TECHNICAL MANUAL

# FcγRIIa-H ADCP Reporter Bioassay, Core Kit

Instructions for Use of Products
**G9991 and G9995**


2/16
TM471

# FcγRIIa-H ADCP Reporter Bioassay, Core Kit

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## 1. Description

Antibody-dependent cell-mediated phagocytosis (ADCP) is an important mechanism of action (MOA) of therapeutic antibodies designed to recognize and mediate the elimination of virus-infected or diseased (e.g., tumor) cells. Unlike antibody-dependent cell-mediated cytotoxicity (ADCC), which is mediated primarily through FcγRIIIa expressed on NK cells, ADCP can be mediated by monocytes, macrophages, neutrophils and dendritic cells via FcγRIIa (CD32a), FcγRI (CD64) and FcγRIIIa (CD16a). In macrophages, the expression level of the various receptors is highly dynamic and influenced by cell lineage, tissue microenvironment and local inflammatory state. All three receptors can participate in antibody recognition, immune receptor clustering and signaling events that result in ADCP; however, blocking studies suggest that FcγRIIa is the predominant FcγR receptor involved in this process (1–4).

Current methods used to measure ADCP rely on the isolation of primary human monocytes, ex vivo differentiation into macrophages, and measurement of target cell engulfment. 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 quality-controlled, drug development settings.

The FcγRIIa-H ADCP Reporter Bioassay[a-c], Core Kit (Cat.# G9991, G9995), 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 FcγRIIa. The assay consists of a genetically engineered Jurkat T cell line that expresses:

- The high-affinity human FcγRIIa-H variant that contains a Histidine (H) at amino acid 131
- A luciferase reporter driven by an NFAT-response element (NFAT-RE)

Compared to the low-affinity FcγRIIa-R variant that contains an arginine (R) at amino acid 131, FcγRIIa-H exhibits higher affinity for IgG2 isotypes. The FcγRIIa-H Effector Cells are provided in a thaw-and-use format, which includes cryopreserved cells that can be thawed, plated and used in an assay without the need for cell propagation.

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

The assay combines (1) a simple, add-mix-read single-day workflow with (2) FcγRIIa-H Effector Cells provided in a frozen, thaw-and-use format, and (3) an optimized protocol that together yield a quantitative bioassay that exhibits low variability and high accuracy. The thaw-and-use cells provided in the FcγRIIa-H ADCP Reporter Bioassay kits are manufactured under stringent quality control to provide high assay reproducibility with the convenience of an assay reagent that eliminates the need for continuous cell propagation.

