

CELLQUANT Calibrator

Kit for leukocyte surface antigen quantitation by flow cytometry

For 5 calibration curves and 50 tests

Ref. 7208



For Research Use Only.

Not for Use in Diagnostic Procedures.

1 INTENDED USE

Calibration kit for the measurement of human leukocyte surface antigen expression level by multiple color analysis.

Leukocytes are stained by indirect immunofluorescence with specific monoclonal antibodies (MAbs) and analyzed by quantitative flow cytometry. Counter-staining reagents can be added to the procedure to gate and analyze sub-populations of interest. The expression level of the tested antigen is determined using the calibration beads.

CELLQUANT Calibrator is designed for purified mouse MAbs of IgG1 and IgG2a isotypes.

CELLQUANT Calibrator is also applicable on isolated cells.

2 REAGENTS

- **Reagent 1:** 1 x 40 mL vial, diluent, 10 fold concentrated.
- **Reagent 2:** 1 x 250 µL vial, calibration beads. The beads are coated with increasing and accurately known quantities of mouse IgG. The number of determinants coated on each bead population is indicated in the calibration flyer provided in the kit. These values may slightly vary from lot to lot.
- **Reagent 3:** 1 x 550 µL vial, staining reagent, polyclonal antibody anti mouse IgG-FITC.
- **Reagent 4:** 1 x 2.75 mL vial, neutralization solution.

CELLQUANT Calibrator contains enough reagent to perform :

- 5 calibration curves
- 50 immunostainings (MAbs to be tested and corresponding negative isotypic controls).

REAGENTS REQUIRED BUT NOT PROVIDED

- Purified mouse monoclonal antibodies of IgG1 and IgG2a isotype.
- Non conjugated mouse negative isotypic control.
- Counter-staining reagent (PE, PE-Cy5...) at a maximum optimal initial concentration of 90 µg/mL.

Recommended BioCytex reagents: Ref. 5104-PE100T: CD4-PE

Ref. 5105-PE100T: CD8-PE

Ref. 5450-PE100T: CD45-PE

For other counter-staining reagents, contact Biocytex technical department.

- Red blood cell lysis solution (for delayed analysis, refer to § 5.2.E.Note)

Recommended BioCytex reagent: CELLQUANT Lysis Ref. 7210, non fixative lysis.

- Anti mouse IgG-FITC reagent (to determine the saturating concentration)

WARNING

All reagents contain sodium azide as a preservative. Reagents containing sodium azide should be discarded with care to prevent the formation of explosive metallic azides. When dumping waste materials into sinks, use copious quantities of water to flush plumbing thoroughly

3 REAGENT PREPARATION AND STORAGE

Intact kits and contents remain stable until the expiration date printed on the box label, when stored at 2-8 °C. Do not freeze the kit.

- **Reagent 1 :** Stability after opening: 2 months at 2-8 °C when free of contamination.
Prepare a **1:10 dilution** with distilled water.
Prepare the appropriate volume required for the series to be tested (prepare 7 mL per cytometric tube).
Stability after dilution: 2 months at 2-8 °C when free of contamination.
The presence of crystals does not affect the quality of the reagent. Incubate at 37 °C until the crystals are completely dissolved.
- **Reagent 2: after resuspension by vortex for 5 seconds**, the reagent is ready for use.
Stability after opening: 2 months at 2-8 °C when free of contamination.
- **Reagents 3 and 4:** Ready for use.
Stability after opening: 2 months at 2-8 °C when free of contamination.

4 SPECIMEN COLLECTION AND TREATMENT

• Sample collection:

- Use non-wettable (plastic or siliconized glass) blood collection tubes.

• Sample preparation

- The test is performed on whole blood drawn on anticoagulant (the anticoagulant of choice is EDTA). For samples containing more than 10×10^6 target cells/mL, the sample numeration must be adjusted with diluted Reagent 1.

- Alternatively, the test can be performed on isolated cells, the cell suspension must be adjusted at 2×10^6 to 10×10^6 cells/mL.

• Sample storage:

- Blood must be preferentially stored at room temperature before testing (18-25 °C). The sample stability is usually 24 hours at room temperature. However it is recommended to check this stability for each tested parameter (antigen).

