FlowCellect™ Human Lymphocyte ZAP-70 Characterization Kit

25 Tests

Cat. No. FCIM025122

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Application

Characterization of lymphocytes and lymphocyte sub-types is essential to understanding the complex nature of the immune system. Activation to antigens, suppression of normal immune activation, and disease states can affect the phenotype of lymphocytes. Multi-parameter phenotypic analysis allows for a greater understanding of the molecular biology associated with these intricate processes.

ZAP-70 is a cytoplasmic protein tyrosine kinase that plays a critical role in the events involved in initiating T-cell responses by the antigen receptor (1). Due to its role in T-cell activation, lack of ZAP-70 expression in T-cells can lead to immune deficiencies as a result of decreased response to antigens.

CD5 is expressed on the cell surface of T-cells, NK cells, and has been identified as a marker for some B-Cell malignancies. Normal B-cells express CD19 but do not express CD5 or ZAP-70 which are typically associated with T-cells; however, there are some disease states in which B-cells have been found to express all three markers (2). In these instances, multi-parameter phenotypic analysis allows for the monitoring of marker expression on lymphocytes within the peripheral blood.

The ability to determine CD5, CD19, and ZAP-70 expression levels simultaneously in mixed populations of cells allows for the characterization of cell phenotypes which gives insight as to the state of the immune response or lack thereof.

Test Principle

We have developed a multi-parameter flow cytometry assay for ZAP-70 protein expression analysis and characterization of human lymphocytes. Millipore's FlowCellect™ Human Lymphocyte ZAP-70 Characterization Kit includes three directly conjugated antibodies: CD5 FITC, CD19 APC, and ZAP-70 PE, along with optimized fixation, permeabilization, wash, and assay buffers to provide researchers the ability to phenotypically distinguish cell types.

Antibodies against CD5 and CD19 are provided in the kit to phenotype cells by their surface marker expression. CD5 is normally a pan-T-cell marker, while CD19 is a mature B-cell marker. Some B-cell malignancies express both of these molecules on their surface, and therefore can be distinguished from the two aforementioned cell types.

ZAP-70 is a molecule involved in T-cell activation, which can also be expressed within B-cells at levels correlating to disease severity. The combinatorial staining of cells for all three of these markers assists the researcher studying the molecular biology of T-cell activation or B-cell disease states.
Sufficient reagents are provided for 25 3-color samples. The kit includes all optimized fluorescent labeled antibodies and buffers necessary for cell preparation and analysis.

Kit Components

- 20X Anti-CD5-FITC, Antibody (Part No. CS205796) One vial containing 150 µL antibody.
- 20X Anti-CD19-APC, Antibody (Part No. CS205795) One vial containing 150 µL antibody.
- 20X Anti-ZAP-70-PE, Antibody (Part No. CS205794) One vial containing 150 µL antibody.
- Fixation Buffer: (Part No. CS202122) One bottle containing 13 mL buffer.
- 10X Wash Buffer: (Part No. CS202123) One bottle containing 13 mL buffer.
- 5X Assay Buffer: (Part No. CS202124) One bottle containing 55 mL buffer.
- 1X Permeabilization Buffer: (Part No. CS203284) Two bottles containing 14 mL buffer.

Materials Not Supplied

1. Flow Cytometer with blue and red laser
2. Cells of interest and/or Ramos and Jurkat cells as positive control
3. Media
4. Tissue culture instruments and supplies.

Precautions

- The instructions provided have been designed to optimize the kit's performance. Deviation from the kit's instructions may result in suboptimal performance and may produce inaccurate data.
- Some assay components included in the kit may be harmful. The kit includes a Fixation Buffer containing formaldehyde. Please refer to the MSDS sheet which can be found at www.millipore.com for specific information on hazardous materials.
- All fluorochrome conjugated antibodies are light sensitive and must be stored in the dark at 2-8°C.
- During storage and shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. For maximum recovery of product, centrifuge vial briefly prior to removing cap.
- Do not use reagents beyond 4 months from date of receipt.

