TECHNICAL MANUAL

ADCC Reporter Bioassay, F Variant, Core Kit

Instructions for Use of Products
G9790 and G9798

Revised 8/19
TM427

ADCC Reporter Bioassay, F Variant, Core Kit

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 antibody drugs in an ADCC mechanism of action (MOA) assay. The assay combines a simple, add-mix-read format and an optimized protocol to provide a bioassay that has low variability and high accuracy. Moreover the bioassay can be performed in a single day. These performance characteristics make the bioassay suitable for application across antibody drug research and development. The thaw-and-use cells provided in the ADCC Reporter Bioassay, Variant, Core Kits are generated under highly controlled conditions that drive low assay variability run to run, while providing the convenience of an assay reagent that eliminates the need to propagate cells each time.

A note on kit formats: We offer the ADCC Reporter Bioassay technology in multiple formats to better meet research needs. In addition to the ADCC Reporter Bioassay, F Variant, Core Kit[a–e] (Cat.# G9790 and G9798), we offer the F Variant Effector Cells for banking and propagation, the ADCC Reporter Bioassay, F Variant, Propagation Model (Cat.# G9302), under a unique purchase agreement. 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 8 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 (V158) and a lower affinity FcγRIIIa variant carrying phenylalanine (F158). 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, due to pathway activation, is high, and assay background is low.

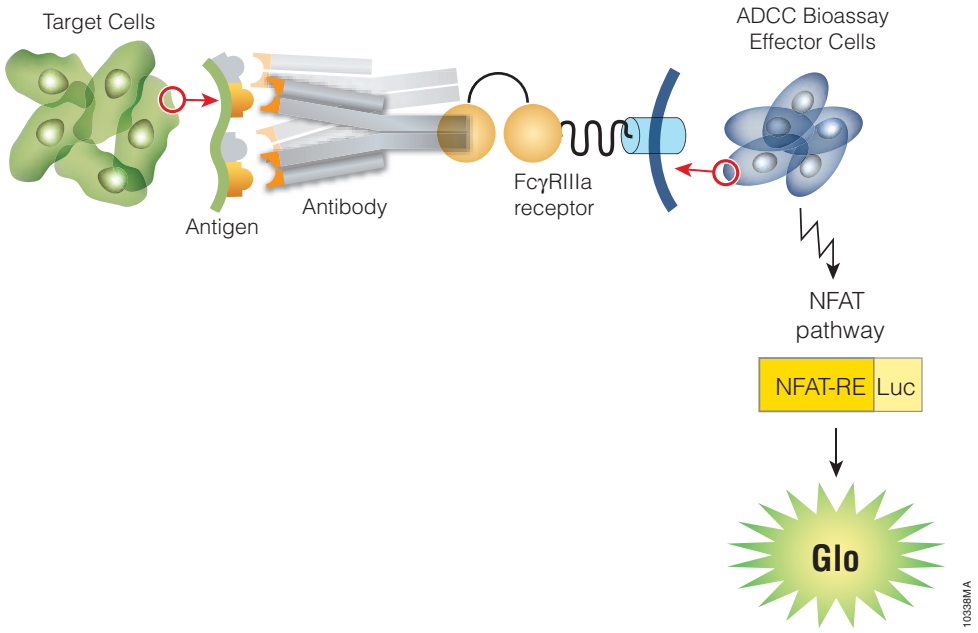

Figure 1. Representation of the ADCC Reporter Bioassay. ADCC Bioassay Effector Cells are genetically engineered Jurkat T cells stabling expressing NFAT response element driving expression of firefly luciferase and FcγRIIIa V158 variant or FcγRIIIa F158 variant. Assay readout is luminescence signal from 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 specific surface antigen, the specific antibody which recognizes the antigen protein on target cells, and effector cells expressing FcγRIIIa are present. If any one of these is missing, there is no response.