- Isolated cells (or cultured cells) must be stored according to their particular characteristics.

5 PROCEDURE

Note : one calibration curve must be performed per sample series.

5.1 Choice of the antibodies

5.1.1 Choice of the specific antibody

- To be used with the kit, the specific antibody must be used at **saturating concentration**. This concentration should be inferior or equal to **5µg/mL final concentration** (corresponds to ≤ 30 µg/mL initial concentration).

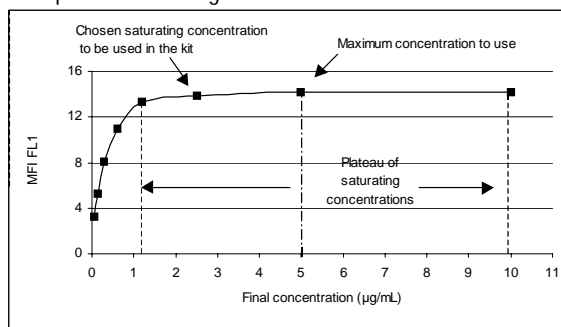
- The determination of the saturating concentration must be performed at room temperature by indirect immunofluorescence with washings :

- 2 by 2 dilutions of the specific antibody
- incubation of the dilutions with the sample for 10 minutes
- washing
- staining with an anti mouse IgG-FITC reagent of your choice
- red blood cell lysis
- washing and cytometric analysis.

On the curve relating the MFI of the stained cells to the final antibody concentration, choose the minimal saturating concentration situated at the plateau of the curve.

- The determined saturating concentration will be the final antibody concentration to be used in the kit protocol.

Example of a saturating curve:



5.1.2 Choice of the negative isotypic control

- The negative isotypic control to use must have the same isotype than the specific antibody and must be adjusted at the same concentration.
- The negative isotypic control must be performed for each blood sample.

5.2 Protocol

Note: For good results exercise great care in the pipetting of small reagent volumes by depositing them at the bottom of the test tubes.

All reagents must be at room temperature.

Example of protocol for the quantitation of 8 antigens using 8 Mabs (MAb1 to MAb8) of IgG1 and IgG2a isotypes on one sample

A/ Setup of antibody and calibrator tubes

• Setup of antibody tubes

In a rack prepare 10 tubes:

MAb1 IgG1	MAb2 IgG1	MAb3 IgG2a	MAbX ...	MAb 8 IgG2a	Ctl IgG1	Ctl IgG2a
T1	T2	T3	Tx	T8	T9	T10

For each sample, in tubes T1 to T10:

- pipette **50 µL** of whole blood or isolated cells.

• Setup of calibration tube

One calibration curve is necessary per sample series.

In a tube labeled T11: pipette **50 µL** of Reagent 2 **after resuspension using a vortex for 5 seconds** and add **10 µL** of diluted reagent1.

B/ Immuno-labelling

For each sample,

- to each of tubes T1 to T8: pipette **10 µL** of the purified MAb.

- to each of tubes T9 and T10: pipette **10 µL** of the non conjugated negative isotypic control.

- Homogenize the tubes using a vortex for 1 to 2 seconds.

- Incubate all tubes at room temperature for **10 minutes**.

C/ Fluorescent Staining

For each sample, to each of tubes T1 to T11:

- Pipette **10 µL** of Reagent 3.

- Homogenize the tubes using a vortex for 1 to 2 seconds.

- Incubate the tubes at room temperature for **10 minutes**.

For a single color protocol, perform directly step E.

D/ Washing- neutralization and counter-staining (if necessary)

Washing and neutralization :

To each of tubes T1 to T11, perform a washing step

- Add **3 mL** of diluted Reagent 1.

- Homogenize the tubes using a vortex for 1 to 2 seconds.

- Centrifuge the tubes for **5 minutes** at **300g** and aspirate the supernatant.

- Resuspend the cell pellet with pipette by adding **50 µL** of Reagent 4.

- Homogenize the tubes using a vortex for 1 to 2 seconds.

Counter-staining :

To each of tubes T1 to T10,

- with BioCytex counter-staining reagent(s), pipette **20 µL**.

