TECHNICAL MANUAL

mFcγRIV ADCC Bioassay Effector Cells, Propagation Model

Instructions for Use of Product
M1212

1/17
TM487

mFcγRIV ADCC Bioassay Effector Cells, Propagation Model

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

Promega

1. Description

Antibody-dependent cell-mediated cytotoxicity (ADCC) is an important mechanism of action (MOA) of antibodies that target virus-infected or diseased (e.g., tumor) cells for destruction by components of the cell-mediated immune system. Mouse FcγRIV (mFcγRIV) is the predominant receptor involved in ADCC in the mouse and is more closely related to human FcγRIIIa, the primary Fc receptor involved in ADCC in humans, than mFcγRIII. Mouse IgG2a, and to a lesser extent IgG2b, are known to mediate ADCC through activation of mFcγRIV. In contrast, mouse IgG1 does not bind to mFcγRIV. In vivo, mouse IgG1-mediated ADCC activity is inhibited via binding to the inhibitory receptor mFcγRIIb at a higher affinity than mFcγRIII (1,2).

Current methods used to measure ADCC rely on the isolation of primary peripheral blood mononuclear cells (PBMCs). These assays are laborious and highly variable due to their reliance on donor primary cells, complex assay protocols and unqualified assay reagents. As a result, these assays are difficult to establish in antibody drug screening and vaccine development programs.

The mFcγRIV ADCC Reporter Bioassay Effector cells, Propagation Model (Cat.# M1212), is a bioluminescent cell-based assay that overcomes the limitations of existing assays and can be used to measure the potency and stability of antibodies and other biologics with Fc domains that specifically bind and activate mFcγRIV (3,4). The assay consists of a genetically engineered Jurkat T cell line that expresses:

- Mouse FcγRIV receptor
- A luciferase reporter driven by an NFAT-response element (NFAT-RE)

mFcγRIV Effector Cells are provided in Cell Propagation Model (CPM) format, which includes cryopreserved cells that can be thawed, propagated and banked for long-term use.

When co-cultured with a target disease cell and relevant antibody, the mFcγRIV Effector Cells bind the antibody Fc domain, resulting in mFcγRIV signaling and NFAT-RE-mediated luciferase activity (Figure 1). The bioluminescent signal is detected and quantified using the Bio-Glo™ Luciferase Assay System (Cat.#G7940, G7941) and a standard luminometer such as the GloMax® Discover System (see Related Products, Section 7).

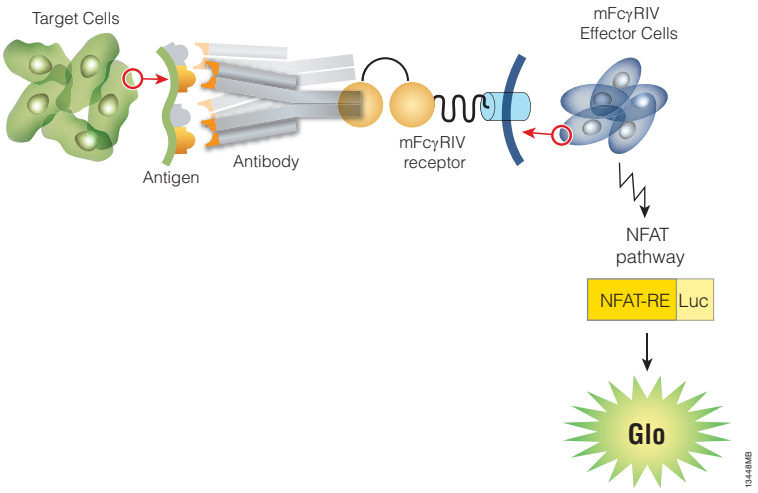

Figure 1. Representation of the mFcγRIV ADCC Reporter Bioassay. The bioassay consists of a genetically engineered cell line (mFcγRIV Effector Cells), an antigen expressing target cell and an antigen-specific antibody. When all components are co-cultured, the antibody simultaneously binds the target cell antigen and FcγRIV receptors on the surface of the effector cells. This results in receptor clustering, intracellular signaling and NFAT-RE-mediated luciferase activity.

The mFcγRIV ADCC Reporter Bioassay reflects the MOA of antibodies that bind and activate mFcγRIV. The bioassay shows high specificity as demonstrated using a mouse anti-HER2 IgG2b antibody and SKBR3 (HER2$^+$) cells, that together activate mFcγRIV Effector Cells but not FcγRIIIa Effector Cells (included in the human ADCC Reporter Bioassay, Cat. #G7015, G7014, G7010, G7018; Figure 2). Importantly, mFcγRIV-mediated luciferase activity is observed only in the presence of: 1) mFcγRIV Effector Cells; 2) an antibody with Fc binding affinity for mFcγRIV; and 3) target cells expressing the relevant antigen (Figure 3). Using a panel of anti-CD20 antibodies and Raji (CD20$^+$) target cells, the mFcγRIV ADCC Reporter Bioassay shows the expected rank ordering of human and mouse antibody subtypes (5; Figure 4).

