Allergenicity Kit, Cellular Analysis of Allergy

REF A17116

∑ 100 Tests
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1. INTENDED USE

**Allergenicity Kit, Cellular Analysis of Allergy** consists of an optimized three-color combination of fluorescent monoclonal antibodies reagent, an activation solution, a positive control for IgE-mediated basophils activation, a stop solution, a lysing and a fixative solution.

It is intended for “For In Vitro Diagnostic Use” for the determination of activated basophils based on accurate basophils gating tool (CRTH2\textsuperscript{pos}CD203c\textsuperscript{pos}CD3\textsuperscript{neg}) performed on whole blood specimen.

2. SUMMARY AND EXPLANATION

The reagents of this kit are designed to identify and differentiate resting and activated basophils by flow cytometry. For this purpose, a 3-color combination is used. It is a mixture of 2-fluorescent murine monoclonal antibodies (CD203c-PE and CD3-PC7) and one-fluorescent rat monoclonal antibody (CRTH2-FITC).

**CRTH2-FITC / CD203c-PE / CD3-PC7 REAGENT**

CRTH2

T helper 1 (Th1) and T helper 2 (Th2) lymphocyte subsets are characterized by their cytokine profile production. While Th1 is reported to be involved in cellular immunity, Th2 profile is known to be responsive for humoral immune responses and allergy. Moreover, Th1- and Th2-like cytokine profiles are known as Type 1 and Type 2 responses (1 - 4).

CRTH2 is a seven-transmembrane molecule known as the Chemotactrant Receptor-homologous molecule that is preferentially expressed on human Th2 and T cytotoxic (Tc2) cells but not on Th1 and Tc1 cells (5).

Two G protein-coupled receptors, Prostaglandin D receptor (DP), and CRTH2 have been identified as receptors for Prostaglandin D2 (PGD2), but they differ in their signaling pathways (6, 7). PGD2 is the major metabolite of arachidonic acid produced by allergen-activated mast cells and has been implicated in various allergic diseases as a proinflammatory lipid mediator (6). CRTH2 is also a reliable surface marker selectively associated with circulating T (Th and Tc) cells able to produce IL-4 (as well as IL-5 and IL-13) but not IFN\(\gamma\) (5, 8).

The monoclonal antibody (mAb) BM16 precipitates a 55 to 70 kDa protein from cells lysates of CRTH2-transfected JURKAT and from established Th2 clone, (e.g. clone 6L21) corresponding to PGD2 receptor (9).

Among normal whole blood leucocytes, CRTH2 is highly expressed on basophils and eosinophils, as well as on Th2 and Tc2 cells that are known to be responsive for humoral immune responses and allergy (10, 11).

**CD203c**

Basophils and mast cells are hematopoietic effector cells involved in allergic and inflammatory reactions (12 - 14). Both cell types highly express the high affinity IgE receptor (FceR1). MAb 97A6 recognizes a surface antigen expressed on human peripheral blood basophils, but not on other blood cells (15). It also reacts with mature mast cells, and with CD34\textsuperscript{pos} bone marrow progenitors of basophils and mast cells. Moreover the 97A6 antigen is up-regulated after activation of basophils by anti-IgE antibodies and various allergens (16).

The basophil activation marker defined by mAb 97A6 is identical to the ecto-nucleotide pyrophosphatase / phosphodiesterase 3 (E-NPP3), a type II transmembrane protein that belongs to a family of ectoenzymes involved in hydrolysis of extracellular nucleotides (17).

MAb 97A6 was assigned to the CD203c cluster of differentiation at the 7\textsuperscript{th} HLDA Workshop on Human Leucocyte Differentiation Antigens, held in Harrogate, England, in 2000 (WS Code: MC10, Section New CD antigens)(17).
The CD3 antigen is a protein complex, which consists of 5 polypeptide chains (γ, δ, ε, ζ, η) linked to TCR (18, 19). The CD3 antigen is expressed only on mature T lymphocytes and on a sub-population of thymocytes (20). In peripheral blood, approximately 67 to 76% of lymphocytes are CD3pos; this percentage is lower in young children and varies according to age (21).

MAb UCHT1 reacts with the ε chain of the CD3 complex (22). MAb UCHT1 was assigned to CD3 during the 1st HLDA Workshop, held in Paris, France, in 1982 (WS Code: 3, Section T) (23).

