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PeliCluster CD16

Monoclonal mouse anti-human reagents for identification of cells expressing CD16 antigen.

Form	REF	Clone
FITC	M1604	CLB-Fc-gran/1, 5D2

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IVD CE

1. INTENDED USE

The PeliCluster antibodies are intended for in vitro diagnostic use. The reagents identify and enumerate cells expressing the CD antigen using a flow cytometer for analysis.
To prevent interference with red cells during analysis, treatment of whole blood with lysing reagent (PeliLyse A1, order number M7101.6) is recommended.
The flow cytometer must be equipped to detect light scatter and the appropriate fluorescence, and be equipped with the appropriate software for data acquisition and analysis. Refer to your instrument user's guide for instructions.

Applications

Enumeration of K and NK cell numbers in peripheral blood and lymfoid tissue.

2. COMPOSITION

Clone CLB-Fc-gran/1, 5D2 has been derived from hybridisation of SP2/0 cells with spleen cells of a BALB/c mouse immunised with human granulocytes. This clone is of a mouse IgG2a subclass. Clone CLB-Fc-gran/1, 5D2, was submitted to CD16 in the Fifth International Workshop on Human Leukocyte Differentiation Antigens. The antibody is conjugated with fluorescein iso-thiocyanate isomer 1 (FITC). The molecular F/P ratio is between 5 and 10.

The antibodies were purified from ascites or tissue culture supernatant using column chromatography (ion exchange and/or affinity chromatography).

Reagent contents.

Each reagent is supplied in 1 ml of 20 mM TRIS plus 150 mM NaCl, pH 8.0, containing BSA 1% (w/v) and NaN_3 0,1% (w/v) as preservative (see table 1).

Table 1. Contents of bottles

FITC	100 tests per ml in TRIS

WARNING:
Sodium azide is harmful if swallowed (R22). Keep out of reach of children (S2). Keep away from food, drink, and animal feedingstuff (S13). Wear suitable protective clothing (S36). If swallowed, seek medical advice immediately and show this container or label (S46). Contact with acids liberates very toxic gas (R32). Azide compounds should be flushed with large volumes of water during disposal to avoid deposits in lead or copper plumbing where explosive conditions can develop.

3. STORAGE AND HANDLING

The antibody reagent is stable until the expiration date shown on the label when stored at 2 to 8°C. Do not use after the expiration date. Do not freeze the reagent or expose it to direct light during storage or incubation with cells. Keep the outside of the reagent vial dry. Reagents should not be used if any evidence of deterioration, such as increase in compensation, or substantial loss of reactivity is observed.

4. REAGENTS OR MATERIALS REQUIRED BUT NOT PROVIDED

- Lysing solution (PeliLyse, order number M7101.6).
- Wash and dilution buffer for mononuclear cells, Phosphate Buffered Saline, containing 0.2% BSA (w/v); PBS/BSA.
- Wash and dilution buffer for platelets, Sequestrine buffer (Seq), storage 1 month at 2 to 8°C. 10 x stock solution, dissolve in 1 litre of distilled water:
  $Na_2HPO_4 . H_2O$:31.3 g
  $Na_2EDTA . 2H_2O$:33.3 g
  NaCl :90.0 g

  Prior to use dilute in distilled water, add BSA till final concentration of 0.2% (w/v). Mix and adjust pH to 6.8.
- Fixation buffer, PFA/BSA (*):
  Para-Formaldehyde 1% in PBS, containing 0.2% BSA (pH 7.2).
- Microwell plates (96 wells, V bottom) or plastic flow cytometry tubes.
- Flow cytometer. Refer to the appropriate instrument user's guide for information.

(*) The procedure employs a fixative, formaldehyde. Contact is to be avoided with skin or mucous membranes.

5. SPECIMEN(S)

Blood samples can be prepared for flow cytometric analysis by using PBMC preparation procedures. PBMC preparation yield more technique-dependent results (1).

Collect blood aseptically by venipuncture (1,2) into sterile K_3EDTA blood collection tube. A minimum of 1 ml of whole blood is required for the whole blood method and a minimum of 2 ml of whole blood is required for PBMC preparation. Store anticoagulated blood at room temperature (18 to 25°C).

