

BASOTEST[®]

TEST KIT FOR THE QUANTITATIVE DETERMINATION OF THE DEGRANULATION OF BASOPHILIC GRANULOCYTES IN HEPARINIZED HUMAN WHOLE BLOOD

**For Research Use Only.
Not for use in diagnostic or therapeutic procedures.**

**ORPEGEN Pharma
Gesellschaft für biotechnologische Forschung, Entwicklung und Produktion m.b.H.
Czerny-Ring 22, D-69115 Heidelberg, Germany
Tel (49) 6221/9105-0, Fax (49) 6221/910510, E-mail: info@orpegen.com**

Catalog No. 10-0500

Test kit with antibodies, allergens and reagents for 100 tests.

Please read the instructions carefully before use!

SUMMARY and EXPLANATION

This test 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 validation assays. Critical steps are in bold letters. A graphic summary of the test is attached.

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_ε 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 preformed 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).

BASOTEST[®] can be used to study immediate-type hypersensitivity (type I reactions). A comparison of the flow cytometric method with a histamine release assay is shown in **Fig. 3**. BASOTEST[®] may also be used to study the effects of immunotherapies (e.g., hyposensitisation). However, this test is not suited to detect any delayed type hypersensitivity (e.g., type IV reactions).

TEST PRINCIPLES

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 STIMULATION BUFFER for 10 min at 37°C, then with allergen at various concentrations for 20 min at 37°C. The chemotactic peptide N-Formyl-Met-Leu-Phe (fMLP) is used as a positive control, addition of WASHING 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 with the 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-gp53-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 LYSING SOLUTION. After one washing step with WASHING SOLUTION, the percentage of activated basophilic granulocytes is determined by flow cytometry.

MATERIAL and REAGENTS

The test kit contains:

1. 1 bottle (2 ml) two-colour antibody reagent (**STAINING REAGENT**), contains two monoclonal antibodies: anti-IgE-PE and anti-gp53-FITC.
2. 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.
3. 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.
4. 1 vial (200 µl) containing the allergenic extract **MITE MIX**, concentrated stock solution. Dilute 10 µl in 1 ml of WASHING SOLUTION for use.
5. 1 vial (200 µl) containing the chemotactic **peptide fMLP** (200 x stock solution, 0.4 mM). Dilute 10 µl in 2 ml of WASHING SOLUTION for use.
6. 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 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 of ultrapure, apyrogenic water, 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® 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. Ultrapure, apyrogenic water or water for injection for reconstitution of WASHING SOLUTION.
6. Reagent-grade (both distilled and deionized) water for dilution of 10 x LYSING SOLUTION.
7. Various other allergens or allergen mixtures.

Required apparatus:

1. Variable volume micropipettes 10 - 200 µl, 200 - 1000 µl and disposable tips.
2. Dispenser pipette and dispenser tips.
3. Bottle-top dispensers for WASHING SOLUTION and 1 x LYSING SOLUTION.
4. Waterbath.
5. 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. The fMLP and allergen working solutions have to be discarded after use. The **STIMULATION BUFFER** has to be stored **in aliquots at -20°C** after reconstitution. The reagents are supplied sterile with a preservative that does not influence activation of basophilic granulocytes.

ASSAY PROCEDURE

1. Preparations:

- 1.1 Dissolve the salts for WASHING SOLUTION in 1000 ml **ultrapure, apyrogenic water**.
- 1.2 Reconstitute lyophilized STIMULATION BUFFER by addition of 2 ml of **ultrapure, apyrogenic water** or thaw one aliquot of frozen STIMULATION BUFFER.
- 1.3 Dilute the stock solutions:
 - LYSING SOLUTION 1 : 10 in distilled water (volume as needed, 2 ml per test)
 - fMLP stock solution 1 : 200 in WASHING SOLUTION (volume as needed, e.g., 10 µl in 2 ml, 100 µl per blood sample).
 - allergen stock solutions 1 : 100 in WASHING 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 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!**

2.2 Degranulation:

A)

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

Add **100 µl** of the **fMLP working solution** to a further test tube as a **positive control** (tube #2)

Add **100 µl** of **allergen** per test to the whole blood (**test sample**, 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 STAINING REAGENT:

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

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

2.5 Lysis and fixation:

The whole blood samples are lysed and fixed with **2 ml** of prewarmed (room temperature, 20 to 25°C) **1 x LYSING SOLUTION**.