1. Description (continued)

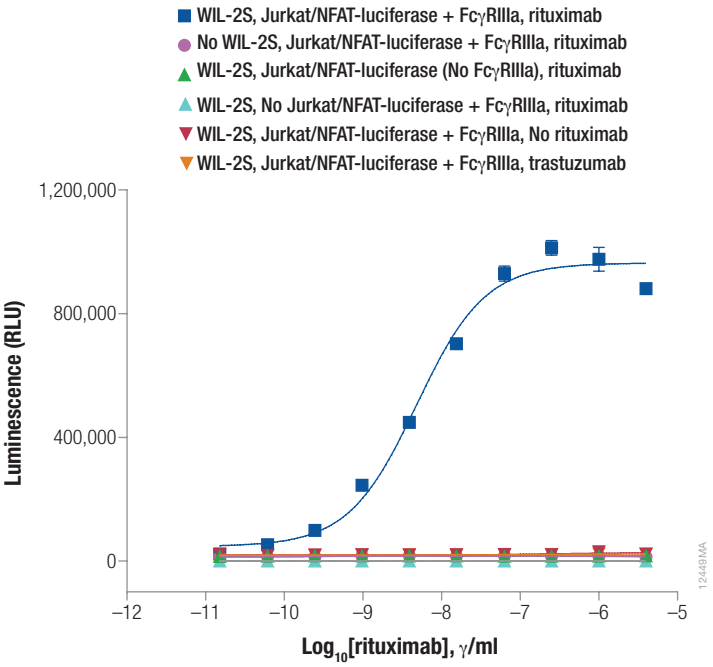

Figure 2. Specificity of the ADCC Reporter Bioassay, F Variant. Serial dilutions of rituximab (anti-CD20 chimeric monoclonal antibody drug), trastuzumab (anti-Her2 humanized monoclonal antibody drug) or assay medium control (no antibody) were incubated for 6 hours at 37°C with engineered Jurkat effector 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[c]. Data were fitted to a 4 Parameter Logistic nonlinear regression (4PL) model 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)

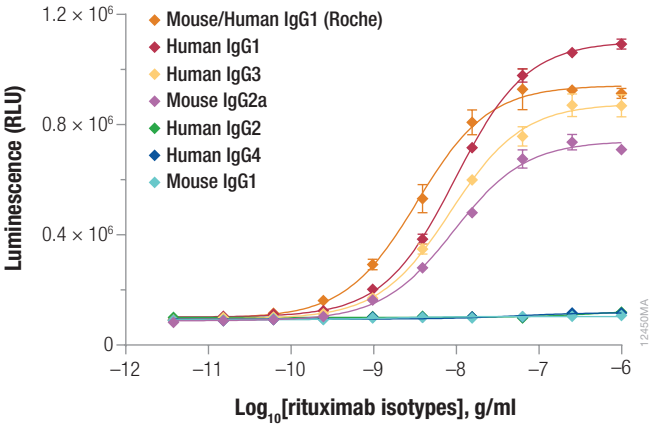

Figure 4. Antibody IgG isotype-specificity. Serial dilutions of various IgG isotopes of anti-CD20 rituximab were incubated for 6 hours at 37°C with engineered Jurkat effector cells (ADCC Bioassay Effector Cells, F Variant) and ADCC Bioassay Target Cells (WIL2-S). The therapeutic biologic, mouse/human 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.