WARNING:
Consider all biological specimens and materials which come in contact with them as biohazardous. Specimens should be handled as potentially infectious (3,4) and disposed in accordance with federal, state an local regulations. Do not pipet by mouth. Wear suitable protective clothing and gloves. Fixation has been reported to inactivate HIV (5).

6. PROCEDURES

A: Method with ficoll purified cells

1 Prepare a mononuclear cell suspension with a concentration of 1 x 10^7 cells/ml.
2 Ad 40 µl of cell suspension to microtiter wells or tubes.
3 Add 10 µl of the undiluted antibody to the microtiter wells or tubes and mix gently.
4 Incubate for 30 minutes at 2 to 8°C.
5 Add 150 µl buffer to the microtiter wells or 2 ml buffer to the tubes and centrifuge at 500 x g for 5 minutes.
6 Aspirate the supernatant from the cell pellet and resuspend the cells.
7 Add 200 µl buffer to the microtiter wells or 2 ml buffer to the tubes and centrifuge at 500 x g for 5 minutes.
8 Aspirate the supernatant from the cell pellet and resuspend the cells.
9 Flowcytometer analysis:
Add 200 µl buffer to the microtiter wells and transfer this final cell suspension to appropriate test tubes, or add 200 µl buffer to the tubes.
10 If analysis within 8 hours is not possible add at no. 9, instead of buffer, 200 µl PFA 1%. Sanquin Reagents recommends then analysing within 24 hours.

B: Whole blood method

1 Draw blood into a blood collection tube containing EDTA.
2 Deliver 100 µl (*) of well mixed whole blood to the bottom of the test tube.
3 Add 10 µl of the undiluted antibodies to the bottom of the test tube, and mix firmly during 30 seconds.
4 Incubate for 15 to 30 minutes at room temperature.
5 Mix the tubes and add 2 ml of lysing solution (PeliLyse A1, 10x diluted).
6 Incubate for 10 to 15 minutes at room temperature until lysing is complete.
7 Analyse the samples within 90 minutes.

If analysis within 90 minutes is not possible, centrifuge the tubes at 500x g for 5 minutes. Aspirate the supernatant from the cell pellet and resuspend the cells in 1 ml buffer when analysed within 8 hours or in 1 ml PFA 1%. Sanquin Reagents recommends then analysing within 24 hours.

* This method was developed for blood samples with a normal white count with the use of PeliLyse A1 (lysing solution, order number M7101.6). It may be necessary to adjust the quantity of blood for samples with very high or low white count.

C: Platelet membrane flow cytometry and microscopy.

1. Transfer 45 µl of platelet suspension (1x10^8 cells/ml) into the microwell plate or tubes and add 5 µl monoclonal antibody*. Mix gently and incubate for 30 minutes at 2 to 8°C.
2. Wash by mixing and adding Seq to the microwell plate (1st wash 150 µl, 2nd wash 200 µl) or tubes (2 ml). Centrifuge at 1000 x g for 5 minutes and aspirate the supernatant, repeat this procedure once more.
3. Prepare cells for analysis:
   For flow cytometry, resuspend the cells by adding 200 µl Seq to the microwell plate or tubes. If a microwell plate was used the contents are transferred to appropriate tubes.
   For fluorescence microscopy, resuspend the cells in 50 µl embedding medium, transfer cells to a microscope slide and place a cover glass.

* In general, 5 µl undiluted monoclonal antibody can be used. Alternatively an optimal dilution can be determined.To determine background fluorescence always use a negative control from the same isotype

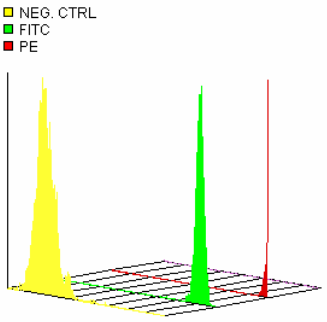
Analytical Results

Abnormal number of cells expressing this antigen or aberrant expression levels of the antigen can be expected in some disease states. It is important to understand the normal expression pattern for this antigen and its relationship to expression of other relevant antigens in order to perform appropriate analysis.