Vortex and incubate for **10 min at room temperature**.

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

2.6 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 (5 min, 250 x g, 2-8°C). Aspirate the supernatant leaving approximately 100 µl. Add 200 µl of WASHING 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, e.g., FACSCalibur™, CELLQuest™ software).

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 gp53** 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).

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. 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 WASHING BUFFER 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 WASHING SOLUTION** is **essential**, because pyrogens may lead - erroneously - to degranulation of basophils. It is essential to use WASHING SOLUTION as negative control and also for the dilution of fMLP and allergen stock solutions. Therefore, only tissue culture grade water or water for injection should be used for this purpose. **LYSING SOLUTION** may be diluted with **reagent-grade (both distilled and deionized 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 WASHING SOLUTION (250 x g, 5 min, 2-8°C) prior to the addition of the STAINING REAGENT.
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.

EXPECTED VALUES

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

Stimulus	% activated (gp53 positive) basophilic granulocytes
WASHING SOLUTION (negative control)	1.8 - 9.5
fMLP (positive control)	25.2 - 59.5

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.

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.

	Negative Control (Incubation with WASHING SOLUTION)	Positive Control (Incubation with fMLP)
Range of values	1.0 - 6.1	20.9 - 76.2
Average CV (%)	22.3	3.6
n	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.
Cells from older and incompletely anticoagulated blood samples may stain nonspecifically. 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.

WARNINGS

1. Blood samples must always be regarded as potentially infectious (hepatitis, HIV etc.!) Wear disposable gloves and protective clothing while handling blood samples.
2. The STAINING REAGENT contains sodium azide as preservative. Sodium azide is harmful if swallowed. Keep out of of children. Keep away from food, drink, and animal feedingstuff. Wear suitable protective clothing. If swallowed, seek medical advice immediately and show this container or label. Contact with acids liberates very toxic gas. Azide compounds should be flushed with large volumes of water during disposal to avoid deposits in lead or copper plumbing where explosive conditions may develop.
3. 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.
4. SALTS f. WASHING 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.

TROUBLESHOOTING

Problem	Possible Causes	Solutions
Positive control (fMLP) does not provide basophil activation	<ol style="list-style-type: none"> 1) fMLP was inactive 2) Analysis gate includes other cells than basophils (giant platelets or platelet aggregates) 3) Basophils from some patients are not recognized by the anti-IgE antibody 4) Patient is under treatment 	<ol style="list-style-type: none"> 1) Use freshly diluted fMLP 2) Reprocess data
Negative control (WASHING SOLUTION) gives positive signal	<ol style="list-style-type: none"> 1) Incubation with STAINING REAGENT was not at 0°C 2) Presence of pyrogen or allergens during incubation 3) Analysis gate include platelets 4) Basophils are activated in vivo 	<ol style="list-style-type: none"> 1) Incubate samples in an ice bath 2) Avoid presence of pyrogen or aeroallergens in sample 3) Reprocess data

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BASOTEST® is a registered trademark of ORPEGEN Pharma. Patents pending.

Manufactured by:

ORPEGEN Pharma

Gesellschaft für biotechnologische Forschung, Entwicklung und Produktion m.b.H.

Czerny-Ring 22, D-69115 Heidelberg, Germany

Tel. (49) 6221-91050, Fax (49) 6221-910510, E-mail: info@orpegen.com

Distributed by:

BioCarta Inc.