**Allergenicity** Positive Control
Basophils express the IgE high affinity receptor (FcεR1). The anti-IgE recognizes the IgE bound to their receptor and consequently induces basophils activation. This reagent provides an IgE-mediated activation positive control.

**Allergenicity** Activation Solution
Is an optimized calcium-enriched buffer that allows the *in vitro* activation of basophils from whole blood using EDTA (ethylenediaminetetraacetic acid) as anti-coagulant.

**Allergenicity** Stop Solution
Is an optimized EDTA-enriched buffer that permits to stop the *in vitro* activation of basophils.

**Allergenicity** Lysing Solution
Flow cytometric procedures require monodispersed cell preparations and the removal of erythrocytes interference. Consequently, specimen for flow cytometric immuno-phenotyping must be appropriately prepared for staining and analysis (24 -26). This can generally be accomplished by two methods: lysis of erythrocytes or gradient density separation of the mononuclear cells from the erythroid cells. This reagent is intended for the lysis of red blood cells in the preparation of biological samples for flow cytometry analysis. The major active ingredient is a cyclic amine which, in contact with carbonic anhydrase present in red blood cells, is transformed into a compound which is highly lytic for these cells. It is compatible with all types of cytometers, as long as properly calibrated fluorescent antibodies are used for the chosen protocol.

**Allergenicity** Fixative Solution
The Allergenicity Fixative Solution allows whole blood specimen preparation by fixing the cell suspension during erythrolysis. It is also used for fixing the preparation before flow cytometry analysis.

### 3. PRINCIPLE OF TEST

The cell population of interest is stained with monoclonal antibodies in the presence of the allergen or controls. Erythrocytes are then lysed prior to flow cytometry analysis. Once excluding T lymphocytes (CD3 positive cells), basophils are analyzed using CRTH2 and CD203c expression. Non activated and resting basophils are identified as CRTH2<sup>pos</sup>CD203c<sup>dim</sup>CD3<sup>neg</sup>, whereas *in vitro* activated basophils are identified as CRTH2<sup>pos</sup>CD203c<sup>bright</sup>CD3<sup>neg</sup>.

For each blood specimen, a minimum of three analysis are performed:
- Tube # “Neg” = Negative Control Tube
- Tube # “Pos” = Positive Control Tube
- Tubes “Test” (it is recommended to use different dilutions of the allergen)

The red blood cells in each sample are lysed with a “Fix-and-Lyse” mixture and the remaining cells analyzed by flow cytometry. The flow cytometer analyzes light diffusion and the fluorescence of cells. It makes possible the delimitation of the population of interest within the electronic window defined on a histogram, which correlates the orthogonal diffusion of light (Side Scatter or SS) and the diffusion of narrow-angle light (Forward Scatter or FS). Histograms combining two of the different parameters available on the cytometer are used as supports in the gating stage for the application (see section 11).

The fluorescence of the delimited cells is analyzed in order to distinguish the positively-stained events from the unstained ones.
4. CLINICAL RELEVANCE

Basophils play a key role in immediate hypersensitivity as primary effector cells. These cells express the high affinity receptor for IgE (FcεR1). Allergens induce basophils activation by cross-linking surface IgE leading to release of mediators and expression of activation markers on the basophils surface. In response to allergen challenge, sensitized patient’s basophils up-regulate CD203c surface expression.

The analysis of CD203c antigen allows the characterization of resting and activated basophils in an IgE-dependent response to allergens in sensitized individuals (16, 27-30).

The set of reagents is intended to determine the in vitro measurement of the IgE-dependent responses to allergen in sensitized individuals by flow cytometry analysis of basophils.

5. REAGENTS AND CONTENTS

5.1 Allergenicity Kit allows to perform 100 tests and contains the following:

- **Allergenicity CRTH2-FITC / CD203c-PE / CD3-PC7** – 1 vial (2 mL, liquid format) – 20 µL/test.
- **Allergenicity Positive Control** – 1 vial [0.2 mg, freeze-dried, in phosphate-buffered saline (PBS) containing 2 mg/mL bovine serum albumin (BSA)] – 20 µL/test.
- **Allergenicity Activation Solution** – 2 vials. (2x5 mL, liquid format containing calcium in buffered solution) – 100 µL/test.
- **Allergenicity Stop Solution** – 1 vial (10 mL, liquid format containing EDTA and 0.1% sodium azide) – 100 µL/test.
- **Allergenicity Lysing Solution** – 3 vials (3x100 mL, liquid format containing cyclic amine as major active ingredient) – 2 mL/test.
- **Allergenicity Fixative Solution** – 1 vial (10 mL, liquid format containing 8% of formic acid).

