



**FLOW-CAST  
Basophil Activation Test (BAT)  
Flow Cytometry**

For Research Use Only. Not For Use In Diagnostic Procedures.

Catalog Number: 01-FK-BAT  
Size: 100 Tests  
Version: 2003-08-04 – ALPCO 08/27/03

American Laboratory Products Company  
PO Box 451 • Windham, NH 03087  
Tel.(800)592-5726 • Fax.(603)898-6854  
[www.alpco.com](http://www.alpco.com) • [email@alpco.com](mailto:email@alpco.com)

**This protocol is for reference purposes only.  
DO NOT use this copy to run your assay;  
use the protocol included with the kit ONLY.**

## INTENDED USE

The Flow-CAST<sup>®</sup> kit is intended for the quantitative determination of expression of CD63 surface marker on basophils upon antigen stimulation by flow cytometry.

## PRINCIPLE OF THE ASSAY

The assay is based on the method first described by Sainte-Laudy *et al.* 1994 and 1996 (1,2) where basophil activation by allergens or controls is flow cytometrically measured by the increase of the CD63 (gp53) at the cellular surface. The method can be used not only for IgE mediated allergies but also for the so-called pseudo-allergies (3-5).

Peripheral blood leukocytes are isolated from patients whole blood samples and primed with Stimulation Buffer containing interleukin 3 (IL-3). Specific allergen is added and the cells are incubated to mimic the *in vivo* situation where, in IgE mediated allergies, specific IgE bound to the cellular surface are bridged by the allergen and activate an intracellular enzymatic cascade leading to the activation of the basophils. During this activation intracellular compounds containing the transmembrane protein CD63 are fused to the cellular membrane and therefore exposed to the extracellular matrix.

A highly specific monoclonal antibody (mAb) recognizing the high affinity IgE binding receptor (FcεRI) is used as a positive control, leading to the activation of the basophils by mimicking the bridging event.

After stopping the reaction the Staining Reagent is added containing a mixture of monoclonal antibodies to human CD63 labeled with phycoerythrin (anti-CD63-PE) and to human IgE labeled with fluorescein isothiocyanate (anti-IgE-FITC). The remaining erythrocytes are removed by a lysing reaction and after blocking the reaction the cells are analyzed by flow cytometry (for flow cytometric analysis see page 4).

## REAGENTS SUPPLIED AND PREPARATION

Reagents	Quantity	Code	Reconstitution
Stimulation Buffer with heparin and IL-3	1 vial lyophilized	B-BAT-STB	Reconstitute with 50 ml of water *
Stimulation Control anti-FcεRI mAb	1 vial lyophilized	B-BAT-STCON	Reconstitute with 1.5 ml of water *
Staining Reagent Mix of anti-CD63-PE and anti-IgE-FITC mAb	1 vial 2.2 ml	B-BAT-SR	Ready to use
Lysing Reagent** 10x concentrated	1 vial 40 ml	B-BAT-LYR	Dilute with 360 ml of deionized water
Blocking Buffer stop of cell lysis	2 vials 100 ml	B-BAT-BLB	Ready to use

Table 1

\* For required water quality, see PROCEDURAL NOTES

\*\* Crystals may be formed during storage at 2-8°C and should be dissolved at 18-28°C prior to dilution.

## STORAGE AND SHELF LIFE OF REAGENTS

Unopened reagents	
Store at 2-8°C. Do not use past kit expiration date.	
Opened / reconstituted reagents	
Stimulation Buffer	Stable at -20°C for 6 months. Aliquot if repeated use is expected.
Stimulation Control	Stable at 2-8°C for 2 months. For longer storage aliquot and freeze at -20°C for up to 6 months.
Staining Reagent	Stable at 2-8°C until expiration date marked on vial.
Lysing Reagent	
Blocking Buffer	

Table 2

This protocol is for reference purposes only.  
DO NOT use this copy to run your assay;  
use the protocol included with the kit ONLY.

---

## ALLERGENS SUPPLIED UPON REQUEST

**Order codes:** see the **BÜHLMANN Allergen list on the webpage** ([www.invitroallergy.com](http://www.invitroallergy.com))

**Protein Allergens:** The BÜHLMANN protein allergens are quality controlled and shipped in liquid, concentrated form (1 µl/vial). The protein allergens need to be stored refrigerated and must be diluted before use.

