Monoclonal Mouse Anti-Human CD45/FITC, Clone T293/34
Monoclonal Mouse Anti-Human CD14/RPE, Clone TÜK4

Code FR700

Intended use
For in vitro diagnostic use.

Monoclonal Mouse Anti-Human, CD45/FITC, Clone T293/34 (Dako Anti-CD45/FITC, T293/33) and Monoclonal Mouse Anti-Human, CD14/RPE, Clone TÜK4 (Anti-CD14/RPE, TÜK4) have been developed for use in flow cytometry. This reagent may be used to optimize the gating of lymphocytes when analyzing peripheral whole blood (lymphocyte preferential blood samples) or peripheral blood mononuclear cell preparations. This reagent is one component of the suggested monoclonal antibody (mAb) combination for routine immunophenotyping of lymphocytes in peripheral blood using flow cytometry (3).

Explanation and example
Anti-CD45, T293/34 was included in the Third and Fourth International Workshops on Human Leucocyte Differentiation Antigens (October 1986, Vienna 1989) (4, 5). At the Third International Workshop on Human Leucocyte Differentiation Antigens (October 1986) the antibody was designated as a CD45 antibody reacting with all the known isotypes (3). Anti-CD14, TÜK4 was developed for the Fourth International Workshop on Human Leucocyte Differentiation Antigens (Vienna 1989) the CD45 family was further divided into four groups of non-restricted and restricted isoforms (CD45R0, CD45RA, CD45RB and CD45RC) (epitope not encoded by either exons A, B or C)(5). The five CD45 glycoproteins are found exclusively on nucleated cells of the hematopoietic lineage (6, 4). Anti-CD45, T293/34 labels the cell membrane of almost all leukocytes. Many plasma cells, macrophages and histiocytes are unreactive (5). Qualitatively, the expression of CD45 on the surface of mature granulocytes is less than that of normal lymphocytes (10). Mature erythrocytes and platelets are unreacted (8). Non-hematopoietic cells are unreactive with this antibody (5).

Anti-CD14, TÜK4 was included in the Fourth and Fifth International Workshops on Human Leucocyte Differentiation Antigens (Vienna 1989, Boston 1993), and studies by a number of laboratories confirmed its reactivity with the CD14 antigen (11). Anti-CD14, TÜK4 labels monocytes (48%) in peripheral blood and bone marrow (13). Anti-CD14, TÜK4 reacts with a 55 kDa single-chain glycoprotein (CD45), that is expressed primarily on monocytes and macrophages but also on granulocytes, dendritic reticulum cells, Langhans' cells and some tissue macrophages (12). The CD14 amino acid sequence is comprised of 256 amino acids and is anchored to the cell membrane by a glycosyl phosphatidyl inositol (GPI) anchor (13, 14). Two soluble forms of CD14 have been documented, one (55 kDa) is found in urine and is probably the result of cleavage with an enzyme specific for PI (1). A smaller, smaller soluble form (48 kDa) has been isolated from normal monocytes and from HL60 cells induced to differentiate to monocyes, which do not contain the GPI anchoring (11).

Principle of procedure
Dako's dual antibody reagent Anti-CD45/FITC, Clone T293/34 and Anti-CD14/RPE, Clone TÜK4 is a combination of two monoclonal antibodies, one antibody is directed against the CD45 unrearranged form, and conjugated with fluorochrome isothiocyanate (FITC) and one antibody is directed against the CD14 epitope, conjugated with phycoerythrin (PE). Each conjugate can be detected by light excitation of the fluorochrome. When bound to the specific blood cell molecule, fluorescent light emission allows measurement of the leukocyte subpopulations by flow cytometry. This reagent contains two fluorochrome-labeled monoclonal antibodies that have bound anti-CD45/FITC at epitope 40 kDa and anti-CD14/RPE at epitope 55kDa, which displays green or those cells reacting with anti-CD14/RPE fluorochrome (570 nm), which displays orange-red.

Subpopulations of white blood cells from peripheral blood can be stained with fluorescence-conjugated antibodies and evaluated after contaminating red blood cells (RBC) are lysed. Alternately, monocytes can be separated from other blood components by a density gradient cell separation method (Ficoll) and stained with the fluorescence-conjugated antibody prior to analysis. Each white blood cell subpopulation is selected for assessment based upon size and forward scatter analysis. Lymphocyte gate purity may be calculated by determining the percentage of non-lymphocytes events within the gate. Non-lymphocytes are CD14 positive. Because each flow cytometer has different operating characteristics, each laboratory must determine its optimal operating procedure.

