QIFIKIT®*

Code K0078

7th edition

For quantitative analysis of indirect immunofluorescence staining in flow cytometry.
The kit contains reagents for 10 calibrations.

For research use only. Not for use in diagnostic procedures.

*Trade name of BIOCYTEX

Contents Page

Recommended Use

For research use only. Not for use in diagnostic procedure.

QIFIKIT® is recommended for the quantitative determination of cell surface antigen by flow cytometry using indirect immunofluorescence assay. The antigen quantity is expressed in Antibody-Binding Capacity (ABC) units.

Summary and Explanation

Numerous immunological methods, including flow cytometry, have been developed for the phenotyping of cells and tissues. In the past, however, little attention has been paid to the quantitative aspects of antibody binding and expression levels of cell-surface and intracellular antigens.

Several studies now indicate that quantitation of cell-surface and intracellular antigens should be emphasized and developed for research applications. Important research areas where absolute quantitation may be of value include (1-9):

- Leukemia/lymphoma
  - Studies of cell differentiation
  - Characterization of leukemia subtypes
  - Measurements of expression levels of adhesion molecules in relation to metastasis
  - Studies of prognostic indicators (CD10, CD24, CD34, CD45)
  - Studies of minimal residual disease
- Investigation of multi-drug resistance (P-glycoprotein) (10)
- AIDS research
  - Quantitation of activation antigens CD25, CD26, CD28, CD38, CD69, and HLA-DR
- Normal cells
  - Studies of antigen density variation, e.g. during stress
- Characterization of new antigens and clustering of monoclonal antibodies (Mab)

Flow cytometry using the QIFIKIT®, which combines measurements of antigen density and percentage of antigen-positive cells, is a more easy and precise way of quantifying surface and intracellular molecules than conventional mass techniques, e.g. radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA).

QIFIKIT® contains a series of beads, 10 µm in diameter and coated with different, but well-defined quantities of mouse Mab molecules (high-affinity anti-human CD5, Clone CRIS-1, isotype IgG2a). The beads mimic cells with different antigen densities which have been labeled with a primary mouse Mab, isotype IgG.

For the calibration of the beads, different subclones of the CCRF-CEM lymphoblast cell line were used. These subclones express different amounts of the CD5 antigen (11).

Principle of the Assay

Cells are labeled with primary mouse monoclonal antibody directed against the antigen of interest. In a separate test tube, cells are labeled with irrelevant mouse monoclonal antibody (control). Then, cells and Set-Up Beads and Calibration Beads of the kit are labeled, in parallel, with fluorescein-conjugated anti-mouse secondary antibody.

The primary antibody used for labeling of the cells is used at saturating concentration. The saturation conditions are determined by performing titration studies on each Mab investigated using the fluorescein-conjugated anti-mouse secondary antibody. The primary antibody may be of any mouse IgG isotype (11). Under these conditions, the number of bound primary antibody molecules corresponds to the number of antigenic sites present on the cell surface.

The secondary antibody is also used at saturating concentration. Consequently, the fluorescence is correlated with the number of bound primary antibody molecules on the cells and on the beads. The samples are then analyzed in the following order:

- Vial 1, Set-Up Beads. This sample is used to establish Window of Analysis. The Set-Up Beads comprise a mixture of blank beads and high-level beads.
- Vial 2, Calibration Beads. This sample is used for the construction of the calibration curve (Mean Fluorescence Intensity (MFI)) against ABC.
- Cells are analyzed on the flow cytometer and ABC is calculated based on the equation of the calibration curve.

Reagents

A. Materials provided

SET-UP BEADS

Set-Up Beads
Vial 1 (1 mL)
Two populations of beads. Blank beads (A) and beads with a high number of Mab molecules.
In 0.01 mol/L phosphate-buffered saline, 0.1% BSA, 15 mmol/L NaN_3, pH 7.4.

CALIBRATION BEADS

Calibration Beads
Vial 2 (1 mL)
Five populations of beads (B, C, D, E, and F) bearing different numbers of Mab molecules. Lot-specific information on the exact number of Mab is presented on the enclosed Analytical Value Sheet.
In 0.01 mol/L phosphate-buffered saline, 0.1% BSA, 15 mmol/L NaN_3, pH 7.4.

FITC CONJUGATE

FITC Conjugate
Vial 3 (0.2 mL)
$F(ab')_2$ Fragment of FITC-Conjugated Goat Anti-Mouse Immunoglobulins (affinity-isolated antibody).
In 0.01 mol/L phosphate-buffered saline, 15 mmol/L NaN_3, pH 7.2.

