Ig Subclasses (2 tubes)

Ref: CYT-IGS-2

RUO

For Research Use Only. Not for use in diagnostics procedures

INTENDED USE

Ig Subclasses (2 tubes) kit contains 2 tubes with a different mixture of 4 antibodies each one. It has been designed for the detection of cell populations expressing immunoglobulins subclasses by flow cytometry (FC). This 2-color panel can be used where Ig subclasses studies could be relevant, such as immunodeficiencies, allergies, autoimmune diseases, etc [1, 2]. This reagent must be used by FC qualified personal.

INTRODUCTION

Circulating peripheral B cell compartment is represented by B lymphocytes having different maturation stages: immature or transitional B cells, naïve B cells, memory B cells and newly generated plasma cells: (plasmablasts) / plasma cells.
Immature and naïve B cells are mostly lymphocytes, coexpressing IgM and IgD, leaving the bone marrow and traveling via peripheral blood to the secondary lymphoid organs/tissues for encountering their specific antigen.
Memory B cells and plasmablasts / plasma cells are B lymphocytes which have increased their BCR (B cell receptor) antigenic affinity by somatic hypermutation and isotype change. These cells will have a different phenotype than the naive cells, which along with their BCR will define the subtypes B: IgM / IgD, IgG, IgA and IgE [3].
It is well known that IgG and IgA have several subclasses, and accordingly, we will have for each a specific mature cell producing them: IgA1, IgA2, IgG1, IgG2, IgG3, IgG4. Each of these immunoglobulin subclasses have different biological properties, being important in the immune response against pathogens [4].

REAGENT COMPOSITION

Material included

Ig Subclasses (2 tubes) kit contains sufficient volume for 20 tests and includes:

- Tube 1:
  - Antibody anti human IgG2/IgG4-FITC, clone: SAG2/SAG4, isotype: IgG1/IgG1
  - Antibody anti human IgG1/IgG2-PE, clone: SAG1/SAG2, isotype: IgG2b/IgG1

TUBE 1				
Fluorochrome	**FITC**		**PE**	
Marker	IgG2	IgG4	IgG1	IgG2
Clone	SAG2	SAG4	SAG1	SAG2

- Tube 2:
  - Antibody anti human IgA1/IgG3-PE, clone: SAA1/SAG3, isotype: IgG1/IgG1
  - Antibody anti human IgA1/IgA2-PE, clone: SAA1/SAA2, isotype: IgG1/IgG1

TUBE 2				
Fluorochrome	**FITC**		**PE**	
Marker	IgA1	IgG3	IgA1	IgA2
Clone	SAA1	SAG3	SAA1	SAA2

- Fixative free ammonium chloride erythrocyte lysing solution (BulkLysis™).

Material required but not included

- Reagents for generic compensation of non-tandems (Cytognos recommends to follow the EuroFlow instrument set-up and compensation [5]).
- Rainbow beads calibration particles [5].
- Suitable antibody cocktail for memory B cells and plasma cells identification.
- Test tubes suitable for obtaining samples in the flow cytometer used. Usually tubes with a rounded bottom for 6 ml, 12x75 mm are used (FACS tubes).
- 50 ml tubes (Falcon tubes).
- Automatic pipette and tips.

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- Micropipette with tips.
- Vortex mixer.
- Roller mixer.
- Chronometer.
- Centrifuge.
- Pasteur pipette or vacuum system.
- Distilled water.
- Erythrocyte fixative lysing solution.
- Wash buffer as phosphate buffered saline (PBS) + 0,09% (m/v) of NaN_3 + 0,5% (m/v) of Bovine Serum Albumin (BSA).

STORAGE CONDITIONS

Ig Subclasses (2 tubes) kit is stable until the expiration date shown on the label, when stored at 2-8º C.
Components should not be frozen or exposed to direct light during storage or during incubation with cells. Keep vials in a dry place. Once opened, vials must be stored in a vertical position to avoid any possible spillage.