**Drug and Chemical Allergens:** The BÜHLMANN low molecular weight allergens are shipped in lyophilized form. The low molecular weight allergens need to be stored refrigerated and must be reconstituted before use. Consult the BÜHLMANN Allergen Booklet for details.

---

## ALLERGEN REAGENTS FROM OTHER SOURCES

Allergens from other sources might be used in the Flow-CAST® assay with the following limitations:

- No matrix-bound allergens (solid or liquid phase).
- No allergen preparations containing cytotoxic compounds (stabilizers, preservatives) such as glycerol, phenol, sodium azide or merthiolate (thimerosal).

For the procedure to establish customer specific allergens for the CAST®-Assays ask your local distributor or contact Bühlmann Laboratories AG.

---

## WARNINGS AND PRECAUTIONS

**Reagents Containing Human Source Material:** Stimulation Buffer (B-BAT-STB) and Stimulation Control (B-BAT-STCON) of this kit contain components of human origin. Each serum used in the preparation of the kit components was tested by a FDA-approved method and found negative for HBV surface antigen, HCV and HIV1/2 antibodies. Although those methods are highly accurate, there is no guarantee that this material cannot transmit Hepatitis or AIDS. *Therefore, all patient specimens and kit components should be handled as if capable of transmitting infections.* All products containing human source material should be handled in accordance with good laboratory practices using appropriate precautions.

---

## MATERIALS REQUIRED BUT NOT PROVIDED

- ACD or K-EDTA tubes, respectively.
  - Centrifuge for centrifugation at 200 - 1200 x g.
  - Disposable, pyrogen-free plastic pipettes or plastic syringes for harvesting the leukocytes fraction.
  - Disposable, pyrogen-free polypropylene or polystyrene test tubes and appropriate test tube racks for the cell separation, stimulation and staining. Optionally, tissue culture grade microtiter plates can be used for stimulation and staining.  
NOTE: Polystyrene tubes should fit with the Flow Cytometer used (e.g. 12 x 75 mm FALCON tubes from Becton Dickinson; order code: 352052).
  - Vortex Mixer.
  - Precision pipettes with disposable, pyrogen-free tips:  
10-100 µl, 100-1000 µl, 1-5 ml adjustable pipette and a 10-50 µl adjustable multishot pipette.
  - Cylinder for preparing the Stimulation Buffer.
  - Sterile, ultrapure and apyrogenic water for preparing the cell stimulation reagents (cf. PROCEDURAL NOTES, page 3).
  - Water bath set at 37°C.
  - Distilled or deionized water as well as beaker or cylinder for the preparation of Lysing Reagent.
  - Bottle-top dispensers for 1 x Lysing Reagent and Blocking Buffer, respectively.
  - Flow Cytometer with 488 nm excitation wavelength (argon-ion laser) including appropriate software.
  - Aspiration device (optional).
  - Blotting paper (optional).
  - Reagent for fixing the labeled cells such as CELL-FIX from Becton Dickinson (order code: 340181) (optional).
- 

## SPECIMEN COLLECTION AND STORAGE

It is recommended that patients should avoid systemically administered antiallergenic drugs such as corticosteroids, chromoglycic acid (DSCG), indomethacin or similar for at least 24 hours prior to blood sampling.

Collect sufficient blood into ACD or K-EDTA tubes. Perform the cell stimulation immediately or store the blood sample refrigerated (2-8°C) for up to 24 hours. **Do not centrifuge or freeze blood samples.**

Determine the minimum amount of blood needed according to the following formula:

$$\frac{(n \text{ allergens} \times n \text{ concentrations}) + 2 \text{ (pos/neg controls)}}{2} = x \text{ ml whole blood}$$

**Example 1:** One allergen in 1 single concentration:

$$\frac{(1 \times 1) + 2}{2} = 1.5 \Rightarrow 2 \text{ ml whole blood}$$

**Example 2:** Two allergens in 3 different concentrations each:

$$\frac{(2 \times 3) + 2}{2} = 4 \Rightarrow 4 \text{ ml whole blood}$$

---

## PROCEDURAL NOTES

- **RECOMMENDED WATER QUALITY FOR THE FLOW-CAST®.** The use of sterile, ultrapure and apyrogenic water for reconstituting cell stimulation reagents, Stimulation Buffer (B-BAT-STB) and Stimulation Control (B-BAT-STCON), is essential for good and reproducible basophil stimulation. The following sources of water may be used: Cell culture grade water, infusion grade water or deionized, double distilled water that is ultra filtered in a periodically sanitized 10 kDa ultra filter.