The reagents of this kit should be used as a set as supplied in the kit and should not be separated. When the tests are performed, care should be taken that all reagents have the same lot number on the vial label.

5.2 Allergenicity CRTH2-FITC / CD203c-PE / CD3-PC7

**Summary of antibodies characteristics**

<table>
<thead>
<tr>
<th>Specificity</th>
<th>CLONE 1</th>
<th>CLONE 2</th>
<th>CLONE 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clonal</td>
<td>CRTH2</td>
<td>CD203c</td>
<td>CD3</td>
</tr>
<tr>
<td>Hybridoma</td>
<td>BM16</td>
<td>97A6</td>
<td>UCHT1</td>
</tr>
<tr>
<td>Immunogen</td>
<td>CRTH2 transfected cell line (TART/B19-12.10)</td>
<td>UT-7 megakaryoblastic cell line</td>
<td>Peripheral blood lymphocytes</td>
</tr>
<tr>
<td>Ig Chain</td>
<td>IgG2a</td>
<td>IgG1</td>
<td>IgG1</td>
</tr>
<tr>
<td>Species</td>
<td>Rat</td>
<td>Mouse</td>
<td>Mouse</td>
</tr>
<tr>
<td>Conjugation</td>
<td>Fluoresceine isothiocyanate (FITC)</td>
<td>R Phycoerythrin (PE)</td>
<td>R Phycoerythrin covalently linked to cyanine 7 (PC7)</td>
</tr>
<tr>
<td>Molar Ratio</td>
<td>5 – 8 moles of FITC per mole of Ig</td>
<td>0.5 – 1.5 moles of PE per mole of Ig</td>
<td>0.5 – 1.5 moles of PC7 per mole of Ig</td>
</tr>
<tr>
<td>Excitation wavelength</td>
<td>488 nm</td>
<td>488 nm</td>
<td>488 nm</td>
</tr>
<tr>
<td>Maximum emission wavelength</td>
<td>525 nm</td>
<td>575 nm</td>
<td>750 – 810 nm</td>
</tr>
<tr>
<td>Source</td>
<td>Ascites fluid</td>
<td>Ascites fluid</td>
<td>Ascites fluid</td>
</tr>
<tr>
<td>Purification</td>
<td>Ion exchange or affinity chromatography</td>
<td>Protein A affinity chromatography</td>
<td>Protein A affinity chromatography</td>
</tr>
<tr>
<td>Main emission color</td>
<td>Green</td>
<td>Orange-red</td>
<td>Far-red</td>
</tr>
<tr>
<td>Buffer</td>
<td>2 mg/mL bovine serum albumin in phosphate-buffered saline containing 0.1% sodium azide.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6. STATEMENT OF WARNING

6.1 All blood samples must be considered as potentially infectious and must be handled with care (in particular: the wearing of protective gloves, gowns and goggles).

6.2 Some reagents of this kit contain 0.1% sodium azide. Sodium azide under acid conditions yields hydrazoic acid, an extremely toxic compound. Azide compounds should be flushed with running water while being discarded. These precautions are recommended to avoid deposits in metal piping in which explosive conditions can develop. If skin or eye contact occurs, wash excessively with water.

6.3 Allergenicity Fixative Solution contains formaldehyde as a fixative. Formaldehyde is harmful at both concentrations of 8% and 0.8%. Avoid inhalation, contact with eyes, skin and clothing. Formaldehyde is toxic and allergenic. It is thought to be a carcinogenic agent.

6.4 Never pipet by mouth and avoid contact of specimens or reagents with skin and mucous membranes.

6.5 Do not use reagents beyond the expiration date on the vial label.

6.6 Do not freeze reagents (except Allergenicity Positive Control, if aliquoted).

6.7 Do not expose reagents to heat during storage or use.

6.8 Incubation or mixing times or temperatures other than those specified may give erroneous results.

6.9 Avoid evaporation and leakage of reagents by capping vials tightly after use, or erroneous results may occur.

6.10 Erroneous results may occur if the flow cytometer is not properly aligned or standardized for fluorescence or if the cell populations are improperly gated.

