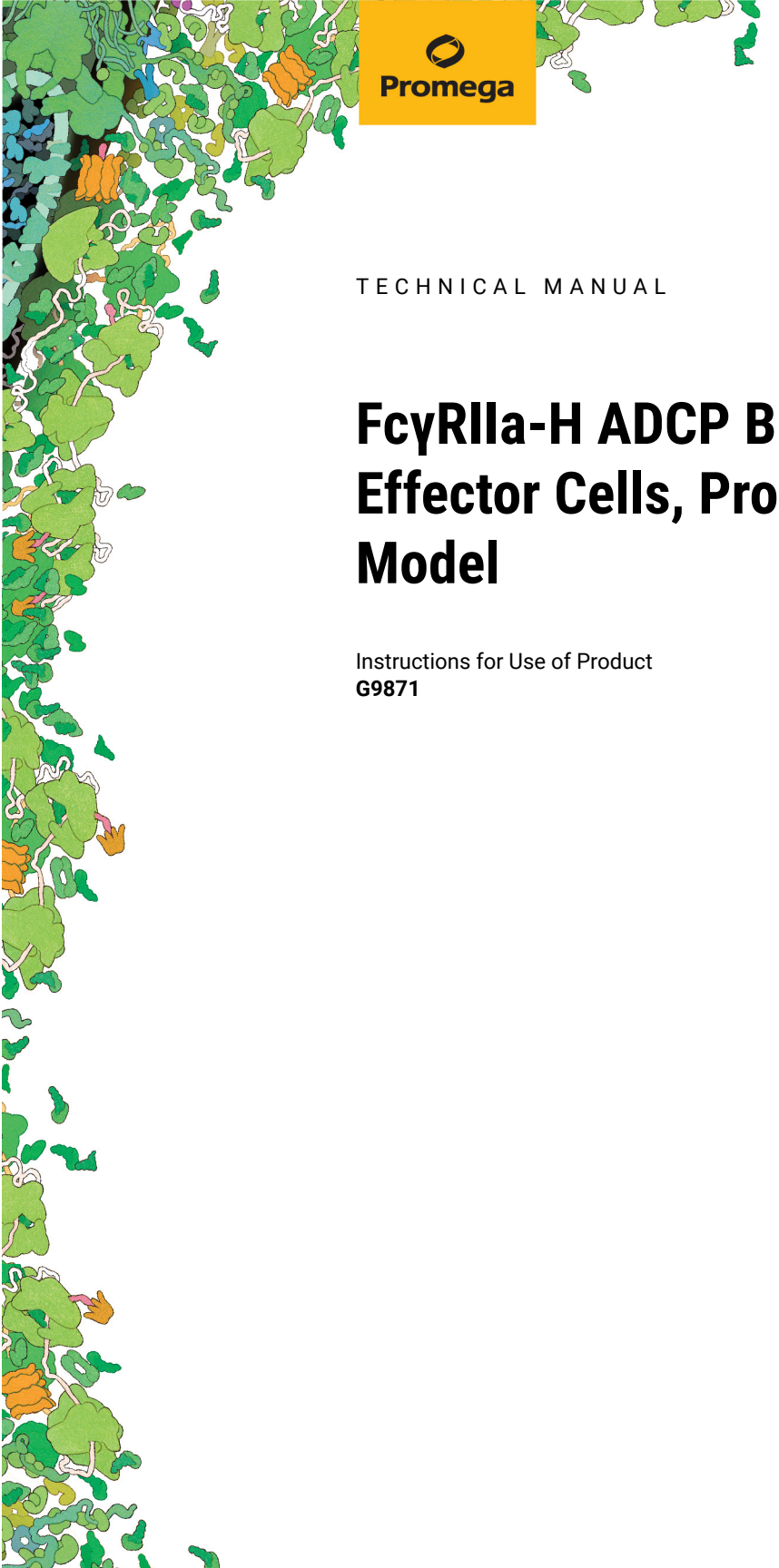


TECHNICAL MANUAL

FcγRIIa-H ADCP Bioassay Effector Cells, Propagation Model

Instructions for Use of Product
G9871

Revised 10/26
TM466

Promega

FcγRIIa-H ADCP Bioassay Effector Cells, Propagation Model

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

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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 Bioassay Effector Cells[a, b], Propagation Model (Cat.# G9871), 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 cell line is provided in a 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 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.