1. Description (continued)

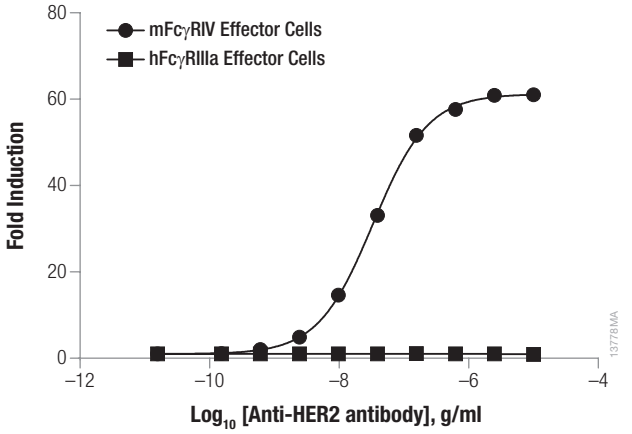

Figure 2. The mFcγRIV ADCC Reporter Bioassay reflects the MOA and specificity of antibodies that bind and activate mFcγRIV. Increasing concentrations of a mIgG2b anti-HER2 antibody were incubated with SKBR3 (HER2$^+$) target cells and either mFcγRIV Effector Cells or hFcγRIIIa Effector Cells (included in the human ADCC Reporter Bioassay, Cat. #G7015, G7014, G7010, G7018), as indicated. Bio-Glo™ Reagent was added, and luminescence was measured. Data were fitted to a 4PL curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

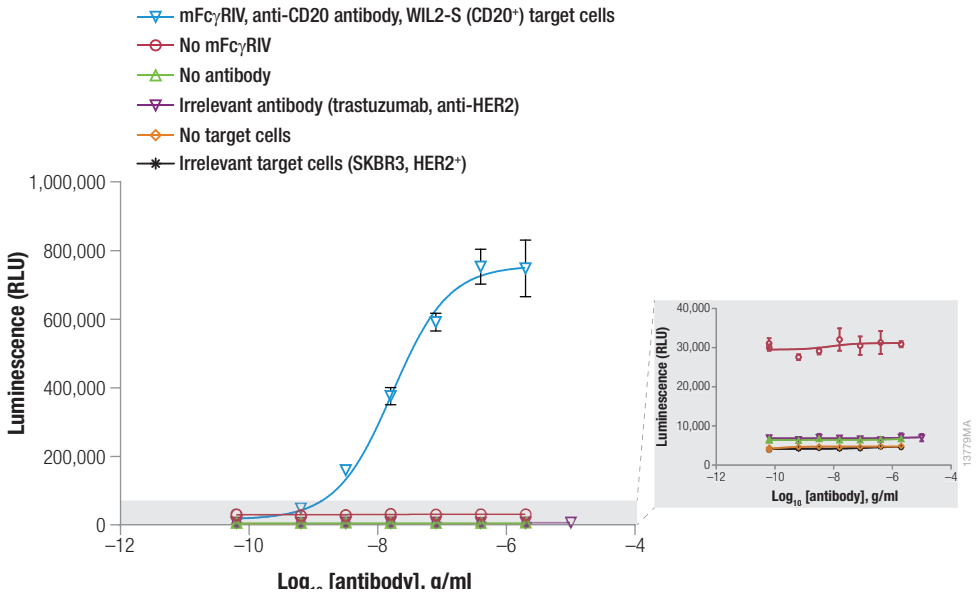

Figure 3. The mFcγRIV ADCC Reporter Bioassay is specific and requires mFcγRIV Effector Cells, an antibody with Fc binding affinity for mFcγRIV, and target cells expressing the relevant antigen. Increasing concentrations of an anti-CD20 antibody (IgG2a) were incubated with WIL2-S (CD20+) target cells and mFcγRIV Effector Cells. Bio-Glo™ Reagent was added, and luminescence was measured. Data were fitted to a 4PL curve using GraphPad Prism® software. No induction of luciferase activity was detected using effector cells that do not express mFcγRI, in the absence of anti-CD20 antibody, or in the presence of an irrelevant antibody (trastuzumab, anti-HER2). No induction of luciferase activity was detected in the absence of target cells or in the presence of an irrelevant target cell (SKBR3, HER2+).