Required reagents

- Whole Blood  Materials required, not provided
- 1. Use peripheral blood containing white blood cells (WBCs) of optimal concentration.
- 3. 4. An additional aliquoted sample is needed for an isotype control: IgG1/FITC and IgG2a/RPE. Repeat steps 1-3 to prepare the control sample.
- 6. 5 mL disposable polystyrene test tubes
- 7. Vortex mixer
- 8. Centrifuge
- 9. Flow cytometer
- 10. Cell counter

Staining procedure

1. Use peripheral blood containing white blood cells (WBCs) of optimal concentration.
2. Add 10 µL of Anti-CD45/FITC and 10 µL of Anti-CD14/RPE (Dako Code FR700), mix by gentle tapping or vortexing.
3. An additional aliquoted sample is needed for an isotype control: IgG1/FITC and IgG2a/RPE. Repeat steps 1-3 to prepare the control sample.
4. Incubate in the dark at room temperature (18-22 °C) for 15-30 minutes.
5. Perform erythrocyte lysing procedure. Follow the reagent manufacturer’s recommended procedure. This procedure may not require a wash step and may include a fixation step, e.g. Dako Utilise (Dako Codes S3325 or S3350).
6. If a fixation step is not included with the erythrocyte lysing procedure, resuspend pellet in 0.5 mL of 0.05 – 1% paraformaldehyde (fixative) in PBS for 30 minutes (21).
7. Analyze samples on a flow cytometer within 48 hours. If lysed and fixed samples are held more than 2 hours, store at 4 °C in the dark until analysis (22, 23).

Flow cytometric analysis

1. Analyze cells on a flow cytometer properly standardized and gated on lymphocytes according to the instrument operating manual.
2. Establish gate purity.
3. Set the cursor using the isotypic control so that 99 ±1% events are in the negative cell population.

For samples containing a low white blood cell count (less than 0.7 x 10^9 cells), cell concentrations by centrifuging by blotting at 18-22 °C for 5 minutes at 500 g. Air dry fully coated with some red cells and plasma to ensure that all white blood cells are collected. Resuspend cells in isotonic medium and adjust white blood cell concentration to approximately 1 x 10^6 cells/mL. Proceed with staining procedure.

Whole Blood

Materials required, not provided
1. Blood collection tubes containing anticoagulant (K-EDTA, Heparin or ACD).
2. Phosphate Buffered Saline (PBS), 0.01 M Phosphate Buffered Saline, pH 7.4 Stock solution
3. 0.05 M NaCl, pH 7.0
- 24 g KCl Add distilled water to 1 liter. Filter through a 0.2 µm filter. Store at 18-22 ° C.
- 21 g NaHCO3 Add 90 mL of distilled water. Mix to dissolve. Store at 18-22 °C. Stable for 2 months.
- 2.5 g Na3HPO4 Add distilled water to 1 liter. Mix to dissolve. Store at 18-22 °C. Stable for up to 2 months.
- 1 mL of 10% Methyl Cellulose (Methylcellulose) (Sigma-Aldrich catalog No. M4508). Mix to dissolve. Store at 18-22 °C. Stable for 2 months.

For samples containing a low white blood cell count (less than 0.7 x 10^9 cells), cell concentrations by centrifuging by blotting at 18-22 °C for 5 minutes at 500 g. Air dry fully coated with some red cells and plasma to ensure that all white blood cells are collected. Resuspend cells in isotonic medium and adjust white blood cell concentration to approximately 1 x 10^6 cells/mL. Proceed with staining procedure.

Primary antibodies

- CD45 Clone
  - Source: Human T-cell Tissue culture supernatant
  - Clone/Ref: T293/34
  - Isotype: IgG1, kappa
  - Antibody MW: 180, 190, 2 @ 205, and 220 kDa
- CD14 Clone
  - Source: Human T-cell Tissue culture supernatant
  - Clone/Ref: TÜK4
  - Isotype: IgG1, kappa
  - Antibody MW: 190, 200 @ 205, 220 kDa

Purification
- Affinity chromatography

Conjugation
- Anti-CD45/FITC - Fluorescein isothiocyanate isomer 1
- Anti-CD14/RPE - Phycoerythrin

Molar ratio
- FITC:Protein is approximately 5.
- RPE:Protein is approximately 1.

