TECHNICAL MANUAL

ADCC Bioassay Effector Cells, F Variant, Propagation Model

Instructions for Use of Product
G9302

Revised 3/16
TM430

ADCC Bioassay Effector Cells, F Variant, 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

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1. Description

Antibody-dependent cell-mediated cytotoxicity (ADCC) is a mechanism of action of antibodies through which virus-infected or other diseased cells are targeted for destruction by components of the cell-mediated immune system, such as natural killer cells. The ADCC Reporter Bioassay, F Variant, is a bioluminescent reporter assay for quantifying biological activity on pathway activation by therapeutic antibodies in an ADCC mechanism of action (MOA) assay. The ADCC Reporter Bioassay, F Variant, can be performed with the ADCC Bioassay Effector Cells, F Variant, Propagation Model[a–c] (Cat.# G9302), described here, a model that allows cell banking and propagation under a unique purchase agreement. The assay combines a simple, add-mix-read format and an optimized protocol to provide a bioassay that has low variability and high accuracy. These performance characteristics make the bioassay suitable for application across antibody drug research and development.

A note on kit formats: We offer the ADCC Reporter Bioassay technology in multiple kit formats to better meet research needs. The ADCC Reporter Bioassay, F Variant, Core Kit (Cat.# G9790 and G9798) contains the key reagent components (ADCC Bioassay Effector Cells, F Variant, RPMI 1640 Medium, Low IgG Serum and Bio-Glo™ Luciferase Assay System) needed to perform an ADCC Reporter Bioassay with user-provided specific target cells and antibody. For users wanting a control target cell line (thaw-and-use format) and a control Ab, we offer two target kits. Target kits contain Target Cells [WIL2-S (Cat.# G7013) or Raji (Cat.# G7016)] and Control Ab, Anti-CD20, and can be used with Core Kits if desired, providing flexibility to the end user. The F Variant products extend the original ADCC Reporter Bioassay product line, which possesses the V158 variant of the FcγRIIIa receptor in the engineered Jurkat effector cell. These products are listed in Section 11 and on the Promega Web site (**www.promega.com/adcc**).

ADCC is a desirable mechanism for killing target cells. The antibody binds to target antigens on the cell surface. When the Fc effector portion of target-bound antibodies also binds to FcγRIIIa receptors on the cell surface of effector cells (natural killer cells predominantly), multiple crosslinking of the two cell types occurs, leading to pathway activation of ADCC MOA (1). The human FcγRIIIa gene displays a polymorphism in the position coding for amino acid residue 158. This translates to a higher affinity FcγRIIIa variant having a valine at amino acid residue 158 (158V) and a lower affinity FcγRIIIa variant carrying phenylalanine (158F). Killing of target cells is an endpoint of this pathway activation and is used in classic ADCC bioassays, which use donor peripheral blood mononuclear cells (PBMC) or the natural killer (NK) cell subpopulation as effector cells (2) isolated from blood donors with FcγRIIIa VV, VF or FF alleles. These cells can be highly variable in response, are tedious to prepare and can result in high background readings. It is highly challenging to quantitatively evaluate the impact of FcγRIIIa polymorphism by classic in vitro ADCC bioassays.

The ADCC Reporter Bioassay uses an alternative readout at an earlier point in ADCC MOA pathway: the activation of gene transcription through the NFAT (nuclear factor of activated T-cells) pathway in the effector cell (3–4). In addition, the ADCC Reporter Bioassay, F Variant, uses engineered Jurkat cells stably expressing the FcγRIIIa receptor, F158 (low affinity) variant, and an NFAT response element driving expression of firefly luciferase as effector cells. Antibody biological activity in ADCC MOA is quantified through the luciferase produced as a result of NFAT pathway activation; luciferase activity in the effector cell is quantified with luminescence readout (Figure 1). Signal is high, and assay background is low.

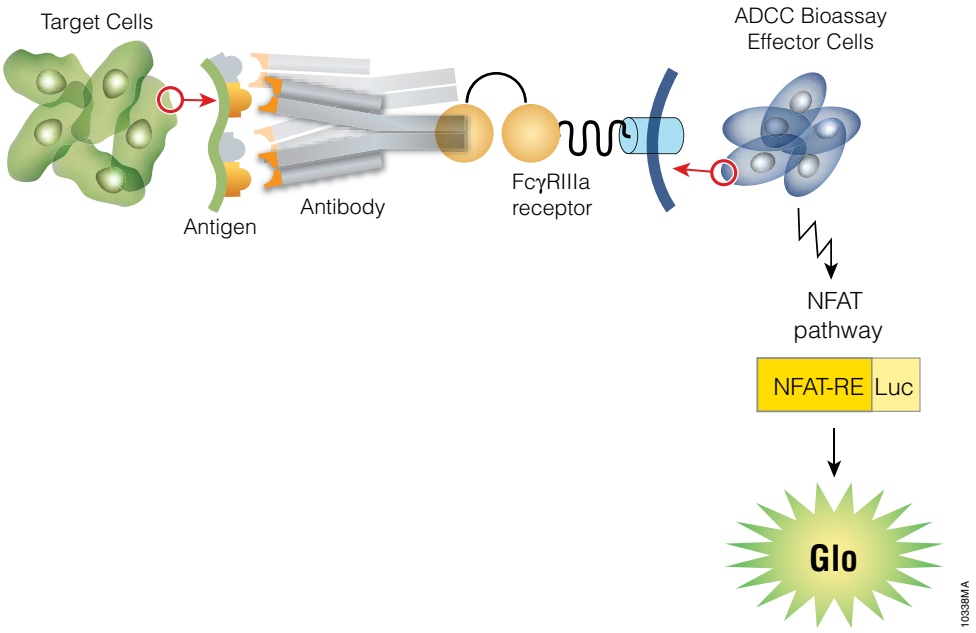

Figure 1. Representation of the ADCC Reporter Bioassay. ADCC Bioassay Effector Cells are genetically engineered Jurkat T cells stabling expressing NFAT response element driving expression of firefly luciferase and FcγRIIIa V158 variant or FcγRIIIa F158 variant. Assay readout is luminescence signal from ADCC Bioassay Effector Cells.