1. Description (continued)

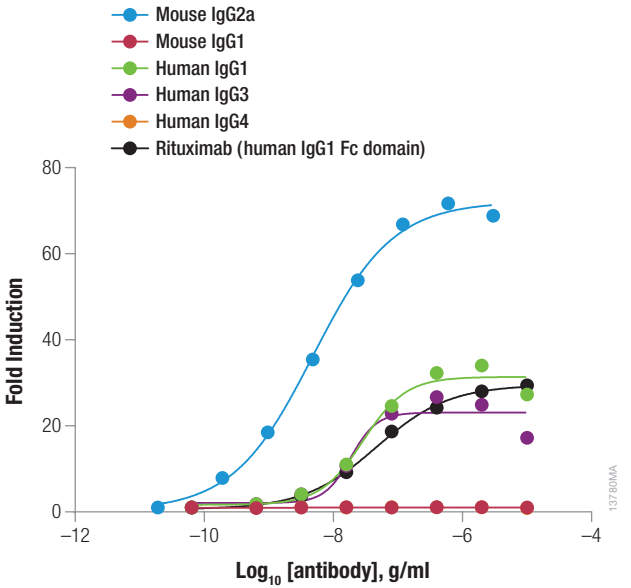

Figure 4. The mFcγRIV ADCC Reporter Bioassay shows the expected rank ordering of human and mouse antibody subtypes. Increasing concentrations of human and mouse anti-CD20 antibodies representing different IgG subtypes were incubated with Raji (CD20$^+$) target cells and mFcγRIV Effector Cells, as indicated. Bio-Glo™ Reagent was added, and luminescence was measured. Data were fitted to a 4PL curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

Table 1. EC_{50} and Maximum Fold Induction for Human and Mouse Antibody Subtypes Analyzed in the mFcγRIV ADCC Reporter Bioassay.

	EC_{50} (ng/ml)	Maximum Fold Induction
mouse IgG2a	4.8	71.7
human IgG3	18.4	26.7
human IgG1	41.0	34.0
Rituximab (human IgG1 Fc domain)	46.7	29.4
human IgG4	NA	1.1
mouse IgG1	NA	1.0

NA=Not applicable

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT. #
mFcγRIV ADCC Bioassay Effector Cells, Propagation Model	**1 each**	**M1212**

Not for Medical Diagnostic Use. Includes:

- 2 vials mFcγRIV Effector Cells (1.0ml)

Note: Thaw and propagate one vial to create cell banks before use in an assay. The second vial should be reserved for future use.

Storage Conditions: Upon arrival, immediately transfer the cell vials to below –140°C (freezer or liquid nitrogen vapor phase) for long-term storage. **Do not** store cell vials submerged in liquid nitrogen. **Do not** store cell vials at –80°C because this will negatively affect cell viability and cell performance.

3. Before You Begin

The mFcγRIV ADCC Reporter Bioassay differs from classic ADCC assays in a number of ways. Assay parameters including effector:target (E:T) cell ratio, cell number per well, antibody dose range, buffer composition and incubation time may differ from those used in classic ADCC assays using primary cells or other cell lines.

Please read through the entire protocol to become familiar with the components and the assay procedure before beginning. Cell thawing, propagation and banking should be performed ***exactly*** as described in Section 3.B. Cell seeding and propagation densities have been optimized to ensure stable cell growth, which is reflected in a steady cell doubling rate to achieve optimal and consistent performance. The recommended cell plating densities, induction time and assay buffer components described in Section 4 were established using a select few research antibodies that bind and activate mFcγRIV. You may need to adjust these parameters and optimize assay conditions for your own antibodies or other biologic samples.

The mFcγRIV ADCC Reporter Bioassay produces a bioluminescent signal and requires a sensitive luminometer or luminescence plate reader for the detection of luciferase activity. Bioassay development and the performance data included in this Technical Manual were generated using the GloMax®-Multi Detection System. An integration time of 0.5 second/well was used for all readings. The bioassay is compatible with most other plate luminometers; however, relative luminescence unit readings may vary due to the sensitivity and settings of each instrument.

3. Before You Begin (continued)

3.A. Materials to Be Supplied by the User

(Compositions of buffers and media are provided in Section 7.A.)

Reagents

- user-defined target cells expressing target antigen recognized by the mAb or derivative
- user-defined reference and test antibodies or derivatives with Fc effector function
- RPMI 1640 Medium (Gibco Cat.#22400)
- MEM nonessential amino acids (Gibco Cat.#11140-050)
- sodium pyruvate (Gibco Cat.# 11360-070)
- fetal bovine serum (e.g., HyClone Cat.# SH30070)
- Super low-IgG FBS (e.g., HyClone Cat.# SH30898)
- DPBS (e.g., Gibco Cat.# 14190)
- Hyrgomycin B (e.g, Gibco Cat.#10687-010)
- G418 sulfate solution (e.g., Gibco Cat.# 10131035)
- DMSO (e.g., Sigma Cat.# D2650)
- Trypan Blue solution (e.g., Sigma Cat.# T8154)
- Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941)

Supplies and Equipment

- sterile bottles for preparation of media and buffer
- sterile, clear 96-well plate with lid (e.g., Costar #3370 or Linbro Cat. #76-223-05)
- white, flat-bottom 96-well assay plates (e.g., Corning Cat.# 3917)
- sterile 15ml and 50ml conical tubes
- sterile reagent reservoirs (e.g., Corning Cat.# 4870)
- 37°C, 5% CO_2 incubator
- 37°C water bath
- plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System)