- with any other reagents, pipette a volume of counter-staining reagent(s), according to the manufacturer's recommendation.

- Homogenize the tubes using a vortex for 1 to 2 seconds.

- Incubate the tubes according to the manufacturer's recommendation.

E/ Red cell lysis (if necessary)

- If the sample is a cell suspension, perform directly step F.

- To each of tubes T1 to T11 :

- with CELLQUANT Lysis ref.7210, pipette **2 mL** of diluted (1X) lysis, vortex immediately, incubate for **10 minutes** at room temperature and vortex again.

- with any other lysis, follow the manufacturer's recommendations .

- Centrifuge the tubes for **5 minutes** at **300g** and aspirate the supernatant.

Notes:

- In case of **automated lysis**, in tube T11 pipette a volume of diluted Reagent 1 (equivalent to the volume of lysis solution added to the other tubes) instead of the lysis.

- A change of lysis solution during a study may affect the results.

- In case of delayed cytometric analysis, a fixing lysis solution or an additional fixation step are recommended.

F/ Washing

To each of tubes T1 to T11,

- Pipette **3 mL** of diluted Reagent 1.

- Homogenize the tubes using a vortex for 1 to 2 seconds.

- Centrifuge the tubes for **5 minutes** at **300g** and aspirate the supernatant.

- Add **500 µL** of diluted Reagent 1.

The technology allows to quantify leukocyte receptor antigen expression up to 48 hours after staining when samples are stored at 2-8°C.

However it is recommended to check the stability of the expression values measured for each studied parameter.

6 Cytometric analysis

Refer to the Operator's Manual of the cytometer for instructions on how to perform cytometric readings.

The selected Mean Fluorescence Intensity (MFI) statistics is the geometric mean (Mn (x) or GeoMean depending upon the cytometer).

For compensation settings, you can refer to your current protocol or to the appendix.

7 RESULTS

Computer data analysis or graphic data analysis

7.1. Computer data analysis:

The result treatment is easily performed using a calculation template available upon request from the BioCytex technical department.

7.2 Graphic data analysis :

If the MFI values are expressed as linearized values or channel numbers, use a log-log or semi-log graph paper, alternatively.

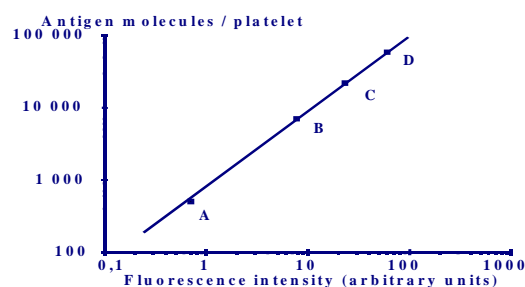
Plot the MFI calibration values (calibration tube) on the abscissa (x-axis) and their corresponding number of monoclonal antibody molecules (as indicated in calibration flyer) on the ordinate (y-axis).

Draw the calibration curve.

Interpolate the MFI values of the test tubes on the calibration curve and read off directly their corresponding molecule numbers.

Specific quantitative values (sABC) of the binding of the selected antibodies are calculated after subtraction of the corresponding negative isotypic control measurement.

Example of calibration curve:



8 PERFORMANCES

8.1.Measurement zone : the measurement zone ranges from 1 000 to 300 000 antigens per cell.

8.2. Repeatability : example, one analysis on CD4⁺ lymphocytes tested 5 times with the same kit:

Antibody	Mean sABC	SD	CV %
CD55	7 676	108	1.4 %
CD5	54 144	804	1.5 %
CD45	254 330	8 304	3.3 %

Values obtained on Coulter XL MCL cytometer.

REFERENCES

1- Poncelet P. *et al.*: "Clinical applications of quantitative Immunophenotyping" in: "Immunophenotyping", C.C. Stewart and J.K.A. Nicholson, (eds) Wiley-Liss 2000, pp 105-132.

BIOCYTEX
140 ch. ARMEE D'AFRIQUE
13010 MARSEILLE
FRANCE
TEL : +33 (0) 4 96 12 20 40
FAX : +33 (0) 4 91 47 24 71

Version September 2002