The ADCC Reporter Bioassay exhibits the clear specificity desired for a bioassay, as shown in Figure 2. A specific assay response is only obtained when target cells with the correct surface antigen, the correct specific antibody, and effector cells expressing FcγRIIIa F158 are present. If any one of these is missing, there is no response.

1. Description (continued)

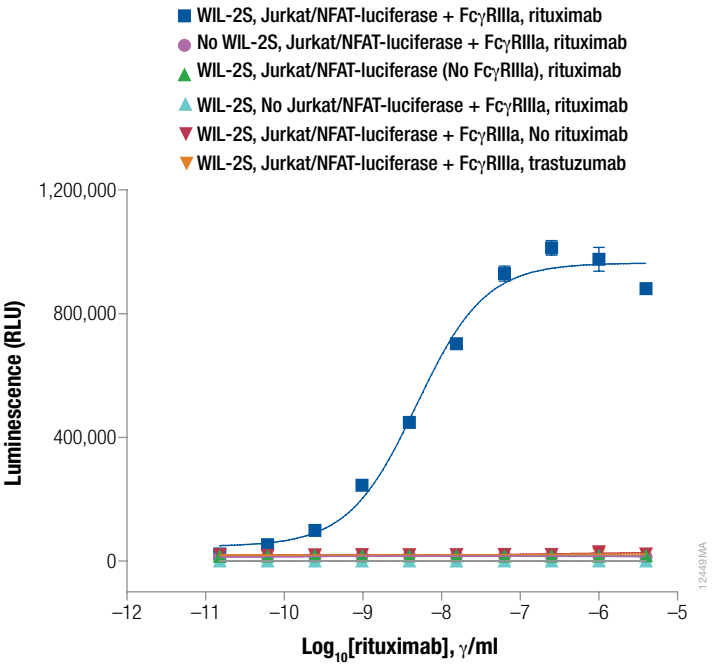

Figure 2. Specificity of the ADCC Reporter Bioassay, F Variant. Serial dilutions of rituximab (anti-CD20 chimeric monoclonal antibody drug), trastuzumab (anti-Her2 humanized monoclonal antibody drug) or assay medium control (no antibody) were incubated for 6 hours at 37°C with engineered Jurkat effector cells (ADCC Bioassay Effector Cells, F Variant), with or without ADCC Bioassay Target Cells (WIL2-S), as indicated. These data were generated using frozen, thaw-and-use cells. Luciferase activity was quantified using Bio-Glo™ Reagent. Data were fitted to a 4PL curve using GraphPad Prism® software.

The ADCC Reporter Bioassay has performance characteristics suitable for many applications of a bioassay used across antibody drug discovery research: It is stability-indicating, precise and accurate (Figure 3). The assay shows appropriate antibody IgG isotype specificity, which is correlated with binding affinities to FcγRIIIa receptors (Figure 4). ADCC Reporter Bioassay, F Variant can be used side by side with its sister product, ADCC Reporter Bioassay (Cat.# G7010, G7018), which uses ADCC Bioassay Effector cells (V Variant). The potency difference for the same antibody in V and F Variant ADCC Reporter Bioassays appropriately reflected the reported impact of FcγRIIIa polymorphism on antibody binding affinities and ADCC activities (see Figure 5).

Parameter		Results	
		WIL2-S Target cells	Raji Target cells
Accuracy	% Expected Relative Potency	% Recovery	% Recovery
	50	87.7	101.3
	75	90.4	99.2
	150	95.1	107.8
	200	103.1	99.3
Repeatability (%CV)	(100% Reference)	3.4	5.7
Intermediate Precision (%CV)		6.2	7.7
Linearity (r^2)		0.997	0.993
Linearity (y = mx + b)		y = 1.071x – 12.04	y = 1.017x + 0.596

Figure 3. Bioassay characterization. The ADCC Reporter Bioassay, F Variant, was characterized in studies that evaluated accuracy, repeatability, intermediate precision and linearity across the 50–200% relative potency range. Dilution ranges for rituximab were selected to ensure good coverage of upper and lower asymptotes and sufficient points in the intermediate dose-range for accurate slope and EC_{50} determinations. A series of relative potency samples, of 50%, 75%, 150% and 200% theoretical relative potency, were evaluated as triplicate dilution series of antibody dose on each of 3 different days. The effector-to-target cell ratio (E:T ratio) was 6:1. The ADCC Reporter Bioassay, F Variant, was characterized using ADCC Bioassay Target Cells (WIL2-S) and ADCC Bioassay Target Cells (Raji). These data were generated using thaw-and-use cells. The data were fitted to a 4PL curve using GraphPad Prism® software, and relative potencies were calculated after parallelism determination using PLA2.1 software from Stegmann Systems GmbH. Relative potencies were calculated using the 100% reference sample run as a triplicate dilution series in the same assay plate as the test sample.

1. Description (continued)

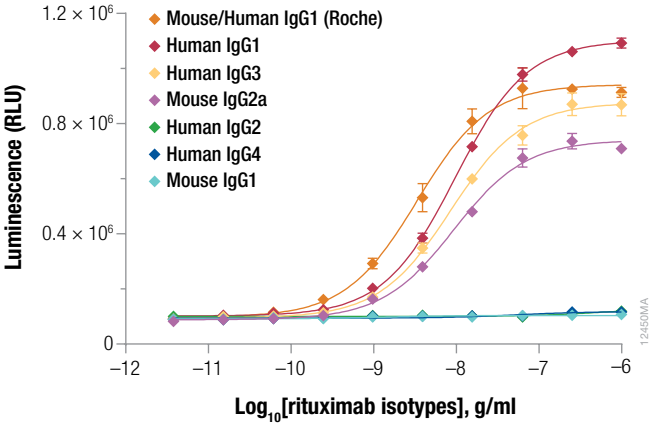

Figure 4. Antibody IgG isotype-specificity. Serial dilutions of various IgG isotopes of anti-CD20 rituximab were incubated for 6 hours at 37°C with engineered Jurkat effector cells (ADCC Bioassay Effector Cells, F Variant) and ADCC Bioassay Target Cells (WIL2-S). The therapeutic biologic, mouse/human IgG1 rituximab (Trade name: Rituxan®) was included as positive control. These data were generated using frozen, thaw-and-use cells. Luciferase activity was quantified using the Bio-Glo™ Reagent. Data were fitted to a 4PL curve using GraphPad Prism® software.