2185 Faraday Avenue, Suite 100

Carlsbad, California 92008, USA

Tel 760.804.1385

Fax 760.804.1395

Email: info@BioCarta.com

FIGURES

Fig. 1: Recommended gating during data acquisition and analysis

- A) Fluorescence **triggering in the FL2 channel (PE)** to gate on basophilic granulocytes expressing high amounts of IgE
- B) **Analysis gate (lin SSC / log FL2 dot plot)** on basophilic granulocytes during data analysis; the **trigger threshold** was set on FL2
- C) **Analysis gate (lin SSC / log FL2 dot plot)** on basophilic granulocytes during data analysis; the **trigger threshold** was set on FSC

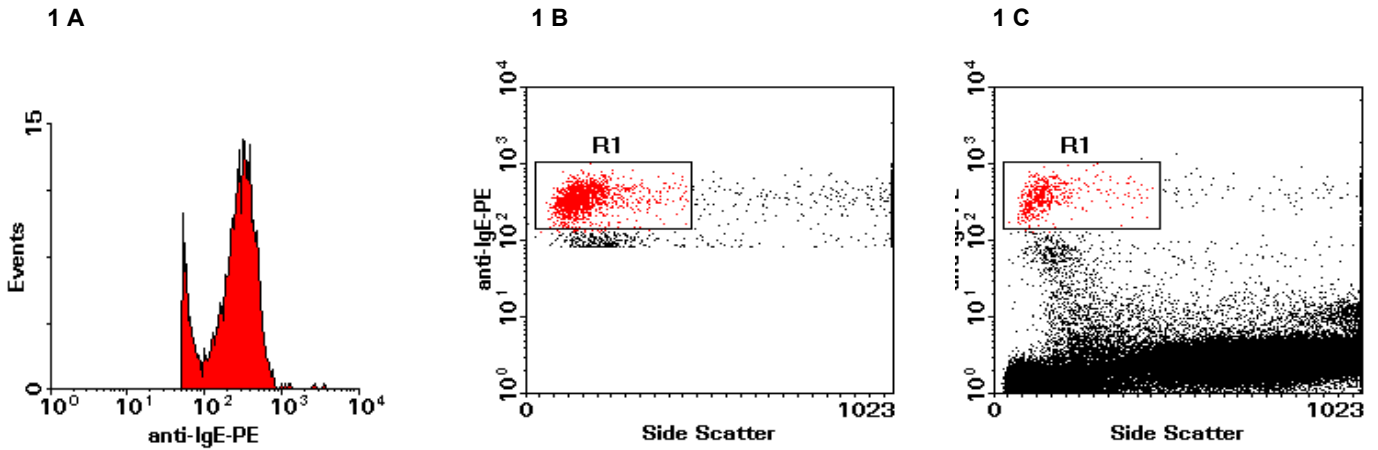


Fig. 2: Typical FL1 histograms during data evaluation, representative experiment with cells from a subject allergic to grass pollens, incubation time of 10 + 20 min at 37°C.

- A) **Negative control** (Incubation with **WASHING SOLUTION**)
- B) **Positive control** (incubation with **fMLP**)
- C) **Test sample** (Incubation with **grass pollen mix**)