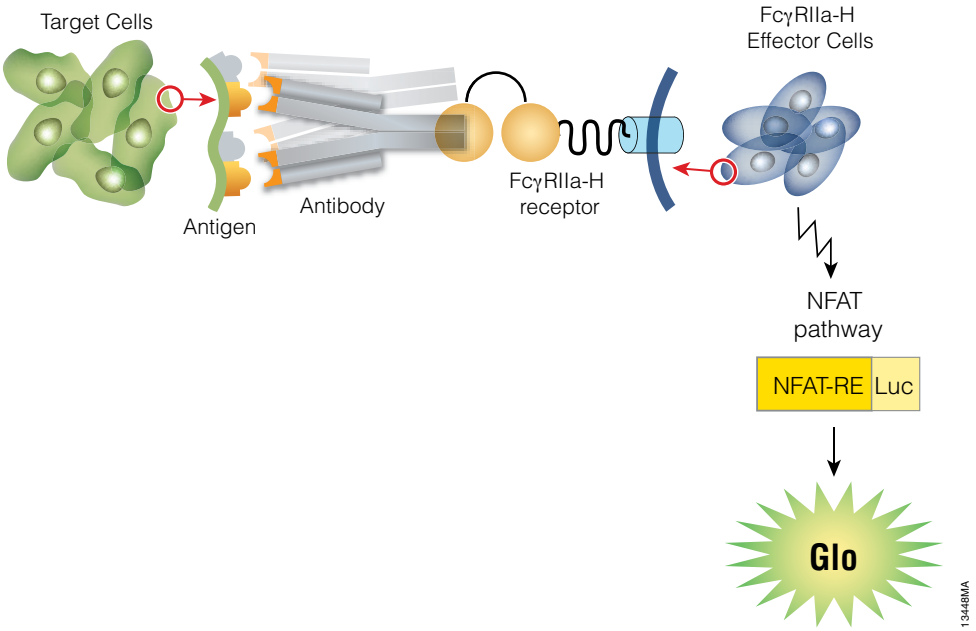


Figure 1. Representation of the FcγRIIa-H ADCP Reporter Bioassay. The bioassay consists of a genetically engineered cell line (FcγRIIa-H Effector Cells), an antigen expressing target cell and an antigen-specific antibody. When all components are co-cultured, the antibody simultaneously binds target cell 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 mechanism of action (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).