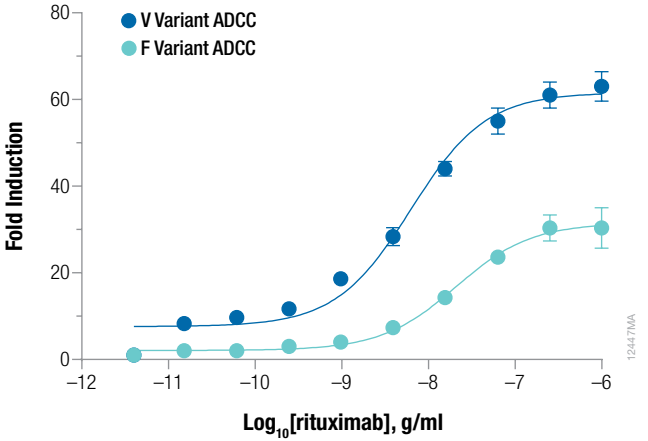

Figure 5. Comparison of V and F Variant ADCC Reporter Bioassays. Serial dilutions of anti-CD20 rituximab were incubated for 6 hours at 37°C with engineered Jurkat effector cells expressing FcγRIIIa V158 or F158 (ADCC Bioassay Effector Cells, V Variant or F Variant) and ADCC Bioassay Target Cells (WIL2-S). These data were generated using frozen, thaw-and-use cells. Luciferase activity was quantified using Bio-Glo™ Reagent. Data were fitted to a 4PL curve using GraphPad Prism® software.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
ADCC Bioassay Effector Cells, F Variant, Propagation Model	**2 vials**	**G9302**

Not for Medical Diagnostic Use.

Includes: two vials at 2×10^7 cells/ml and 1.0ml/vial. One vial should be thawed, propagated and cells frozen to create a cell bank. The remaining vial should be reserved as backup.

Storage Conditions: Upon arrival, immediately transfer vials of ADCC Bioassay Effector Cells, F Variant, for long-term storage below –140°C (freezer or liquid nitrogen vapor phase). For safety reasons do not store cell vials submerged in liquid nitrogen.

3. General Considerations

The ADCC Reporter Bioassay with ADCC Bioassay Effector Cells, F Variant, Propagation Model, differs from classic ADCC assays in a number of ways. **Please read through the entire protocol for this system to become familiar with the assay, the components and the protocol in general before beginning.** The ADCC Bioassay Effector Cells, Propagation Model, should be handled as described in Section 5 for initial culture and for propagation. Cell seeding densities and propagation cell density range have been selected to provide good assay performance and consistency. Cell freezing protocols also are described in Section 5 and should be followed carefully.

The effector:target (E:T) cell ratio, the cell number per well for target and effector cells, the antibody dose range, assay buffer and incubation times may differ from those used in a classic ADCC assay with PBMCs or natural killer cells as effector cells. **We recommend that you evaluate these parameters rigorously and select the best conditions for your target system.**

Because the ADCC Reporter Bioassay produces a bioluminescent readout, the assay requires a sensitive luminometer or luminescence plate reader for the detection of signal. See Related Products, Section 11, for a list of GloMax®-Multi+ Detection Systems available from Promega. The bioassay produces a strong signal; therefore, an integration time of 0.5sec/well should be sufficient. If your luminometer/plate reader requires gain adjustment for luminescence, use the well with the highest Ab concentration. Finally, if you have the ability to select the multiwell plate type in your reader's software and that multiwell plate is not listed in the software, a generic 96-well plate selection will suffice. We recommend white, flat-bottom 96-well assay plates (e.g., Corning Cat.# 3917).

4. Before You Begin

4.A. Materials to Be Supplied by the User

(Composition of buffers and media are provided in Sections 4.B and 9.)

Reagents

- RPMI 1640 with L-glutamine and HEPES (Gibco Cat.# 22400)
- FBS (HyClone Cat.# SH300070)
- super low IgG FBS (HyClone Cat.# SH30898)
- DPBS (Gibco Cat.# 14190)
- hygromycin (Gibco Cat.# 10687-010)
- Antibiotic G-418 Sulfate Solution (Cat.# V8091)
- sodium pyruvate (Gibco Cat.# 11360), 1mM
- MEM nonessential amino acids (Gibco Cat.# 1114)
- DMSO (Sigma Cat.# D2650)
- trypan blue solution (Sigma Cat.# T8154)
- monoclonal antibody or derivative with Fc effector function
- user-defined target cells expressing target antigen recognized by the mAb or derivative
- Bio-Glo™ Luciferase Assay System (Cat.# G7940 or Cat.# G7941)

Supplies and Equipment

- bottles for preparation of media and buffer [We recommend Nalgene square media bottles (PETG).]
- sterile clear 96-well, V-bottom plate with lid (Linbro Cat.# 76-223-05 or equivalent) for preparing antibody dilutions
- white, flat-bottom 96-well assay plates (Corning Cat.# 3917 or equivalent)
- pipettes (single channel and 12-channel)
- sterile 15ml and 50ml conical tubes
- sterile reagent reservoirs (Corning Cat.# 4870 or equivalent)
- 37°C CO_2 incubator
- 37°C water bath
- plate reader with glow luminescence read capability or luminometer (e.g., GloMax® Discover Detection System)
- cryogenic vials

4.B. Composition of Required Media and Buffers

Cell Growth Medium

90%	RPMI 1640 with L-glutamine
10%	FBS
100µg/ml	hygromycin
250µg/ml	Antibiotic G-418 Sulfate Solution
1mM	sodium pyruvate
0.1mM	MEM nonessential amino acids

Cell Freezing Medium

85%	RPMI 1640 with L-glutamine
10%	FBS
5%	DMSO

ADCC Assay Buffer

99.5%	RPMI 1640 with L-glutamine
0.5%	low IgG FBS

5. Preassay Cell Procedures for ADCC Bioassay Effector Cells, F Variant, Propagation Model

5.A. Thawing and Initial Culture Procedure for ADCC Bioassay Effector Cells, F Variant, Propagation Model

Note: Always follow proper aseptic technique when working with cells in culture.