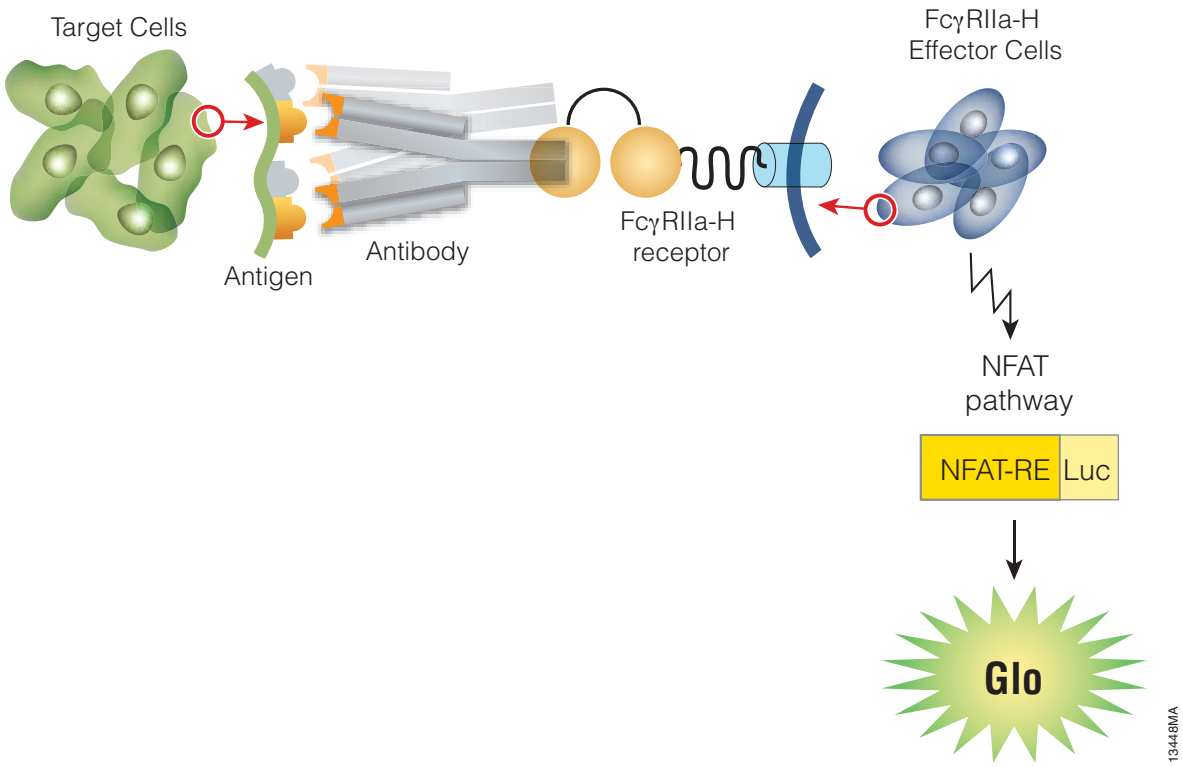


**Figure 1. Representation of the FcγRIIa-H ADCP Reporter Bioassay.** The bioassay consists of a genetically engineered cell line (FcγRIIa-H Effector Cells), an antigen expressing target cell and an antigen-specific antibody. When all components are co-cultured, the antibody simultaneously binds the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

The FcγRIIa-H ADCP Reporter Bioassay reflects the MOA of biologics designed to bind and activate FcγRIIa. The bioassay shows high specificity as demonstrated using trastuzumab (anti-HER2) or rituximab (anti-CD20) antibodies and the relevant target cells SKBR3 ($HER2^+$) or Raji ($CD20^+$), respectively (Figure 2). In response to trastuzumab, FcγRIIa-mediated luciferase activity is detected using SKBR3 target cells, but not Raji cells. Conversely, rituximab-induced luciferase activity is detected using Raji target cells but not SKBR3 cells. No antibody response occurs using NFAT-RE effector cells that do not express FcγRIIa. The bioassay shows precision, accuracy and linearity required for potential validation and routine use in potency and stability studies (Figure 3 and Table 1). Finally, FcγRIIa-H ADCP Reporter Bioassay is compatible with both adherent and non-adherent target cells (Figure 4).

