RAT IgG Calibrator

Calibration kit for the quantitation of Rat IgG on whole blood or on cell lines by flow cytometry

For 10 calibration curves and 50 tests on Human whole blood samples
Or 20 calibration curves and 200 tests on Mouse whole blood samples CP011

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

1 INTENDED USE

Calibration kit for the measurement of rat IgG expression level by multiple color analysis.
Whole blood is stained by indirect immunofluorescence 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 IgG is determined using the calibration beads.
RAT IgG Calibrator is also applicable on isolated cells.

2 REAGENTS

- **Reagent 1:** 2 x 45 mL vial, diluent, 10 fold concentrated.
- **Reagent 2:** 1 x 0.5 mL vial, calibration beads. The beads are coated with increasing and accurately known quantities of Rat 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 1.1 mL vial, staining reagent, polyclonal antibody anti Rat IgG-FITC.
- **Reagent 4:** 1 x 2.5 mL vial, neutralization solution.
- **Reagent 5 :** 2 x 10 mL vial, lysing solution, 10 fold concentrated.
- **Reagent 6 :** 1 x 60 mL vial, fixative solution.

RAT IgG Calibrator contains enough reagents to perform:
- 10 calibration curves and 50 stainings on human whole blood.
- 20 calibration curves and 200 stainings on mouse whole blood.

REAGENTS REQUIRED BUT NOT PROVIDED

- Non conjugated Rat monoclonal antibodies.
- Non conjugated Rat negative isotypic control.
- Counter-staining reagent from Mouse or Rat origin (other than FITC).
- Anti Rat IgG-FITC reagent (to determine the saturating concentration).
- Lysing solution (to determine the saturating concentration).
- Washing buffer (to determine the saturating concentration).

WARNING

- Reagent 6 contains 1% paraformaldehyde.
  R40 - Limited evidence of a carcinogenic effect.
  S36/37 - Wear suitable protective clothing and gloves.
- 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.

- **Reagents 1 and 5:** 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: 2 months at 2-8 °C when free of contamination.
  The presence of crystals in Reagent 1 does not affect the quality of the reagent. Incubate at 37 °C until the crystals are completely dissolved.
- **Reagent 2: <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.
- **Reagents 3, 4 and 6 :** 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 Human or Mouse whole blood drawn on EDTA or heparin.
For samples containing more than $5x10^6$ 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 $3x10^6$ to $5x10^6$ 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.
- Isolated cells (or cultured cells) must be stored according to their particular characteristics.

5 PROCEDURE

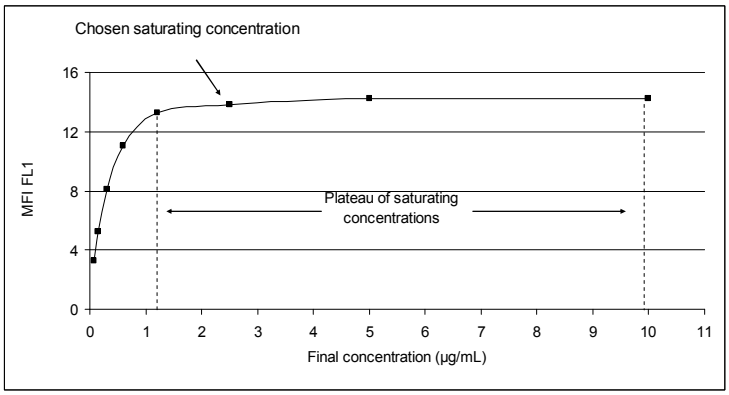
Note: one calibration curve must be performed per sample series.

5.1 Choice of the antibodies

<u>5.1.1 Choice of the specific antibody</u>

- To be used with the kit, the specific antibody must be used at **saturating concentration**.
The determination of the saturating concentration must be performed by indirect immunofluorescence with washings:
- 2 by 2 dilutions of the specific antibody.
- incubation of the dilutions with the sample for a specific time and temperature to apply later on the kit protocol.
- red blood cell lysis.
- washing.
- staining with an anti Rat IgG-FITC reagent of your choice.
- 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 multiplied by 6 will be the initial antibody concentration to be used in the kit protocol.

Example of a saturating curve:



<u>5.1.2 Choice of the negative isotypic control</u>

- 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.
- If you do not have access to a negative isotypic control, use diluted Reagent 1 instead.

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.