B. Materials required but not provided

Unconjugated Primary Antibody.
Mouse monoclonal antibody to cell surface antigen. Isotype IgG.

Unconjugated Negative Control Reagent.
Irrelevant mouse monoclonal antibody of the same isotype as the unconjugated primary antibody.

Erythrocyte lysing solution.

Bovine serum albumin (BSA).

Sodium azide (NaN_3).

Phosphate-Buffered Saline (PBS).

PBS Stock Solution (10 x concentrated).
80.0 g NaCl
0.2 g KH_2PO4
14.4 g Na_2HPO4, $2H_2O$
2.0 g KCl
Add distilled water to 1 liter. Check pH. At a dilution of 1:10 the pH should be 7.4 ± 0.1.

PBS Working Solution (0.01 mol/L PBS).
Add 100 mL of PBS Stock Solution to 900 mL of distilled water.

PBS-BSA-Azide.
0.01 mol/L PBS, 0.1% BSA, 15 mmol/L NaN_3, pH 7.4.

PBS-Azide.
0.01 mol/L PBS, 15 mmol/L NaN_3, pH 7.4.

1 % Paraformaldehyde in PBS.
Add 1.0 g paraformaldehyde to 90 mL of 70 °C distilled water. Leave at 70 °C for 15-30 minutes in a fume hood. Add 10 mL of PBS Stock Solution and allow to cool. Store at 4 °C. Stable for no longer than three weeks.

General laboratory equipment for flow cytometry procedures.

Semi-logarithmic and double logarithmic 5-cycle graph paper.

Calculator.

Spreadsheet programs. These programs are optional.

Precautions

1. The device is not intended for clinical use including diagnosis, prognosis, and monitoring of a disease state, and it must not be used in conjunction with patient records or treatment.
2. This product contains sodium azide (NaN_3), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, sodium azide may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing.
3. As with any product derived from biological sources, proper handling procedures should be used.

Storage

Store in the dark at 2-8 °C. Do not use after expiration date stamped on vial. If reagents are stored under any conditions other than those specified, the conditions must be verified by the user. There are no obvious signs to indicate instability of this product. Therefore, positive and negative controls should be run simultaneously with sample specimens. If unexpected results are observed which cannot be explained by variations in laboratory procedures and a problem with the kit is suspected, contact Dako Technical Services.

Assay Procedure

A. Indirect Immunofluorescence Staining of Cell Surface Antigens Using Whole Blood

1. Collect venous blood in a test tube containing an anticoagulant.
2. Isolate mononuclear cells by centrifugation on a separation medium. Alternatively, lyse the red cells after step 10.
3. Mix 100 µL cell suspension with 10 µL of an unconjugated primary mouse monoclonal antibody. Negative control: Replace the unconjugated antibody with an irrelevant mouse monoclonal antibody of the same isotype and adjusted to the same concentration.
   Note: Ensure that the primary antibody is used at saturating concentration.
4. Incubate at 4 °C for 30 to 60 minutes.
5. Add 3 mL of PBS-BSA-Azide. Mix gently with a vortex mixer to ensure that the cells are in suspension.
6. Centrifuge 300 x g for 5 minutes. Aspirate and discard the supernatant, leaving approximately 50 µL of fluid in the tube.
7. Repeat steps 5 and 6.
8. Add 100 µL of Vial 3, FITC Conjugate, diluted 1:50 in 0.01 mol/L PBS. Mix gently with a vortex mixer to ensure that the cells are in suspension.
9. Incubate in the dark at 4 °C for 45 minutes.

10. Add erythrocyte-lysing solution to the tube and Mix gently with a vortex mixer to ensure that the cells are in suspension. Follow the manufacturer's recommendations for volumes, time, and temperature of incubation.
11. Centrifuge at 300 x g for 5 minutes. Aspirate and discard the supernatant, leaving approximately 50 µL of fluid in the tube.
12. Add 3 mL of PBS-Azide (or PBS-BSA-Azide if fixation is not needed). Mix gently with a vortex mixer to ensure that the cells are in suspension.
13. Centrifuge at 300 x g for 5 minutes. Aspirate and discard the supernatant, leaving approximately 50 µL of fluid in the tube.
14. Repeat steps 12 and 13.
15. If fixation is not needed:
    a. Resuspend the pellet in 500 µL of PBS-BSA-Azide. Store test tubes at 4 ℃ for no more than 2 hours before analysis.
    b. Analyze on a flow cytometer.
16. If fixation is needed:
    a. Resuspend the pellet in 500 µL of 1% paraformaldehyde in PBS for 2 hours at room temperature or overnight at 4 ℃.
    b. Add 3 mL of PBS-Azide. Vortex gently.
    c. Centrifuge at 300 x g for 5 minutes. Aspirate and discard the supernatant, leaving approximately 50 µL of fluid in the tube.
    d. Resuspend in 500 µL of PBS-Azide. Store test tube at 4 ℃ in the dark if it cannot be analyzed immediately.
    e. Analyze on a flow cytometer.