Fluorescence
- FITC (Green): Excited at 488 nm; Emits around 530 nm
- RPE (Orange-Red): Excited at 488 nm; Emits around 570 nm

Amount per vial:
- 50 tests (10 µL antibody to 10^6 cells) Reagents are not considered sterile.

Storage
- Store the dark at 2-8 °C. Do not freeze.
- After the appearance of precipitation, such as precipitation, indicates instability or deterioration. In such cases, the reagent is not to be used.

Precautions

1. For in vitro diagnostic use.
2. Wear disposable gloves when handling any human material.
3. Specimen, before and after fixation, and all materials exposed to them, should be handled as if capable of transmitting infection and disposed of with proper precautions.
4. The sodium azide (NaN3) used as a preservative is toxic if ingested. Sodium azide binds organic compounds with metals. Upon disposal, flush with large volumes of water to prevent azide build-up in pluming.
5. Never pipette reagents by mouth and avoid their contact with skin and mucous membranes.
6. Avoid microbial contamination of reagents or incorrect results may occur.
7. Incubation times or temperatures other than those specified may give erroneous results; any such change must be validated by the user.
8. Do not use reagents beyond expiration date for prescribed storage method. If reagents are stored under any conditions other than those specified in the specification sheet, the conditions must be validated by the user.
9. Avoid contact with eyes when heated. Formaldehyde is toxic, allergic, and is known to cause cancer. If skin or eye contact occurs, flush with plenty of water and contact a physician. Inhalation or ingestion is harmful and may be fatal. If swallowed, induce vomiting and contact a physician immediately. Dispose of according to federal, state and local regulations.

Lymphocytes from peripheral blood labeled with Anti-CD45/FITC, T293/33, and Anti-CD14/RPE, TÜK4 (99.14% labeled lymphocytes).
Expected values
Blood samples from 149 apparently healthy males and females at three geographically separate locations. The population included adults from a variety of ages ranging in age from 19 to 80 years. Samples were stained with Anti-CD45/FITC, T29/33 and Anti-CD14/RPE, TÜK4 monoclonal antibodies. Normal CD45-positive and CD14-positive cell populations were assessed in the following table. Values are expressed as a percentage of the total cell count, rather than absolute cell counts and are intended as representative values only. Due to uncontrollable variance among the different laboratory methods for determining absolute lymphocyte counts, an assessment of the accuracy of the method used is necessary (30). Each laboratory should establish its own expected values from the local population of normal donors.

### Quality Assurance
Performance of a normal, apparently healthy donor should be run as a positive control to ensure proper working conditions. Normal ranges should be established within a local population of normal, apparently healthy donors.

### Interference
Interference in the counts of percent positive cells by monocytes and granulocytes with Fc receptor bound antibody may be reduced by proper flow cytometric gating on lymphocytes. Dako's Modified Phosphate Buffered Saline (PBS) (without calcium and magnesium) (Dako Code 53024) can be substituted for PBS working solution.

### Limitations
Samples from certain patients may present special problems due to abnormal erythrocyte or leucocyte populations that are a result of illness or drug usage. Blood samples from abnormal donors may not show abnormal values for the percent of lymphocytes stained with a monoclonal antibody. Results obtained from flow cytometric analysis should be reviewed with results from other diagnostic procedures.

### Reproducibility
AnticD45/FITC and Anti-CD14/RPE Specificity

### Flow cytometric analysis

1. Obtain a washed isolation of mononuclear cells and adjust cell concentration to approximately 1 x 10^6 cells per mL.
2. Add 50 mL of Anti-CD45/FITC and Anti-CD14/RPE (Dako Code FR700). Mix by gentle tapping or vortexing.
3. Incubate the cells for 15 min.
4. Wash in approximately 2 mL of PBS. Centrifuge at 200 x g for 15 min. Aspirate and discard the supernatant.
5. Resuspend pellet in 0.3 mL of 1% paraformaldehyde (fixative) in PBS.
6. Analyze on a flow cytometer within 48 hours. If samples are held more than 2 hours, store at 2-8 °C in the dark until analysis.

### Expected blood values
Blood samples were collected from 149 apparently healthy males and females at three geographically separate locations. The population included adults from a variety of ages ranging in age from 19 to 80 years. Samples were stained with Anti-CD45/FITC, T29/33 and Anti-CD14/RPE, TÜK4 monoclonal antibodies. Normal CD45-positive and CD14-positive cell populations were assessed in the following table. Values are expressed as a percentage of the total cell count, rather than absolute cell counts and are intended as representative values only. Due to uncontrollable variance among the different laboratory methods for determining absolute lymphocyte counts, an assessment of the accuracy of the method used is necessary (30). Each laboratory should establish its own expected values from the local population of normal donors.