**This protocol is for reference purposes only.  
DO NOT use this copy to run your assay;  
use the protocol included with the kit ONLY.**

The Lysing Reagent (B-BAT-LYR) can be reconstituted with deionized, double distilled water or the same water quality that is used for the cell stimulation reagents.

- PRECAUTIONS TO AVOID ALLERGEN CONTAMINATION DURING CELL STIMULATION Aeroallergens in the laboratory may contaminate open blood samples and cell suspensions from patients potentially causing an elevated background release. Therefore, care must be taken to cover blood samples and cell stimulation tubes. Avoid dust mites, pollinating plants, latex gloves or equipment potentially containing latex and open windows in the laboratory where the cell stimulation is performed. Therefore, we recommend carrying out the cell preparation and stimulation steps in a laminar flow hood.
- For cell stimulation and labeling reaction, the use of tissue culture grade MICROTITER PLATES are possible.

## ASSAY PROCEDURE

1. Mix the anti-coagulated blood sample by inverting the venipuncture tube several times. Centrifuge the venipuncture tube for 5 minutes at 200 x g.

After the centrifugation step, two phases can be observed from top to the bottom of the tube:

- i) plasma fraction containing the leukocytes
- ii) erythrocyte fraction

### Alternative procedure for cell separation:

Mix the anti-coagulated blood sample by inverting the venipuncture tube several times.

Let the samples sediment (erythrocytes) at room temperature for one hour. Collect the upper phase (plasma fraction containing the leukocytes) and continue with step 2.

If the erythrocytes sedimentation is not complete after one hour of incubation, the centrifugation step above is recommended.

2. Collect the plasma fraction with the leukocytes on the top of the erythrocytes with a disposable plastic syringe or plastic pipette and transfer it into a fresh and pyrogen-free 5 ml polypropylene or polystyrene tube.  
NOTE: Because of the low amount of basophils in the leukocyte fraction it is important to collect the entire plasma fraction. Contamination with a low amount of erythrocytes does not significantly influence the results.
3. Centrifuge for 10 minutes at 500 x g. Remove the plasma fraction on the top of the cell pellet.
4. Add 100 µl of Stimulation Buffer per ml of blood used at the beginning (*i.e.* for 5 ml blood sample used, the pellet has to be solved with 500 µl of Stimulation Buffer). Resuspend the leukocytes pellet carefully.
- 5a. For each patient, label pyrogen-free polystyrene tubes suited for Flow Cytometry measurements (e.g., PB = patient background, PC = stimulation control with anti-FcεRI Ab, A1-1 for antigen 1 with dilution 1, A1-2 for allergen 1 with dilution 2, etc.) or use tissue culture grade microtiter plates.
- 5b. Pipet 50 µl of **Stimulation Buffer (background)** into the PB tube of each patient.
- 5c. Pipet 50 µl of **Stimulation Control** into the PC tube of each patient.
- 5d. Pipet 50 µl of **Allergen** into the corresponding patient tubes.
6. Pipet 50 µl of each **patient's cell suspension** into the corresponding tubes.
7. Vortex gently, cover the tubes and incubate for 40 ± 5 minutes at 37°C in a water bath.
8. Add 50 µl of cold Blocking Buffer to each tube.
9. Add 20 µl of cold Staining Reagent to each tube. Vortex gently.
10. Incubate for 30 ± 5 minutes at 2-8°C.
11. Vortex gently the sedimented cells, add 3.5 ml of pre-warmed (18-28°C) Lysing Reagent to each tube and incubate for 5 ± 1 minutes at 18-28°C.
12. Stop the reaction with 1 ml of Blocking Buffer.
13. Centrifuge for 5 minutes at 1000 - 1200 x g.
14. Decant or aspirate the supernatant and resuspend the cell pellet with 500 µl of Blocking Buffer. Vortex gently.
15. Proceed to the analysis by Flow Cytometry within 2 hours.