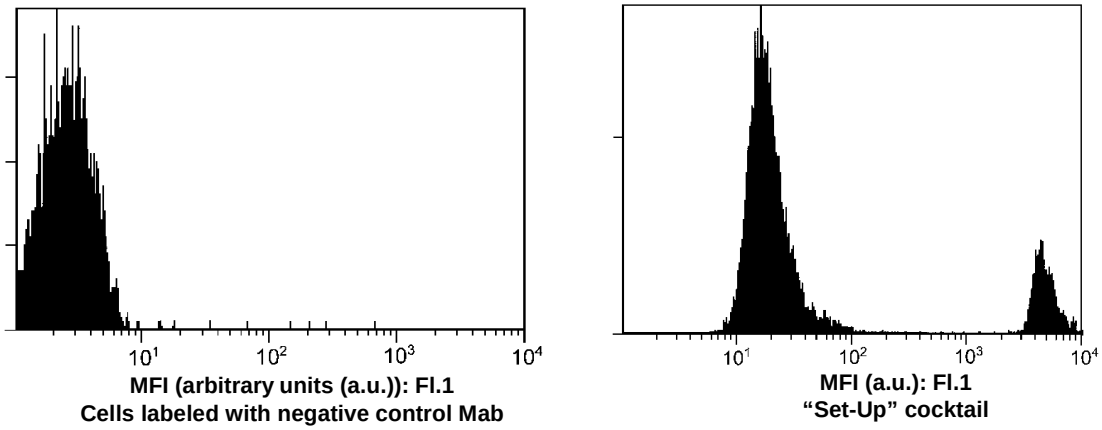
B. Indirect Immunofluorescence Staining of the QIFIKIT® Beads

1. Place 100 µL of resuspended (vortex) beads from Vial 1 and Vial 2, respectively, in two separate test tubes.
2. Add 3 mL of PBS-BSA to each tube. Mix gently with a vortex mixer to ensure that the cells are in suspension.
3. Centrifuge at 300 x g for 5 minutes. Aspirate and discard the supernatant, leaving approximately 50 µL of fluid in each tube.

From this point follow Assay Procedure A from step 8. The beads should be treated the same way as the cells.

Data Acquisition

1. Set-up the flow cytometer using standard operating procedures.
2. Select logarithmic amplification for the fluorescence parameter detecting FITC.
3. Establishment of the Window of Analysis. Since the autofluorescence of the QIFIKIT® beads tends to be higher than that of the lymphocytes, it might be necessary to make pre-adjustments of the PMT of the corresponding fluorescence detector to assure that both negative cells and populations of the Set-Up Beads are displayed on scale as shown in Figure 1a and 1b, respectively.



1a 1b

Figure 1. Establishment of Window of Analysis using the QIFIKIT® Set-Up Beads. The PMT has been adjusted to assure that both negative cells (Figure 1a) and populations of Set-Up Beads (Figure 1b) are displayed on scale.

4. Acquire the data from the Set-Up Beads.
   Fluorescence analysis is confined to bead singlets clear of debris, as defined on a forward scatter versus side scatter dot plot (see Figure 2).

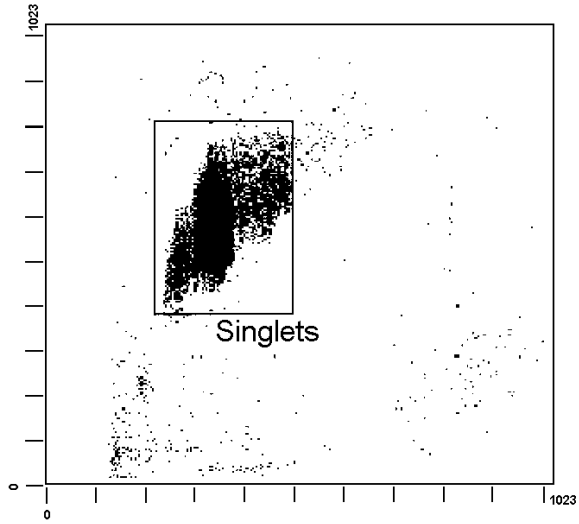


Figure 2. Forward scatter versus side scatter of Set-Up Beads. The gate has been set to collect bead singlets.

5. Without changing the Window of Analysis acquire data from the Calibration Beads and the sample.

Data from beads and cell specimens have to be collected at the same time.