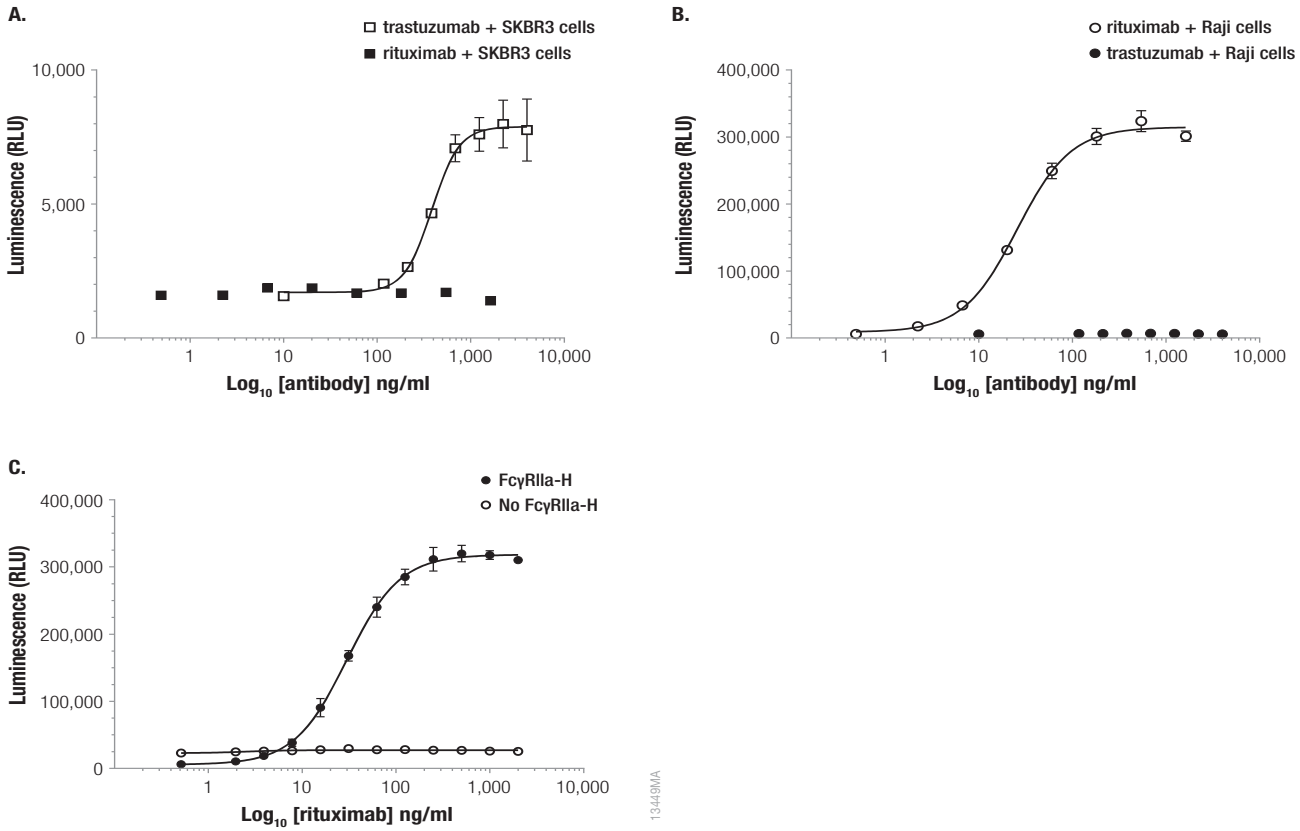


**Figure 2. The FcγRIIa-H ADCP Reporter Bioassay reflects the MOA and specificity of antibodies designed to bind and activate FcγRIIa-H. Panels A and B.** Increasing concentrations of trastuzumab (anti-HER2) or rituximab (anti-CD20) were incubated with either SKBR3 (HER2$^+$) or Raji (CD20$^+$) target cells and FcγRIIa-H Effector Cells, as indicated. **Panel C.** Increasing concentrations of rituximab were incubated with Raji target cells and NFAT-RE effector cells either with or without FcγRIIa-H expression. Bio-Glo™ Reagent was added, and luminescence was measured with a luminometer. Data were fitted to a 4PL curve using GraphPad Prism® software. With the exception of SKBR3 cells, data were generated using thaw-and-use cells.

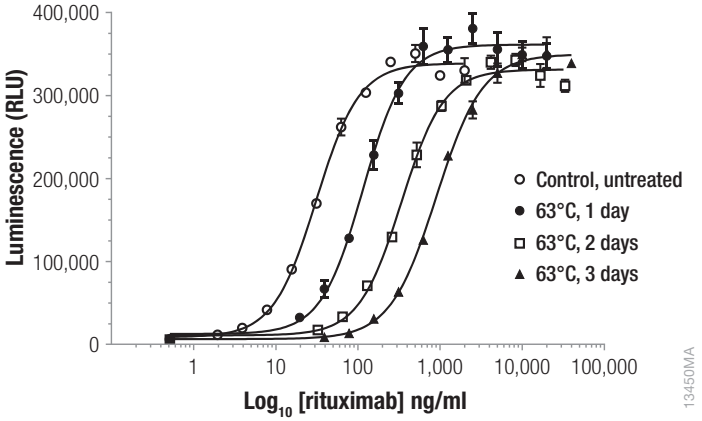


**Figure 3. The FcγRIIa-H ADCP Reporter Bioassay is stability-indicating.** Samples of rituximab (anti-CD20) were maintained at 4°C (control) or heat denatured at 63°C for the indicated times and analyzed using the FcγRIIa-H ADCP Reporter Bioassay. The $EC_{50}$ values were 32ng/ml (control) and 116ng/ml, 339ng/ml, and 904ng/ml across the three time points. Data were fitted to a 4PL curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

**Table 1. The FcγRIIa-H ADCP Reporter Bioassay Demonstrates Accuracy, Repeatability, Intermediate Precision and Linearity.**

| Parameter | Results | |
|---|---|---|
| | % Expected Relative Potency | % Recovery |
| Accuracy | 50 | 99.1 |
| | 71 | 102.7 |
| | 140 | 105.0 |
| | 200 | 99.1 |
| Repeatability (% CV) | 100% reference of 3 days | 3.5 |
| Intermediate Precision (% CV) | | 6.5 |
| Linearity ($r^2$) | | 0.997 |
| Linearity (y = m*x* + b) | | y = 0.997*x* + 1.95 |

A 50–200% theoretical relative potency series of the ADCP Control Ab, Anti-CD20 (IgG1), was analyzed in triplicate in three independent experiments performed on three days. Luciferase activity was quantified using the Bio-Glo™ Reagent. Data were analyzed and relative potencies calculated after parallelism determination using JMP® software. Data were generated using thaw-and-use cells.