3.B. Preparing mFcγRIV Effector Cells

Cell Thawing and Initial Cell Culture (Day 1)

1. Prepare 25ml of cell thawing medium.
2. Remove one vial of mFcγRIV Effector Cells from storage at –140°C and thaw in a 37°C water bath with gentle agitation (no inversion) until just thawed.
3. Transfer all of the cells (approximately 1ml) into the 50ml conical tube containing 9ml of prewarmed cell thawing medium.
4. Centrifuge at 130 x *g* for 10 minutes.
5. Carefully aspirate the medium, and resuspend the cell pellet in 12ml of prewarmed cell thawing medium.
6. Transfer the cell suspension to a T75cm tissue culture flask, and place the flask horizontally in a 37°C, 5% CO_2 incubator.
7. Incubate for approximately 24 hours.

Cell Maintenance and Propagation (Day 2)

8. Remove a sample and measure the cell viability and density by Trypan Blue staining.
9. Centrifuge at 130 x *g* for 10 minutes.
10. Resuspend cells to a concentration of is $1x10^6$ cells/ml in growth medium containing hygromycin B and antibiotic G418 sulfate solution.
11. Place the flask horizontally in a 37°C, 5% CO_2 incubator.
12. Incubate the cells for approximately 24 hours.

3.B. Preparing mFcγRIV Effector Cells (continued)

Cell Maintenance and Propagation (Day 3 and Beyond)

Note: For cell maintenance and propagation starting from the second cell passage, use the cell growth medium containing antibiotics and monitor cell viability and doubling rate during propagation. The cell growth rate will stabilize by 7–14 days post-thaw, at which time cell viability is typically >80% and the average cell doubling rate is 35–55 hours. Passage number should be recorded for each passage. Cells should be banked appropriately soon after growth rate stabilization. The mFcγRIV Effector Cells normally grow as small-to-medium clusters. After gentle disruption for counting, passage and harvest purposes, the cells will begin to reassociate rapidly (3–4 hours). It is not uncommon to observe large macrosopic clumps of cellular debris during the first 24–48 hours of culture.

13. To passage the cells, gently mix the cells with a pipette to create a homogeneous cell suspension.
14. Measure the cell viability and density by Trypan Blue staining.
15. Seed the cells at a density of 7.5×10^5 viable cells/ml if passaging every two days (e.g., Mon.–Wed., Wed.–Fri.) or 5.0×10^5 viable cells/ml if passaging every three days (e.g., Fri.–Mon.) by adding fresh cell growth medium to the cell suspension in the original flask or by transferring the cells to a new flask. Always maintain the flasks in a horizontal position in the incubator.
16. Maintain the cell culture by adding fresh cell growth medium to the cell suspension in the original flask or by transferring cells to a new flask while maintaining a consistent ratio of culture volume to flask surface area (e.g., 25–30ml volume per T75cm flask or 50–60ml volume per T150cm flask).
17. Place the flasks horizontally in a 37°C, 5% CO_2 incubator.

Cell Freezing and Banking

18. On the day of cell freezing, prepare new cell freezing medium and keep it on ice.
19. Gently mix the cells with a pipette to create a homogeneous cell suspension.
20. Remove a sample for cell counting by Trypan Blue staining. Calculate the volume of cell freezing medium needed based on desired cell freezing density between $4 \times 10^6 - 2 \times 10^7$ cells/ml.
21. Transfer the cell suspension to 50ml sterile conical tubes or larger size centrifuge tubes and centrifuge at 130 x *g* for 10–15 minutes.
22. Gently aspirate the medium, taking care not to disturb the cell pellet.
23. Carefully resuspend the cell pellet in ice-cold cell freezing medium to a final cell density between $4 \times 10^6 - 2 \times 10^7$ cells/ml. Combine the cell suspension into a single tube and dispense into cryovials.
24. Freeze the cells using a controlled-rate freezer (preferred), Mr. Frosty® or a Styrofoam® rack in a –80°C freezer. Transfer the vials to at or below –140°C for long-term storage.

4. Assay Protocol

This assay protocol illustrates the use of the mFcγRIV ADCC Bioassay Effector cells, Propagation Model to test two antibody samples against a reference sample in a single assay run. Each test and reference antibody is run in triplicate, in a 10-point dilution series, in a single 96-well assay plate using the inner 60 wells. Other experimental and plate layouts are possible but may require further optimization.

