PLATELET Calibrator

Kit for platelet antigen quantitation by flow cytometry
For 5 calibration curves and 50 tests

Ref. 7011

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 1.25 mL vial, staining reagent, polyclonal antibody anti mouse IgG-FITC.
- **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 1.25 mL 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)

**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.

- **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.
- **Reagent 3** **: 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.

**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

5.1.1 Determination of the saturating concentration of the specific antibodies
The selected specific antibody must be used at 10µg/mL in the kit protocol provided that this antibody is saturating at a concentration ≤ 5µg/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").

5.1.2 Determination of the isotype of the specific antibodies
Refer to the documentation of the manufacturer.

5.1.3 Choice of the negative isotypic control
- 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.

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

**A/ Setup of antibody, calibrator and sample tubes**

- **Setup of antibody tubes**
  In a rack, per sample, prepare the following series:

<table>
<thead>
<tr>
<th>MAb1/IgG1</th>
<th>MAb2/IgG2a</th>
<th>MAb3/IgG2b</th>
<th>Ctl IgG1</th>
<th>Ctl IgG2a</th>
<th>Ctl IgG2b</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>T2</td>
<td>T3</td>
<td>T4</td>
<td>T5</td>
<td>T6</td>
</tr>
</tbody>
</table>

  - 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).

- **Setup of calibration tube**
  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.

- **Setup of sample tubes**
  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 | Ctrl 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:

| MAb1 IgG1 | MAb2 IgG1 | MAb3 IgG2a | MAb4 IgG2a | MAb5 IgG2b | Ctrl IgG1 | Ctrl IgG2a | Ctrl 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”.

- 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”.

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 LOG10 of the MFI calibration values for the 3 calibration beads on the abscissa (x-axis), and their corresponding LOG10 number of monoclonal antibody molecules as indicated on the calibration flyer, on the ordinate (y-axis).
Draw the optimal calibration curve of the type LOG10(ABC) = a x LOG10(MFI) + b. Interpolate the LOG10 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:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Mean sABC</th>
<th>SD</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD63 (CLB Gran/12)</td>
<td>309</td>
<td>20</td>
<td>6.6</td>
</tr>
<tr>
<td>CD41 (P2)</td>
<td>48,795</td>
<td>402</td>
<td>0.8</td>
</tr>
</tbody>
</table>

REFERENCES
Determination of the saturating concentration of a monoclonal antibody to be used in the PLATELET Calibrator kit

Recommended protocol

Definition of the saturating concentration of a monoclonal antibody (MAb): MAb concentration which enables to saturate all accessible antigenic sites at the surface of the cells of interest.

A/ Setup of the dilutions of the specific monoclonal antibody:
For one specific MAb to be tested, prepare 6 dilutions according to the following table:

<table>
<thead>
<tr>
<th>Dilutions</th>
<th>Volume of the specific MAb</th>
<th>Volume of diluted R1</th>
<th>Concentration of the specific MAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>300 μL</td>
<td></td>
<td>20 μg/mL</td>
</tr>
<tr>
<td>D2</td>
<td>150 μL of D1 + 150 μL</td>
<td></td>
<td>10 μg/mL</td>
</tr>
<tr>
<td>D3</td>
<td>150 μL of D2 + 150 μL</td>
<td></td>
<td>5 μg/mL</td>
</tr>
<tr>
<td>D4</td>
<td>150 μL of D3 + 150 μL</td>
<td></td>
<td>2.5 μg/mL</td>
</tr>
<tr>
<td>D5</td>
<td>150 μL of D4 + 150 μL</td>
<td></td>
<td>1.25 μg/mL</td>
</tr>
<tr>
<td>D6</td>
<td>150 μL of D5 + 150 μL</td>
<td></td>
<td>0.6 μg/mL</td>
</tr>
</tbody>
</table>

B/ Setup of the staining reagent (R4)
For one specific MAb: mix 70 μL of Reagent 4 and 630 μL of diluted Reagent 1. Homogenize using a vortex for 1 to 2 seconds.

C/ Protocol
Label 6 test tubes T1 to T6. Prepare a platelet rich plasma (PRP) from a citrated whole blood sample (trisodium citrate 0.109 M or 0.129 M using a ratio 9:1 volumes) or drawn on CTAD / Diatube® (Becton-Dickinson).
PRP preparation: Centrifuge the blood sample, at 170g for 15 minutes at room temperature.
Collect the PRP in a test tube and homogenize the tube by inverting it gently up and down.

To each tube T1 to T6:
- Pipette 5 μL of PRP and add 100 μL of the dilutions prepared according to paragraph A. Homogenize the tubes using a vortex for 1 to 2 seconds.
- Incubate the tubes at room temperature (18-25 °C) for 15 minutes.
- Perform one washing:
  - Add to tubes T1 to T6, 3 mL of diluted Reagent 1.
  - Centrifuge the tubes for 5 minutes at 1900 g.
  - Discard the supernatant.
- Add 100 μL of diluted Reagent 4 prepared according to paragraph B. Homogenize the tubes using a vortex for 1 to 2 seconds.
- Incubate the tubes at room temperature for 15 minutes.
- Perform 2 more washings as described above.
- Add 500 μL of diluted Reagent 1.

D/ Cytometric analysis
Vortex each tube before analysis.
Refer to the Operator’s Manual of the cytometer for instructions on how to perform cytometric readings.

Create a FS LOG vs SS LOG cytogram.
Add a discriminator on FS to minimize the artefactual background.
Isolate the platelet population from other whole blood cells by an analysis region “PLT” (fig.a).
Create a FL1 LOG histogram gated by the “PLT” region.
Note the mean fluorescence intensity (MFI) for each dilution of the specific MAb (fig.b).

E/ Results
With a calculation software, draw the saturation curve converting the MFI to the concentration of the antibody.
To achieve this plot the 6 tested concentrations on the abscissa (X axis) and their corresponding MFI values on the ordinate (Y axis).

The saturation curve presents a plateau corresponding to the range of saturating concentrations of the MAb.
Check that the concentration of 5μg/mL give a MFI situated on the plateau of the saturation curve. If this is confirmed, the MAb can be used in the protocol of the PLATELET Calibrator at the concentration of 10μg/mL.

Note: The use of specific MAb not saturating at a concentration ≤ 5μg/mL in the kit protocol will lead to incorrect results.

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