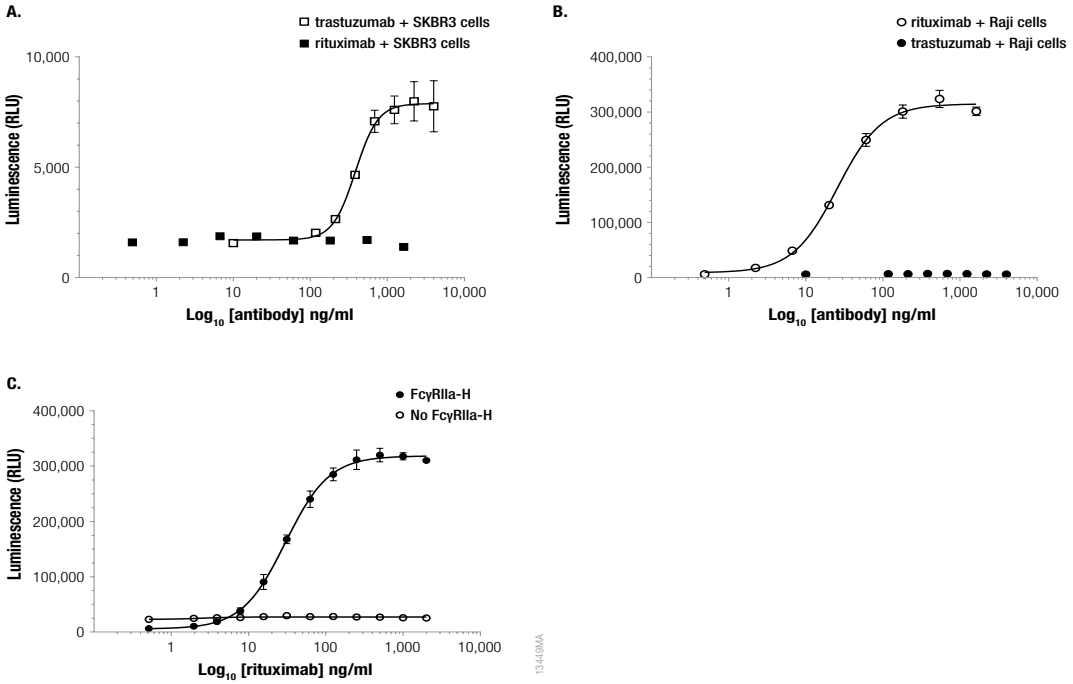


Figure 2. The FcγRIIa-H ADCP Reporter Bioassay reflects the MOA and specificity of antibodies designed to bind and activate FcγRIIa-H. Panels A and B. Increasing concentrations of trastuzumab (anti-HER2) or rituximab (anti-CD20) were incubated with either SKBR3 ($HER2^+$) or Raji ($CD20^+$) target cells 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. 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.

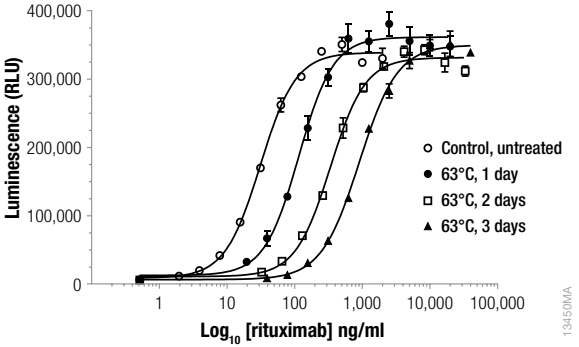


Figure 3. The FcγRIIa-H ADCP Reporter Bioassay is stability-indicating. Samples of rituximab (anti-CD20) were maintained at 4°C (control) or heat denatured at 63°C for the indicated times and analyzed using the FcγRIIa-H ADCP Reporter Bioassay. The EC_{50} values were 32ng/ml (control) and 116ng/ml, 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.

