CD203c Reagent Set

Flow CAST®

Basophil Activation Test (BAT)
Flow Cytometry

For Research use only

B-CCR-203SET 100 tests

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INTENDED USE

The CD203c Reagent Set is intended for the use within an extended protocol for the Flow CAST® kit. It contains an additional staining reagent allowing the measurement of both activation markers, CD63 and CD203c, on basophilic granulocytes in whole blood by flow cytometry after antigen stimulation.
In addition, the reagent set contains stimulation buffer without IL-3 which allows the optimal stimulation of the CD203c marker.

REAGENTS SUPPLIED AND PREPARATION

Reagents	Quantity	Code	Reconstitution
CD203c Staining Reagent Anti-CD203c-PE-DY647 mAb	1 vial 2.2 ml	B-CCR-SR203	Ready to use
Cellular Stimulation Buffer Contains calcium and heparin	1 vial lyoph.	B-CCR-CSB	Reconstitute with 50 ml of water[1)]

Table 1

[1)] For required water quality, see Chapter Procedural Notes

STORAGE AND SHELF LIFE OF REAGENTS

Unopened reagents	
Store at 2-8℃. Do not use past expiration date.	
Opened/reconstituted reagents	
Stimulation Buffer	Stable at –20℃ for 6 months. Aliquot if repeated u se is expected.
Staining Reagent	Stable at 2-8℃ until expiration date.

Table 2

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents	Supplier	Code	Processing
Flow CAST® Kit	Bühlmann Laboratories AG	FK-CCR	See instruction for use

- K-EDTA venipuncture tubes.
- Flow Cytometer with 488 nm (blue) excitation wavelength including appropriate software.

SPECIMEN COLLECTION AND STORAGE

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

Collect sufficient blood in **K-EDTA venipuncture tubes**. Fill the venipuncture tubes up to the specified volume. In tubes which are filled < 50 %, the EDTA concentration in the sample is higher and thus may give false negative results.
1 ml of whole blood is sufficient for about 18 tests.
Perform the cell stimulation immediately or store the blood sample refrigerated (2-8℃) for up to 24 hours**.** In order to be able to detect responses to drugs, use blood samples only for up to 3 hours after collection. **Do not centrifuge or freeze blood samples**.

PROCEDURAL NOTES

- PRECAUTIONS TO AVOID ALLERGEN CONTAMINATION DURING CELL STIMULATION. Aeroallergens in the laboratory may contaminate unsealed blood samples and cell suspensions and thus potentially cause elevated background stimulation. Therefore, blood samples and cell stimulation tubes must be carefully covered. Avoid dust mites, pollinating plants, latex gloves or equipment potentially containing latex and open windows in the laboratory where the cell stimulation is carried out. Therefore, we recommend preparing and stimulating the cells in a laminar flow hood.
- RECOMMENDED WATER QUALITY FOR THE FLOW CAST®. The use of sterile, ultrapure and apyrogenic water for reconstituting Stimulation Buffer (B-CCR-CSB) is essential for sufficient and reproducible basophil stimulation. Water may be used from the following sources: Cell culture grade water, infusion grade water or deionized water that is ultra filtrated in a periodically sanitized 10 kDa ultra filter.

ASSAY PROCEDURE

Important: The following procedure is optimized for whole blood specimen collected in EDTA tubes.

1. Mix the blood sample by inverting the EDTA coated venipuncture tube several times.
2. Prepare fresh and pyrogen-free 3.5 ml polypropylene or polystyrene tubes suitable for Flow Cytometry measurements.
3. For each patient, label tubes for:
   PB = patient background
   PC1 = stimulation control with anti-FcεRI Ab
   PC2 = stimulation control with fMLP
   A1-1 for allergen 1 with dilution 1
   A1-2 for allergen 1 with dilution 2
   etc.

Stimulation and Staining

4. Pipet 50 µl of the following “stimulators” into each tube
   a) PB tube: 50 µl of **Stimulation Buffer (background)**
   b) PC1 tube: 50 µl of **Stimulation Control**
   c) PC2 tube: 50 µl of **Stimulation Control fMLP**
   d) Ax-y tube: 50 µl of **Allergen**

   Repeat the sequence a)-d) for each patient.
5. Add 100 µl of Stimulation Buffer into each tube.
6. Add 50 µl of patient’s whole blood into each tube (a-d). Make sure that the side wall and top of the tube are free of blood.
7. Mix gently.
8. Add 20 µl Staining Reagent (CCR-SR) into each tube.
9. Add 20 µl CD203c Staining Reagent (CCR-SR203) into each tube.
10. Mix gently, cover the tubes and incubate them for 15 minutes at 37℃ in a **water bath**.
    (If an incubator is used, the incubation time will have to be extended for about 10 minutes due to a less efficient heat transfer).

Lysing

11. Add 2 ml Lysing Reagent, adjusted to 18-28 °C, into each tube, mix gently.
12. Incubate for 5 -10 minutes at 18-28℃.

13. Centrifuge the tubes for 5 minutes at 500 x g.
14. Decant the supernatant on blotting paper.
15. Resuspend the cell pellet with 300 µl of Wash Buffer.