Note: Prior to routine use of the mFcγRIV ADCC Reporter Bioassay with your own antibody and target cell lines, we recommend optimizing the Effector:Target cell (E:T) ratio and cell densities. Fix the number of mFcγRIV Effector Cells (75,000 cells/well for a 96-well plate), and vary the number of target cells (5,000–25,000 cells/well for a 96-well plate). This will help establish an E:T ratio and cell density that give a strong signal response and fold induction. As a preliminary experiment, this can be simplified by using a single concentration of antibody (e.g., 2–5µg/ml). Additional optimization of the antibody dose-range and dilution series may be needed to achieve a full dose-response curve with proper upper and lower asymptotes and sufficient points throughout the dose range. Induction times of 16–24 hours are a good starting point for the assay. You can vary the induction time further to determine an optimal or convenient time. We recommend that you evaluate these parameters rigorously and select the best conditions for your target system.

4.A. Preparing Bio-Glo™ Reagent, Assay Buffer, and Test and Control Ab Samples

1. **Bio-Glo™ Reagent:** For your reference, 10ml of Bio-Glo™ Reagent is sufficient for 120 wells in a 96-well assay format. Thaw the Bio-Glo™ Luciferase Assay Buffer in a refrigerator overnight or in a room-temperature water bath on the day of assay. Equilibrate the Bio-Glo™ Luciferase Assay Buffer to ambient temperature, protected from light. Transfer the Bio-Glo™ Luciferase Assay Buffer into the amber bottle containing the Bio-Glo™ Luciferase Assay Substrate and mix by inversion until the Substrate is thoroughly dissolved. Equilibrate the reconstituted Bio-Glo™ Reagent to ambient temperature before adding to assay plates. Approximate stability of Bio-Glo™ Reagent after reconstitution is 18% loss of luminescence over 24 hours at ambient temperature.

2. **Assay Buffer:** Prepare an appropriate amount of assay buffer on the day of assay. Thaw the low-IgG serum in a 37°C water bath, taking care not to overheat it. Add an appropriate amount of FBS to RPMI 1640 medium to yield 96% RPMI 1640/4% low-IgG serum. Mix well and warm to 37°C before use. For reference, 50ml of assay buffer is typically sufficient for 120 wells in a 96-well assay format using the inner 60 wells.

   Note: The recommended assay buffer contains 4% low-IgG serum This concentration of FBS works well for most antibodies and target cells that we have tested. If you experience target cell viability or assay performance issues using this assay buffer, we recommend testing different serum concentrations in the range of 0.5–10%.

3. **Test and Reference Samples:** Using assay buffer as the diluent, prepare starting dilutions (dilu1, 3X final concentration; see Figure 5) of two test antibodies (minimum 200µl each) and one reference antibody (minimum of 400µl) in 1.5ml tubes. Store the tubes containing antibody starting dilutions appropriately before making antibody serial dilutions.

   Note: Select starting antibody concentrations (1X final concentration) based on previous experimental results, if available.

4.B. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 5 as a guide. The protocol describes serial replicate dilutions (n = 3) of test and reference antibodies to generate two 10-point dose-response curves in each plate.

Recommended Plate Layout Design													
	1	2	3	4	5	6	7	8	9	10	11	12	
A	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)
B	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ab
C	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
D	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ab
E	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
F	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ab
G	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
H	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)

Figure 5. Example plate layout showing non-clustered sample locations of the reference antibody dilution series and a single test antibody dilution series.

4.C. Preparing and Plating Target Cells

Note: Target cells for use in the mFcγRIV ADCC Reporter Bioassay should be maintained in culture according to established protocols for each individual cell type. Cell viability, antigen expression and assay reproducibility require that the target cells are cultured within an optimal cell density range and window of passage stability. Both adherent and non-adherent target cells have been used successfully with the mFcγRIV ADCC Reporter Bioassay (Figures 2 and 3).

Preparing Non-Adherent Target Cells

1. Estimate the quantity of target cells needed.
2. Sample and count the target cells by Trypan Blue staining, and harvest ~2–3 times the required number of cells by centrifuging in a 50ml tube for 10 minutes at 130–170 × *g*.
3. Gently resuspend the cell pellet in warm assay buffer at approximately 2X the original cell density. Count cells by Trypan Blue staining, and adjust the cell density by adding warm assay buffer to achieve a final cell density of 0.2–1 × 10^6 viable cells/ml (5,000–25,000 cells per 25µl).
4. Transfer the cell suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 25µl of the cell suspension to each of the inner 60 wells of a white 96-well, flat-bottom assay plate.
5. Add 75µl of assay buffer to each of the outside wells of the assay plates. Cover the plates with their lids.
6. Equilibrate target cells about 15 minutes in a 37°C, 5% CO_2 humidified incubator while preparing antibody dilution series.

Preparing and Plating Adherent Target Cells

1. Estimate the quantity of target cell numbers needed.
2. Eighteen to twenty-four hours before performing the assay, harvest adherent target cells from the propagation flasks by trypsinization (or other appropriate procedure), and centrifuge the cells at 130–200 × *g* for 10 minutes.
3. Resuspend the cells in fresh culture medium, count by Trypan Blue staining, and adjust the cell density so that the desired quantity of cells will be present in 100µl (approximately 5,000–20,000 cells).
4. Transfer the cell suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 100µl of the cell suspension to each of the inner 60 wells of a white 96-well, flat-bottom assay plate.