6.11 Do not expose reagents to strong light during storage or incubation.

6.12 Avoid microbial contamination of reagents or incorrect results might occur.

6.13 Results determined using flow cytometers, lysing systems, or antibodies that are different from those used to determine the expected results may not fall within the expected range.

6.14 Blood tubes and disposable material used for handling should be disposed of in ad hoc containers intended for incineration.

6.15 Use general good laboratory practices when handling the reagents.

6.16 Allergenicity Lysing Solution must be stored at room temperature. At receipt, if stored at 4°C, bring the Lysing Solution to room temperature before use to avoid erroneous results.

6.17 If a packaging problem occurs or if erroneous results appear, contact the Customer Service of Beckman Coulter.

7. STORAGE CONDITIONS AND STABILITY

7.1 Storage

All reagents are stored at 2 – 8°C except:

- Allergenicity Lysing Solution: store at 18 – 25°C. Store the Lysing Solution at room temperature at receipt of the kit. The Lysing Solution must be used at room temperature. If stored at 4°C, bring the Lysing Solution to room temperature before use.

- Allergenicity Positive Control: store at –20°C if resuspended and aliquoted (see section 7.3.2).

7.2 Stability

Allergenicity Kit is stable up to the expiry date stated on the label when stored at 2 – 8°C. Do not use after the expiration date. Once components of the kit are opened, the stability of the kit is 90 days.

7.3 Reagents Preparation

Bring all reagents to room temperature (18 – 25°C) prior to experiment.

7.3.1 Ready-for-use reagents

- CRTH2-FITC / CD203c-PE / CD3-PC7.
- Allergenicity Activation Solution.
- Allergenicity Stop Solution.

No reconstitution is necessary and reagents may be used directly from the vial.

7.3.2 Preparation of other reagents

7.3.2.1 Allergenicity Positive Control

Reconstitute freeze-dried Positive Control by addition of 1 mL of 0.22 μm filtered deionized water in order to obtain the concentration of 0.2 mg/mL. We recommend to aliquot and store at −20°C. Before use, thaw one aliquot and dilute it 1:20 to obtain the working solution.
Prepare a sufficient amount of the Positive Control for the total number of sample preparations. See the following table as an example:

<table>
<thead>
<tr>
<th>Allergenicity</th>
<th>Positive Control</th>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>For 10 tubes</td>
<td>10 µL</td>
<td>190 µL</td>
</tr>
<tr>
<td>For 20 tubes</td>
<td>20 µL</td>
<td>380 µL</td>
</tr>
<tr>
<td>For 50 tubes</td>
<td>50 µL</td>
<td>950 µL</td>
</tr>
</tbody>
</table>

7.3.2.2 “Fix-and-Lyse” preparation

Allergenicity Lysing Solution (3x100 mL). Use reagent directly from the vial.

Allergenicity Fixative Solution (1x10 mL). Use reagent directly from the vial.

A “Fix-and-Lyse” mixture using Allergenicity Lysing Solution and Allergenicity Fixative Solution must be prepared daily for the total number of sample preparation. Do not refrigerate the working “Fix and Lyse” solution. Discard the unused “Fix-and-Lyse” mixture at the end of the day.

Prepare a sufficient amount of the “Fix-and-Lyse” mixture for the total number of sample preparation. See the following table as an example:

<table>
<thead>
<tr>
<th>Allergenicity Lysing Solution</th>
<th>Allergenicity Fixative Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>For 10 tubes</td>
<td>20 mL</td>
</tr>
<tr>
<td>For 20 tubes</td>
<td>40 mL</td>
</tr>
<tr>
<td>For 50 tubes</td>
<td>100 mL</td>
</tr>
</tbody>
</table>

7.3.2.3 PBS 0.1% formaldehyde preparation

Prepare PBS 0.1% formaldehyde by mixing 1 mL of PBS with 12.5 µL (example for two tubes) of Allergenicity Fixative Solution. Prepare a sufficient amount of PBS 0.1% formaldehyde for the total number of sample preparation.