Note: Depending on Flow cytometry instrumentation, it might be necessary to increase the required Wash Buffer volume (e.g. 800 µl).

16. Vortex gently.
17. Acquire the data on the flow cytometer within the same day. If the samples are stored for several hours before the analysis (<16 h) they should be kept protected from light at 2-8°C.

FLOW CYTOMETRIC DATA ACQUISITION

Flow cytometric data acquisition can be performed on any flow cytometer equipped with an argon laser diode at 488 nm (blue-green excitation light).
The flow cytometer must be equipped with Forward Scatter (FSC), Side Scatter (SSC), and suitable detection channels for the fluorochromes FITC, PE and PE-DY647 (analogue to PC5).
Ensure that the flow cytometer is properly aligned and colour compensation is set.
During data acquisition of samples, make sure that the leukocyte population is separated into three discrete populations on the FSC/SSC histogram. Adjust the amplification (gain) of FSC and SSC signals to obtain a cell distribution like the one shown in Figure 1. Refer to the flow cytometer manual for further information.

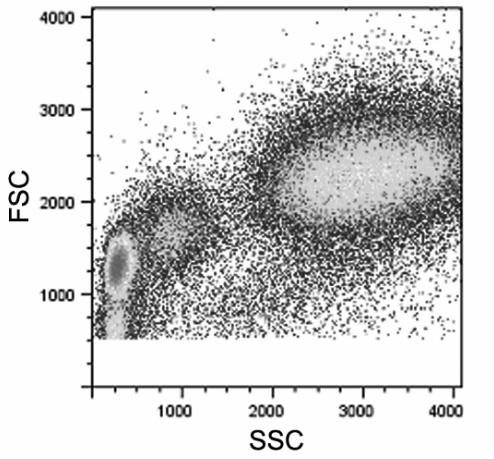


Figure 1: Three discrete populations (lymphocytes, monocytes and granulocytes) in FSC/SSC histogram.

Typically, the acquisition can be stopped, after having acquired 500-600 basophilic cells (gated as shown in figure 2). At least 200 basophilic cells must be analyzed to obtain reliable results. This requires a total amount of 50'000-100'000 leukocytes per sample to be acquired. The analysis of drug allergies is more difficult, because the activation of basophils in these patients is lower than with other allergies. Thus the limit of basophilic cells analyzed should be set to 300 or more. We recommend each laboratory define its own confidence limits.

DATA ANALYSIS

The analysis of acquired data can be performed with any flow cytometry analysis software e.g. FlowJo, FloMax, CellQuest or others.
The analysis is done in two steps:

1. Include the entire basophil population CCR3 ^{pos} by setting the gate R1 with the low Side Scatter SSC ^{low} (see Figure 2). Eosinophils located at SSC ^{high} position (upper right side) will be excluded.
2. Display the cells selected in R1 in a graph with CCR-PE on the x-axis and CD63-FITC on the y-axis. Set a quadrant gate so that the ratio of cells in the upper right quadrant for the base activation (PB-tube) is below 5% (eg. between 2 and 2.5%) compared to the total amount of basophilic cells gated in R1 (see Figure 3). Do the same for CD203c-PE-DY647 in an additional graph window (Figure 3 right side).

Note: Set a new quadrant threshold for each patient. Once the quadrant is set with the measurement of the patient background (PB), use the same quadrant position for the analysis of all other tubes to be tested from the respective patient (positive controls and allergens. See Figure 4).

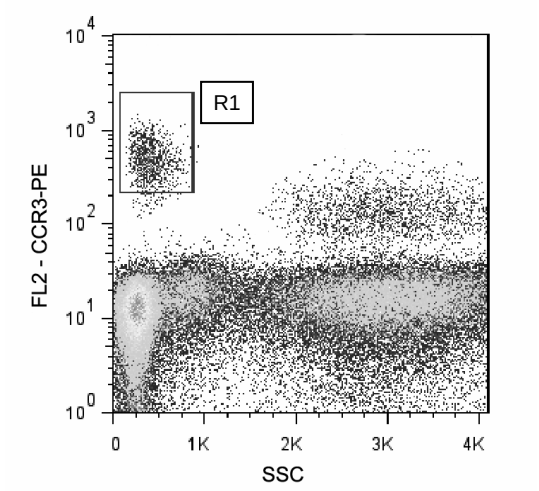
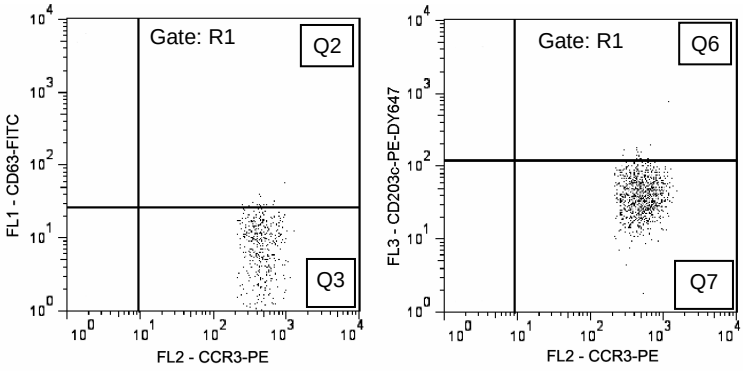
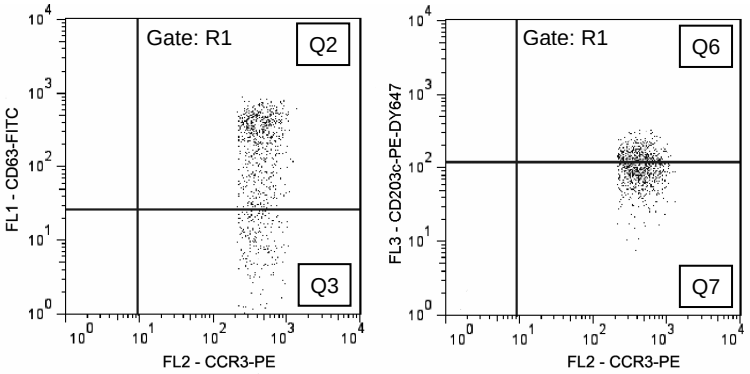