5.2.1 HUMAN whole blood and isolated cells sample

A/ Sample preparation

Prepare 2 x 15 mL tubes labeled T1 and T2.

In tube T1:
- pipette **100 µL** of whole blood or isolated cells.
- add **20 µL** of the Rat IgG Mab.

In tube T2:
- pipette **100 µL** of whole blood or isolated cells.
- add **20 µL** of the negative isotypic control Mab (or diluted Reagent 1).

- homogenize T1 and T2 tubes using a vortex for 1 to 2 seconds.
- incubate for the appropriate time and temperature determined at § 5.1.1.

B/ Sample lysis

For whole blood:
In T1 and T2 tubes:
- pipette **4 mL** of diluted Reagent 5.
- homogenize the tubes using a vortex for 1 to 2 seconds.
- incubate the tubes on ice for **5 minutes**.
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- incubate the tubes on ice for **5 minutes**.
- homogenize the tubes using a vortex for 1 to 2 seconds.
- centrifuge the tubes at **4°C** for **5 minutes** at **300 g** and discard the supernatant (turn the tube upside down and remove the remaining last drop on tissue paper).
- add **4 mL** of diluted Reagent 1.
- homogenize the tubes using a vortex for 1 to 2 seconds.
- centrifuge the tubes at **4°C** for **5 minutes** at **300 g** and discard the supernatant (turn the tube upside down and remove the remaining last drop on tissue paper).

For isolated cells:
In T1 and T2 tubes:
- pipette **4 mL** of diluted Reagent 1.
- homogenize the tubes using a vortex for 1 to 2 seconds.
- centrifuge the tubes at **4°C** for **5 minutes** at **300 g** and discard the supernatant (turn the tube upside down and remove the remaining last drop on tissue paper)
- add **4 mL** of diluted Reagent 1.
- homogenize the tubes using a vortex for 1 to 2 seconds.
- centrifuge the tubes at **4°C** for **5 minutes** at **300 g** and discard the supernatant (turn the tube upside down and remove the remaining last drop on tissue paper).

In case of non specific binding from the Rat IgG Mab, proceed twice to the following steps in tubes T1 and T2:
- add **4 mL** of diluted Reagent 1.
- homogenize the tubes using a vortex for 1 to 2 seconds.
- centrifuge the tubes at **4°C** for **5 minutes** at **300 g** and discard the supernatant (turn the tube upside down and remove the remaining last drop on tissue paper).

For whole blood or isolated cells:
In T1 and T2 tubes:
-resuspend the cell pellet with pipette by adding **100 µL** of diluted Reagent 1.
- homogenize the tubes using a vortex for 1 to 2 seconds.

C/ Fluorescent staining

Calibration tube:
In a 4 mL tube labeled T3:
- pipette **50 µL** of Reagent 2 **after resuspension using a vortex for 5 seconds**
- add **10 µL** of Reagent 3.
- homogenize the tube using a vortex for 1 to 2 seconds.

In the meantime for the sample:
In T1 and T2 tubes:
- add **20 µL** of Reagent 3.
- homogenize the tubes using a vortex for 1 to 2 seconds.

For T1, T2 and T3 tubes:
- incubate the tubes on ice for **10 minutes**.
- add **4 mL** of diluted Reagent 1.
- homogenize the tubes using a vortex for 1 to 2 seconds.
- centrifuge the tubes at **4°C** for **5 minutes** at **300 g** and discard the supernatant (turn the tube upside down and remove the remaining last drop on tissue paper).

Store tube T3 (Calibration) on ice until step E.

For a single color protocol, perform directly step E.

D/ Neutralization- counter-staining and washing (if necessary)

In T1 and T2 tubes:
- resuspend the cell pellet with pipette by adding **50 µL** of Reagent 4.
- homogenize the tube using a vortex for 1 to 2 seconds.
- 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.
- add **4 mL** of diluted Reagent 1.
- homogenize the tubes using a vortex for 1 to 2 seconds.
- centrifuge at **4°C** the tubes for **5 minutes** at **300g** and discard the supernatant (turn the tube upside down and remove the remaining last drop on tissue paper).

E/ Fixation and flow cytometric analysis

In T1, T2 and T3 tubes:
- homogenize the tubes using a vortex for 1 to 2 seconds.
- add **500 µL** of Reagent 6.
- homogenize the tubes using a vortex for 1 to 2 seconds.
- incubate either **1 hour at room temperature** or **overnight at 2-8°C.**
- perform flow cytometric analysis.