   Note: White, clear-bottom tissue-culture plates can be used if observation of adherent target cells is desired the following day, but luminescence will be lower.
5. Dispense 100µl of culture medium into the outermost wells, labeled "B" in Figure 5.
6. Allow the target cells to attach by incubating overnight in a 37°C, 5% CO_2 incubator.

Immediately before beginning the assay, hold the assay plate at a 45° angle and use a multichannel pipette to carefully remove approximately 95µl of culture medium from each well. Immediately add 25µl assay buffer (prewarmed to 37°C) to the inner 60 wells of both assay plates. Make additions so that pipette tips touch the wall of the well and leave the cells undisturbed. Dispense 75µl of assay buffer into the outermost wells, labeled "B" in Figure 5, of both assay plates. Cover the plates with lids, and place them in a 37°C, 5% CO_2 incubator while preparing the antibody dilution series.

4.D. Preparing Antibody Serial Dilutions

The instructions described here are for preparation of a single stock of 5-fold serial dilutions of a single antibody for analysis in triplicate (100µl of each dilution provides a sufficient volume for analysis in triplicate for two plates). Alternatively, you can prepare three independent stocks of serial dilutions to generate triplicate samples. To prepare 5-fold serial dilutions, you will need 125µl of reference antibody at 3X the highest concentration for each dose-response curve (already prepared in Section 4.A). You will need at least 125µl of each test antibody at 3X the highest concentration in each of the test-antibody dose-response curves. For other dilution schemes, adjust the volumes accordingly.

1. Using a sterile, clear 96-well plate, add 125µl of reference antibody starting dilution (dilu1, 3X final concentration) to wells A11 and B11.
2. Add 125µl of test antibodies 1 and 2 starting dilution (dilu1, 3X final concentration) to wells C11 and D11, respectively (see Figure 6).
3. Add 100µl of assay buffer to other wells in these four rows, from column 10 to column 2.
4. Transfer 25µl of the antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
5. Repeat equivalent 5-fold serial dilutions across the columns from right to left, through column 3. Do not dilute into column 2.

Note: Wells A2, B2, C2 and D2 contain 120µl of assay buffer without antibody as a negative control.

Recommended Plate Layout for Antibody Dilutions Prepared from a Single Antibody Stock

	1	2	3	4	5	6	7	8	9	10	11	12	
A		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference Ab
B		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference Ab
C		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test Ab 1
D		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test Ab 2
E													
F													
G													
H													

Figure 6. Example plate layout showing antibody serial dilutions.

4.E. Adding Antibodies to Plated Target Cells

1. Using a multichannel pipette, add 25µl of the appropriate antibody dilution to the pre-plated target cells according to the plate layout in Figure 5.
2. Cover the assay plates with lids and keep the plates at ambient temperature before adding mFcγRIV Effector Cells.

4.F. Preparing and Plating mFcγRIV Effector Cells

While maintaining the mFcγIV Effector Cells, follow the recommended cell seeding density as changes in cell culture volume or seeding density may affect the cell growth rate and assay performance. Only use the cells after the cell doubling rate has stabilized during propagation. Do not use cells that have poor viability or that have exceeded the maximum recommended density of 2.2×10^6 cells/ml.

1. Passage the cells 2 to 3 days before performing the assay as described in Section 3.B. To ensure optimal and consistent assay performance, maintain cell density upon harvest in the range of $1.5–2.2 \times 10^6$ cells/ml and cell viability at greater than 90%.
2. Count the mFcγIV Effector Cells by Trypan Blue staining, and calculate the cell density and viability.
3. Transfer an appropriate amount of mFcγIV Effector Cells from the culture vessel to a 50ml conical tube or larger sized centrifuge tube.
4. Pellet the cells at $130 \times g$ for 10 minutes at ambient temperature and resuspend in assay buffer at approximately 2–3X the original cell culture density.
5. Count the cells again, and adjust the volume of assay buffer to achieve a final cell density of 3×10^6 cells/ml, for a final concentration of 7.5×10^5 cells/well. You will need at least 6ml of mFcγIV Effector Cells to fill the inner 60 wells of two assay plates.
6. Gently mix the mFcγRIV Effector Cell suspension, add to a sterile reagent reservoir and dispense 25µl of the cell suspension into each well that contains antibody and target cells.
7. Cover the assay plate with a lid and incubate in a 37°C, 5% CO_2 incubator for 6–24 hours.

4.G. Adding Bio-Glo™ Reagent

Note: The Bio-Glo™ Reagent should be at ambient temperature when added to assay plates.

1. Remove the assay plates from the incubator and equilibrate to ambient temperature (22–25°C) for 15 minutes.
2. Using a manual multichannel pipette, add 75µl of Bio-Glo™ Reagent to the inner 60 wells of the assay plates, taking care not to create bubbles.
3. Add 75µl of Bio-Glo™ Reagent to wells B1, C1, and D1 of each assay plate to measure background signal.
4. Incubate at ambient temperature for 5–30 minutes.