### PBMC (Separated cells)

#### Materials required, not provided
1. Blood samples containing autologous T(LEU, HEPDS or THC)
2. phosphate buffered saline (PBS), 0.01 M Phosphate Buffered Saline, pH 7.4
3. 2.0% NaN3, 2.0% EDTA, 2.0% KCI, 2.0% HEPES, 2.0% MgCl2

#### Working Solution

1. Add 100 µl of whole blood to 900 mL of distilled water. Store at 18-32 °C. Stable for up to 2 months.

#### Flow cytometry

1. # Paraformaldehyde in PBS
2. Add 1 g paraformaldehyde (Sigma catalog no. P0814) to 90 mL of distilled water at 70 °C for 15-30 minutes in a fume hood. Add 10 mL PBS block solution and cool down. Filter through a 0.2 µm filter. Store at 4 °C. Stable for up to three weeks.
3. # Fc receptors containing receptor (Fc-PA)
4. # Transfer pipettes (use pipettes with non-sticking surfaces)
5. # 5 mL disposable polyethylene test tubes
6. # Vortex mixer
7. # Centrifuge
8. # Flow cytometer
9. # Anti-CD14: FITC or IgG1/FITC
10. # Anti-CD45: FITC or IgG2a/RPE
11. # Isotype control: FITC or IgG1/RPE
12. # 5 µL anti-CD14 antibodies (Pharmingen goat-anti-mouse-RPE)
13. # Isotonic medium (e.g., PBS with 1% w/v BSA and 0.1% NaN3)
14. # Trypan Blue

#### Working Solution

1. Dissolve Trypan Blue in PBS to a final concentration of 5 g/L.
2. Mix equal volumes of cell suspension and Trypan Blue solution.
3. Place a sample of the mixture in a hemocytometer chamber. Do not overfill or underfill the chamber.
4. Count the live and dead (blue) cells. Calculate the percentage of viable cells from the number of live and dead cells.

#### Cell viability

1. Add 0.5 mL of red blood cells (DB1 t 1) to with isotonic medium on top of 3 mL of Ficoll-Paque in a 15 mL centrifuge tube.
2. Centrifuge at 180 g for 20 min. Mononuclear cell suspension could show a fine, clear interface between the plasma and Ficoll-Paque.
3. Collect the layer of cells by inserting a thin pipette through the plasma. Place collected cells in an 18 ml test tube containing 5 mL isotonic medium. Centrifuge at 200 g for 10 minutes.
4. Wash in approximately 2 mL of isotonic medium and centrifuging at 200 g for 10 minutes.
5. Resuspend cells in the isotonic medium. The cells can be counted and evaluated for viability background and further studies.

#### Specificity

1. Specificity for red blood cells
2. Specificity for granulocytes
3. Specificity for platelets

#### Linear relationship

1. Dissolve Trypan Blue in PBS to a final concentration of 5 g/L.
2. Mix equal volumes of cell suspension and Trypan Blue solution.
3. Place a sample of the mixture in a hemocytometer chamber. Do not overfill or underfill the chamber.
4. Count the live and dead (blue) cells. Calculate the percentage of viable cells from the number of live and dead cells.

#### Flow cytometric analysis

1. Obtain a washed isolation of mononuclear cells and adjust cell concentration to approximately 1 x 10^6 cells per mL.
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#### Correlation

1. Obtain a washed isolation of mononuclear cells and adjust cell concentration to approximately 1 x 10^6 cells per mL.
2. Add 50 mL of Anti-CD45/FITC and Anti-CD14/RPE (Dako Code FR700). Mix by gentle tapping or vortexing.
3. Incubate the cells for 15 min.
4. Wash in approximately 2 mL of PBS. Centrifuge at 200 x g for 15 min. Aspirate and discard the supernatant.
5. Resuspend pellet in 0.3 mL of 1% paraformaldehyde (fixative) in PBS.
6. Analyze on a flow cytometer within 48 hours. If samples are held more than 2 hours, store at 2-8 °C in the dark until analysis.
References


Explanation of symbols

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<th>Symbol</th>
<th>Description</th>
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<td>BD</td>
<td>In vitro diagnostic medical device</td>
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<tr>
<td>ND</td>
<td>Temperature limitation</td>
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<tr>
<td>IC</td>
<td>Use by</td>
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