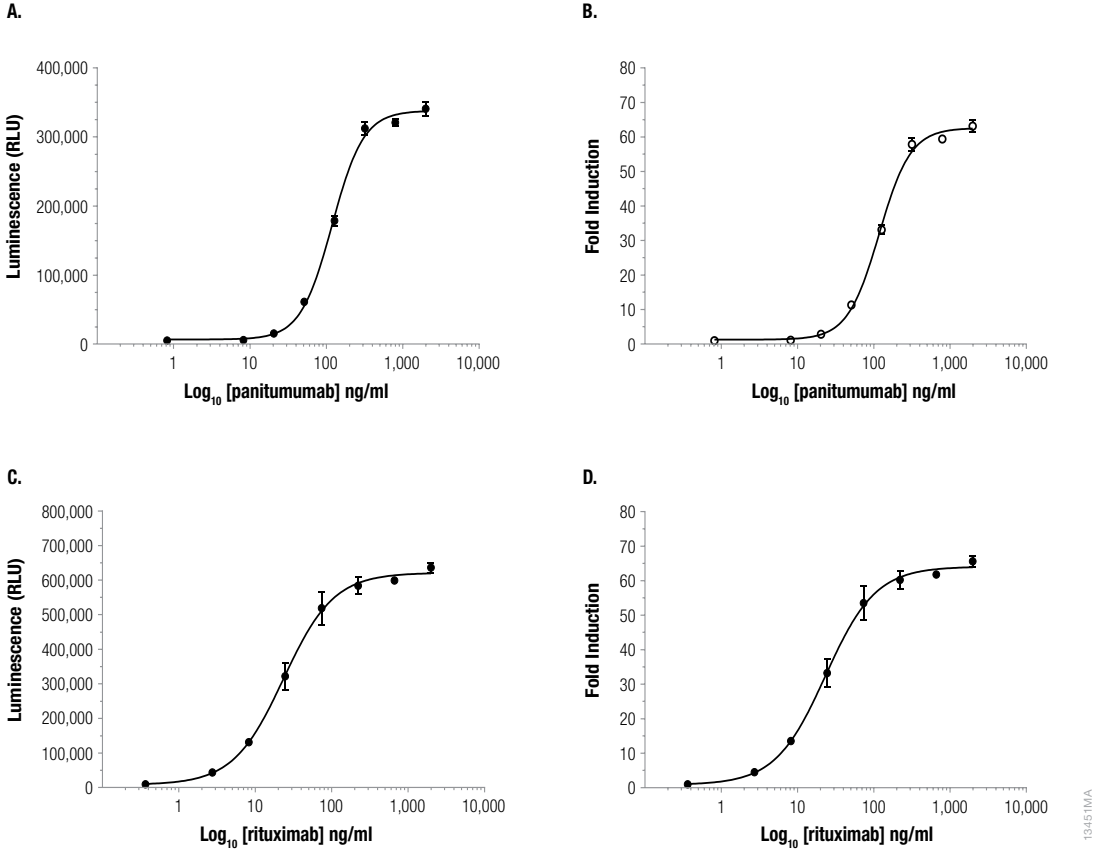


Figure 4. The FcγRIIa-H ADCP Reporter Bioassay is compatible with both adherent and non-adherent, continuous culture target cells. Increasing concentrations of panitumumab (anti-EGFR, IgG2) or rituximab (anti-CD20, IgG1) were incubated with either A431 (EGFR$^+$, adherent) or Raji (CD20$^+$, non-adherent) continuous culture target cells and FcγRIIa-H Effector Cells from continuous culture, as indicated. Bio-Glo™ Reagent was added, and luminescence was measured. Data were fitted to a 4PL curve using GraphPad Prism® software.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
FcγRIIa-H ADCP Bioassay Effector Cells, Propagation Model	**1 each**	**G9871**

Not for Medical Diagnostic Use. Includes:

- 2 vials FcγRIIa-H Effector Cells, 1.5 x 10^7 cells/ml (1.0ml per vial)

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 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. Cell thawing, propagation and banking should be performed ***exactly*** as described in Section 4. 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. By contrast, 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 FcγRIIa. You may need to adjust these parameters and optimize assay conditions for your own antibodies or other biologic samples.

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 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.A. Materials to Be Supplied by the User

(Composition of buffers and solutions is 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 [high glucose, with pyruvate (e.g., ATCC Cat.# 30-2001 or Invitrogen Cat.# A10491-01)]
- RPMI 1640 Medium with HEPES [normal glucose (e.g., Corning® Cat.# 10-041-CV)]
- MEM nonessential amino acids (e.g., Corning® Cat.# 25-025-CI)
- fetal bovine serum (e.g., HyClone Cat.# SH30070)
- super low-IgG FBS (e.g., HyClone Cat.# SH30898)
- DPBS (e.g., GIBCO® Cat.# 14190)
- hygromycin B (e.g, Invitrogen #10687-010)
- Antibiotic G-418 Sulfate Solution (Cat.# V8091)
- DMSO (e.g., Sigma Cat.# D2650)
- Trypan Blue solution (e.g., Sigma Cat.# T8154)
- monoclonal antibody or derivative with Fc effector function
- 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® 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 (e.g., GloMax® Discover System)

3.B. Preparing FcγRIIa-H Effector Cells

Cell Thawing and Initial Cell Culture

1. Prepare 25ml of cell thawing medium.
2. Remove one vial of FcγRIIa-H 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 170 × *g* for 5 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, **vertically** in a 37°C, 5% CO_2 incubator.
7. Incubate for approximately 24 hours.

Cell Maintenance and Propagation (Day 2)

8. Add 12ml of Day 2 cell growth medium to the T75cm tissue culture flask with the FcγRIIa-H Effector Cells.
9. Place the flask **horizontally** in a 37°C, 5% CO_2 incubator.
10. Incubate the cells for approximately 24 hours.