WARNINGS AND RECOMMENDATIONS

1. For research use only. Not for use in diagnostics procedures.
2. If components of this kit are altered by addition of other components, such conditions must be validated by the user.
3. The kit is stable until the expiration date shown on the label if it is properly stored. Do not use after the expiration date shown on the label. If the reagents are stored in conditions different from those recommended, such conditions must be validated by the user.
4. Alteration in the appearance of the reagents, such as the precipitation or discoloration indicates instability or deterioration. In such cases, the reagents should not be used.
5. It contains 0,09% (m/v) sodium azide (CAS-No. 26628-22-8) as a preservative, but even so care should be taken to avoid microbial contamination of reagent or incorrect results may occur.

   Indication(s) of danger:

H302	Harmful if swallowed

   Safety advice:

P264	Wash thoroughly after handling.
P270	Do not eat, drink or smoke when using this product.
P301+P312	If swallowed, call a poison center or doctor/physician if you feel unwell.
P301+P330	If swallowed, rinse mouth.
P501	Dispose of contents/container in accordance with local/regional/national/international regulation.

6. All patient specimens and materials with which they come into contact are considered biohazards and should be handled as if capable of transmitting infection [6], and disposed according to the legal precautions established for this type of product. Also recommended is handling of the product with appropriate protective gloves and clothing, and its use by personnel sufficiently qualified for the procedures described. Avoid contact of samples with skin and mucous membranes. After contact with skin, wash immediately with plenty of water.
7. Use of the reagents with incubation times or temperatures different from those recommended may cause erroneous results. Any such changes must be validated by the user.
8. Any serious incident relating to the product must be reported to Cytognos SL as well as the competent professional authority of the Member State in which the user is established.

PROCEDURE

Preparation

Since some cellular subpopulations have a very low frequency in peripheral blood, it is recommended to use the macro-volume lysis technique with a hypotonic solution without fixative, prior to labeling. If Bulk lysis has been already done for another study, the excess sample can be used in the present panel.
Sample must be collected in commercially available anticoagulant-treated tube (use of EDTA is recommended) [7, 8].

1. Determine the absolute count of leukocytes per µl for the sample to be processed.
2. Transfer 1.5 ml (nucleated cells > $8x10^6$/ml) or 2.0 ml (nucleated cells < $8x10^6$/ml) of sample in a 50 ml-Falcon tube.
Do not use more than 2 ml of sample per 50 ml of lysing solution. If larger volumes of sample need to be processed (i.e. starting cells concentration is low), use several 50 ml tubes.
3. Fill the tube up to reach 50 ml volume with BulkLysis™ (CYT- BL) (diluted to 1X in distilled water and at room temperature (RT)).
4. Mix well and incubate for 15 min in a roller or sample-shaker device.
5. Centrifuge at 800 g for 10 min and remove the supernatant using a Pasteur pipette or a vaccum system without disturbing the cell pellet. Typically, 300 µl of cell suspension should remain in the tube.
6. Add 2 ml of PBS + 0,09% (m/v) of NaN3 + 0,5 % (m/v) of BSA and resuspend the cell pellet vigorously.
7. Complete the volume of the tube containing the cell suspension up to 50 ml final volume with PBS + 0,09% (m/v) of NaN3 + 0,5 % (m/v) of BSA.
8. Mix well.
9. Centrifuge at 800 g for 5 min and remove the supernatant using a Pasteur pipette or a vaccum system, without disturbing the cell pellet.
10. Resuspend the cell pellet in 2 ml of PBS + 0,09% (m/v) of NaN3 + 0,5 % (m/v) of BSA. Mix well and transfer this volume to a 5 ml “FACS tube”.

11. Wash the 50 ml Falcon tube with 2 mL of PBS + 0,09% (m/v) of NaN3 + 0,5 % (m/v) of BSA more to recover cells that might have left in the original tube. Add this volume to the 5 ml tube containing the rest of the sample transferred in step 10.
12. Centrifuge at 540 g for 5 min and remove the supernatant by decanting or using a Pasteur pipette. If the remaining cell volume is lower than 300 µl, PBS + 0,09% (m/v) of NaN3 + 0,5 % (m/v) of BSA will be added to reach a volume of at least 300 µl.
13. In case multiple 50 ml tubes were used (because it was needed to lyse large sample volumes) the cell suspensions from the same sample should be combined at this moment, before adjusting cell concentration. Try to keep the final volume low, so that, in case that cell concentration needs to be adjusted as indicated in the next step, it can be easily done by diluting with the recommended buffer.
14. Adjust the final cells concentration to 1 x 10^5 cells/ µl, by resuspending the pellet with PBS + 0,09% (m/v) of NaN3 + 0,5 % (m/v) of BSA.
15. Adjust the volume in order to obtain 200 µl containing 2 x10^6 cells of the cell suspension per each tube to be stained/acquired.