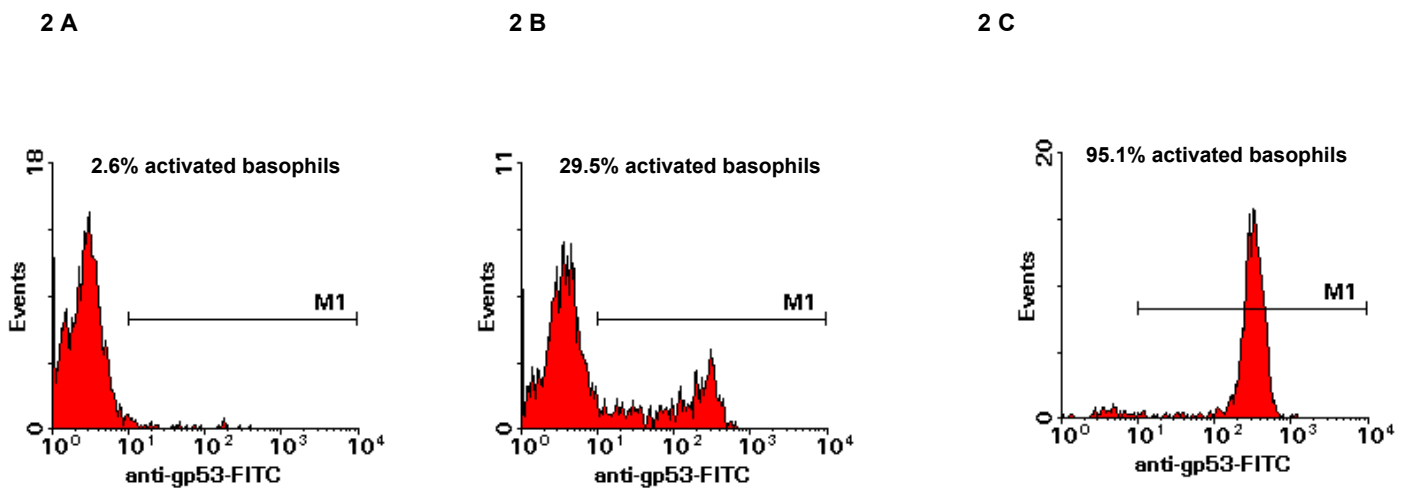
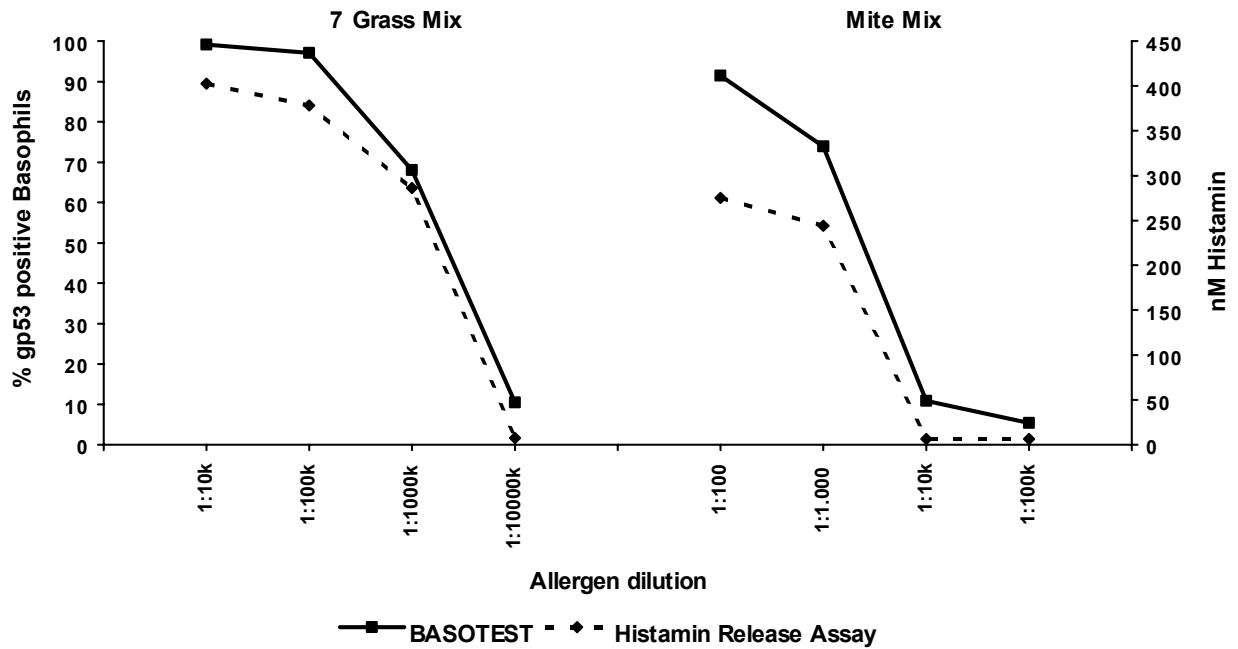


Fig. 3: Comparison of BASOTEST® and histamine release assay. Whole blood samples from a subject allergic to grass pollens and mite allergens were analysed in parallel. The results are expressed as percentage of gp53 positive basophils (left) or as amount of released histamine (right).