NOTE: If the samples cannot be measured within 2 hours, add 500 µl of cell fixation solution (*cf.* MATERIALS REQUIRED BUT NOT PROVIDED, page 3) and store at 2-8°C for up to 24 hours. However, immediate reading of the labeled cells is recommended, since a greater number of cells will be recovered. *Due to the small amount of basophils in the leukocytes fraction, recovery is a limiting factor of the assay.*

## FLOW CYTOMETRIC ANALYSIS

Flow cytometric analysis can be performed on any flow cytometer containing a 488 nm argon laser (blue-green excitation light) and the corresponding software.

At least 150 basophilic cells must be analyzed, requiring a total amount of 50-80'000 leukocytes to analyze. Because of the lower activation percentage in drug allergies each laboratory has to define its own confidence limits (*i.e.* in drug allergies the limit of basophilic cells analyzed should be set to 200 or more)

The analysis is based on three steps:

1. Set a gate 1 (R1) by including the entire lymphocyte population (see **Figure 1**).
2. Within the lymphocyte population gate (R2) the brightly fluorescent FITC, IgE positive, cells (see **Figure 2**).
3. Calculate the percentage of brightly fluorescent PE cells (CD63 positive cells) compared to the total amount of brightly fluorescent FITC cells, by the cytometer software (see **Figure 3-5**).

For a typical example of results obtained see Appendix I figures 1 to 5 and corresponding tables 11-13.

## INTERPRETATION OF RESULTS

To obtain an optimal sensitivity and specificity, slightly different cut-off values should be applied for each allergen since:

- i) The negative controls show variable values, usually below 5 % (see next chapter).
- ii) Some allergens (e.g. foods) may cause non specific *in vitro* stimulation. The BÜHLMANN allergens are controlled in this respect.

**This protocol is for reference purposes only.  
DO NOT use this copy to run your assay;  
use the protocol included with the kit ONLY.**

- iii) Some allergens (e.g. drugs) yield lower stimulation percentages in positive cases than others (e.g. inhalant allergens).  
For these, a lower cut-off has to be chosen.

As a rule, the cut offs are determined by Receiver operating characteristic (ROC) curves enabling to achieve highest possible sensitivity by an optimal specificity (5). On the basis of extensive studies we propose the following cut-off:

Inhalant allergens:	≥15%	
Food allergens:	≥15%	
Hymenoptera venoms:	≥10%	
Betalactams*:	≥5%	SI ≥2
Analgesics*:	≥5%	SI ≥2

\* Drugs usually give lower activation percentages than other allergens. Therefore, a lower cut-off should be taken, but the stimulation index (SI = allergen stimulation divided by negative control) must be equal or superior to 2 in order to consider the result as positive.

---

#### QUALITY CONTROL

A thorough understanding of this package insert is necessary for the successful use of the product. Reliable results will be obtained only by using precise laboratory techniques (current GLP guidelines) and accurately following this package insert. For appropriate evaluation of results, different values should be taken in account:

- The appearance of the three **typical leukocyte populations** (lymphocytes, monocytes and granulocytes) in the FSC/SSC plot can be regarded as a criterion for the quality of the blood sample (time of collection, storage).
- The **absolute number of basophils** recovered and evaluated, indicating that the test has been properly performed and that a sufficient number of basophils have been counted for achieving statistical difference from the controls. In our experience, the number of basophils analyzed should not be below 150 cells.
- The **percentage of activated basophils**. In the negative control the percentage of activated basophils is usually below 5%. Sometimes, however, the percentage of activated basophils in the negative control may appear much higher. This may be due to some *in vivo* basophil activation, indicating recent allergen exposure and ensuing allergic reaction *in vivo* (e.g. pollen allergic patient during the pollen season, blood sampling following food allergen or drug allergen exposure). Exceptionally, it may also be due to some technical mishap such as contact of the basophils *in vitro* with inappropriate plastic or reagent resulting in non-specific basophil activation.
- The **positive control** may be quite variable from patient to patient. Atopic patients with high IgE levels usually react more strongly than healthy non atopic donors. In contrast to positive controls with anti-IgE Ab, by which up to 30% of people may not react, the percentage of non reactors with anti-FcεRI mAb is below 5% (5).