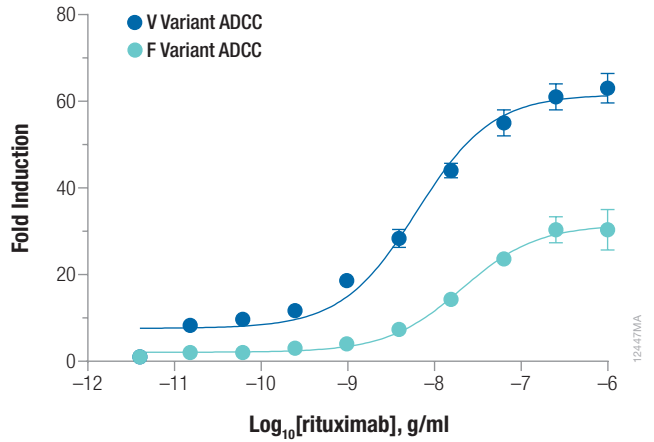

Figure 5. Comparison of V and F Variant ADCC Reporter Bioassays. Serial dilutions of anti-CD20 rituximab were incubated for 6 hours at 37°C with engineered Jurkat effector cells expressing FcγRIIIa V158 or F158 (ADCC Bioassay Effector Cells, V Variant or F Variant) and ADCC Bioassay Target Cells (WIL2-S). 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

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

PRODUCT	SIZE	CAT.#
ADCC Reporter Bioassay, F Variant, Core Kit	**1 kit**	**G9790**

Each system contains sufficient reagents for 120 assays using the inner 60 wells of two 96-well plates. Includes:

- 1 vial ADCC Bioassay Effector Cells, F Variant (0.65ml)
- 4ml Low IgG Serum
- 10ml Bio-Glo™ Luciferase Assay Buffer
- 1 vial Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 36ml RPMI 1640 Medium

PRODUCT	SIZE	CAT.#
ADCC Reporter Bioassay, F Variant, Core Kit 5X	**5 kits**	**G9798**

Each system contains sufficient reagents for 600 assays using the inner 60 wells of ten 96-well plates. Includes:

- 5 vials ADCC Bioassay Effector Cells, F Variant (0.65ml)
- 5 × 4ml Low IgG Serum
- 5 × 10ml Bio-Glo™ Luciferase Assay Buffer
- 5 vials Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 5 × 36ml RPMI 1640 Medium

Storage Conditions: Upon arrival, immediately transfer the vials of ADCC Bioassay Effector Cells, F Variant, for long-term storage below –140°C (freezer or liquid nitrogen vapor phase). The cells are sensitive, and care should be taken when handling. For safety reasons do not store cell vials submerged in liquid nitrogen. Low IgG Serum should be stored at –20°C. Avoid multiple freeze-thaw cycles of the serum. Bio-Glo™ Luciferase Assay Buffer and Bio-Glo™ Luciferase Assay Substrate should be stored at –20°C. For optimal performance, reconstituted Bio-Glo™ Luciferase Reagent should be used on the day of preparation. However, once reconstituted, Bio-Glo™ Luciferase Assay Reagent can be stored –20°C for up to 6 weeks. RPMI 1640 Medium should be stored at 4°C protected from fluorescent light.

3. General Considerations

The ADCC Reporter Bioassay differs from classic ADCC bioassays in a number of ways. Please read through the entire protocol for this kit to become familiar with the assay, the components and the protocol in general before beginning. The ADCC Bioassay Effector Cells, F Variant, when thawed and diluted as instructed, will be at the proper concentration for the bioassay. The effector:target (E:T) cell ratio, the antibody dose range, assay buffer and incubation times may differ from those used in a classic ADCC bioassay with PBMCs or natural killer cells as effector cells. We recommend that you evaluate these parameters with your target cells 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 8, for a list of GloMax® 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. Protocol for ADCC Reporter Bioassay, F Variant, Core Kit

4.A. Before You Begin

Materials to Be Supplied by the User

- user-defined target cells expressing target antigen recognized by the mAb or derivative
- reference antibody
- test antibody
- 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)

4.B. Overview

One recommended protocol, 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.