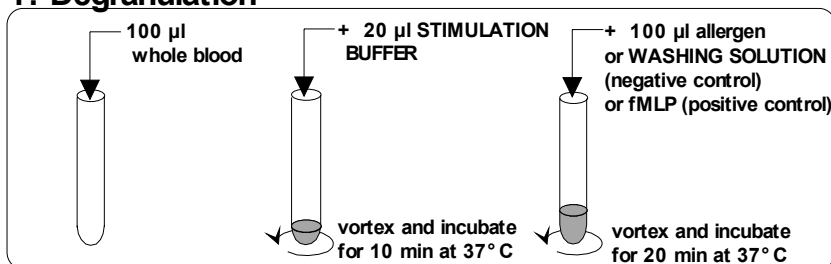
Summary of Assay Procedure

Preparations

1. Dissolve the salts for **WASHING SOLUTION** in 1000 ml of **ultrapure, apyrogenic water**.
2. Reconstitute lyophilized **STIMULATION BUFFER** by addition of 2 ml of **ultrapure, apyrogenic water** or thaw one aliquot of frozen **STIMULATION BUFFER**.
3. Dilute the stock solutions (**LYSING SOLUTION**, fMLP and allergen extracts).
4. Prepare ice bath (ice in water).
5. Prewarm water bath to 37°C (**precise temperature control!**).
6. Switch on and calibrate flow cytometer.

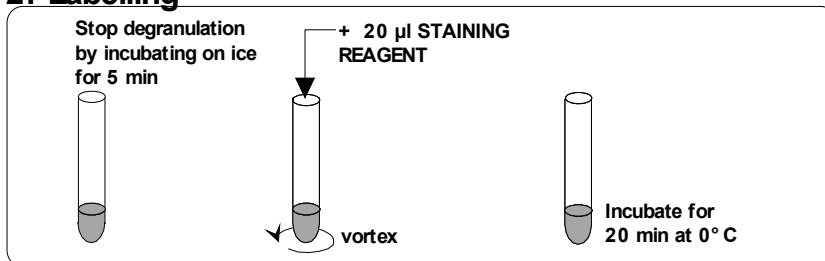
BASOTEST set up

1. Degranulation



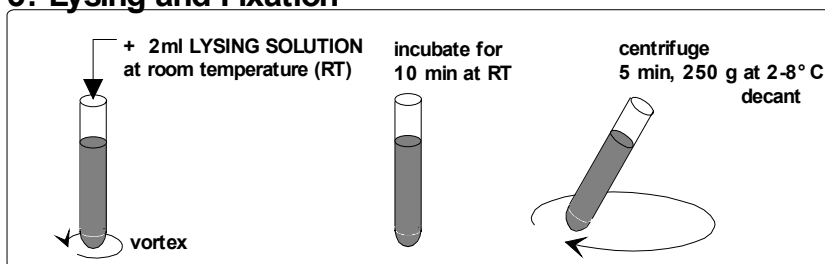
Use only **heparinized** whole blood.
Avoid smearing blood down the side of the tube.

2. Labelling



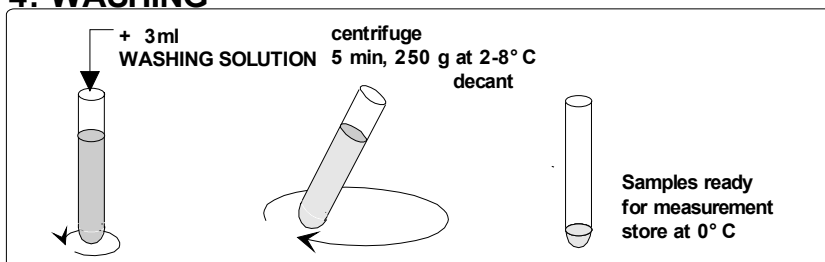
It is important to **stop degranulation** by **incubating the samples on ice!**

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



Measure samples within 2 hours.

* 1200 rpm for 16 cm rotor