Surface Staining:

1. Add **12 µl** of antibody mixture of tube 1 to a tube containing the 100 µl cell suspension (1 x 10^6 cells per tube) and **12 µl** of antibody mixture of tube 2 to other tube containing the 100 µl cell suspension (1 x 10^6 cells per tube). Add proper volume of monoclonal antibodies for B cell subsets identification (not included in the mixture).
2. Mix well. For optimal staining conditions, complete with PBS until a final volume of 200 µl.
3. Incubate for 30 min at RT protected from light.
4. Add 2 ml of fixative Lysing Solution.
5. Mix well.
6. Incubate for 10 min at RT protected from light.
7. Centrifuge for 5 min at 540 g.
8. Discard the supernatant using a Pasteur pipette or vaccum system without disturbing the cell pellet, leaving approximately 100 µl residual volume in each tube.
9. Add 2 ml of PBS+0,5% (m/v) BSA + 0,09% (m/v) NaN_3 to the cell pellet.
10. Mix well.
11. Centrifuge for 5 min at 540 g.
12. Discard the supernatant using a Pasteur pipette or vaccum system without disturbing the cell pellet, leaving approximately 100 µl residual volume in each tube.
13. Resuspend the cell pellet in 300 µl PBS + 0,5% (m/v) BSA (without NaN_3).
14. Acquire the cells immediately after staining or (if not immediately acquired) store at 4°C (for 1h maximum) until measured in the flow cytometer.
15. Acquire the sample in medium flow rate (acquire 5x10^6 cells).

LIMITATIONS

- Blood samples should be stored at 18-22ºC and be tested within the 24 hours after they are obtained.
- It is advisable to acquire stained samples on the cytometer as soon as possible to optimize the results. Non-viable cells may stain nonspecifically. Prolonged exposure of whole blood samples to lytic reagents may cause white cell destruction and loss of cells from the target population.
- When using whole blood procedures, all red blood cells may not lyse under following conditions: nucleated red blood cells, abnormal protein concentration or hemoglobinopathies. This may cause falsely decreased results due to unlysed red blood cells being counted as leucocytes.
- Results obtained by FC may be erroneous if the cytometer laser is misaligned or the gates are improperly set.
- Each laboratory should establish a normal range for lymphocyte subsets. We recommend to follow the EuroFlow antibody panels together with the EuroFlow instrument set-up, sample preparation and data analysis procedures [(5)].
- Cells separated from whole blood by means of density gradients may not have the same relative concentrations of cells as unseparated blood. This may be relatively insignificant for samples from individuals with normal white blood cell counts. In leucopenic patients, the selective loss of specific subsets may affect the accuracy of the determination.

QUALITY CONTROL

- Pipettes precision and cytometer calibration should be verified to obtain optimal results.
- In multicolour panels, fluorochromes emit in wavelengths that can show certain spectral overlap which must be corrected by electronic compensation. Optimal compensation levels can be established by analysing cells from healthy individuals stained with mutually exclusive monoclonal antibodies conjugated with appropriate fluorochromes.
- This product has been manufactured in accordance with standards of production and quality system of the ISO 9001:2008 standard.

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WARRANTY

This product is warranted only to conform to the quantity and contents stated on the label. There are no warranties that extend beyond the description on the label of the product. Cytognos's sole liability is limited to either replacement of the product or refund of the purchase price.

EXPLANATION OF SYMBOLS

	Use by (YYYY-MM)
	Storage temperature limitation
	Keep out of sunlight
	Consult instructions for use
	For research use only
	Batch code
	Catalogue number
	Manufacter

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