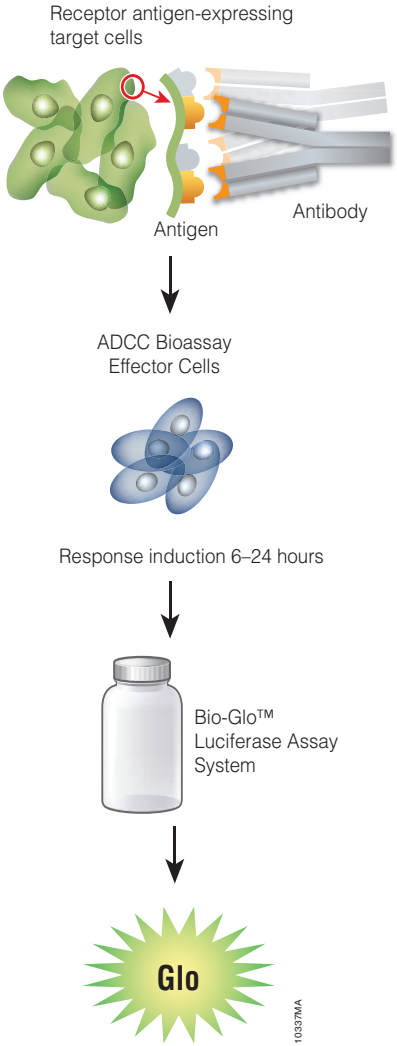

Figure 6. Schematic protocol for the ADCC Reporter Bioassay.

4.C. Preparation of Components, Reagents and Bioassay Starting Materials

1. **Bio-Glo™ Luciferase Assay Reagent:** Prepare Bio-Glo™ Luciferase Assay Reagent 4–6 hours before use on the day of assay. First, thaw one bottle of Bio-Glo™ Luciferase Assay Buffer. Equilibrate Bio-Glo™ Luciferase Assay Buffer and Bio-Glo™ Luciferase Assay Substrate to ambient temperature. Transfer the buffer into the amber bottle containing Substrate. Mix by inversion until the Substrate is thoroughly dissolved. Store reconstituted Bio-Glo™ Luciferase Assay Reagent at ambient temperature (22–25°C), protected from light. Approximate stability of Bio-Glo™ Reagent after reconstitution: 18% loss of luminescence over 24 hours at ambient temperature.
2. **ADCC Assay Buffer:** On the day of assay, thaw the Low lgG Serum in a 37°C water bath. In a 50ml conical tube, add 1.4ml Low IgG Serum to 33.6ml of RPMI 1640 Medium to make 35ml of ADCC Assay Buffer for two assay plates. Mix well and warm to 37°C prior to use.
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 1–5 µg/ml as starting concentration, and adjust later based on the assay results; this starting concentration has worked for both rituximab and trastuzumab in the ADCC Reporter Bioassay, F Variant.

   Make 400µl of starting dilution for 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.

4.D. 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	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	Test Ab
E	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Ref. 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 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.

4.E. 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, F Variant, 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 $1–5 \times 10^{-6}$g/ml, fourfold serial dilution for rituximab, and $1–5 \times 10^{-6}$g/ml, threefold serial dilution for trastuzumab. These provide full dose-response curves in both cases.

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 target 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		Ref. Ab
B		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Ref. 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.

4.F. Preparing and Plating Target Cells

Follow institutional guidelines for handling, including use of personal protective equipment (PPE), and waste disposal for biohazardous material.

Recommendations for Plating Target Cells

To prepare the target cells for use with the ADCC Bioassay Effector Cells, F Variant, Core Kit, 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 an example of the results are shown in Figure 11.

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)]. 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 75,000 cells per well for ADCC Bioassay Effector Cells, F Variant, and an E:T ratio of 6:1 when working with ADCC Bioassay Target Cells and an anti-CD20 antibody.

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.

Note: This ADCC Reporter Bioassay, F Variant, protocol recommends using ADCC Assay Buffer, which contains 4% Low IgG Serum (FBS, low IgG). If you experience any cell viability or assay performance issues with the recommended ADCC Assay Buffer, we suggest that you test several other serum concentrations (in the range of 1–10%) to determine the optimal serum concentration for your test antibody and target cells.