Note: Varying the incubation time will impact the raw RLU values but should not significantly change the EC_{50} and fold induction.

5. Measure luminescence using a luminometer or luminescence plate reader.

4.H. Data Analysis

1. Measure plate background by calculating the average relative light units (RLU) from wells B1, C1 and D1.
2. Calculate fold induction = RLU (induced – background) / RLU (no antibody control – background).

Note: When calculating fold induction, if the sample RLUs are equal to or greater than 100X higher than the plate background RLU, there is no need to subtract the plate background from the sample RLU.

3. Graph data as RLU versus Log_{10} [antibody] and fold induction versus Log10 [antibody]. Fit curves, and determine EC_{50} of antibody response using appropriate curve fitting software (such as GraphPad Prism® software).

5. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptom	Possible Causes and Comments
Poor viability of mFcγRIV Effector Cells during cell culture	Confirm that the serum and antibiotic concentrations are correct. Ensure that all reagents are within their indicated expiration dates.
	Confirm that incubator temperatures and CO_2 levels are correctly set.
	Confirm the passage cell density (5.0×10^5 cells/ml if passaging every 3 days or 7.5×10^5 cells/ml if passaging every 2 days). Confirm previous passage harvest density.
Weak assay response	Confirm that the antibody is a subtype with affinity for the mFcγRIV receptor.
	Confirm, if known, the antibody affinity for the target cell antigen.
	Make sure to use the optimal concentration range for the antibody, which can provide a full dose response with complete upper and lower asymptotes. Note that the antibody EC_{50} in the mFcγRIV ADCC Reporter Bioassay will not necessarily be the same as determined from other ADCC bioassays. Thus, some adjustment to the antibody starting concentration and serial dilution schemes may be needed to achieve maximal response in the assay.
	Increase the target cell density while maintaining the same effector cell density. Since the readout of the assay is from the effector cells, improvement of the response can be achieved by increasing the number of target cells per well.
	Increase the mFcγRIV Effector Cell density and target cell density.
	Vary induction times within a range of 4–24 hours, and choose the induction time that gives the optimal response.
	If applicable, verify that the target cells still express antigen at the relevant passage number and method of harvesting.
	If applicable, verify that the target cells remain viable, and ensure that you are following recommended pre-assay culture directions.
	Optimize the composition of the assay buffer by varying the concentration of low-IgG serum in a range of 0.5–10%, and choose the serum concentration that gives the optimal assay response.

5. Troubleshooting (continued)

Symptom	Possible Causes and Comments
Weak assay response (continued)	If the untreated control RLU is less than tenfold above plate reader background RLU, subtract the plate reader background RLU from all samples prior to calculating fold induction.
Poor or low luminescence measurements (RLU readout)	Choose a sensitive instrument designed for plate-reading luminescence detection. Instruments primarily designed for fluorescence are not recommended.
	Luminometers measure and report luminescence as relative values, and actual numbers will vary among instruments. Some plate-reading luminometers provide the ability to adjust the photomultiplier tube (PMT) gain to expand the signal range.
	Solid-white assay plates will return the most luminescence; clear-bottom plates will show a significant reduction in luminescence, which can be partially remedied by adding white tape to the bottom of the plate.
Possible issues with matrix effect	IgG, serum complement or other components from serum, supernatant of phage display or hybridoma culture could nonspecifically affect antibody binding to the mFcγRIV receptor or affect the NFAT-RE signaling pathway directly, causing a matrix effect. Use low-IgG serum or perform further dilution of antibody starting preparation to minimize this effect.
Will I see the same ranking of antibody potency in the Promega mFcγRIV ADCC Reporter Bioassay as in a classic ADCC bioassay?	The mFcγRIV ADCC Reporter Bioassay will measure antibody Fc-mediated signaling specifically through the mFcγRIV receptor, which data suggest is the primary Fc receptor through which antibodies mediate mouse ADCC in vivo (3-5). However, mFcγRI and mFcγRIII may have some contribution to mouse ADCC function in vivo, and those receptors are not represented in the mFcγRIV ADCC Reporter Bioassay.

6. References

1. Nimmerjahn, F, and Ravetch, J.V. (2006) Fcγ receptors: Old friends and new family members. *Immunity* **24**, 19–28.
2. Guilliams, M. *et al.* (2014) The function of Fcγ receptors in dendritic cells and macrophages. *Nat. Rev. Immunol.* **14**, 94–108.
3. Parekh, B.S. *et al.* (2012) Development and validation of an antibody-dependent cell-mediated cytotoxicity reporter gene assay. *mAbs* **4**, 310–8.
4. Surowy, T. *et al.* (2012) Low variability ADCC bioassay: Novel NFAT reporter bioassay for Fc effector function. *GEN* **32**, 28–9.
5. Overdijk, M.B. *et al.* (2012) Crosstalk between human IgG isotypes and murine effector cells. J. *Immunol.* **189**, 3430–8.