Flow cytometry

Vortex the cells thoroughly at low speed to reduce aggregation before running the cells on the flow cytometer (6). Acquire and analyse list-mode data using appropriate software. Before acquiring samples, adjust the threshold to minimise debris and ensure populations of interest are included. Fig 1 displays representative data performed on gated lymphocytes. Laser excitation is at 488 nm.

Fig. 1: Fluorescence profile, scatter gates set on the granulocyte fraction (R1)



NOTE: Improper gate setting on the sample data can give incorrect results.

Internal Quality Control

The use of a negative control (see Sanquin Reagents catalogue) is recommended to determine background fluorescence produced due to Fc binding capacities by mononuclear cells.
The concentration and F/P ratio of these controls have been adjusted to the conjugated monoclonal antibodies of Sanquin Reagents.

7. PERFORMANCE CHARACTERISTICS

Specificity

The monoclonal antibody is directed against the CD16 antigen (the Fc gamma Receptor III), which is expressed on neutrophil granulocytes, monocytes (weak), macrophages (weak) and NK cells (molecular mass 45 – 72 kDa). It is absent in patients with PNH. The mobility of the CD16 antigen is dependent on the NA_1 /NA_2 allotype of the neutrophil donor. The monoclonal antibody inhibits the binding of human IgG to the Fc gamma Receptor III (7-11).

Sensitivity

Sensitivity is defined as a resolution of the CD negative population from the different CD positive population. Sensitivity was measured by evaluating a range of antibody concentrations. Each concentration was tested on whole blood. The separation of CD positive from CD negative was determined from each sample and averaged within each concentration. The bottled antibody concentration for each reagent provided optimum sensitivity in resolving the CD positive cells from the negative.

Reproducibility/Repeatability.

The CDs were submitted in one of the International Workshops on Human Leukocyte Differentiation Antigens or meet the Workshop specifications (see composition).

To determine the repeatability of staining with each reagent, samples were stained with multiple lots of reagents. The different samples used in the evaluation provided an average mean fluorescence intensity (MFI) value as shown in table 2. For each sample, two different lots of reagents generated a pair of results. Individual SDs were determined from the paired results for each sample. The SDs were combined to derive a pooled SD for each reagent that provides an estimate of within-sample repeatability.

Table 2. **Repeatability of mean fluorescence intensity (MFI) of target cells across different lots (N) and across multiple donors.**

	N*	Average MFI	Pooled SD	Pooled %CV
FITC	6	2141	278.1	12.99%

* N = number of samples

8. LIMITATIONS

Conjugates with brighter fluorchromes (PE, PE-Cy5) will give a greater separation then those with other dyes (FITC). When populations overlap, calculation of the percentage positive for the markers can be affected by choice of fluorchrome.

Use of monoclonal antibodies in patient treatment can interfere with recognition of target antigens by this reagent. This should be considered when analysing samples from patients treated in this fashion. Sanquin Reagents has not characterised the effect of the presence of therapeutic antibodies on the performance of this reagent.
Single reagents can provide only limited information in the analysis of leukaemia and lymphomas. Using combination of other reagents and application of other diagnostic procedures may provide more information than application of these reagents only. Multicolour analysis using relevant combination of reagents is highly recommended.

As reagents can be used in different combinations, laboratories need to become familiar with the properties of each antibody in conjunction with other markers in normal and abnormal samples.

Reagent data performance was collected typically with EDTA-treated blood. Reagent performance can be affected by the use of other anticoagulants.

TROUBLESHOOTING

Problem	Possible Cause	Solution
Poor resolution between debris and lymphocytes	Cell interaction with other cells and platelets	Prepare and stain another sample.
	Rough handling of cell preparation	Check cell viability; centrifuge cells at lower speed.
	Inappropriate instrument settings	Follow proper instrument set-up procedures; optimise instrument settings as required.
Staining dim or fading	Cell concentration too high at staining step	Check and adjust cell concentration or sample volume; stain with fresh sample
	Insufficient reagent	Repeat staining with increased amount of antibody.
	Cells not analysed within 8 hours of staining	Repeat staining with fresh sample; analyse promptly.
	Improper medium preparation (preservative omitted)	Use preservative in staining medium and washing steps.
Few or no cells	Cell concentration too low	Resupend fresh sample at a higher concentration ; repeat staining and analysis.
	Cytometer malfunction	Troubleshoot instrument.

REFERENCES

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