4.G. Adding Antibody to Target Cells in Assay Plates

1. Using a multichannel pipette, add 25µl per well of antibody dilution series from the antibody dilution plates you prepared in Section 4.D to the white, 96-well assay plates already containing target cells, according to the plate layout in Figure 7.
2. Cover plates with lids and keep the plates on the bench before adding ADCC Bioassay Effector Cells, F Variant, at the next step.

4.H. Plating ADCC Bioassay Effector Cells, F Variant

1. Label a sterile 15ml conical tube, "ADCC Bioassay Effector Cells, F Variant". Add 3.6ml of ADCC Assay Buffer (prewarmed to 37°C) to the tube.
2. Remove 1 vial of ADCC Bioassay Effector Cells, F Variant, from –140°C freezer storage or vapor phase of liquid nitrogen to dry ice for transport to the bench on day of use. Thaw vial in a 37°C water bath until cells are just thawed (about 2–3 minutes). While thawing, gently agitate and visually inspect. Do not invert.

   Note: The recommended thawing protocol above is important to the performance of the cells. No further handling is required or recommended.
3. Gently mix the cell suspension by pipetting 1 or 2 times. Transfer 630µl of cells to the 15ml "ADCC Bioassay Effector Cells, F Variant" tube containing 3.6ml of ADCC Assay Buffer. Mix well by gently inverting the tube 2 times.
4. Transfer cell suspension to a sterile reagent reservoir. Immediately, using a multichannel pipette, add 25µl of cells to the inner 60 wells of the 96-well assay plates already containing target cells and antibody.
5. Cover plates with lids, and incubate the plates for 6 hours at 37°C in a humidified CO_2 incubator. Do not stack plates within the incubator.

4.I. 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.

4.J. 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).

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
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).
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.

5. Troubleshooting (continued)

Symptoms	Possible Causes and Comments
Weak ADCC response	Optimize the E:T ratio while keeping the effector cell number constant at 75,000 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.
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.

Symptoms	Possible Causes and Comments
Some of the cells in the vial provided with the kit were dead	There will be some dead cells in the vial upon thawing, but we designed and tested the bioassay such that this will not affect performance of the bioassay as long as instructions for handling cells are followed carefully. Carefully follow the instructions on gentle thawing and handling of the cells as outlined in the protocol (Section 4.G). Use the thawed cells immediately in the assay.

6. 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.

7. Appendix: Representative Assay Results

The following data were generated using the ADCC Bioassay Effector Cells, F Variant, with the ADCC Bioassay Target Cells (Figures 9 and 10) in the thaw-and-use format or with adherent target cells grown in continuous culture prior to bioassay (Figure 11).

