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## PeliDuo CD4 FITC / CD8 PE

Mixture of monoclonal mouse anti-human reagents for identification of cells expressing these antigens

REF	M1599	Form	Clone
CD4 FITC			CLB-T4/2, 6D10
CD8 PE			CLB-T8/4, 4H8

X0033-521eng 0110031626



### 1. INTENDED USE

The PeliDuo antibody mixture is intended for in vitro diagnostic use. The reagent identifies and enumerates cells expressing the CD antigens, using a flow cytometer for analysis.

To prevent interference with red cells during analysis treatment of whole blood 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 T helper/inducer and T cytotoxic/suppressor lymphocytes subset in peripheral blood. Characterisation and monitoring of some forms of immunodeficiency, acquired immune deficiency syndrome (AIDS) and autoimmune diseases. Immunophenotyping of leukemia and lymphomas.

### 2. COMPOSITION

Clone CLB-T4/2, 6D10 has been derived from hybridisation of SP2/0 cells with spleen cells of a (BALB/c x A/J) mouse immunised with human T lymphocytes. This clone is of a mouse IgG1 subclass. The antibody is conjugated with fluorescein iso-thiocyanate isomer 1 (FITC). The molecular F/P ratio is between 5 and 10.

Clone CLB-T8/4, 4H8 has been derived from hybridisation of SP2/0 cells with spleen cells of a (BALB/c x A/J) mouse immunised with human T lymphocytes. This clone is of a mouse IgG2b subclass. The antibody is conjugated with R-phycoerythrin (PE). The molecular F/P ratio is between 1.0 – 2.0.

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

### Reagent contents.

The reagent is supplied as an optimally formulated mixture of both conjugated antibodies in 1 ml of 20 mM TRIS and 150 mM NaCl buffer, pH8.0, containing a stabilising protein (0.4%) and NaNs 0.1% (w/v) as preservative.

Table 1. Contents of bottles

PeliDuo	50 tests per ml in TRIS buffer

### WARNING:

Sodium azide is harmful if swallowed (R22). Keep out of reach of children (S2). Keep away from food, drink, and animal feedstuff (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 reagent is stable until the expiration date shown on the label when stored at 2 to 8°C in the dark. 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, Sequesterine buffer (Seq), storage 1 month at 2 to 8°C. 10 x stock solution, dissolve in 1 litre of distilled water:
 

Na <sub>2</sub> HPO <sub>4</sub> · H <sub>2</sub> O	:	15.65 g
Na <sub>2</sub> EDTA · 2H <sub>2</sub> O	:	16.65 g
(Complexon)		
NaCl	:	450.0 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<sub>2</sub>EDTA 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 or 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  $1 \times 10^7$  cells/ml.
- 2 Add 30  $\mu$ l of cell suspension to microtiter wells or tubes.
- 3 Add 20  $\mu$ l of the undiluted antibodies to the microtiter wells or tubes and mix gently.
- 4 Incubate for 30 minutes at 2 to 8°C.
- 5 Add 150  $\mu$ l 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  $\mu$ l 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 Flow cytometer analysis: Add 200  $\mu$ l buffer to the microtiter wells and transfer this final cell suspension to appropriate test tubes, or add 200  $\mu$ l buffer to the tubes.
- 10 If analysis within 8 hours is not possible add at no. 9, instead of buffer, 200  $\mu$ l PFA 1%. Sanquin Reagents recommends analysing then within 24 hours.

#### B: Whole blood method

- 1 Draw blood into a blood collection tube containing EDTA.
- 2 Deliver 100  $\mu$ l (\*) of well mixed whole blood to the bottom of the test tube.
- 3 Add 20  $\mu$ l 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.

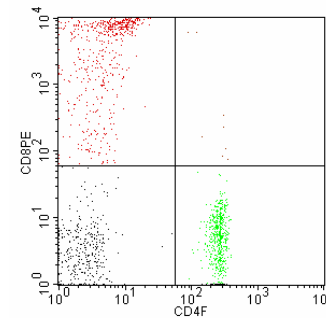
### Analytical Results

Abnormal number of cells expressing these antigens or aberrant expression levels of the antigens can be expected in some disease states. It is important to understand the normal expression pattern for these antigens 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 lymphocyte fraction (R1)



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

### Internal Quality Control

The use of a negative control (order numbers M1627 or M2251) 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 CD 4 is directed against the CD4 antigen (T4 antigen), which is expressed on human T lymphocytes. (molecular mass is 20, 25, 28 kDa). The monoclonal antibody reacts with 50 - 60% human peripheral T lymphocytes and 80% of thymocytes. The monoclonal antibody reacts on a low level with human monocytes and macrophages. The monoclonal antibody does not react with B cells, granulocytes and thrombocytes.

The monoclonal antibody CD8 is directed against the CD8 antigen (T8 antigen), which is expressed on human T lymphocytes. (molecular mass is 45 - 72 kDa). The antibody reacts with 20 - 30% of human peripheral T lymphocytes. The monoclonal antibody does not react with B cells, monocytes, granulocytes and platelets.

### 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
CD4 FITC	9	227.26	11.94	5.25%
CD8 PE	9	3454.5	402.05	11.64%

\* N = number of samples

### 8. LIMITATIONS

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

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 Rough handling of cell preparation Inappropriate instrument settings	Prepare and stain another sample.  Check cell viability; centrifuge cells at lower speed. Follow proper instrument set-up procedures; optimise instrument settings as required.
Staining dim or fading	Cell concentration too high at staining step  Insufficient reagent  Cells not analysed within 8 hours of staining Improper medium preparation (preservative omitted)	Check and adjust cell concentration or sample volume; stain with fresh sample Repeat staining with increased amount of antibody. Repeat staining with fresh sample; analyse promptly. Use preservative in staining medium and washing steps.

Few or no cells	Cell concentration too low  Cytometer malfunction	Resuspend fresh sample at a higher concentration ; repeat staining and analysis. Troubleshoot instrument.
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## REFERENCES

- 1 *Clinical Applications of Flow Cytometry: Quality Assurance and Immunophenotyping of Lymphocytes; Approved Guideline.* Wayne PA: National Committee for Clinical Laboratory Standards;1998. NCCLS document H42-A.
- 2 *Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture, Fourth Edition;Approved Standard.* Wayne, PA: National Committee for Clinical Laboratory Standards; 1998. NCCLS document H3-A4.
- 3 *Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids and Tissue: Tentative Guideline.* Villanova, PA: National Committee for Clinical Laboratory Standards; 1991. NCCLS document M29-T2.
- 4 Centers for Disease Control. Update: universal precautions for prevention of transmission of human immunodeficiency virus, hepatitis B virus, and other bloodborne pathogens in health-care settings. *MMWR.* 1988;37;377-388.
- 5 Nicholson JK, Browning SW, Orloff SL, McDougal JS. Inactivation of HIV-infected H9 cells in whole blood preparations by lysing/fixing reagents used in flow cytometry. *J Immunol Methods.* 1993;160:215-218.
- 6 Jackson AL, Warner NL. Preparation, staining and analysis by flow cytometry of peripheral blood leukocytes. Rose NR, Friedman H, Fahey JL eds. *Manual of Clinical Laboratory Immunology.* 3<sup>rd</sup> ed. Washington, DC; American Society for Microbiology; 1986:226-235.