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 7–10 days post-thaw, at which time cell viability is typically >95% and the average cell doubling rate is 24–40 hours. Passage number should be recorded for each passage. Cells will maintain their functionality for 25 passages (or 58 doublings if passaging is performed on a Monday-Wednesday-Friday schedule). Cells should be banked appropriately soon after growth rate stabilization. The FcγRIIa-H 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.

11. To passage the cells, gently mix the cells with a pipette to create a homogeneous cell suspension.
12. Measure the cell viability and density by Trypan Blue staining.
13. Seed the cells at a density of 4×10^5 viable cells/ml if passaging every 2 days (e.g., Mon.–Wed., Wed.–Fri.) or 2.5×10^5 viable cells/ml if passaging every 3 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.

3.B. Preparing FcγRIIa-H Effector Cells (continued)

Cell Maintenance and Propagation (Day 3 and Beyond; continued)

14. 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).
15. Place the flasks horizontally in a 37°C, 5% CO_2 incubator.

Cell Freezing and Banking

16. On the day of cell freezing, prepare new cell freezing medium and keep it on ice.
17. Gently mix the cells with a pipette to create a homogeneous cell suspension.
18. 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 4×10^6 – 2×10^7 cells/ml.
19. Transfer the cell suspension to 50ml sterile conical tubes or larger size centrifuge tubes and centrifuge at 170 *× g* for 10–15 minutes.
20. Gently aspirate the medium, taking care not to disturb the cell pellet.
21. Carefully resuspend the cell pellet in ice-cold cell freezing medium to a final cell density between 4×10^6–2×10^7 cells/ml. Combine the cell suspension into a single tube and dispense into cryovials.
22. 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 FcγRIIa-H ADCP 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 ten-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 FcγRIIa-H ADCP Bioassay Effector Cells, Propagation Model with your antibody and target cell line, we recommend optimizing the E:T (Effector:Target cell) 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 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 Reference Samples

1. **Bio-Glo™ Reagent:** Prepare Bio-Glo™ Reagent according to the manufacturer's instructions. For 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.

   If you are using a large (100ml) Bio-Glo™ Luciferase Assay System, you may dispense the reconstituted Bio-Glo™ Reagent into 10ml aliquots and store at –20°C for up to six weeks. Avoid repeated freeze-thaw cycles. On the day of the assay, thaw the appropriate amount of reconstituted Bio-Glo™ Reagent in a room temperature water bath for at least 1–2 hours before use. 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 FBS 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 FBS. 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 FBS. 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:** Prepare starting dilutions (dilu1, 3X final concentration) of two test antibodies (minimum 250µl each) and one reference antibody (minimum of 500µl). Using assay buffer as the diluent, prepare 500µl of reference antibody starting dilution and 250µl of each test antibody starting dilution 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. 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 layouts illustrated in Figure 5 as a guide. The protocol describes serial replicate dilutions (n = 3) of test and reference antibodies to generate two ten-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	Test Ab
E	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference 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 test and reference 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. Place the target cell suspension at 37°C to maintain the temperature while preparing effector cells and 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 the inner 60 wells of a 96-well white, 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. 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 sidewalls of the wells, leaving 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 at while preparing effector cells and antibody dilution series.

4.D. Preparing FcγIIa-H Effector Cells

While maintaining the FcγRIIa-H Effector Cells, follow the recommended cell seeding density because 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 1.8×10^6 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 the cell density, upon harvest, in the range of $1.0–1.4 \times 10^6$ cells/ml and cell viability at greater than 95%.
2. Count the FcγRIIa-H Effector Cells by Trypan Blue staining, and calculate the cell density and viability.
3. Transfer an appropriate amount of FcγRIIa-H Effector Cells from the culture vessel to a 50ml conical tube or larger sized centrifuge tubes.
4. Pellet the cells at 170 *× 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 $8 \times 10^5–2 \times 10^6$ cells/ml. You will need at least 6ml of FcγRIIa-H Effector Cells to fill the inner 60 wells of two assay plates.
6. Place the FcγRIIa-H Effector Cell suspension at 37°C to maintain the temperature while plating target cells and preparing antibody dilution series.

