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Technical Manual

FcγRIIIa ADCC Reporter Bioassay

RD00887 GS-J2C/CD16A-158F

For research use only

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1 Description

Fc receptor-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) is an important mechanism of cell-mediated immune defense whereby an effector cell of the immune system actively lyses a target cell, whose membrane-surface antigens have been bound by specific antibodies. The ADCC is mediated primarily through binding of the FcγRIIIa (CD16a) receptor on natural killer (NK) cells, in which CD16a occurs in two variants, 158V or 158F, that elicit high or low binding affinity, respectively, to the Fc domain of IgG1 antibodies.

To evaluate FcγRIIIa-mediated ADCC, primary cell-based assay is a classic method to measure the potency of ADCC using peripheral blood mononuclear cells (PBMCs) or purified natural killer (NK) cells. These endpoint assays are a much more laborious process and rely on donor PBMCs or the natural killer (NK) cell subpopulation as effector cells, which result in high assay failure rates, especially in lot release testing due to inherent donor variability.

To eliminate these problems in ADCC Assays, ~~Genscript~~GenScript has developed an FcγRIIIa ADCC Reporter Bioassay that enables rapid and reproducible measurement of ADCC for drug candidate screening and validation. The cell-based assay utilizes an engineered cell line rather than primary cells as the effector cells that stably expressing FcγRIIIa and a luciferase reporter driven by an NFAT response element (NFAT-RE). When co-cultured with a target disease cell and relevant antibody, the FcγRIIIa Effector cells bind the Fc domain of the antibody, resulting in FcγRIIIa signaling and NFAT-RE-mediated luciferase activity. The luminescence signal is detected and quantified using Bio-Glo™ Luciferase Assay System.

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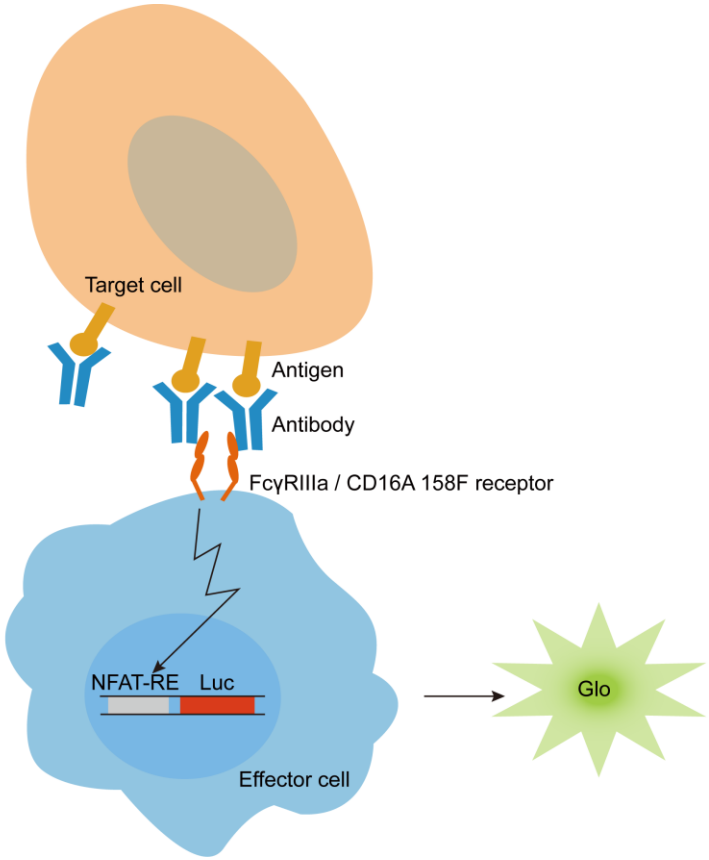

Figure 1. Principle of FcγRIIIa-158F Reporter Bioassay. ADCC reporter cells (Effector cells) express human FcγRIIIa and a luciferase reporter driven by an NFAT response element (NFAT-RE). Target cells are either with endogenously expressed antigens or exogenously expressed antigens. When co-culturing the Effector cells with Target cells, antigen-specific antibody in the culture simultaneously binds the antigen on the target cells via Fab region and FcγRIIIa receptors on the effector cells via Fc region. This ligation results in receptor clustering, intracellular signaling and NFAT-RE-mediated luciferase activity.