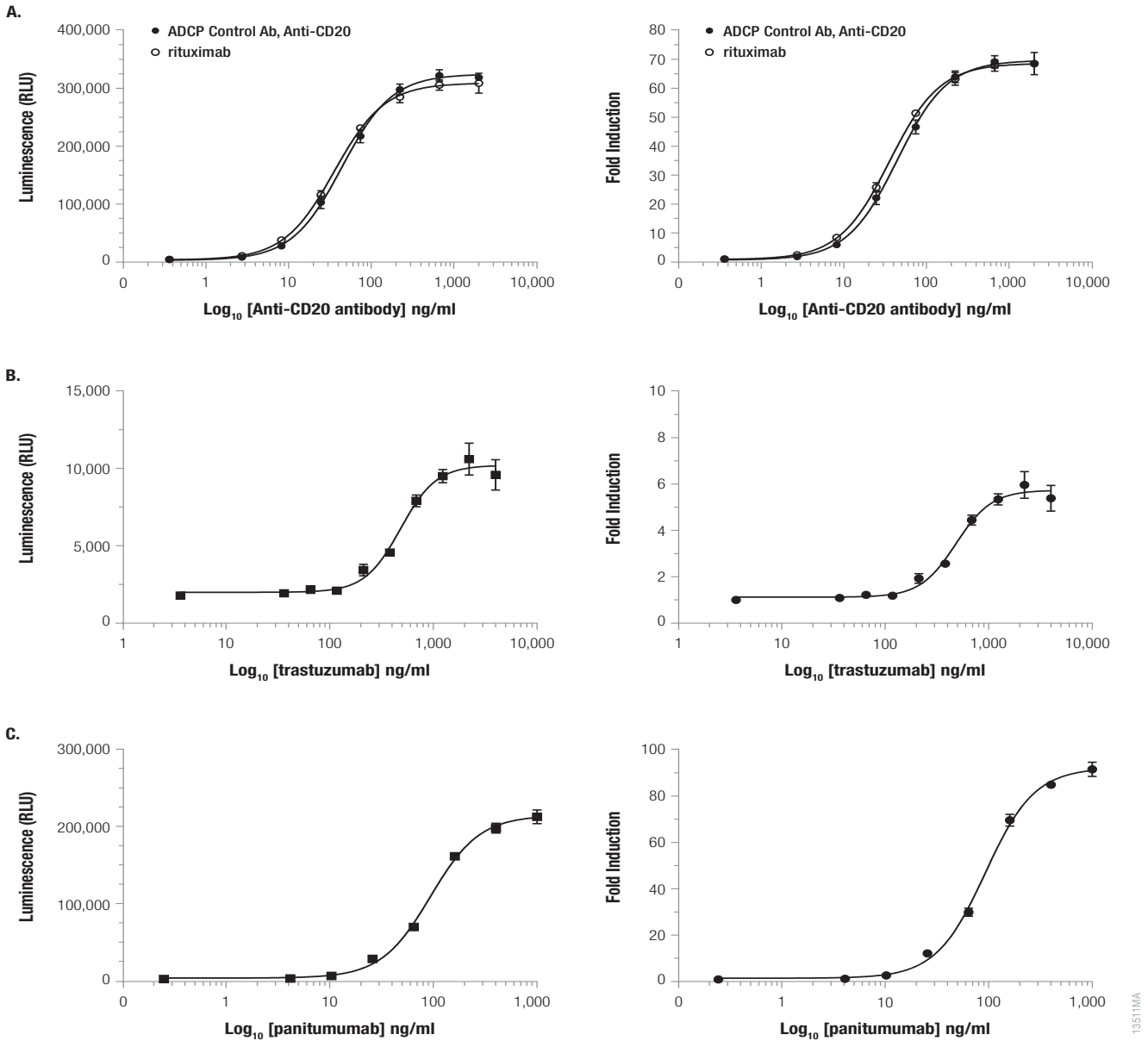


**Figure 4. The FcγRIIa-H ADCP Reporter Bioassay is compatible with both adherent and non-adherent target cells. Panel A.** Increasing concentrations of anti-CD20 antibody (ADCP Control Ab, Anti-CD20, or rituximab, both IgG1, as indicated), or (**Panel B**) trastuzumab (anti-HER2, IgG1) or (**Panel C**) panitumumab (anti-EGFR, IgG2) were incubated with either Raji ($CD20^+$, non-adherent, Panel A), SKBR3 ($HER2^+$, adherent, Panel B) or A431 ($EGFR^+$, adherent, Panel C) target cells and FcγRIIa-H Effector Cells. Bio-Glo™ Reagent was added, and luminescence was measured using a luminometer. Data were fitted to a 4PL curve using GraphPad Prism® software. With the exception of SKBR3 and A431 cells, data were generated using thaw-and-use cells. All data were obtained following a 6-hour induction.

## 2. Product Components and Storage Conditions

| PRODUCT | SIZE | CAT.# |
|---|---|---|
| **FcγRIIa-H ADCP Reporter Bioassay, Core Kit** | **1 each** | **G9991** |

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 120 assays using the inner 60 wells of two 96-well plates. Includes:

- 1 vial FcγRIIa-H Effector Cells (0.62ml)
- 36ml RPMI 1640 Medium
- 4ml Low IgG Serum
- 1 vial Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 10ml Bio-Glo™ Luciferase Assay Buffer

| PRODUCT | SIZE | CAT.# |
|---|---|---|
| **FcγRIIa-H ADCP Reporter Bioassay, Core Kit (5X)** | **1 each** | **G9995** |

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 600 assays using the inner 60 wells of ten 96-well plates. Includes:

- 5 vials FcγRIIa-H Effector Cells (0.62ml)
- 5 × 36ml RPMI 1640 Medium
- 5 × 4ml Low IgG Serum
- 5 vials Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 5 × 10ml Bio-Glo™ Luciferase Assay Buffer

**Note:** The FcγRIIa-H ADCP Reporter Bioassay components are shipped separately because of temperature requirements. The FcγRIIa-H Effector Cells are shipped on dry ice. The Bio-Glo™ Luciferase Assay System and Low IgG Serum are shipped on dry ice, separately from the cells. The RPMI 1640 Medium is shipped at ambient temperature.

**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.
- Store the Bio-Glo™ Luciferase Assay Substrate, Bio-Glo™ Luciferase Assay Buffer and Low IgG Serum at −20°C. Avoid multiple freeze-thaw cycles of the serum.