Prepare a sufficient amount of buffer for the total number of sample preparation. See the following table as an example:

<table>
<thead>
<tr>
<th>PBS</th>
<th>Allergenicity Fixative Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>For 10 tubes</td>
<td>5 mL</td>
</tr>
<tr>
<td>For 20 tubes</td>
<td>10 mL</td>
</tr>
<tr>
<td>For 50 tubes</td>
<td>25 mL</td>
</tr>
</tbody>
</table>

8. MATERIAL REQUIRED BUT NOT SUPPLIED

8.1 Deionized water.
8.2 PBS buffer (e.g. Beckman Coulter PN 6602489).
8.3 Evacuated blood collection tubes with EDTA anticoagulant (if heparin is used, see section “Procedure for Specimen Processing”).
8.4 Flow-Set™ Fluorospheres (e.g. Beckman Coulter Ref. 6607007)
8.5 PC7 Set-up Kit (e.g. Beckman Coulter PN 6607121)
8.6 Reagents for FITC, PE, PC7 compensation adjustment.
8.7 Appropriate Isotypic Controls :
   a. Rat IgG2a-FITC (e.g. Beckman Coulter PN IM1271)
   b. Mouse IgG1-PE (e.g. Beckman Coulter Ref. A07796)
   c. Mouse IgG1-PC7 (e.g. Beckman Coulter PN 6607099)
8.8 Plastic test tubes (12 x 75 mm).
8.9 37°C water bath.
8.10 Vortex mixer.
8.11 Timer.
8.12 Calibrated repeater pipet (20 µL, 100 µL, 2 mL) and tips.
8.13 Tubes for freezing.
8.14 Flow cytometer.
8.15 Allergens.
9. SPECIMEN COLLECTION AND PREPARATION

9.1 Specimen Requirements

9.1.1 Collect peripheral blood samples aseptically into a sterile evacuated blood collection tube with EDTA as anticoagulant. EDTA-collected specimens should be stored at room temperature (18 – 25°C) until processing.

9.1.2 EDTA-collected specimens should be less than 24 hours old for processing.

9.1.3 The volume of collected peripheral blood samples shall be optimum (i.e. more than half of total volume acceptance).

9.1.4 Heparinized whole blood may be used with a modified procedure (see section 9.3). Fresh specimens shall be used.

9.1.5 All specimens shall be processed with the kit reagents.

9.1.6 The specimen shall be kept at room temperature (18-25°C) and not shaken. The specimen should be homogenized by gentle agitation prior pipetting. Samples must be processed within 24 hours of taking them.

9.2 Procedure For Instrument Adjustment

9.2.1 The flow cytometer must be equipped to detect Forward Scatter, Side Scatter and three fluorescence channels allowing the analysis of FITC-, PE-, and PC7- conjugated antibodies (respectively 504 – 541 / 568 – 590 / 750 – 810 nm maximal peak emission).

9.2.2 Ensure that the flow cytometer is properly aligned and standardized for fluorescence intensity (according to the manufacturer recommendations).

9.2.3 Ensure that the flow cytometer is properly adjusted for fluorescence compensation.

Example of photomultiplier (PMT) settings on Beckman Coulter instrument (EPICS® XL/ XL-MCL™ flow cytometer equipped with EXPO32™ analysis software).

While running Flow-Set Fluorospheres, adjust the PMT settings to place the peaks of Scatter and fluorescence intensity distributions within the following target ranges:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS</td>
<td>116 – 132</td>
</tr>
<tr>
<td>SS</td>
<td>370 – 432</td>
</tr>
<tr>
<td>LOG FL1 (FITC)</td>
<td>22.5 – 24.2</td>
</tr>
<tr>
<td>LOG FL2 (PE)</td>
<td>37.5 – 50.5</td>
</tr>
<tr>
<td>LOG FL4 (PC7)</td>
<td>235 – 270</td>
</tr>
</tbody>
</table>

Refer to the instruction for use of Flow-Set Fluorospheres and PC7 Set-up Kit (see section 8) for optimized standardization.

9.3 Procedure for Specimen Processing

**IMPORTANT:**

- The following procedure is optimized for whole blood specimen collected with EDTA as anticoagulant.
- Fresh heparinized whole blood may be used following this procedure while eliminating step 3 (without 100 µL of Activation Solution).
- Bring all reagents to room temperature (18 – 25°C) prior to experiment.
- Each laboratory shall establish the right range of allergens dilutions to be used for the experiment.