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2 Product Components and Storage Instructions

Component	Size	Amount
GS-J2C/CD16A-158F	3 × 10^6 cells/vial	2 vials

- ❖ Check all containers for leakage or breakage.
- ❖ Remove the frozen cells from the dry ice packaging and immediately place the cells at a temperature below -140°C, preferably in liquid nitrogen vapor, until ready for use.

3 Materials to Begin

3.1 Suggested Reagents to Be Prepared by the Users

Materials	Company	Cat. No.
RPMI 1640	Gibco	22400089
FBS	Gibco	10099141
Hygromycin B	Gibco	10687010
Puromycin	Gibco	A11138-03
DPBS	Gibco	14190144
DMSO	Sigma	D2650
Low IgG FBS	Wisent	086-750
MycoAlert™ PLUS Mycoplasma Detection Kit	Lonza	LT07-710

3.2 Suggested Supplies and Equipment to Be Prepared by the Users

Materials	Company	Cat. No.
96-well assay plate	Corning	3788
96-well assay plate	Corning	3917
Luminometer	BMG	PHERAstar FSX

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3.3 Composition of Required Media

Medium/Buffer Type	Medium/Buffer Recipe
Complete Growth Medium	RPMI 1640, 10% FBS
Culture Medium	RPMI 1640, 10% FBS, 1 μg/mL Puromycin, 400 μg/mL Hygromycin B
Freezing Medium	90% Complete Growth Medium, 10% DMSO
Assay Buffer	RPMI 1640, 4% Low IgG FBS

4 Instruction for Preparing Stable Cell Lines

4.1 Preparing ADCC-158F Effector Cells

4.1.1 Cell Recovery

The cells were maintained in complete growth medium (**see medium recipe in Section 3.3**).

1. Prewarm a water bath to 37°C and bring the complete growth medium to 37°C in the water bath.
2. Remove the cryovial from the liquid nitrogen tank and thaw it by gentle agitation in the 37°C water bath until ice crystals are melted (no inversion), usually within 2-3 minutes.
3. Remove the vial from the water bath and sterilize it with 75% ethanol.
4. Unscrew the vial and transfer the cells to a 15 mL sterile conical centrifuge tube containing 9.0 mL prewarmed complete growth medium.

   ★ ***CRITICAL*** *20% FBS is recommended for quick cell recovery.*
5. After centrifugation at 100 × g for 4 minutes, discard the supernatant and resuspend the cell pellet in 2.0 mL prewarmed complete growth medium. Pipette gently to loosen the pellet and break apart clumps.
6. Transfer the cell suspension to the T25 flask with prewarmed complete growth medium and mix thoroughly. Incubate cells at 37°C, 5% CO_2.
7. Incubate for approximately 48 hours before subculturing.

★ ***Important Reminder*** *Upon cell arrival, if immediate thawing and recovery are not intended, promptly store the cells in a liquid nitrogen tank (-196°C) for long-term preservation. This will ensure maximum viability and performance when the cells are eventually thawed for use.*

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4.1.2 Cell Maintenance and Subculturing

The cells were maintained in culture medium (**see medium recipe in Section 3.3**).

1. Bring the fresh culture medium to 37°C in a water bath.
2. Count the GS-J2C/CD16A effector cells by a cell counter, then seed cells in a T25 flask at a density of 4 × 10^5 cells/mL if passaging every two days or 2.5 × 10^5 cells/mL if passaging every three days.
3. Add fresh culture medium to the cell suspension in the flask to maintain the cell culture.