Storage

Upon receipt, all antibodies and buffers should be stored at 2-8°C. ***Caution:*** *Fluorochrome conjugated antibodies should always be stored at 2-8°C. Do not freeze fluorescent antibodies. Any deviation in temperature for long periods of time may compromise the performance of the antibodies.*

Preparation of Buffers

To prepare sufficient buffers for 10 samples (scale up or down as necessary):

- Make 10 mL of Wash Buffer. Mix 1 mL of 10X Wash Buffer and 9 mL of deionized water.
- Make 10 mL of 1X Fixation buffer. Mix 1 mL of 10X Wash Buffer, 2.5 mL of Fixation buffer and 6.5 mL of deionized water.
- Make 30 mL of 1X Assay Buffer. Mix 6 mL of 5X Assay Buffer and 24 mL of deionized water.
- Place Permeabilization Buffer on ice. *Permeabilization buffer must be ice cold for optimal results*.

Note: Prepared 1X Fixation Buffer is stable up to one month if stored at 2-8ºC. Prepared 1X Wash and Assay Buffers are stable up to six months if stored at 2-8ºC, and can be prepared when you receive the kit or when you start the first assay.

Example Cell Staining Protocol

Note: Each sample consists of 500,000 cells. This protocol can be used to generate 5 samples. Scale up or down as necessary.

1. Centrifuge 3 million cells in a 15 mL conical tube at 600Xg for 5 minutes.
2. Decant the supernatant and add 10 mL of 1x DPBS. Centrifuge at 600Xg for 5 minutes.
3. Aspirate supernatant and resuspend cell pellet in 1.5 mL of 1x Assay Buffer. Aliquot 250 µL of cells for each sample needed (5 samples for this protocol = 5 wells with 250 µL each) into a guava approved 96 well plate. **If done correctly the plate should have a total of 5 wells with 250 µL each at this point.**
4. Centrifuge the plate at 600Xg for 5 minutes.
5. Decant the supernatant, and resuspend each pellet in 90 µL 1x Assay Buffer. Add 5 µL anti-CD5 FITC and 5 µL of anti-CD19 APC to each well. Mix contents of each well by pipetting up and down gently 3 times.
6. Incubate the plate on ice in the dark for one hour, after which centrifuge the plate at 600Xg for 5 minutes and decant the supernatant.
7. Add 250 µL 1x Assay Buffer to each well, mix by pipetting, and centrifuge tubes at 600Xg for 5 minutes. Decant supernatant.
8. Repeat Step 7 one time.

9. After washing, resuspend each pellet in 250 µL 1x Fixation Buffer. Pipet up and down to mix, and incubate samples at room temperature for 20 minutes to fix cells. **Note: keep cells in the dark at this time to prevent bleaching of surface staining.**

10. Centrifuge the plate at 600Xg for 5 minutes. Decant the supernatant.

11. Add 250 uL 1x Wash Buffer to each well, mix by pipetting, and centrifuge each sample at 600Xg for 5 minutes.

12. Decant the supernatant and repeat step 11.

13. Decant the supernatant, and resuspend each well in 250 µL of ice cold 1x Permeabilization Buffer. Resuspend the cells 5 times by pipeting up and down. Incubate plate on ice for 20 minutes to permeabilize cells.

14. Centrifuge each sample at 600Xg for 5 minutes.

15. Decant the supernatant, and wash each sample with 250 µL 1x Assay Buffer. Repeat this step one time.

16. Decant the supernatant and resuspend each pellet in 95 µL 1x Assay Buffer. Add 5 µL of anti-ZAP70 PE 20x stock to each well, mixing by pipetting 3 times up and down, and incubate the plate on ice in the dark for 1 hour.

17. Centrifuge each sample at 600Xg for 5 minutes. Decant the supernatant.

18. Add 250 µL of 1x Assay Buffer to each tube. Mix by pipetting up and down 3 times, and centrifuge samples at 600Xg for 5 minutes.