---

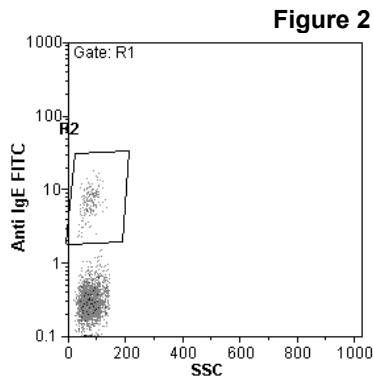
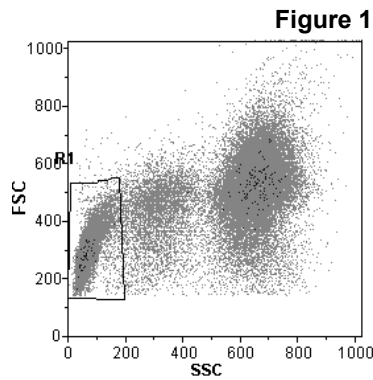
#### PERFORMANCE CHARACTERISTICS

**Intra-Assay Precision: 2.7% (PC).** The intra-assay precision was calculated from a blood sample of a normal donor stimulated with negative (PB) and positive control (PC) and consecutively analyzed ten times by flow cytometry (*cf. Table 6*)

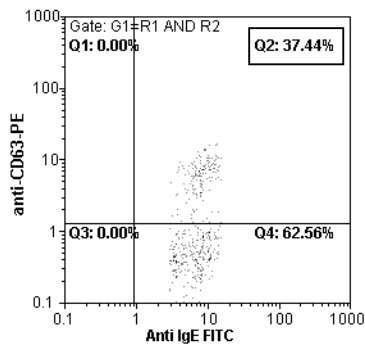
**Inter-Assay Precision: 8.8% (PC).** The inter-assay precision was calculated from the blood samples of three normal donors stimulated ten times and consecutively analyzed by flow cytometry (*cf. Table 7*)

**Range of Values:** The normal range of activated basophilic granulocytes was determined from fresh whole blood samples of allergic and non-allergic individuals after stimulation with positive and negative control (*cf. Table 8*).

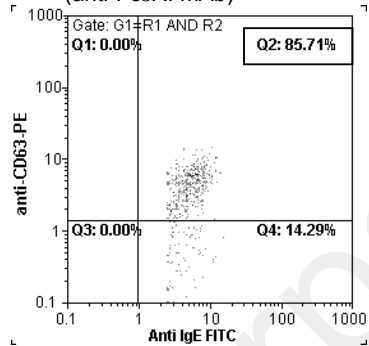
**APPENDIX I  
TABLES**



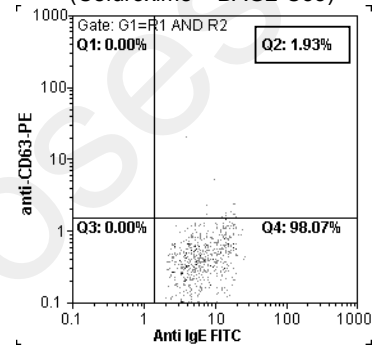
**Figure 3**  
Patient Background



**Figure 4**  
positive Control (anti-FcεRI mAb)



**Figure 5**  
spec. Allergen (Cefuroxime – BAG2-C33)



**Table 3**

Region	Gate	Count	%Gated
R1	--	16497	32.9
R2	R1	569	4.1
Q2	G1	11	1.9
Q4	G1	558	98.1

**Table 4**

Region	Gate	Count	%Gated
R1	--	18902	37.8
R2	R1	497	2.6
Q2	G1	426	85.7
Q4	G1	71	14.3

**Table 5**

Region	Gate	Count	%Gated
R1	--	14259	28.5
R2	R1	441	2.9
Q2	G1	165	37.4
Q4	G1	275	62.2

**Table 6** Intra-Assay Precision

Sample	Range of values	Mean	SD	% CV
P1 PB	1.9 - 3.4	2.7	0.5	2.7
PC	68.4 - 74.5	71.3	1.9	

**Table 7** Inter-Assay Precision

Sample	Range of values	Mean	SD	% CV
P2 PB	0.0-2.1	1.1	0.6	13.4
PC	27.8-47.8	41.1	5.5	
P3 PB	0.0-3.7	1.4	1.1	9.6
PC	43.0-58.8	49.2	4.7	
P4 PB	0.9-4.6	2.4	1.3	3.4
PC	69.2-76.7	73.8	2.5	
Mean				8.8

