PLATELET Calibrator

Kit for platelet antigen quantitation by flow cytometry

For 5 calibration curves and 50 tests **Ref. 7011**

BioCytex

For Research Use Only.
Not for Use in Diagnostic Procedures.

1 INTENDED USE

Calibration kit for the measurement of platelet glycoprotein expression level or any other human platelet surface molecules.
Platelets are stained by no wash indirect immunofluorescence with specific monoclonal antibodies (MAbs) and analyzed by quantitative flow cytometry.
The expression level of the tested antigen is determined using the calibration beads.
This protocol is designed for purified mouse MAbs of IgG1, IgG2a and IgG2b isotypes.

2 REAGENTS

- **Reagent 1:** 1 x 35 mL vial, diluent, 10 fold concentrated.
- **Reagent 2a:** 1 x 500 µL vial, negative isotypic control IgG1.
- **Reagent 2b:** 1 x 500 µL vial, negative isotypic control IgG2a.
- **Reagent 2c:** 1 x 500 µL vial, negative isotypic control IgG2b.
- **Reagent 3:** 1 x 200 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.
- **Reagent 4:** 1 x 1.25 mL vial, staining reagent, polyclonal antibody anti mouse IgG-FITC.

This kit contains enough reagent to perform:
- 5 calibration curves
- 2 determinations of saturating concentration
- 50 antibody tests (antibody to be tested and corresponding negative isotypic control)

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.*

- **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.
  Stability after dilution: 15 days at 2-8 °C.
- **Reagent 2a, 2b, 2c and 4:** Ready for use.
  Stability after opening: 2 months at 2-8 °C when free of contamination.
- **Reagent 3: <u>after resuspension by vortex for 5 seconds</u>,** the reagent is ready for use.
  Stability after opening: 2 months at 2-8 °C when free of contamination.

Notes: ***** do not freeze the kit.
****** the presence of crystals does not affect the quality of the reagent. Incubate at 37 °C until the crystals are completely dissolved.

4 SPECIMEN COLLECTION AND TREATMENT

- **Sample collection:**

- Use non-wettable plastic blood collection tubes.
- In order to maintain platelet integrity, exercise utmost care to avoid platelet activation during the collection procedure.
- Anticoagulant: trisodium citrate 0.109 M or 0.129 M (using a ratio 9:1 volumes) or CTAD / Diatube®H (Becton-Dickinson).

- **Sample storage:**

- Blood sample must be treated within **8 hours** after collection.
- Blood must be stored at room temperature before testing (18-25 °C).
- The test can be performed either on whole blood or on plasma rich platelet (PRP).

5 PROCEDURE

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

5.1 Choice of the antibodies

<u>5.1.1 Determination of the saturating concentration of the specific antibodies</u>
The selected specific antibody must be used at **10 µg/mL** in the kit protocol provided that this antibody is saturating at a concentration **≤ 5 µg/mL.**
If necessary, check the saturating concentration of the antibody (cf. appendix "Determination of the saturating concentration of a monoclonal antibody to be used in the Platelet Calibrator kit").

<u>5.1.2 Determination of the isotype of the specific antibodies</u>
Refer to the documentation of the manufacturer.

<u>5.1.3 Choice of the negative isotypic control</u>
- Three negative isotypic controls IgG1, IgG2a and IgG2b are provided with the kit and are ready to use.
- The negative isotypic control to use must have the same isotype than the specific antibody.
- The negative isotypic control must be performed for each blood sample.

5.2 Examples of protocol

Note: For good results exercise great care in the pipetting of small reagent volumes (20 µL) by depositing them at the bottom of the test tubes.
All reagents must be at room temperature.

