0 – 6.1</b>	<b>20.9 – 76.2</b>
<b>Average CV (%)</b>	<b>22.3</b>	<b>3.6</b>
<b>n</b>	<b>6</b>	<b>6</b>

### EXPECTED RESULTS

The **normal range** of of activated basophilic granulocytes was determined on **fresh heparinized whole blood samples** from allergic and non-allergic individuals after stimulation with wash solution (negative control) and fMLP (positive control).

<b>Stimulus</b>	<b>% activated basophilic granulocytes</b>
<b>Reagent A (Negative Control)</b>	<b>1.8 – 9.5</b>
<b>Reagent E (Positive Control)</b>	<b>25.2 – 59.5</b>

#### **Interpretation of results after stimulation with allergens:**

Individuals with more than 15 % of activated basophils should be regarded as allergic for the allergen tested. Testing several dilutions of the allergen (e.g., 1:100; 1:1,000; 1:10,000; 1:100,000 or 1:1,000,000) should lead to a concentration-dependent activation of basophils.

### 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-

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

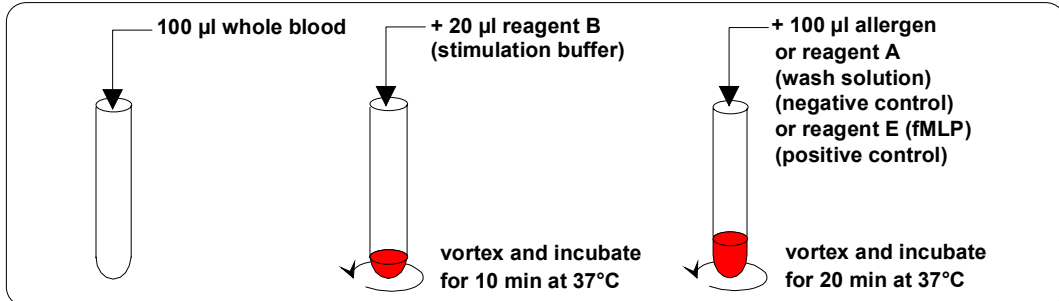
3. Dilution of allergen extracts leads to loss of potency. Therefore, very dilute extracts can not be stored and should be prepared as needed.
4. Patients should avoid systemically administered antiallergic drugs such as antihistaminics for at least 48 h prior to blood sampling, corticosteroids should be off for more than 2 weeks.
5. It may be possible that basophilic granulocytes from some individuals are not recognized by this anti-IgE antibody.

### REFERENCES

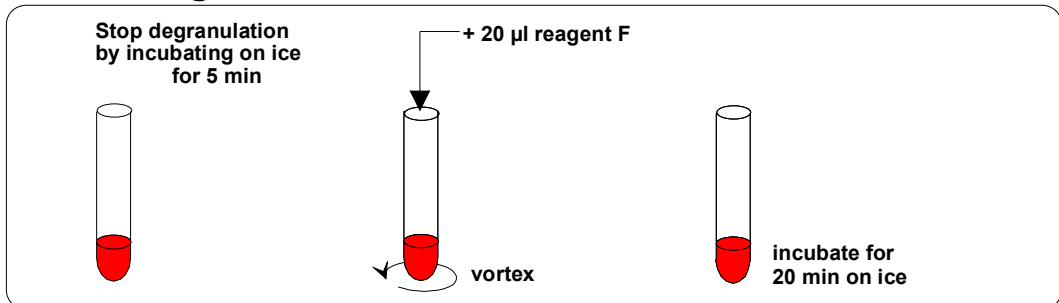
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## BASOTEST™ - Sample Preparation Procedure

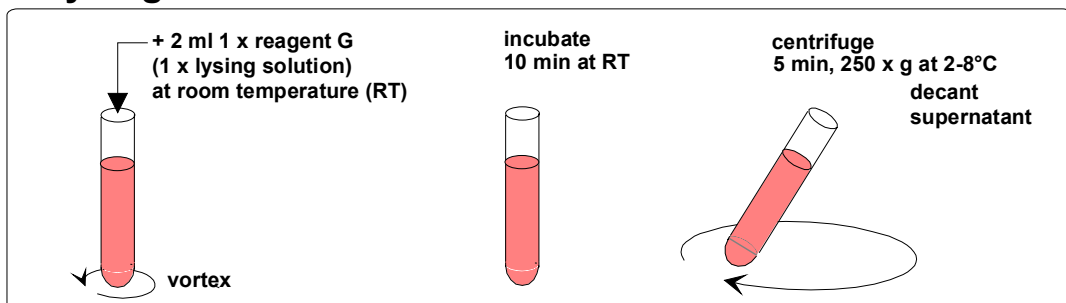
### 1. Degranulation



### 2. Labelling



### 3. Lysing and Fixation



### 4. Washing