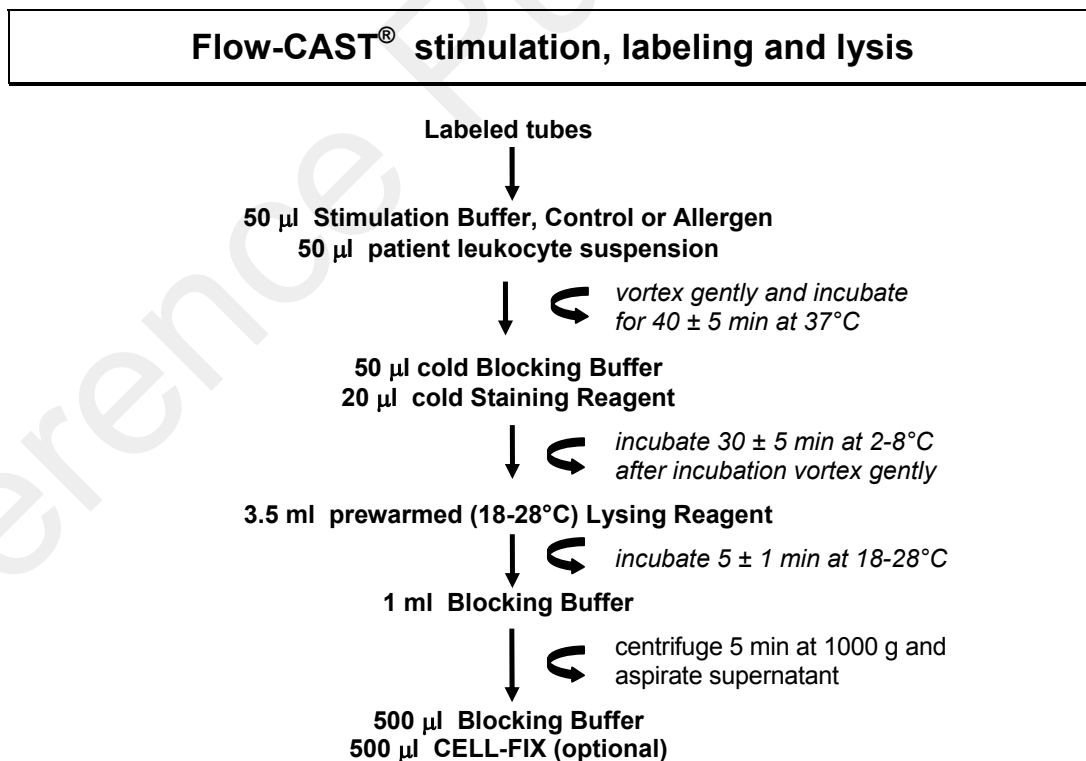
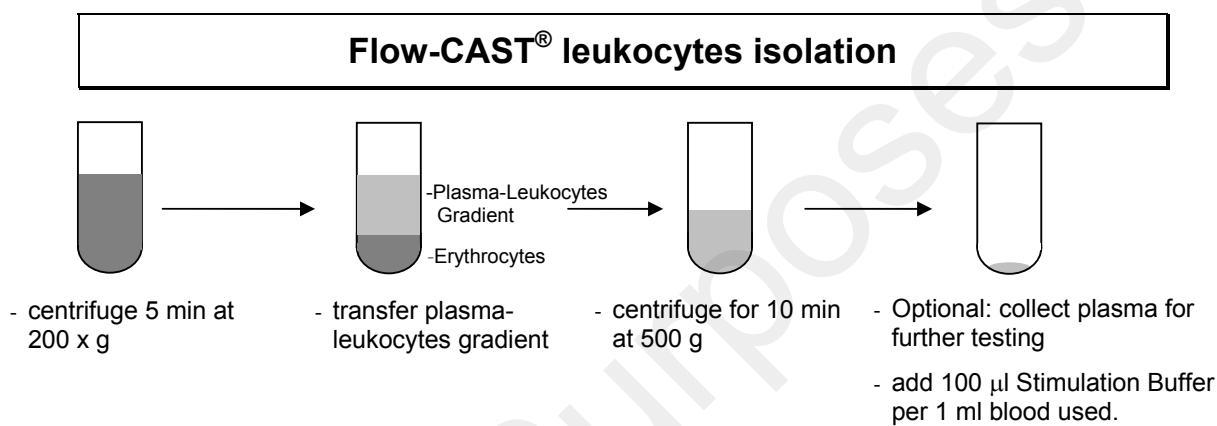
**Table 8** Expected Values