1. Rapidly thaw the cells by placing cryovials in a 37°C water bath with gentle agitation for 1–2 minutes.
2. Decontaminate the vial by wiping with 70% ethanol before opening in a class II biological safety cabinet.
3. Gently transfer the contents of the vial into 10ml of Cell Growth Medium in a sterile 15ml conical tube.
4. Centrifuge cells at **90 × *g* for 10 minutes** at ambient temperature.

   Note: Higher speeds may reduce cell viability.
5. Aspirate the supernatant, and resuspend the cell pellet in 10ml of Cell Growth Medium (prewarmed to 37°C).
6. Transfer resuspended cells to a T75 flask, and add 15ml of prewarmed Cell Growth Medium for initial culture volume of **25ml** in a T75 flask. Place flasks horizontally, and allow the cells to grow 2 days at 37°C and 5% CO_2 in a humidified incubator; monitor cell density until it reaches 1.2–2.2 × 10^6 cells/ml.

   Note: Lower culture volumes may reduce cell viability.

5.B. Propagation of ADCC Bioassay Effector Cells

For subculture, we recommend a cell seeding density of 3–4.5 × 10^5 cells/ml and maintaining cells in Cell Growth Medium in the range of **3 × 10^5 cells/ml to 2.2 × 10^6 cells/ml**. For two-day passage, we recommend seeding at **4.5 × 10^5 cells/ml**, and for three-day passage, we recommend seeding at **3 × 10^5 cells/ml.** The culture can be maintained by addition of fresh Cell Growth Medium to the cell suspension in a new flask while keeping the culture volume to flask surface area ratio consistent for each size of propagation flask (**25ml** total culture per T75 flask, or 50ml total culture per T150 flask). Always keep flasks in a **horizontal** position in the CO_2 incubator during culture.

Note: Changing culture volumes, seeding densities or propagation density range may affect cell growth rate and performance of the cells in the assay.

5.B. Propagation of ADCC Bioassay Effector Cells (continued)

1. Determine cell density in the flask after 2–3 days in culture, and include trypan blue staining during counting to monitor cell viability.
2. Determine appropriate volume of cell suspension to use, based on seeding density at 3–4.5 × 10^5 cells per milliliter, and transfer this volume of cells to a new flask.
3. Add appropriate volume of fresh Cell Growth Medium to the flask to achieve the desired volume.
4. Place flasks horizontally in a 5% CO_2, 37°C humidified incubator.

   Note: Wait to use cells in an ADCC bioassay or for cell banking until the cell doubling rate has stabilized, which should occur within 7–10 days. We recommend that you demonstrate stabilization across two consecutive doublings. The typical stabilized doubling rate is 26 ± 8 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 2-day/2-day/3-day repeating schedule). Typical viability is 95% during propagation.

5.C. Freezing Cells to Create Cell Banks

1. Determine cell density, and include trypan blue staining during counting for viability determination.
2. Transfer cell suspension to a 50ml conical tube, and pellet cells at 130 × *g* for 10 minutes at room temperature.
3. Resuspend cells in 1X DPBS, and recentrifuge at 130 × *g* for 10 minutes.
4. Resuspend cells in appropriate volume of Cell Freezing Medium (prechilled on ice) so that the final cell density is 5 × 10^6 cells/ml.
5. Dispense 1.0ml of cells per cryogenic vial.
6. Freeze cells using a controlled-rate freezer following a recommended program for vials. Alternatively, place vials in a suitable container (e.g., Mr. Frosty, Thermo Scientific/Nalgene, Cat.# 5100-0001) for slow cooling, and store overnight at –80°C.
7. Transfer vials to a –140°C freezer or into a liquid nitrogen storage tank, and store cells in the vapor phase of liquid nitrogen.

6. Protocol for ADCC Bioassay Effector Cells, Propagation Model

Our recommended protocol for using the Propagation Model cells, described here as an example protocol, is designed to test two antibody samples in a single assay run. Each test antibody and a reference antibody are run in triplicate in a ten-point dilution series in a single 96-well assay plate using the same target cells for a total of two plates. Other protocols and plate layouts are possible and may need to be optimized for your specific target cells and antibody.

To routinely use the ADCC Bioassay Effector Cells, F Variant, Propagation Model, with the antibody and target cell line of your choice in the ADCC Reporter Bioassay, we recommend first optimizing the E:T ratio by fixing the ADCC Bioassay Effector Cells at 150,000 per well for a 96-well plate and varying the cell number of the target cells. You should establish an E:T ratio that gives a good signal response. You also may need to optimize the dose-range of antibody and serial dilutions of antibody you use to achieve a full dose-response curve with proper upper and lower

asymptotes and sufficient points in the middle of the dose range to achieve reliable values for the fitted slope and the EC_{50}. Induction for 16–24 hours is a good starting place for the assay and has given optimal results for most antibodies we have tested. You can vary the induction time in a range of 6–24 hours to determine the optimal induction time for your antibody. These parameters may differ from those used in a classic ADCC assay with PBMCs or NK cells as effector cells. We recommend that you evaluate these parameters rigorously and select the best conditions for your target system.

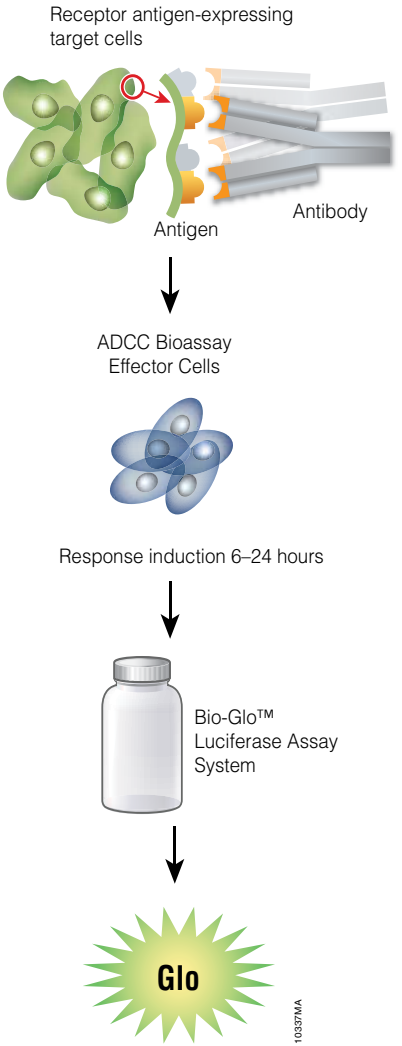

Figure 6. Schematic protocol for the ADCC Reporter Bioassay. For the Cell Propagation Model, we recommend 16–24 hours for induction.