19. Decant the supernatant and repeat step 18 one time.

20. Decant supernatant and resuspend each pellet in 250 µL 1x Assay Buffer. Analyze sample using a guava 2 laser system. (8HT or 6HT 2 laser).

Sample Data

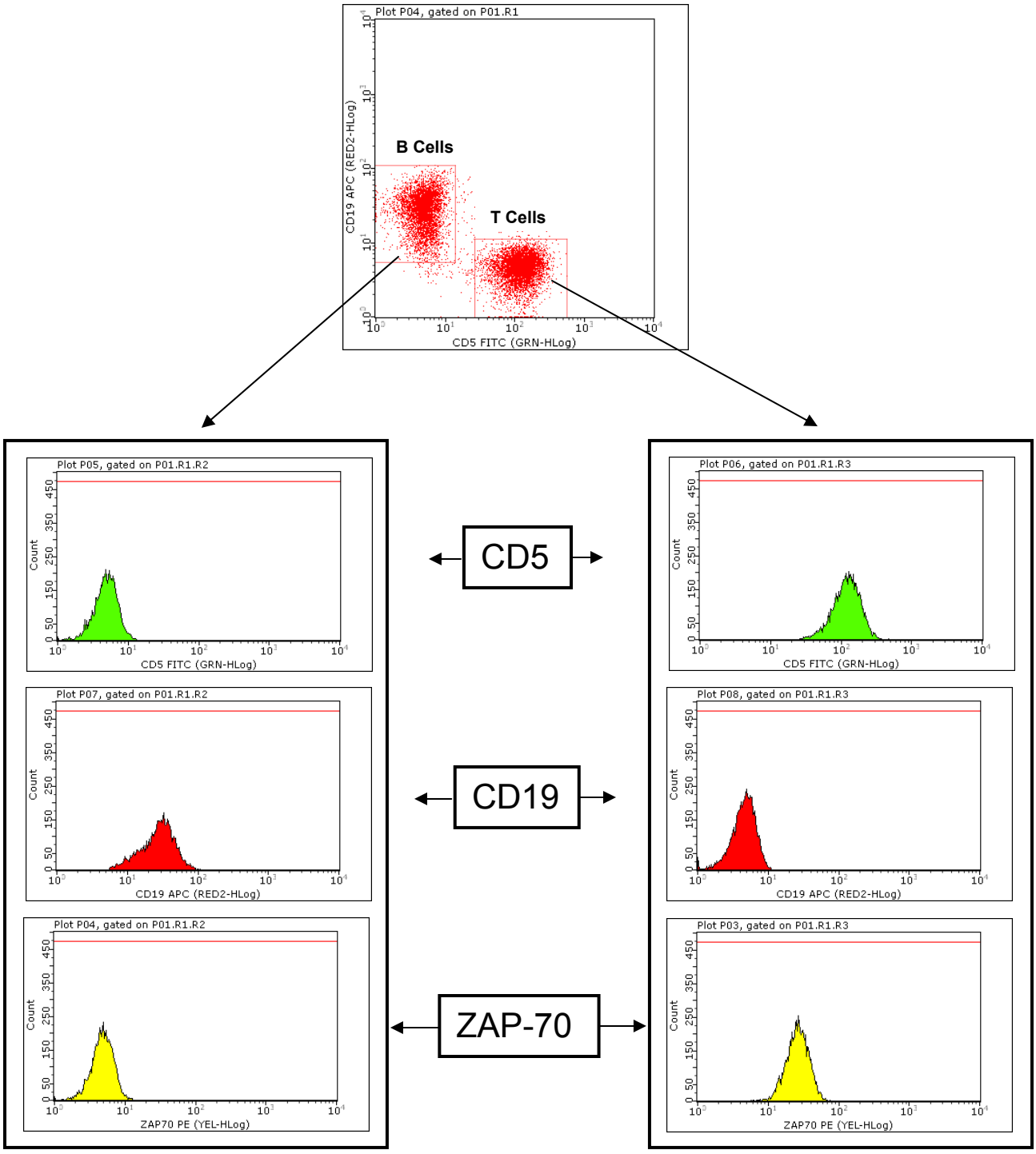

Figure1. Mixture of Ramos (B cell line) and Jurkat (T cell line) cells were used as a model system. The cells were then stained with CD5-FITC, CD19-APC, and ZAP-70 PE. A dot plot showing CD5 vs. CD19 is used to determine the B cells from T cells and is used to draw two gates which allow for the analysis of the expression levels of all three markers within each population.