<u>5.2.1 Example of protocol for the quantitation of 3 antigens using 3 Mabs (MAb1 to Mab3) of different isotypes</u>

A/ Setup of antibody, calibrator and sample tubes

- <u>Setup of antibody tubes</u>

In a rack, per sample, prepare the following series:

MAb1/IgG1	MAb2/IgG2a	MAb3/IgG2b	Ctl IgG1	Ctl IgG2a	Ctl IgG2b
T1	T2	T3	T4	T5	T6

- In tube T1: pipette **20 µL** of MAb1 at **10 µg/mL.**
- In tube T2: pipette **20 µL** of MAb2 at **10 µg/mL.**
- In tube T3: pipette **20 µL** of MAb3 at **10 µg/mL.**
- In tube T4, pipette **20 µL** of corresponding negative isotypic control (in this example, Reagent 2a IgG1).
- In tube T5, pipette **20 µL** of corresponding negative isotypic control (in this example, Reagent 2b IgG2a).
- In tube T6, pipette **20 µL** of corresponding negative isotypic control (in this example, Reagent 2c IgG2b).

- <u>Setup of calibration tube</u>

One calibration curve is necessary per sample series.
In a tube labeled T7: pipette **40 µL** of Reagent 3 **after resuspension using a vortex for 5 seconds.**

- <u>Setup of sample tubes</u>

For each sample, in a labeled tube:
- Pipette **50 µL** of whole blood. (Alternatively, pipette 25 µL of PRP and add 25 µL of diluted Reagent 1).
- Add **150 µL** diluted Reagent 1. Homogenize using a vortex for 1 to 2 seconds.

B/ Immuno-labelling

For each sample, to each of tubes T1 to T6:
- Add **20 µL** of diluted sample.
- 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 T7:
- Pipette **20 µL** of Reagent 4.
- Homogenize the tubes using a vortex for 1 to 2 seconds.
- Incubate the tubes at room temperature for **10 minutes**.
- Pipette **2 mL** diluted Reagent 1.
- Homogenize the tubes using a vortex for 1 to 2 seconds.

Thus treated, the contents of all tubes may be stored for **2 hours** at 2-8 °C before cytometric analysis.

5.2.2 Example of protocol for the quantitation of 3 antigens using 3 MAbs of the same isotype

For each sample, prepare the following series:

MAb1/IgG2a	MAb2/IgG2a	MAb3/IgG2a	Ctl IgG2a
T1	T2	T3	T4

Follow the protocol described at paragraph 5.2.1 adapting the number of tubes.

5.2.3 Example of protocol for the quantitation of 5 antigens using a combination of 5 MAbs of various isotypes (e.g. 2 IgG1, 2 IgG2a, 1 IgG2b)

For each sample, prepare the following series:

MAb1IgG1	MAb2 IgG1	MAb3 IgG2a	MAb4 IgG2a	MAb5 IgG2b	Ctl IgG1	Ctl IgG2a	Ctl IgG2b
T1	T2	T3	T4	T5	T6	T7	T8

Follow the protocol described at paragraph 5.2.1 adapting the number of tubes.

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).
Vortex each tube before analysis.

- **Calibration analysis (Figs 1)**

Create a FS LOG vs SS LOG cytogram. Add a discriminator on FS Log to minimize the artefactual background. Set up a gate "CAL" around the main single bead population (Fig. 1a).
Create a FL1 LOG histogram gated by the "CAL" window. Note the MFI for each of 3 fluorescence peaks (Fig. 1b: A, B and C cursors) corresponding to the 3 calibration beads.
For optimum analysis conditions, the peak of the third bead fluorescence intensity (FL1) must be set at the beginning of the fourth decade. To achieve this adjust the FL1 PMT voltage.
The "A" cursor must include the first channel.
For a correct analysis, at least 8,000 beads must be gated on the window "CAL".