6.A. Preparation of Components, Reagents and Bioassay Starting Materials

1. **Bio-Glo™ Luciferase Assay Reagent:** Prepare an appropriate amount of Bio-Glo™ Luciferase Assay Reagent according to the manufacturer's instructions the day before starting the assay. Thaw the Bio-Glo™ Luciferase Assay Buffer in a room-temperature water bath, and equilibrate Bio-Glo™ Luciferase Assay Substrate to ambient temperature, protected from light. Transfer the buffer into the amber bottle containing the Substrate, and mix by inversion until the Substrate is thoroughly dissolved. Dispense 10ml aliquots of reconstituted Bio-Glo™ Luciferase Assay Reagent, and store at −20°C for up to six weeks. Avoid frequent freeze-thaw cycles.

   For the assay, thaw the appropriate amount of reconstituted Bio-Glo™ Luciferase Assay Reagent in a room-temperature water bath at least 1−2 hours before use on the day of assay. For your reference, 10ml of Bio-Glo™ Luciferase Assay Reagent is enough for 120 assay wells in a 96-well assay format. Approximate stability of Bio-Glo™ Luciferase Assay Reagent after reconstitution: 18% loss of luminescence over 24 hours at ambient temperature.

2. **ADCC Assay Buffer:** Prepare the appropriate amount of ADCC Assay Buffer first thing on the day of the assay. Thaw the low IgG FBS in a 37°C water bath, and add to RPMI 1640 medium to make ADCC Assay Buffer (RPMI 1640/0.5% low IgG FBS). Mix well and warm to 37°C prior to use. For your reference, 35−50ml of ADCC Assay Buffer is enough for 120 wells in 96-well assay format.

   Note: The recommended ADCC Assay Buffer contains 0.5% low IgG FBS. This works well for the majority of target cells that we have tested in ADCC Reporter Bioassay. If you experience any target cell viability or assay performance issues with this ADCC Assay Buffer, we suggest that you test several other serum concentrations (in the range of 0.5−10%) to determine the optimal serum concentration for your test antibody and target cells. Alternatively you can substitute low IgG FBS with BSA in the assay buffer. Further optimization is needed to determine the range of BSA needed in the assay buffer.

3. **Starting dilutions** (dilu1, 3X final concentration) **for Reference antibody and two Test antibodies:** Decide the starting concentration (1X) for reference antibody and two test antibody samples based on previous testing results in conventional ADCC cytotoxicity assay if available. If the working concentration of test antibody is unknown, use 1−5µg/ml as starting concentration, and adjust later based on the assay results; this concentration range has worked for both rituximab and trastuzumab in the ADCC Reporter Bioassay.

   Make 400µl of starting dilution for the reference antibody (dilu1, 3X final concentration) and make 200µl of starting dilution for each of the test antibodies (dilu1, 3X final concentration). Use ADCC Assay Buffer to prepare and dilute antibodies in 1.5ml microcentrifuge tubes. Store the tubes containing the antibody starting dilutions appropriately before making antibody serial dilutions.

6.B. Plate Layout Design

We recommend orienting samples within an assay plate in a non-clustered fashion to help minimize any well positional effects on the response. For the protocol we describe here, use the plate layouts in Figure 7 as a guide. **The protocol uses serial replicate dilutions (n = 3) of reference antibody and each of the test 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	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 7. Example plate layout showing non-clustered sample locations of reference antibody dilution series, a single test antibody dilution series, and ADCC Assay Buffer control, color coded for location.

6.C. Preparing and Plating Target Cells

Recommendations for Plating Target Cells

To prepare the target cells for use with the ADCC Bioassay Effector Cells, F Variant, Propagation Model, the cells need to be cultured using standard practices to maintain viability of cells and density in a range satisfactory for good performance in a conventional ADCC assay. Several suspension target cell lines and adherent target cell lines grown in continuous culture have been tested in the ADCC Reporter Bioassay and the results are shown in Figures 9 and 10.

As a possible alternative that may fit your needs, we have identified appropriate cell growth and freezing conditions that allow specific target cells to be used directly in bioassay without cell culture after thaw. Two suspension B-cell lines (WIL2-S and Raji) have been prepared in frozen, thaw-and-use formats and have demonstrated good results in the ADCC Reporter Bioassay. These Target B-cell lines are currently available from Promega as components in ADCC Reporter Bioassay, Target Kits [Cat.# G7013 (WIL2-S) and Cat.# G7016 (Raji)] or ADCC Reporter Bioassay Complete Kits [Cat.# G7014 (WILS2-S) or Cat.# G7015 (Raji)]. If your target is expressed on these cells, you have the option to use one of the frozen, thaw-and-use target cell lines as provided in these kits.

For assay optimization, try E:T ratios in the range of 2.5:1 to 25:1. Keep the cell density of the ADCC Bioassay Effector cells constant, and vary the cell density of the target cells. As a reference, we use 150,000 cells per well for ADCC Bioassay Effector Cells and an E:T ratio of 6:1 when working with ADCC Bioassay Target Cells and an anti-CD20 antibody.

The target cells are the first assay component added to the assay plate in the ADCC Reporter Bioassay. Antibody is added next, then the ADCC Bioassay Effector Cells. Assay plates are then incubated for induction of reporter gene expression.