7. Appendix

7.A. Composition of Buffers and Solutions

Cell Thawing Medium

Note: Cell thawing medium does not contain antibiotics

88%	RPMI 1640
10%	FBS
1%	MEM nonessential amino acids
1%	Sodium pyruvate

Cell Growth Medium

Note: Cell growth medium should be **prepared fresh every two weeks**.

88%	RPMI 1640
10%	FBS
1%	MEM nonessential amino acids
1%	Sodium pyruvate
500µg/ml	Antibiotic G-418 Sulfate Solution
100µg/ml	Hygromycin B

Cell Freezing Medium

Note: Cell freezing medium should be **prepared fresh and maintained at 4°C during use**.

80%	RPMI 1640
15%	FBS
5%	DMSO

Assay Buffer

96%	RPMI 1640
4%	Low-IgG serum

Promega

7.B. Related Products

Fc Effector Bioassays

Product	Size	Cat.#
mFcγRIV ADCC Reporter Bioassay, Core Kit	1 each	M1211
mFcγRIV ADCC Reporter Bioassay, Core Kit, 5X	1 each	M1215
mFcγRIV ADCC Reporter Bioassay, Complete Kit	1 each	M1201
ADCC Reporter Bioassay, Complete Kit (Raji)	1 each	G7015
ADCC Reporter Bioassay, Target Kit (Raji)	1 each	G7016
ADCC Reporter Bioassay, Core Kit	1 each	G7010
ADCC Reporter Bioassay, Core Kit, 5X	1 each	G7018

Not For Medical Diagnostic Use. Additional kit formats are available.

Note: Additional mouse and human Fc Effector bioassays are available. Please visit **www.promega.com/products/biologics** for a complete listing of catalog and custom assay options.

Immune Checkpoint Bioassays

Product	Size	Cat.#
PD-1/PD-L1 Blockade Bioassay	1 each	J1250
PD-1/PD-L1 Blockade Bioassay, 5X	1 each	J1255
Control Ab, Anti-PD-1	1 each	J1201
PD-L1 Negative Cells	1 each	J1191
PD-L1 Negative Cells , 5X	1 each	J1195

Not for Medical Diagnostic Use. Additional kit formats are available.

Note: Additional Immune Checkpoint bioassays are available from Promega Custom Assay Services. To view and order products from Custom Assay Services visit: **www.promega.com/CAS** or e-mail: **CAS@promega.com**

Detection Reagents

Product	Size	Cat.#
Bio-Glo™ Luciferase Assay System	10ml	G7941
	100ml	G7940

Not For Medical Diagnostic Use.

Luminometers

Product	Size	Cat.#
GloMax® Discover System	1 each	GM3000

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

[a]NOT FOR MEDICAL DIAGNOSTIC USE. FOR IN VITRO USE ONLY. BY USE OF THIS PRODUCT, RECIPIENT AGREES TO BE BOUND BY THE TERMS OF THIS LIMITED USE STATEMENT. If the recipient is not willing to accept the conditions of this limited use statement, and the product is unused, Promega will accept return of the unused product and provide the recipient with a full refund.

This product may not be further sold or transferred by the recipient and may be used only by the recipient, and then only for (1) research use, (2) discovery, development and monitoring of biologic drugs and vaccines, (3) quality assurance testing of biologic drugs and vaccines, and (4) product release assays for biologic drugs and vaccines. No other commercial use is allowed. "Commercial use" means any and all uses of this product by recipient for monetary or other consideration, including providing a service, information or data to unaffiliated third parties, and resale of this product for any use. Recipient has no right to modify, derivatize, genetically engineer or otherwise create variations of the cells or genes stably transfected within the cells except that recipient may propagate and store the cells for long-term use. In addition, recipient must use Bio-Glo™ Luciferase Assay System purchased from Promega Corporation for all luminescence assays using this product or contact Promega to obtain a license for use of this product with reagents other than Promega's. PROMEGA MAKES NO REPRESENTATIONS OR WARRANTIES OF ANY KIND, EITHER EXPRESSED OR IMPLIED, INCLUDING AS TO MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE WITH REGARDS TO THIS PRODUCT. The terms of this agreement shall be governed under the laws of the State of Wisconsin, USA.

[b]U.S. Pat. No. 8,008,006 and European Pat. No. 1341808.

[c]Licensed from Lonza Cologne GmbH under U.S. Pat. Nos. 7,700,357, 8,192,990 and 8,003,389, European Pat. Nos. 1297119, 1522587, 1607484 and 1741778 and other pending and issued patents.

GloMax is a registered trademark of Promega Corporation. Bio-Glo is a trademark of Promega CorporaƟon. GraphPad Prism is a registered trademark of GraphPad Software, Inc.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more informaƟon. All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.