Figure 2: Selection of basophilic cells CCR3 ^{pos} / SSC ^{low}



Gated Region	Count (n=)	%	%
Total	82394	100	
R1	585	0.71	100
Q2 (CD63 ^{pos})	12		**2.05**
Q6 (CD203c ^{pos})	14		**2.39**

Figure 3: Patient Background (PB) for CD63 and CD203c with STB only



Gated Region	Count (n=)	%	%
Total	82503	100	
R1	561	0.68	100
Q2 (CD63 ^{pos})	392		**69.9**
Q6 (CD203c ^{pos})	250		**44.5**

Figure 4: Stimulation Control (STCON) for CD63 and CD203c

QUALITY CONTROL

For the appropriate evaluation of results, reference parameters should be looked at:

a) 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).

b) The **absolute number of basophils** recovered and evaluated, indicates whether the test has been properly performed and a sufficient number of basophils has been counted in order to achieve a statistically relevant difference from the controls. To our experience, the number of basophils to be analyzed should be at least 200.

c) The **percentage of activated basophils**. In the **negative control** (background) is usually below 5%. Sometimes, however, the percentage of activated basophils in the negative control might be much higher. This may be due to *in vivo* basophil activation indicating recent allergen exposure (*e.g.* latex gloves, pollen allergic patient during the pollen season, blood sampling following food or drug allergen exposure). Occasionally, it may also be caused by technical mishap such as contact of the basophils *in vitro* with inappropriate plastic material or reagents which result in non-specific basophil activation.

INTERPRETATION OF RESULTS

In order to obtain optimal sensitivity and specificity, different cut-off values should be applied for different groups of allergens. Each laboratory should determine their own cut-off values for CD63 and CD203c.

Flow CAST® with CD203c staining

Labeled tubes

↓

50 µl Stimulation Buffer, Control or Allergen
add 100 µl Stimulation Buffer (CCR-CSB)
add 50 µl patients whole blood

↓ *mix gently*

add 20 µl Staining Reagent (CCR-SR)
add 20 µl CD203c Staining Reagent (CCR-SR203)

↓ *mix gently and incubate for 15 min at 37°C (waterbath)*

add 2.0 ml pre-warmed (18-28°C) Lysing Reagent (C CR-LYR)

↓ *incubate 5 - 10 min at 18-28°C*

centrifuge 5 min at 500 x g and decant or aspirate supernatant

add 300 µl Wash Buffer[1)]

↓ *vortex gently*

Proceed to analysis by Flow Cytometry (within 8 hours)

[1] Depending on Flow cytometry instrumentation more Wash Buffer (e.g. 800 µl) might be necessary

Symbol	Explanation
	Use By Verwendbar bis Utiliser jusqu'au Utilizzare entro Fecha de caducidad
REF	Catalogue number Bestellnummer Référence du catalogue Numero di catalogo Número de catálogo
LOT	Batch code Chargenbezeichnung Code du lot Codice del lotto Codigo de lote
	Contains sufficient for <n> tests Ausreichend für „n" Ansätze Contenu suffisant pour „n" tests Contenuto sufficiente per „n" saggi Contenido suficiente para <n> ensayos

Symbol	Explanation
	Consult Instructions for Use- Gebrauchsanweisung beachten Consulter le mode d'emploi Consultare le istruzioni per l'uso Consulte las instrucciones de uso
	Temperature limitation Zulässiger Temperaturbereich Limites de température Limiti di temperatura Limite de temperatura
BUF STIM	Stimulation Buffer Stimmulations-Puffer Tampon de stimulation tampone di' stimulazione Tampón de estimulación
REAG STAIN	Staining Reagent Färbe-Reagenz Réactif de coloration Reagente di colorazione Reactivo de coloración

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