6.C. Preparing and Plating Target Cells (continued)

Preparation and plating of suspension target cell lines from continuous culture: On the day of assay, first estimate the target cell numbers needed. Harvest enough target cells (two to three times the required cell number) by centrifugation at 130–200 × *g* for 10 minutes, wash cells once with 10ml of DPBS, and recentrifuge. Resuspend cells in ADCC Assay Buffer (prewarmed to 37°C) to give a cell volume of approximately 70% of the final required and to provide the appropriate cell density that results in the required target cell number in 25µl in each assay well. Count the cells, and evaluate the viability using trypan blue staining. After cell counting, adjust the volume of cells to generate the cell density you need for bioassay. Transfer the cell suspension to a sterile reagent reservoir. Using a multichannel pipette immediately add 25µl of cells to the inner 60 wells of white 96-well assay plates. Dispense 75µl of ADCC Assay Buffer into outermost wells, labeled "B" in Figure 7. Cover plates with lids, and keep the plates in a 5% CO_2 tissue culture incubator at 37°C before adding antibody dilutions and ADCC Bioassay Effector Cells.

Preparation and plating of adherent target cell lines from continuous culture: Twenty to twenty four hours before the assay, remove the cells from the propagation flasks by trypsinization (or other standard procedure), and resuspend the cells in the appropriate volume of fresh culture medium. Count the cells and evaluate cell viability using trypan blue. Centrifuge cells at 130–200 × *g*, depending on established conditions for your target cells. Resuspend cells in fresh culture medium at an appropriate cell density, so that there will be the appropriate cell number required for each well in the ADCC bioassay when you plate 100µl of cells per well. Transfer the cells to a sterile reagent reservoir and immediately add 100µl of cells to the inner 60 wells of white 96-well assay plates using a multichannel pipette. Dispense 100µl of culture medium into those outermost wells, labeled "B" in Figure 7. Place lids on the plates, and incubate overnight in a CO_2 incubator at 37°C.

On the morning of the assay, use a multichannel pipette to remove 95µl of culture medium from each well. Add 25µl per well of ADCC Assay Buffer (prewarmed to 37°C) to the inner 60 wells of both assay plates. Always allow the pipette tips to touch the wall of the well, and add buffer gently to the wells to minimize disruption of cells. Dispense 75µl of ADCC Assay Buffer into those outermost wells, labeled "B" in Figure 7, of both assay plates. Cover the plates with lids, and keep the plates in a CO_2 incubator at 37°C before adding antibody dilutions and ADCC Bioassay Effector Cells.

6.D. Preparing Antibody Serial Dilutions

Preparing Serial Dilutions From a Single Antibody Dilution Stock to Generate Triplicates

Note: Alternatively, you can make three independent antibody dilution stocks to generate triplicates of each dose-response curve.

To establish a full dose-response range for any antibody to be tested in the ADCC Reporter Bioassay, we suggest that you first determine the starting concentrations and serial dilution schemes optimal for the antibody based on previous test results if possible (e.g., data from ADCC cytotoxicity assay). For your reference, when tested in ADCC Reporter Bioassay, F Variant, with various target cells, the starting concentrations and serial dilution schemes are 1–5 × 10^{-6}g/ml, fourfold serial dilution for rituximab, and 1–5 × 10^{-6}g/ml, threefold serial dilution for trastuzumab. These provide full dose-response curves in both cases.

The instructions below are for generating threefold serial dilution series for the reference antibody and test antibodies and serve as an example dilution series. If different serial dilution schemes are needed, please adjust the volumes accordingly. If each of the antibodies requires a different dilution scheme, please make separate serial dilutions for each antibody. Prepare 100µl for each antibody dilution to provide sufficient volume for triplicates in the assay.

1. Use a sterile clear V-bottom 96-well plate for preparing antibody serial dilutions. For threefold serial dilutions, perform the dilutions described in Steps 2–8 below. You will need 400µl of reference antibody at 3X the highest antibody concentration in your dose-response curve. You will need 200µl of each test antibody at 3X the highest antibody concentration in each of the dose-response curves. Adjust all volumes accordingly for other dilution schemes.
2. Add 150µl of reference antibody starting dilution (dilu1, 3X final concentration) to both well A11 and well B11.
3. Add 150µl of test antibody 1 and 150µl of test antibody 2 starting dilution (dilu1, 3X final concentration) to well E11 and well G11, respectively (see Figure 8).
4. Add 100µl of ADCC Assay Buffer to other wells in these four rows, from column 10 to column 2.
5. Transfer 50µl from the antibody starting dilutions in column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
6. Repeat equivalent threefold serial dilutions across columns from right to left until you reach column 3.

   Note: Wells A2, B2, E2 and G2 will contain 100µl of ADCC Assay Buffer as a "no-antibody" control.
7. Place the plate with antibody dilutions on the bench during preparation of ADCC Bioassay Effector Cells at the next step. Cover with a lid.

Recommended Plate Layout 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													
D													
E		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test Ab 1
F													
G		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test Ab 2
H													

Figure 8. Example plate layout showing serial dilutions of antibodies. Reference and test antibodies for serial dilutions from a single antibody stock to generate triplicates.

6.E. Preparing ADCC Bioassay Effector Cells

Grow ADCC Bioassay Effector Cells for use in the assay under the propagation protocol we recommend. Do not exceed the recommended maximum cell density, and adhere to the 2- to 3-day regimen for splitting and to the specified seeding density range for expansion. Use cells in assay only after cell doubling during propagation has stabilized. Stabilization after initial seeding should occur within 7 to 10 days. We recommend that you demonstrate stabilization across two consecutive doublings. Doubling rates of 26 hours ± 33% should be expected if you follow the propagation protocol. The propagation protocol provides assay consistency.

1. Transfer ADCC Bioassay Effector Cells from the propagation flask to a 50ml conical tube, and count the cells to determine cell density. Use Trypan Blue staining to evaluate cell viability. To ensure appropriate functional performance in the ADCC Reporter Bioassay, the ideal density upon harvest is 1.4–2.1 × 10^6 cells/ml, and the cell viability is greater than 95%.
2. Pellet cells at 130 × *g* for 10 minutes at ambient temperature, wash cells with 10ml of 1X DPBS and recentrifuge at 130 × *g* for 10 minutes. Resuspend cells in ADCC Assay Buffer at 70% of full volume needed to generate a cell suspension at 6 × 10^6 cells/ml, based on the cell counts determined in Step 1.
3. Count the cells again to evaluate whether there has been any cell loss during the centrifugation. Adjust the volume of ADCC Assay Buffer based on the second cell count to make a final cell suspension at a density of 6 × 10^6 cells/ml.