Sample	n	range	Mean	SD
Normal donors	30	PB	0.2 - 11.6	2.8
		PC	14.8 - 81.1	43.4
Bee/Wasp allergics	15	PB	0.0 - 9.4	2.9
		PC	30.8 - 90.0	64.7
other allergics	15	PB	0.4 - 7.6	3.0
		PC	20.1 - 87.1	49.3

**Table description:** cf. "Flow cytometric analysis" (page 4) and "Performance Characteristics" (page 5)

1. Sainte-Laudy, J, *et al.* [Analysis of membrane expression of the CD63 human basophil activation marker. Applications to allergologic diagnosis]. *Allerg Immunol (Paris)* **26**, 211-4. (1994).
2. Sabbah, A and Sainte-Laudy, J. *Flow Cytometry applied to the analysis of Lymphocyte and Basophil activation.* *ACI International* **8**, 116-9 (1996).
3. Sanz, ML, *et al.* Use of flow cytometry to assess basophil activation in patients allergic to betalactam antibiotics. Correlation between flow cytometric allergen stimulation test (FAST) and other *in vivo* and *in vitro* tests. *Int Arch Allergy Immunol* **124**, 307-8 (2001).
4. Sanz, ML, *et al.* Flow cytometric basophil activation test by detection of CD63 expression in patients with immediate-type reactions to betalactam antibiotics. *Clin Exp Allergy* **32**, 277-86. (2002).
5. DeWeck, AL and Sanz, ML. *Flow cytometric cellular allergen stimulation Test (FAST/Flow-CAST): technical and clinical evaluation of a new diagnostic test in allergy and pseudo-allergy.* *ACI International* **14**, 204-215 (2002).

**APPENDIX III  
SHORT PROTOCOL**






Proceed to analysis by Flow Cytometry (within 2 hours)


**TIME TO RESULT: ~ 2 HOURS**

**This protocol is for reference purposes only.  
DO NOT use this copy to run your assay;  
use the protocol included with the kit ONLY.**

**APPENDIX III**

**SYMBOLS**

Symbol	Explanation
	Use By Verwendbar bis Utiliser jusqu'au Utilizzare entro Fecha de caducidad
<b>REF</b>	Catalogue number Bestellnummer Référence du catalogue Numero di catalogo Número de catálogo
<b>LOT</b>	Batch code Chargenbezeichnung Code du lot Codice del lotto Codigo de lote
<b>IVD</b>	<i>In Vitro</i> Diagnostic Medical Device <i>In Vitro</i> Diagnostikum Dispositif médical de diagnostic <i>in vitro</i> Dispositivo medico-diagnostico <i>in vitro</i> Producto sanitario para diagnóstico <i>in vitro</i>
	Contains sufficient for <n> tests Ausreichend für „n“ Ansätze Contenu suffisant pour „n“ tests Contenuto sufficiente per „n“ saggi Contenido suficiente para <n> ensayos
	Consult Instructions for Use- Gebrauchsanweisung beachten Consulter le mode d'emploi Consultare le istruzioni per l'uso Consulte las instrucciones de uso

Symbol	Explanation
	Temperature limitation Zulässiger Temperaturbereich Limites de température Limiti di temperatura Limite de temperatura
<b>BUF STIM</b>	Stimulation Buffer Stimulations-Puffer Tampon de stimulation tampone di stimolazione Tampón de estimulación
<b>CONTROL STIM</b>	Stimulation Control Stimulationskontrolle Contrôle de stimulation Controllo di stimolazione Control de estimulación
<b>REAG STAIN</b>	Staining Reagent Färbe-Reagenz Réactif de coloration Reagente di colorazione Reactivo de coloración
<b>REAG LYS</b>	Lysing Reagent Lyse Reagenz Réactif de lyse Reagente di lisi Reactivo de lisis
<b>BUF BLOCK</b>	Blocking Buffer Blockierungs-Puffer Tampon Bloquant tampone bloccante Tampón bloqueante



This protocol is for reference purposes only.  
DO NOT use this copy to run your assay;  
use the protocol included with the kit ONLY.