- For optimal performance, use reconstituted Bio-Glo™ Luciferase Assay Reagent only on the day of preparation. However, once reconstituted, Bio-Glo™ Luciferase Assay Reagent can be stored at −20°C for up to 6 weeks.
- Store the RPMI 1640 Medium at 4°C protected from fluorescent light.

## 3. Before You Begin

The FcγRIIa-H ADCP Reporter Bioassay, Core Kit, is intended to be used with user-provided target cells and antibodies. If your target antigen is CD20 or another antigen expressed on Raji cells, you may analyze your own test and reference antibodies using the FcγRIIa-H ADCP Reporter Bioassay, Complete Kit (Cat.# G9901). The Complete Kit contains Target Cells (Raji) and ADCP Control Ab, Anti-CD20 and is intended to be used as a starter kit to gain familiarity with the FcγRIIa-H Reporter Bioassay. However, if your antibodies are designed to recognize CD20 or another antigen expressed on Raji cells, the Complete Kit also may be used for routine analysis.

The FcγRIIa-H ADCP Reporter Bioassay differs from classic ADCP 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 ADCP assays using primary macrophages or other cell lines.

**Please read through the entire protocol to become familiar with the components and the assay procedure before beginning.** The FcγRIIa-H Effector Cells are provided in frozen, thaw-and-use format and are ready to be used without any additional cell culture or propagation. When thawed and diluted as instructed, the cells will be at the appropriate concentration for the assay. The cells are sensitive and care should be taken to follow the cell thawing and plating procedures **exactly** as described in Section 4, taking care not to over mix or over-warm the cell reagents.

The FcγRIIa-H ADCP 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 in all analyses. 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.