6.F. Adding Antibody and ADCC Bioassay Effector Cells to Target Cells in Assay Plates

1. Using a multichannel pipette, add 25µl per well of the antibody dilution series from the antibody dilution plates you prepared in Section 6.D to the white, 96-well assay plates already containing target cells, according to the plate layout in Figure 7.
2. Transfer the ADCC Bioassay Effector cells prepared in Section 6.E to a sterile reagent reservoir. Using a multichannel pipette, plate 25µl of ADCC Bioassay Effector Cells to those wells in the assay plate already containing target cells and antibody to yield 150,000 ADCC Bioassay Effector Cells per well.
3. Cover the assay plate with a lid, and incubate the plate in a 37°C CO_2 tissue culture incubator for the recommended 16–24 hours.

6.G. Adding Bio-Glo™ Luciferase Assay Reagent

1. Remove assay plates from the 37°C incubator and equilibrate to ambient temperature (22–25°C) on the bench for 15 minutes.
2. Using a manual multichannel pipette, add 75µl of Bio-Glo™ Luciferase Assay Reagent to the inner 60 wells of both assay plates; avoid creating any bubbles.

   Note: Bio-Glo™ Luciferase Assay Reagent should be at ambient temperature when added.
3. Add 75µl of Bio-Glo™ Luciferase Assay Reagent to wells B1, C1 and D1 in each assay plate to determine plate background.
4. Incubate at ambient temperature for 5–30 minutes.
5. Measure luminescence using a plate reader with glow-type luminescence reading capabilities.

6.H. Data Analysis

1. Determine Plate Background by calculating the average RLU from wells B1, C1 and D1.
2. Calculate Fold of Induction = RLU (induced–background) /RLU (no antibody control–background)

   Note: When calculating Fold of Induction, if the sample RLUs are equal to or greater than 100 times higher than the plate background RLU, there is no need to subtract plate background from sample RLU.
3. Graph data as RLU versus Log_{10} [antibody] and Fold of Induction versus Log_{10} [antibody]. Fit curves and determine EC_{50} of antibody response using appropriate curve fitting software (such as GraphPad Prism® software).

7. 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
High background	As a bioluminescent assay, the ADCC Reporter Bioassay generally gives low assay background and high signal response. There are multiple possible causes for high background such as a matrix effect from assay buffer or antibody stock solution, signal cross talk from neighboring wells due to use of unsuitable assay plates or improper settings for the detection instrument. See also the "Possible issues with matrix effect" comments below.
Poor or low luminescence measurements (RLU readout)	Choose a sensitive instrument designed for luminescence detection. Instruments primarily designed for fluorescence detection are not recommended. Luminometers measure and report luminescence as relative values, and actual numbers will vary between instruments. See General Considerations, Section 3, for more recommendations on how to set up the luminometer. If you must use an instrument primarily designed for fluorescence detection, ensure no filters are used.
	Insufficient effector cells could lead to low RLU. Handle and plate the effector cells appropriately according to the instructions in this protocol to ensure that there are sufficient viable effector cells per well in the assay.
	Low activity of Bio-Glo™ Luciferase Assay Reagent also leads to low RLU. Store and handle Bio-Glo™ Luciferase Assay Reagent appropriately according to the instructions in the protocol.
	Weak ADCC response (see section below).

7. Troubleshooting (continued)

Symptoms	Possible Causes and Comments
Possible issues with matrix effect	IgG, serum complement or other components from serum, supernatant of phage display or hybridoma culture could nonspecifically impact antibody binding to the FcγRIIIa receptor or affect NFAT-RE signaling pathway directly and cause a matrix effect. Use Low IgG Serum or perform further dilution of antibody starting preparation to minimize any matrix effect. The use of heat-inactivated or Low IgG Serum for growth of target cells also helps.
Weak ADCC response	Optimize the E:T ratio while keeping the effector cell number constant at 150,000 cells per well. Since the readout of the ADCC Reporter Bioassay is from the effector cells, improvement of the response can be achieved by increasing the number of target cells per well.
	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 EC_{50} of antibody in ADCC Reporter Bioassay is not necessarily the same as that from other ADCC bioassays, thus some adjustment on the antibody starting concentration and serial dilution schemes may be needed to achieve the maximal response in the ADCC Reporter Bioassay.
	Optimize assay incubation time within a range of 6–24 hours, and choose the incubation time that gives optimal ADCC response.
	Optimize the composition of ADCC 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 ADCC response.
Will I see the same ranking of therapeutic Abs in the Promega ADCC Reporter Bioassay as in a classic ADCC bioassay?	The ADCC Reporter Bioassay and classic ADCC assays show the same expected relative potency differences for Ab variants known to differ in ADCC efficiencies. This has been observed in several different studies using antibodies that differ in glycosylation, including fucosylation, and amino acid sequence. **Note:** Assays should be performed using conditions that can differentiate activities in the ranges expected. These conditions may not be the same or both assays.

Symptoms	Possible Causes and Comments
EC_{50} for Ab varies between classic and ADCC bioassay and Promega ADCC Reporter Bioassay	EC_{50} refers to the concentration of the substance (mAb in in this assay) that gives 50% of the maximal biological response. The EC_{50} value is determined not only by the binding affinity of the antibody but also by the assay conditions used in that particular assay such as the E:T ratio, incubation time and assay buffer in the case of ADCC bioassays. The EC_{50} value of any antibody can differ dramatically between different assays and is not an intrinsic property of the antibody. It is normal if the EC_{50} value for an Ab differs between ADCC Reporter Bioassay and other ADCC bioassays.
Low viability of target cells or effector cells grown in continuous culture	Propagate and freeze the ADCC Bioassay Effector Cells in the exact manner described in this manual. Keep the viability of your target cells and ADCC Bioassay Effector Cells high and constant. This provides the best inter-assay precision and consistent data for your reference antibody. Some dead cells are acceptable, and the assay response should be robust within a defined range of cell density and viability. Should a low number of viable cells be evident, do not use them because your assay performance will be compromised.