For each experiment, label the appropriate number of tubes:

- Tube # “Neg” = Negative Control Tube
- Tube # “Pos” = Positive Control Tube
- Tubes “Test” (different dilutions of the appropriate allergen)
Add:
- 20 µL of PBS on tube # “Neg” (Negative Control)
- 20 µL of Allergenicity Positive Control on tube # “Pos” (Positive Control)
- 20 µL of tested allergen on tubes “Test”

2. Add 20 µL of CRTH2-FITC / CD203c-PE / CD3-PC7 reagent into each tube.
3. Add 100 µL of Activation Solution into each tube.
4. Pipet 100 µL of whole blood into each tube.
5. Gently vortex each tube and incubate for 15 minutes at 37°C on water bath, protected from light.
6. Add 100 µL of Stop Solution into each tube, and gently vortex for 5 seconds.

**Warning:** Allergenicity Lysing Solution must be stored and used at room temperature otherwise improper lysis may occur. If stored at 4°C, bring the Lysing Solution to room temperature prior to use.

7. Add 2 mL of “Fix-and-Lyse” mixture to each tube, vortex.
8. Incubate at room temperature (18 – 25°C) for 10 minutes, protected from light.
9. Centrifuge the tubes for 5 minutes at 200 g, and aspirate the supernatant.
10. Add 3 mL of PBS in each tube.
11. Centrifuge the tubes for 5 minutes at 200 g, and aspirate the supernatant.
12. Resuspend the cell pellet on 0.5 mL of PBS 0.1 % formaldehyde.
13. Acquire on the flow cytometer.

If not analyzed within one hour, processed samples must be stored between 2-8°C, protected from light, and analyzed within 4 hours.

### 10. PROCEDURE

<table>
<thead>
<tr>
<th></th>
<th>Negative Control Tube</th>
<th>Positive Control Tube</th>
<th>Test Tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>20 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allergens</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRTH2-FITC/CD203c-PE/CD3-PC7</td>
<td>20 µL</td>
<td>20 µL</td>
<td>20 µL</td>
</tr>
<tr>
<td>Activation Solution</td>
<td>100 µL</td>
<td>100 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>EDTA whole blood</td>
<td>100 µL</td>
<td>100 µL</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

**Gently Vortex**

Incubate for 15 minutes at 37°C , protected from light (**WATER BATH PREFERED**)

<table>
<thead>
<tr>
<th></th>
<th>Negative Control Tube</th>
<th>Positive Control Tube</th>
<th>Test Tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stop Solution</td>
<td>100 µL</td>
<td>100 µL</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

**Gently Vortex**

“Fix-and-Lyse” mixture

**Warning:** Allergenicity Lysing Solution must be stored and used at room temperature. If stored at 4°C, bring the Lysing Solution to room temperature before use.

<table>
<thead>
<tr>
<th></th>
<th>Negative Control Tube</th>
<th>Positive Control Tube</th>
<th>Test Tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 mL</td>
<td>2 mL</td>
<td>2 mL</td>
</tr>
</tbody>
</table>

**Gently Vortex**

Incubate for 10 minutes at 18 – 25°C , protected from light

<table>
<thead>
<tr>
<th></th>
<th>Negative Control Tube</th>
<th>Positive Control Tube</th>
<th>Test Tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

|                      | 3 mL                  | 3 mL                  | 3 mL       |

**Wash:**

Centrifuge for 5 minutes at 200 g
Aspirate supernatant
Resuspend in 0.5 mL PBS 0.1 % formaldehyde

Flow Cytometry Analysis

Stop acquisition when 500 basophils are acquired (Region C)
11. EXAMPLE OF MANUAL ANALYSIS METHOD

11.1 Required parameters

The flow cytometer must be equipped to detect Forward Scatter, Side Scatter and the three following fluorochromes: FITC, PE, and PC7.

11.2 Histogram Creation:

Create histograms as follows in order to characterize resting and activated basophils.

1. Create Histogram 1 as Forward Scatter vs Side Scatter.
2. Create Histogram 2 as CD3-PC7 vs Side Scatter.
3. Create Histogram 3 as CRTH2-FITC vs CD203c-PE.
4. Create Histogram 4 as CRTH2-FITC vs CD203c-PE.