### Materials to be Supplied by the User

- user-defined test and reference antibodies or derivatives with Fc effector function
- user-defined target cells
- sterile clear 96-well plate with lid (e.g., Costar Cat. #3370 or Linbro Cat.# 76-223-05)
- white, flat-bottom 96-well assay plates (e.g., Corning Cat.# 3917)
- pipettes (single channel and 12-channel)
- 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 reading luminometer with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System)

## 4. Assay Protocol

This assay protocol illustrates the use of the FcγRIIa-H ADCP Reporter Bioassay 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 plate layouts are possible but may require further optimization.

**Note:** Prior to routine use of the FcγRIIa-H ADCP Reporter Bioassay with your own antibody and target cell line, we recommend optimizing the Effector:Target cell (E:T) ratio and cell densities. Fix the number of FcγRIIa-H Effector Cells (20,000–50,000 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 6–18 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 Reference Samples

1. **Bio-Glo™ Reagent:** Prepare Bio-Glo™ Reagent according to the manufacturer's instructions. 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 the 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:** On the day of assay, monitor and thaw the Low IgG Serum in a 37°C water bath until the cystals have just melted. Add 1.5ml of Low IgG Serum to 36ml of RPMI 1640 Medium to make 37.5ml 96% RPMI 1640/4% Low IgG Serum (sufficient for two assay plates). Mix well and warm to 37°C before use.

   **Note:** The recommended assay buffer contains 4% Low IgG Serum. This concentration of serum 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 the antibody starting dilutions appropriately before making antibody serial dilutions.

   **Note:** Select starting antibody concentrations (1X final concentration) based on previous experimental results, if available. Otherwise, we recommend starting with a concentration of 1–5µg/ml, which has worked well for rituximab, panitumumab and trastuzumab in the FcγRIIa-H ADCP Reporter Bioassay.

### 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 | Ref. 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 | Ref. 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 | Ref. 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 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 FcγRIIa-H ADCP 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 FcγRIIa-H ADCP Reporter Bioassay (Figure 4).

#### 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 96-well, white, 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, while holding the assay plate at a 45° angle, use a multichannel pipette to carefully remove approximately 95µl of culture medium from each of the wells. Immediately add 25µl per well of 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 2.5-fold serial dilutions of a single antibody for analysis in triplicate (120µl of each dilution provides a sufficient volume for analysis in triplicate). Alternatively, you can prepare three independent stocks of serial dilutions to generate triplicate samples. To prepare 2.5-fold serial dilutions, you will need 400µl of reference antibody at 3X the highest antibody concentration for each dose-response curve (two). You will need 200µl of each test antibody at 3X the highest antibody concentration in each of the test antibody dose-response curves. For other dilution schemes, adjust the volumes accordingly. The FcγRIIa-H ADCP Reporter Bioassay works well with rituximab and panitumumab using 2.5-fold serial dilutions at 0–2µg/ml final concentration.

1. Using a sterile clear 96-well plate, add 200µl of reference antibody starting dilution (dilu1, 3X final concentration) to wells A11 and B11.
2. Add 200µl of test antibodies 1 and 2 starting dilution (dilu1, 3X final concentration) to wells C11 and D11, respectively (Figure 6).
3. Add 120µl of assay buffer to other wells in these four rows, from column 10 to column 2.
4. Transfer 80µl of the antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
5. Repeat equivalent 2.5-fold serial dilutions across the columns from right to left until you reach 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 | | Ref. Ab |
| B | | no Ab | dilu9 | dilu8 | dilu7 | dilu6 | dilu5 | dilu4 | dilu3 | dilu2 | dilu1 | | Ref. 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 plate with a lid and incubate it in a 37°C incubator for 15 minutes.

### 4.F. Preparing and Plating FcγRIIa-H Effector Cells

**Note:** The thaw-and-use FcγRIIa-H Effector Cells included in this kit are sensitive, and care should be taken to follow the cell thawing and plating procedures exactly as described, taking care not to over mix or over-warm the cell reagents. No additional cell culture or manipulation is required or recommended. We recommend thawing and diluting a maximum of 2 vials of thaw-and-use cells at any one time.