4.1.3 Cell Cryopreservation

1. Prepare a freezing medium (**see medium recipe in Section 3.3**) and keep it on ice.
2. Gently aspirate the cells and measure cell viability and density by a cell counter.
3. Harvest cells by centrifugation at 100 × g for 4 minutes and resuspend cells with the freezing medium at a density of 3 × 10^6 cells/mL.
4. Label the cryovials with the name of the cell line, then add 1.0 mL of the cell suspension to each of the vials and seal the cap.
5. Place the vials into a pre-cooled (4°C) Mr. Frosty and place the chamber in a -80°C freezer for at least 24 hours.
6. Quickly transfer the vials to a liquid nitrogen tank.
7. After 24 hours in liquid nitrogen, take one vial and recover the cells to determine the cell viability.

5 Assay Protocol

The protocol presents a bioassay workflow for FcγRIIIa ADCC reporter cell line. The experiment is conducted in a 96-well plate.

Assay Procedure:

1. Prepare antibody serial dilutions: choose an appropriate dilution scheme to achieve a full dose-response curve with proper upper and lower asymptotes and sufficient points on the slope.
2. Plate target cells.
3. Add antibodies and FcγRIIIa Effector cells.

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4. Incubate at 37°C for 6 hours

5. Add Detection reagent and incubate at room temperature for 5 minutes, then read the plate in a microplate reader.

5.1 Plate Layout Design (For 96-well plate)

Following the described serial dilutions of antibody to yield 8-point dose-response curves.

Plate 01	1	2	3	4	5	6	7	8	9	10	11	12
A	Raji + GS-J2C/CD16A + Rituxan (Conc.1)	Raji + GS-J2C/CD16A + Rituxan (Conc.1)-2	Raji + GS-J2C/CD16A + Rituxan (Conc.1)-3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
B	Raji + GS-J2C/CD16A + Rituxan (Conc.2)-1	Raji + GS-J2C/CD16A + Rituxan (Conc.2)-2	Raji + GS-J2C/CD16A + Rituxan (Conc.2)-3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
C	Raji + GS-J2C/CD16A + Rituxan (Conc.3)-1	Raji + GS-J2C/CD16A + Rituxan (Conc.3)-2	Raji + GS-J2C/CD16A + Rituxan (Conc.3)-3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
D	Raji + GS-J2C/CD16A + Rituxan (Conc.4)-1	Raji + GS-J2C/CD16A + Rituxan (Conc.4)-2	Raji + GS-J2C/CD16A + Rituxan (Conc.4)-3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
E	Raji + GS-J2C/CD16A + Rituxan (Conc.5)-1	Raji + GS-J2C/CD16A + Rituxan (Conc.5)-2	Raji + GS-J2C/CD16A + Rituxan (Conc.5)-3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
F	Raji + GS-J2C/CD16A + Rituxan (Conc.6)-1	Raji + GS-J2C/CD16A + Rituxan (Conc.6)-2	Raji + GS-J2C/CD16A + Rituxan (Conc.6)-3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
G	Raji + GS-J2C/CD16A + Rituxan (Conc.7)-1	Raji + GS-J2C/CD16A + Rituxan (Conc.7)-2	Raji + GS-J2C/CD16A + Rituxan (Conc.7)-3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
H	Raji + GS-J2C/CD16A + Rituxan (Conc.8)-1	Raji + GS-J2C/CD16A + Rituxan (Conc.8)-2	Raji + GS-J2C/CD16A + Rituxan (Conc.8)-3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Figure 2. Plate Layout represents 8 serial dilutions of antibody (n = 3).

5.2 Preparing Antibody Solution

Preparation scheme of Antibody (2X)

Antibody	Serial Dilution	Stock Conc. (μg/mL)	Final Conc. (μg/mL)	Note
Conc. 1	Mix the appropriate volume of assay medium with the stock solution	200	100	Dilution Factor = 10
Conc. 2	Mix 20 μL -Conc.1 + 180 μL buffer	20	10	
Conc. 3	Mix 20 μL -Conc.2 + 180 μL buffer	2.	1	
Conc. 4	Mix 20 μL -Conc.3 + 180 μL buffer	0.2	0.1	
Conc. 5	Mix 20 μL -Conc.4 + 180 μL buffer	0.02	0.01	
Conc. 6	Mix 20 μL -Conc.5 + 180 μL buffer	0.002	0.001	
Conc. 7	Mix 20 μL -Conc.6 + 180 μL buffer	0.0002	0.0001	
Conc. 8	Mix 20 μL -Conc.7 + 180 μL buffer	0.00002	0.00001	

5.3 Plating Target Cells

1. Prepare assay buffer. Add 2 mL of Low IgG FBS to 48 mL of RPMI 1640 medium to make assay buffer. Mix well and prewarm in a 37°C water bath before use.