Note: PMT voltage from the corresponding fluorescence detector has to be the same, whereas scatter settings may vary from cell to QIFIKIT® bead data acquisition.

Data Analysis

Individual markers are used to determine the MFI of each bead population of the Calibration Beads (see Figure 3).

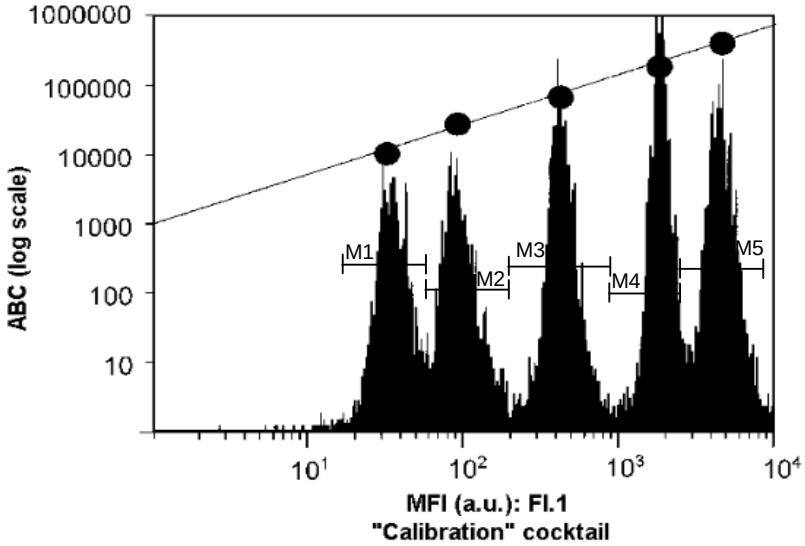


Figure 3. Histogram of QIFIKIT® Calibration Bead populations. The calibration curve has been overlaid.

Calculation of Antigen Density

Definitions and units:

Antibody-Binding Capacity (ABC) is the number of primary mouse monoclonal antibodies per cell or microbead (12).

Background Antibody Equivalent (BAE) is the apparent ABC of the negative control for cells or blank beads (A) due to background fluorescence. It can be deduced from the calibration curve.

Specific Antibody-Binding Capacity (SABC) is the number of primary mouse monoclonal antibodies per cell after corrections for background (BAE):

$$SABC = ABC - BAE$$

Most often, BAE remains negligible compared with ABC, but for low antigen densities SABC is significantly lower than ABC. If the use of saturating Mab concentrations has been ensured, SABC corresponds to the mean number of accessible antigenic sites per cell, referred to as *antigen density* and expressed in sites/cell.

A.1 Manual calculation. For instruments expressing MFI in linear values

Construct the calibration curve and calculate cell antigen density as follows, see Figure 4:

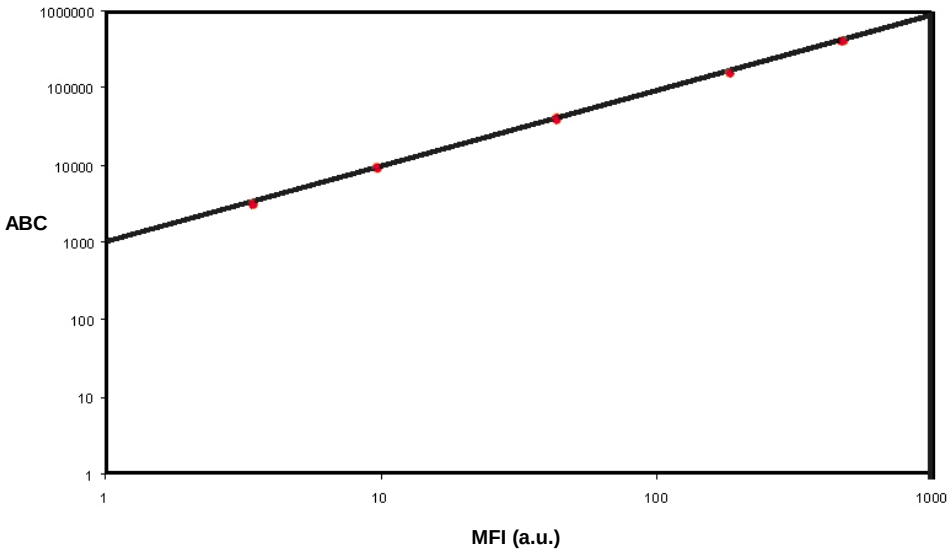


Figure 4. Calibration curve showing MFI versus ABC (log-log scale) for the 5 Calibration Bead populations.