1. Add 5.3ml of prewarmed (37°C) assay buffer to a 15ml conical tube.
2. Remove one vial of FcγRIIa-H Effector Cells from storage at −140°C and transfer to the bench on dry ice.
3. Thaw the cells in a 37°C water bath with gentle agitation (no inversion) until just thawed (about 2 minutes).
4. Immediately mix the cell suspension by pipetting gently 3 times. Transfer 0.55ml of the cell suspension into the 15ml tube containing prewarmed assay buffer. Immediately mix by inversion several times.
5. Hold briefly at 37°C or transfer the cell suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 25µl of the cell suspension to each of the wells of the 96-well plates already containing target cells and antibody.
6. Cover the assay plates with a lid and incubate in a 37°C, 5% $CO_2$ incubator for 6 hours. Do not stack the plates inside the incubator.

### 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 affect 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 $Log_{10}$ [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**

| Symptoms | Possible Causes and Comments |
|---|---|
| Weak assay response | Confirm, if known, the antibody affinity to the FcγRIIa receptor. |
| | 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 FcγRIIa-H ADCP Reporter Bioassay will not necessarily be the same as determined from other ADCP 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 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 FcγRIIa-H Effector Cell density together with an increase in target cell density. |
| | Vary induction times within a range of 4–24 hours, and choose the induction time that gives the optimal response. |
| | Verify that the target cells still express antigen at the relevant passage number and method of harvesting. |
| | Verify that the target cells remain viable, and ensure that you are following recommended pre-assay culture directions. |

| Symptoms | Possible Causes and Comments |
|---|---|
| Weak assay response (continued) | Optimize the composition of the assay buffer by varying the concentration of low-IgG FBS in a range of 0.5–10%, and choose the serum concentration that gives the optimal assay response. |
| 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. |
| | Increase the integration time when reading samples. |
| | 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 FcγRIIa receptor or affect the NFAT-RE signaling pathway directly, causing a matrix effect. Use low-IgG FBS or perform further dilution of antibody starting preparation to minimize impact. |
| Will I see the same ranking of antibody potency in the Promega FcγRIIa-H ADCP Reporter Bioassay as in a classic ADCP bioassay? | The FcγRIIa-H ADCP Reporter Bioassay will measure antibody Fc-mediated signaling specifically through the FcγRIIa-H receptor, which data suggest is the primary Fc receptor through which antibodies mediate ADCP in vivo (1–4). However, FcγRIIIa and FcγRI may also contribute to ADCP function in vivo, and those receptors are not represented in the FcγRIIa-H ADCP Reporter Bioassay. |

## 6. References


1. Richards, J.O. *et al.* (2008) Optimization of antibody binding to FcγRIIa enhances macrophage phagocytosis of tumor cells. *Mol. Cancer Ther.* **7**, 2517–27.
2. Dugast, A.S. *et al.* (2011) Decreased Fc-Receptor expression on innate immune cells is associated with impaired antibody mediated cellular phagocytic activity in chronically HIV-1 infected individuals. *Virology* **415**, 160–7.
3. Ackerman, M.E. *et al.* (2013) Enhanced phagocytic activity of HIV-specific antibodies correlates with natural production of immunoglobulins with skewed affinity for FcγR2a and FcγR2b. *J. Virol.* **87**, 5468–76.
4. Tebo, A.E. *et al.* (2002) Fcγ receptor-mediated phagocytosis of *Plasmodium falciparum*-infected erythrocytes *in vitro*. *Clin. Exp. Immunology* **130**, 300–6.


## 7. Related Products

### Fc Effector Bioassays

| Product | Size | Cat.# |
|---|---|---|
| FcγRIIa-H ADCP Reporter Bioassay, Complete Kit[1] | 1 each | G9901 |
| ADCC Reporter Bioassay, Target Kit (Raji)[1] | 1 each | G7016 |
| ADCC Reporter Bioassay, Complete Kit (Raji)[1] | 1 each | G7015 |
| ADCC Reporter Bioassay, Core Kit[1] | 1 each | G7010 |
| ADCC Reporter Bioassay, Core Kit (5X)[1] | 1 each | G7018 |
| ADCC Reporter Bioassay, F Variant, Core Kit[2] | 1 each | G9790 |
| ADCC Reporter Bioassay, F Variant, Core Kit (5X)[2] | 1 each | G9798 |

[1]For Research Use Only. Not for Use in Diagnostic Procedures. Additional kit formats available.

[2]Not for Medical Diagnostic Use. Additonal kit formats available.

### Immunotherapy 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 available.

## 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.

**Note:** Additional Fc effector and immunotherapy 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**

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