Fig.1a: Calibration cytogram

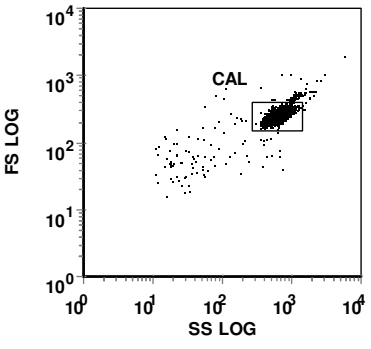
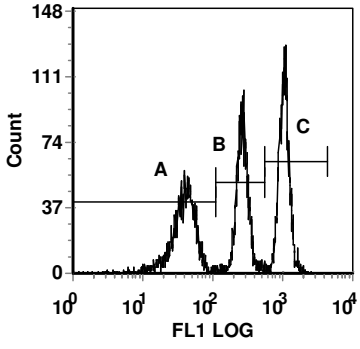


Fig.1b: Cursor settings in gated fluorescence histogram



- **Sample analysis (Figs 2)**

Do not change the acquisition procedure for FL1 (PMTv).
On the FS LOG x SS LOG cytogram (Fig. 2a) platelets are isolated from other whole blood cells by an analysis region " PLT ".
Check that the discriminator setting does not cut the platelet cloud.
In the corresponding gated FL1 LOG histogram, note the mean fluorescence intensity of the positive peak of each assay (Fig. 2b).
Analyse at least 3,000 events on the window "PLT".

Fig. 2a: Whole blood cytogram and platelet window gating

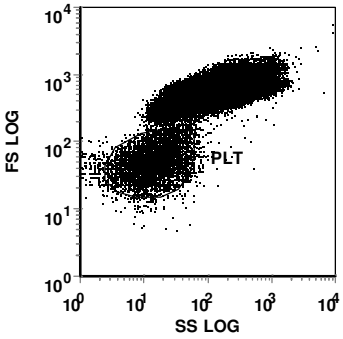
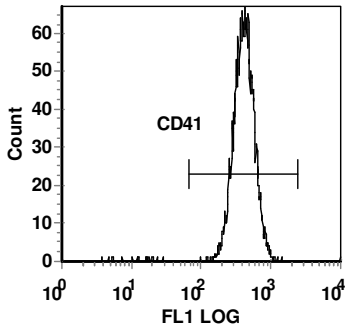


Fig. 2b: example : CD41 immuno-labelling



7 RESULTS

Computer data analysis or graphic data analysis

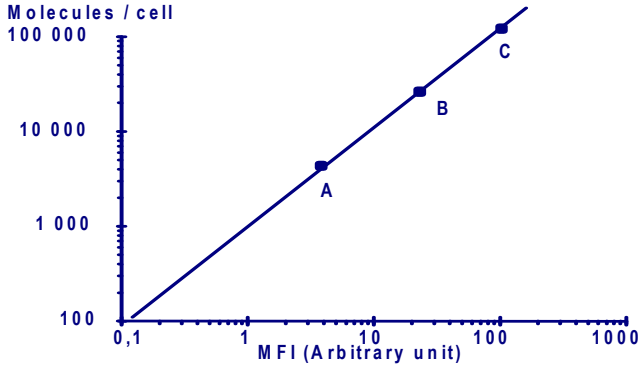
7.1. Computer data analysis:

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

7.2 Graphic data analysis:

Plot the LOG_{10} of the MFI calibration values for the 3 calibration beads on the abscissa (x-axis), and their corresponding LOG_{10} number of monoclonal antibody molecules as indicated on the calibration flyer, on the ordinate (y-axis).
Draw the optimal calibration curve of the type $LOG_{10}(ABC) = a \times LOG_{10}(MFI) + b$. Interpolate the LOG_{10} of the MFI values of the sample tubes on the calibration curve and read directly their corresponding numbers of bound Mab molecules (ABC : Antibody Binding Capacity).
The specific quantitative values (sABC: specific Antibody Binding Capacity) are determined by subtracting the negative isotypic control ABC value as follows:

Example of calibration curve:



8 PERFORMANCES

Repeatability: example, 1 sample treated 5 times with the same kit:

Antibody	Mean sABC	SD	CV %
CD63 (CLB Gran/12)	309	20	6.6
CD41 (P2)	48,795	402	0.8

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