Technical Hints

- If minor precipitate is detected in the 10X Wash Buffer place the bottle in a warm water bath for 30 minutes, followed by mixing the contents on a mechanical vortex.
- For cellular staining and analysis to be most effective, make sure that test cells have good viability prior to use.
- Within certain cell cultures cell pellets may become hazy or transparent following the fixation step, making them difficult to see. After the washing steps they will become easier to visualize.
- Do not mix or interchange reagents from various kit lots.

Troubleshooting

Potential Problem	Experimental Suggestions
Acquisition rate decreases dramatically Instrument clogging Too many cells	• Cell concentration too high - Decrease the number of cells per microliter by diluting sample to 200 – 400 cells per microliter. The guava easyCyte™ 8HT and 6HT 2 laser give the most accurate data when the flow rate is less 500 cells per microliter. • Run three Quick Cleans (for guava instruments) to rinse out the flow cell. This procedure can be performed during or after an assay. This will wash away any material forming within the glass capillary walls.
Too few cells	• Spin down cells and resuspend in a smaller volume. The assay instructions are optimized to give you a range of cells between 100 and 500 cells per µL in the final sample volume. However, cell loss is common during washing steps in the assay procedure. A substantial decrease in cell numbers can lead to difficulty in adjusting settings. Make sure to leave the cell pellet intact when discarding buffer. If the cells are not generating a compact pellet after centrifugation, increase the time to 5 minutes and/or increase the speed by 100Xg until a compact and visible cell pellet forms.
Background staining and/or non-specific staining of cells	• Although the assay procedure has been optimized to function utilizing many different cell types, further antibody titrations may be necessary for some cell types to capture the ideal staining concentration. Non-specific staining and background may indicate that less antibody will need to be used during the staining procedure.

Variability in day to day experiments	• Monitor experimental cell cultures to ensure that cell viability and cell numbers being analyzed are consistent. Any changes in culture conditions or viability can influence experimental results. •When using the guava easyCyte™ 8HT or 6HT 2 laser instrument for flow analysis, make sure that a quality check on the instrument (e.g. Easy Check) is performed on a daily basis prior to use.
Staining is weak	• Some cell lines may require higher concentrations of fixation buffer to stain correctly. Use twice as much Fixation Buffer.
Staining is weak	• Some cell lines may require higher concentrations of conjugated antibodies. Try using more antibody per sample.
Staining is too bright	• Some cell lines may require lower concentrations of conjugated antibodies. Try using less antibody per sample.

***For further support, please contact Millipore's Technical services at +1(800) 437-7500*

References

1. Haopeng Wang, Theresa A. Kadlecek, Byron B. Au-Yeung, Hanna E. Sjölin Goodfellow, Lih-Yun Hsu, Tanya S. Freedman, and Arthur Weiss. *ZAP-70: An Essential Kinase in T-cell Signaling.* Cold Spring Harb Perspect Biol. 2010 May; 2(5): a002279.

2. Adrian Wiestner, Andreas Rosenwald, Todd S. Barry, George Wright, R. Eric Davis, Sarah E. Henrickson, Hong Zhao, Rachel E. Ibbotson, Jenny A. Orchard, Zadie Davis, Mayalice Stetler-Stevenson, Mark Raffeld, Diane C Arthur, Gerald. E. Mari, Wynham H. Wilson, Terry J. Hamblin, David G. Oscier and Louis M. Staudt. Zap-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome and distinct gene expression profile. Blood. DOI 10.1182/blood-2002-10-3306 (2003).

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