8. References

1. Hogarth, P.M. and Pietersz, G.A. (2012) Fc receptor-targeted therapies for the treatment of inflammation, cancer and beyond. *Nat. Rev. Drug Discov.* **11**, 311–31.
2. Chung, S. *et al.* (2014) Characterization of in vitro antibody-dependent cell-mediated cytotoxicity activity of therapeutic antibodies - impact of effector cells. *J Immunol Methods* **407**, 63–75.
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. Cheng, Z.J. *et al.* (2014) Development of a robust reporter-based ADCC assay with frozen, thaw-and-use cells to measure Fc effector function of therapeutic antibodies. *J. Immunol. Methods.* **414**, 69–81.

9. Composition of Buffers and Media

Cell Growth Medium

90%	RPMI 1640 with L-glutamine
10%	FBS
100µg/ml	hygromycin
250µg/ml	Antibiotic G-418 Sulfate Solution
1mM	sodium pyruvate
0.1mM	MEM nonessential amino acids

Cell Freezing Medium

85%	RPMI 1640 with L-glutamine
10%	FBS
5%	DMSO

ADCC Assay Buffer

99.5%	RPMI 1640 with L-glutamine
0.5%	low IgG FBS

10. Appendix: Representative Assay Results

The following data were generated using the ADCC Bioassay Effector Cells, F Variant, Propagation Model, with either suspension (Figure 9) or adherent target cells (Figure 10) grown in continuous culture prior to bioassay.

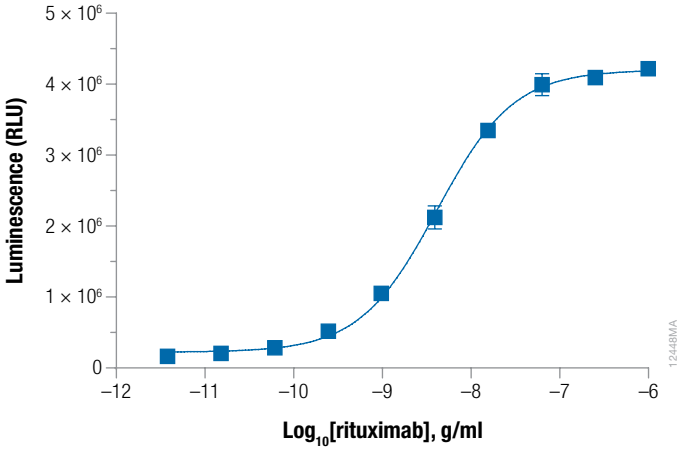

Figure 9. ADCC Reporter Bioassay response to rituximab (RITUXAN®) using ADCC Bioassay Effector Cells, F Variant. CD20+ WIL2-S cells were harvested and plated in a 96-well white assay plate at 20,000 cells per well, followed by addition of a series of concentrations of rituximab. ADCC Bioassay Effector Cells, F Variant (150,000 cells per well) were then added to the assay plate. After 20 hours of induction at 37°C, Bio-Glo™ Luciferase Assay Reagent was added and luminescence was determined using a GloMax®-Multi+ Luminometer. The data were fitted to a 4PL curve using GraphPad Prism® software.

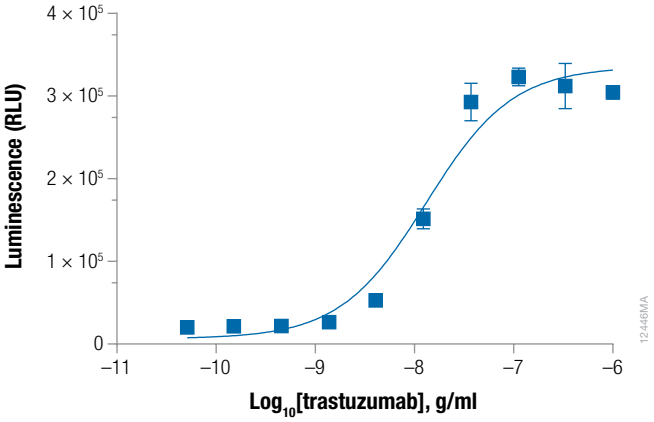

Figure 10. ADCC Reporter Bioassay response to trastuzumab (trade name: HERCEPTIN®) using ADCC Bioassay Effector Cells, F Variant. Her2+ SK-BR-3 cells were plated in 96-well assay plate at 10,000 cells per well in culture medium overnight before bioassay. On the day of assay, the culture medium was removed carefully and replaced with a series of concentrations of trastuzumab. After 5–10 minutes of incubation on the bench top, 150,000 cells per well of ADCC Bioassay Effector Cells, F Variant, were added to the assay plate already containing SK-BR-3 cells and antibody. After 20 hours of induction at 37°C, Bio-Glo™ Luciferase Assay Reagent was added and luminescence determined using a GloMax®-Multi+ Luminometer. Data were fitted to a 4PL curve using GraphPad Prism® software

11. Related Products

ADCC Reporter Bioassays and Detection Reagent

Product	Size	Cat.#
ADCC Reporter Bioassay, Core Kit**	1 each	G7010
ADCC Reporter Bioassay, Core Kit (5X)**	1 each	G7018
ADCC Reporter Bioassay, F Variant, Core Kit**	1 each	G9790
ADCC Reporter Bioassay, F Variant, Core Kit (5X)**	1 each	G9798
ADCC Reporter Bioassay, Target Kit (WIL2-S)**	1 each	G7013
ADCC Reporter Bioassay, Complete Kit (WIL2-S)**	1 each	G7014
ADCC Reporter Bioassay, Target Kit (Raji)**	1 each	G7016
ADCC Reporter Bioassay, Complete Kit (Raji)**	1 each	G7015
Bio-Glo™ Luciferase Assay System*	100ml	G7940
	10ml	G7941

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

11. Related Products (continued)

Luminometers

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

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

12. Summary of Changes

The following changes were made to the 3/16 revision of this document:

1. Added new legal disclaimer statement.

[a]**ADCC Bioassay Effector Cells Propagation Limited Use Label License**

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