1. Determine the MFI for each population of the QIFIKIT® Calibration Beads (B to F) and plot MFI versus ABC on double logarithmic paper.
2. Draw the “best-fit” straight line.
3. Note that the blank beads serve as an indicator of the detection threshold of the instrument.
4. For cell samples, determine the MFI of each cell population of interest and then determine ABC by interpolation on the calibration curve.
5. For the negative control sample, determine the MFI and then determine the apparent ABC = BAE by interpolation on the calibration curve.
6. Calculate antigen density: SABC = ABC – BAE.

A.2 Manual calculation. For instruments expressing MFI in channel numbers

Construct the calibration curve and calculate cell antigen density as follows, see Figure 5:

1. Determine the MFI for each population of the QIFIKIT® Beads (B to F), and plot MFI versus ABC on single logarithmic paper.
2. Draw the “best-fit” straight line.
3. Note that the blank beads serve as an indicator of the detection threshold of the instrument.
4. For samples, determine the MFI of each cell population of interest and then determine ABC by interpolation on the calibration curve.
5. For the negative control sample, determine the MFI and then determine the apparent ABC = BAE by interpolation on the calibration curve.
6. Calculate antigen density: SABC = ABC - BAE.

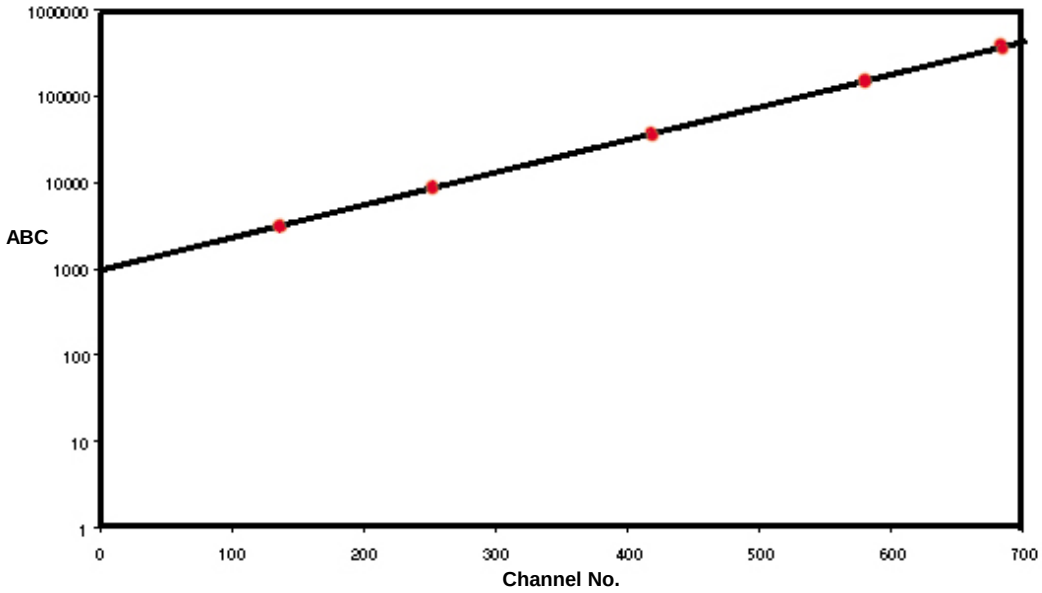


Figure 5. Calibration curve showing Channel No. versus ABC (lin-log scale) for the 5 calibration bead populations.

B. Calibration using a calculator with statistical functions or a spreadsheet program

1. Record the MFI value for each population of the QIFIKIT® Calibration Beads (B to F).
2. Log transform each MFI and the corresponding ABC.
3. Calculate the parameters of linear regression:
   log (ABC) = a x log (MFI) + b.
4. For samples, determine the MFI of each cell population of interest. Log transform MFI.

5. Calculate: log (ABC) = a x log (MFI) + b.
6. Back transform log (ABC): ABC = $10^{(\log (ABC))}$.
7. Repeat steps 4-6 for the negative control.
8. Calculate antigen density by subtracting the BAE of the negative control from the ABC of the population of interest: SABC = ABC - BAE.

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Explanation of symbols

REF	Catalogue number		Keep away from sunlight (consult storage section)		Use by
	Consult instructions for use	Σ	Contains sufficient for <n> tests		Manufacturer
2 °C 8 °C	Temperature limitation	LOT	Batch code		

Produced by:
Dako Denmark A/S
Produktionsvej 42
DK-2600 Glostrup
Denmark
Tel. +45 44 85 95 00
Fax +45 44 85 95 95

www.dako.com