4.E. Plating Non-Adherent Target Cells

Note: Skip this section if only adherent target cells (prepared in section 4.C) are used.

1. Gently mix the previously prepared target cell suspension (Section 4.C). Add the cell suspension to a sterile reagent reservoir. Immediately dispense 25µl of the cell suspension to each of the inner 60 wells of a 96-well flat-bottom, white assay plate using a multichannel pipette.
3. Add 75µl of assay medium to each of the outside wells of the assay plates.
4. Equilibrate the target cells for approximately 15 minutes in a 37°C, 5% CO_2 incubator.

4.F. 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 (150µl 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 500µl of reference antibody at 3X the highest antibody concentration for each dose-response curve (two). You will need 250µl 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 250µl of reference antibody starting dilution (dilu1, 3X final concentration) to wells A11 and B11.
2. Add 250µl of test antibodies 1 and 2 starting dilution (dilu1, 3X final concentration) to wells C11 and D11, respectively (Figure 6).
3. Add 150µl of assay buffer to other wells in these four rows, from column 10 to column 2.
4. Transfer 100µl 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 through column 3. Do not dilute into column 2.

Note: Wells A2, B2, C2 and D2 contain 150µl 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.G. Adding Antibodies to Plated Target Cells

1. Using a multichannel pipette, add 25µl of the appropriate antibody dilution to the preplated 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 15–25 minutes.

4.H. Adding FcγRIIa-H Effector Cells

1. Gently mix the FcγRIIa-H Effector Cells suspension, add to a sterile reagent reservoir, and dispense 25µl of the cell suspension into each well that contains antibody and target cells.
2. Cover the assay plate with a lid and incubate the plate in a 37°C, 5% CO_2 incubator for 6–24 hours.

4.I. 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.J. 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**. Email: **techserv@promega.com**

Symptoms	Causes and Comments
Poor viability of FcγRIIa-H 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 (2.5×10^5 cells/ml if passaging every 3 days or 4×10^5 cells/ml if passaging every 2 days). Confirm previous passage harvest density.
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.

Symptoms	Causes and Comments
Weak assay response (continued)	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.
	Verify the viability and density of the FcγRIIa-H Effector Cells at harvesting (Section 4.D).
	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.

5. Troubleshooting (continued)

Symptoms	Causes and Comments
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

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4. Tebo, A.E., Kremsner, P.G. and Luty, A.J.F. (2002) Fcγ receptor-mediated phagocytosis of *Plasmodium falciparum*-infected erythrocytes in vitro. *Clin. Exp. Immunology.* **130**, 300–6.

7. Appendix

7.A. Composition of Buffers and Solutions

Cell Thawing Medium

Note: Cell thawing medium does not contain antibiotics

89%	RPMI 1640 (high glucose, with pyruvate)
10%	FBS
1%	MEM nonessential amino acids

Day 2 Cell Growth Medium

Note: Day 2 cell growth medium contains 2X antibiotics and should be used only for cell propagation on Day 2.

89%	RPMI 1640 (high glucose, with pyruvate)
10%	FBS
1%	MEM nonessential amino acids
1.0mg/ml	Antibiotic G-418 Sulfate Solution
400µg/ml	hygromycin B

Cell Growth Medium

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

89%	RPMI 1640 (high glucose, with pyruvate)
10%	FBS
1%	MEM nonessential amino acids
500µg/ml	Antibiotic G-418 Sulfate Solution
200µg/ml	hygromycin B

Cell Freezing Medium

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

79%	RPMI 1640 (high glucose, with pyruvate)
14%	FBS
7%	DMSO

Assay Buffer

96%	RPMI 1640 (normal glucose, with HEPES)
4%	low-IgG FBS

8. Summary of Changes

The following changes were made to the 10/26 revision of this document:

1. Removed Section 7.B, Related Products.
2. Updated the LULL and removed a patent statement.
3. Made minor text edits.

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