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2. Count the Raji cells by a cell counter. Transfer an appropriate amount of Raji cells from T-75 flask to a 15 mL conical tube or larger sized centrifuge tube.

3. Count the cells suspension and resuspend target cells in complete culture medium to achieve the desired cell seeding density at 4 × 10^5 cells/mL.

4. Using a multichannel pipette, immediately dispense 25 µL cell suspension to each of the dedicated wells of a 96-well flat-bottom assay plate. The final target cell number in each well should be 1 × 10^4 cells/well.

5.4 Adding Antibody and ADCC Effector Cells to Assay Plates

1. Using a multichannel pipette, add 50 µL of the appropriate antibody dilution to the pre-plated cells according to the plate layout in Figure 2.

2. Count the ADCC cells by a cell counter. Transfer an appropriate amount of ADCC cells from the T-75 flask to a 15.0 mL conical tube or larger sized centrifuge tube.

3. Pellet the cells at 100 × g for 4 minutes at ambient temperature. Resuspend effector cells in medium to achieve the desired cell seeding density of 2 × 10^6 cells/mL.

4. Using a multichannel pipette, immediately dispense 25 µL cell suspension to each of the dedicated wells of a 96-well flat-bottom assay plate. The final effector cell number in each well should be 5 × 10^4 cells/well.

5. Cover the assay plates and incubate at 37°C, 5% CO_2 for 6 hours

5.5 Adding Detection Reagent and Data Analysis

1. Add 100 µL of Detection Reagent to wells.

2. Incubate at ambient temperature for 5 - 10 minutes.

3. Measure luminescence using a microplate reader.

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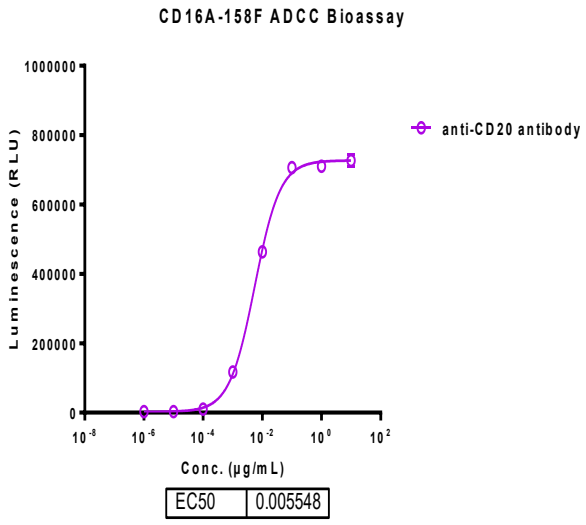
6 Assay Results

6.1 Evaluation by Functional Assay

The following dose-response curve was generated by FcγRIIIa ADCC Reporter Bioassay with anti-CD20 antibody.



Best-fit values	
Sample ID	anti-CD20 antibody
Bottom	2227
Top	726979
$LogEC_{50}$	-2.2560
HillSlope	1.0140
EC_{50} (μg/mL)	0.0055
Span	724753
Windows	326.4387

Figure 3. The anti-CD20 antibody shows a dose-dependent ADCC Bisoassay. Anti-CD20 antibody was added in the culture of Raji (CD20+) target cells, followed by addition of FcγRIIIa effector cells. After 6-hour incubation, luciferase substrate reagent was added and luminescence signal measured by PHERAStar FSX. Data were analyzed by GraphPad Prism® software.

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7 Reference

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