Depending upon the experimental conditions, the lysis step could also be performed either at the beginning or at the end of the protocol.
Please contact us if you require further information.

5.2.2 MOUSE whole blood sample

A/ Sample preparation

Prepare 2 x 4 mL tubes labeled T1 and T2.

In tube T1:
- pipette **25 µL** of whole blood.
- add **5 µL** of the Rat IgG Mab.

In tube T2:
- pipette **25 µL** of whole blood or isolated cells.
- add **5 µL** of the negative isotypic control Mab (or diluted Reagent 1).

- homogenize T1 and T2 tubes using a vortex for 1 to 2 seconds.
- incubate for the appropriate time and temperature determined at § 5.1.1.

B/ Sample lysis

In T1 and T2 tubes:
- pipette **1 mL** of diluted Reagent 5.
- homogenize the tubes using a vortex for 1 to 2 seconds.
- incubate the tubes on ice for **5 minutes**.
- homogenize the tubes using a vortex for 1 to 2 seconds.
- incubate the tubes on ice for **5 minutes**.
- homogenize the tubes using a vortex for 1 to 2 seconds.
- centrifuge the tubes at **4°C** for **5 minutes** at **300 g** and discard the supernatant (turn the tube upside down and remove the remaining last drop on tissue paper).
- add **1 mL** of diluted Reagent 1.
- homogenize the tubes using a vortex for 1 to 2 seconds.
- centrifuge the tubes at **4°C** for **5 minutes** at **300 g** and discard the supernatant (turn the tube upside down and remove the remaining last drop on tissue paper).
- homogenize the tubes using a vortex for 1 to 2 seconds.

C/ Fluorescent staining

Calibration tube:
In a 4 mL tube labeled T3:
- pipette **25 µL** of Reagent 2 **after resuspension using a vortex for 5 seconds**
- add **5 µL** of Reagent 3.
- homogenize the tube using a vortex for 1 to 2 seconds.

In the meantime for the sample:
In T1 and T2 tubes:
- add **5 µL** of Reagent 3.
- homogenize the tubes using a vortex for 1 to 2 seconds.

For T1, T2 and T3 tubes:
- incubate the tubes on ice for **10 minutes**.
- add **1 mL** of diluted Reagent 1.
- homogenize the tubes using a vortex for 1 to 2 seconds.
- centrifuge the tubes at **4°C** for **5 minutes** at **300 g** and discard the supernatant (turn the tube upside down and remove the remaining last drop on tissue paper).

Store tube T3 (Calibration) on ice until step E.

For a single color protocol, perform directly step E.

D/ Neutralization- counter-staining and washing (if necessary)
In T1 and T2 tubes:
- resuspend the cell pellet with pipette by adding **25 µL** of Reagent 4.
- homogenize the tube using a vortex for 1 to 2 seconds.
- 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.
- add **1 mL** of diluted Reagent 1.
- homogenize the tubes using a vortex for 1 to 2 seconds.
- centrifuge at **4°C** the tubes for **5 minutes** at **300g** and discard the supernatant (turn the tube upside down and remove the remaining last drop on tissue paper).

E/ Fixation and flow cytometric analysis
In T1, T2 and T3 tubes:
- homogenize the tubes using a vortex for 1 to 2 seconds.
- add **500 µL** of Reagent 6.
- homogenize the tubes using a vortex for 1 to 2 seconds.
- incubate either **1 hour at room temperature** or **overnight at 2-8°C.**
- perform flow cytometric analysis.

Depending upon the experimental conditions, the lysis step could also be performed either at the beginning or at the end of the protocol.
Please contact us if you require further information.

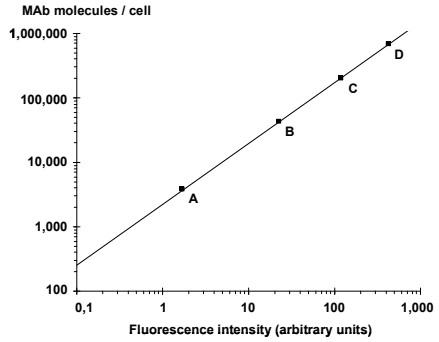
6 CYTOMETRIC ANALYSIS
Refer to the Operator's Manual of the cytometer for instructions on how to perform cytometric readings.
The selected Mean Fluorescence Intensity (MFI) statistics is the geometric mean (Mn (x) or GeoMean depending upon the cytometer).

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:



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