11.3 Region Creation

Create regions as follows:
1. Histogram 1 – Create an amorphous/polygonal Region A on Histogram 1 to include all leucocytes and eliminate red blood cell debris, and aggregates.
2. Histogram 2 – Create a rectilinear Region B on Histogram 2 to include all the lymphocytes and monocytes while excluding CD3bright events (i.e. T lymphocytes).
3. Histogram 3 – Create a rectilinear Region C on Histogram 3 to include all clustered CRTH2posCD203cposCD3neg events.
4. Histogram 4 – Create Quadstat Region D to include all clustered CRTH2posCD203cposCD3neg events.

11.4 Gate Creation

Create gates as follows:
1. Histogram 1 – Ungated to display all events.
2. Histogram 2 – Assign “A” to Histogram 2 to display all leucocytes while eliminating red blood cell debris, and aggregates.
3. Histogram 3 – Assign “A” and “B” (AB) to Histogram 3 to display all basophils CRTH2posCD203cposCD3neg.
4. Histogram 4 – Assign “A”, “B” and “C” (ABC) to Histogram 4 to display all CRTH2posCD203cposCD3neg events.

11.5 Analysis Example

See Appendix

11.6 Calculation of Analyzed Basophils

See Appendix

12. LIMITATIONS OF THE TECHNIQUE

12.1 Flow cytometry may produce false results if the cytometer has not been aligned perfectly, if fluorescence leaks have not been correctly compensated for and if the regions have not been carefully positioned.

12.2 The conjugated antibody of this reagent is calibrated so as to offer the best specific signal/non-specific signal ratio. Therefore, it is important to adhere to the reagent volume/number of cells ratio in every test.

12.3 Accurate and reproducible results will be obtained as long as the procedures used are in accordance with the technical insert leaflet and compatible with good laboratory practices.

12.4 All reagents must be brought to room temperature (18 – 25°C) before use.

12.5 Verify the preparations using the naked eye to assess the efficacy of lysis. If they are cloudy or if the light diffraction histograms are unusual, it may be that lysis is incomplete.

12.6 The erythroblasts may be incompletely lysed and appear on a light diffraction histogram in the same location as the leucocytes.

12.7 Acetazolamide, an inhibitor of carbonic anhydrase can completely inhibit the action of the lysing solution.
13. PERFORMANCE

13.1 Linearity

To test the linearity of staining of this reagent, a positive cell line (KU812 or MOLT16) and a negative cell line (KG-1a) were mixed in different proportions with a constant final number of cells, so that the positive/negative line ratio of the mixture ranged from 0 to 100%.

Aliquots were stained with the 3-color combination. After analysis the data were plotted and linear regression between the expected values and the observed values was calculated. The parameters of the equation of the linear regression may be used to determine the linearity as well as the range of measurement.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Cell Line Tested</th>
<th>Linearity (R²)</th>
<th>Range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRTH2-FITC</td>
<td>MOLT16 + KG-1a</td>
<td>0.99</td>
<td>0.1 – 90</td>
</tr>
<tr>
<td>CD203c-PE</td>
<td>KU812 + KG-1a</td>
<td>0.99</td>
<td>0.1 – 97</td>
</tr>
<tr>
<td>CD3-PC7</td>
<td>MOLT16 + KG-1a</td>
<td>0.99</td>
<td>2.0 – 98</td>
</tr>
</tbody>
</table>

13.2 Expected values

Each laboratory must compile a list of reference values based upon a group of healthy donors from the local population. This must be done by taking age, sex and ethnic group into account, as well as any other potential regional differences.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Cell Line Tested</th>
<th>Linearity (R²)</th>
<th>Range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRTH2-FITC</td>
<td>MOLT16 + KG-1a</td>
<td>0.99</td>
<td>0.1 – 90</td>
</tr>
<tr>
<td>CD203c-PE</td>
<td>KU812 + KG-1a</td>
<td>0.99</td>
<td>0.1 – 97</td>
</tr>
<tr>
<td>CD3-PC7</td>
<td>MOLT16 + KG-1a</td>
<td>0.99</td>
<td>2.0 – 98</td>
</tr>
</tbody>
</table>

13.3 Intra-Laboratory Reproducibility

On the same day, using the same cytometer, 12 measurements of the percentage of total non-activated and activated basophils (after anti-IgE in vitro stimulation) were carried out on blood taken from the same donor. The results obtained are summarized in the following table:

<table>
<thead>
<tr>
<th>Basophils</th>
<th>Number</th>
<th>Mean of (%)</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated</td>
<td>12</td>
<td>94.26</td>
<td>1.06</td>
<td>1.1</td>
</tr>
<tr>
<td>Non activated</td>
<td>12</td>
<td>5.39</td>
<td>1.33</td>
<td>24.6</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>0.54</td>
<td>0.05</td>
<td>8.5</td>
</tr>
</tbody>
</table>

13.4 Inter-Laboratory Reproducibility

On the same day and on blood from the same donor, 12 measurements of the percentage of total non-activated and activated basophils (after anti-IgE in vitro stimulation) were carried out by two technicians and the preparations analyzed using two different cytometers. The results obtained are summarized in the following tables:

Cytometer No 1:

<table>
<thead>
<tr>
<th>Basophils</th>
<th>Number</th>
<th>Mean of (%)</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated</td>
<td>12</td>
<td>95.63</td>
<td>0.55</td>
<td>0.6</td>
</tr>
<tr>
<td>Non activated</td>
<td>12</td>
<td>5.42</td>
<td>1.02</td>
<td>18.9</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>0.53</td>
<td>0.06</td>
<td>11</td>
</tr>
</tbody>
</table>

Cytometer No 2:

<table>
<thead>
<tr>
<th>Basophils</th>
<th>Number</th>
<th>Mean of (%)</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated</td>
<td>12</td>
<td>94.26</td>
<td>1.06</td>
<td>1.1</td>
</tr>
<tr>
<td>Non activated</td>
<td>12</td>
<td>5.39</td>
<td>1.33</td>
<td>24.6</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>0.54</td>
<td>0.05</td>
<td>8.5</td>
</tr>
</tbody>
</table>

14. REFERENCES

See Appendix
11.5 Analysis Example

The following histograms display examples in an ascending number order as displayed on the protocol. The following histograms correspond to the analysis of the whole blood from an allergic patient stained with CRTH2-FITC / CD203c-PE / CD3-PC7 reagents.

**NOTE:** Below are histograms obtained on a BECKMAN COULTER CYTOMICS™ FC 500 flow cytometer equipped with the CYTOMICS™ RXP Software.

**Histogram 1:**
- Histogram 1 displays all events.
- Position Region “A” to include all leukocytes while excluding debris.

**Histogram 2:**
- Display events from region “A”.
- Adjust Region “B” to include lymphocytes and monocytes while excluding CD3^{pos} events (i.e. T lymphocytes).
Negative control | Positive control | Allergen (grass pollen)
---|---|---
![Histograms 3a, 3b and 3c](image)
- Display events from gate “A” and “B” (AB).
- Adjust Region C to include all clustered CRTH2\(^{\text{pos}}\)CD203\(^{\text{pos}}\)CD3\(^{\text{neg}}\) events.
- Region C allows the determination of the Y-Median fluorescence intensity parameter of non-activated and activated basophils.

Negative control | Positive control | Allergen (grass pollen)
---|---|---
![Histograms 4a, 4b and 4c](image)
- Display all events from gate “A” and “B” and “C” (ABC).
- Adjust Quadstat to include CRTH2\(^{\text{pos}}\)CD203\(^{\text{pos}}\)CD3\(^{\text{neg}}\) cluster of cells (95 – 98% of cells) on the lower quadrant D4 from the non-activated whole blood sample (Negative Control Tube, Histogram 4a). The x-axis of D4 region must be the same for the negative control, positive control and activated sample with allergen (Histograms 4a, 4b and 4c). Quadrants D1 and D3 are aligned on the y axis of the histogram 4.
- Lower quadstat (i.e. D4 region) defines the region of non-activated basophils. Lower quadstat gives the percentage of non activated basophils on the statistic printout.
- Upper quadstat (i.e. D2 region) defines the region of activated basophils. Upper quadstat gives the % of activated basophils on the statistic printout.

### 11.6 Calculation of Analyzed Basophils
- Histograms 3a, 3b and 3c allows the analysis of activated basophils using the Y-Median fluorescence intensity as parameter for data interpretation.
- Histograms 4a, 4b and 4c allow the analysis of activated basophils using the percentage of activated basophils as parameter for data interpretation.
14. REFERENCES


Manufactured by:
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