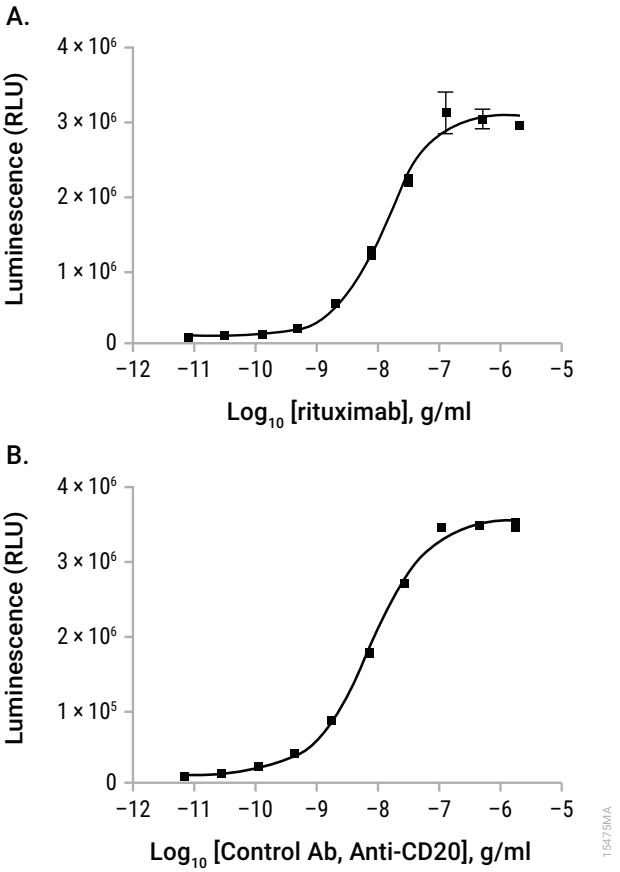

Figure 9. ADCC Reporter Bioassay response to rituximab (Panel A; trade name: RITUXAN®) or Control Ab, Anti-CD20 (Panel B). ADCC Bioassay Target Cells (WIL2-S) were incubated with a series of concentrations of rituximab or Control Ab, Anti-CD20, followed by addition of ADCC Bioassay Effector Cells, F Variant. The E:T (effector:target) ratio was 6:1. After 6 hours of induction at 37°C, Bio-Glo™ Luciferase Assay Reagent was added and luminescence was determined using a GloMax®-Multi+ Luminometer. Data were fitted to a 4PL curve using GraphPad Prism® software. The EC_{50} response determined was 12.1ng/ml for rituximab and 8.5ng/ml for the Control Ab, Anti-CD20.

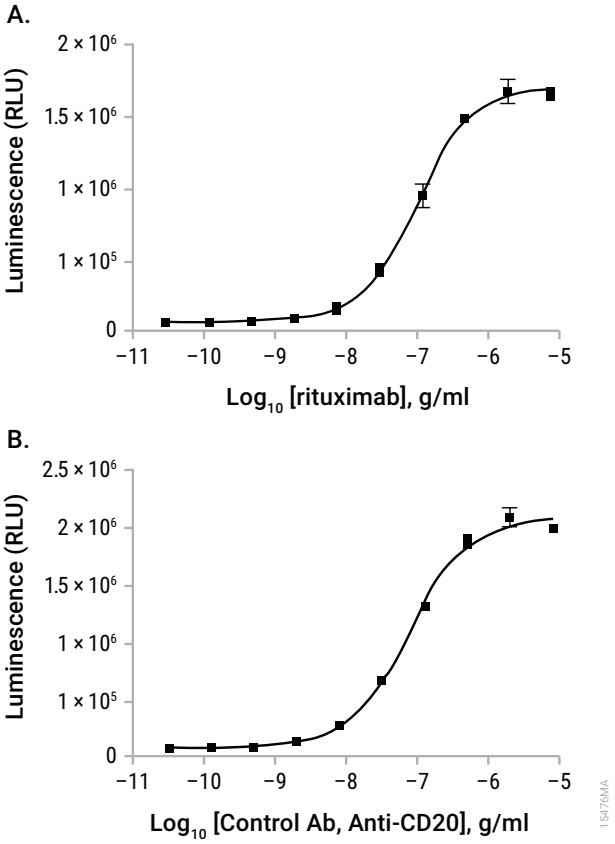

Figure 10. ADCC Reporter Bioassay response to rituximab (Panel A; trade name: RITUXAN®) or Control Ab, Anti-CD20 (Panel B). ADCC Bioassay Target Cells (Raji) were incubated with a series of concentrations of rituximab or Control Ab, Anti-CD20, followed by addition of ADCC Bioassay Effector Cells, F Variant. The E:T ratio was 6:1. After 6 hours of induction at 37°C, Bio-Glo™ Luciferase Assay Reagent was added and luminescence was determined using a GloMax®-Multi+ Luminometer. Data were fitted to a 4PL curve using GraphPad Prism® software. The EC_{50} response determined was 102.4ng/ml for rituximab and 75.2ng/ml for the Control Ab, Anti-CD20.

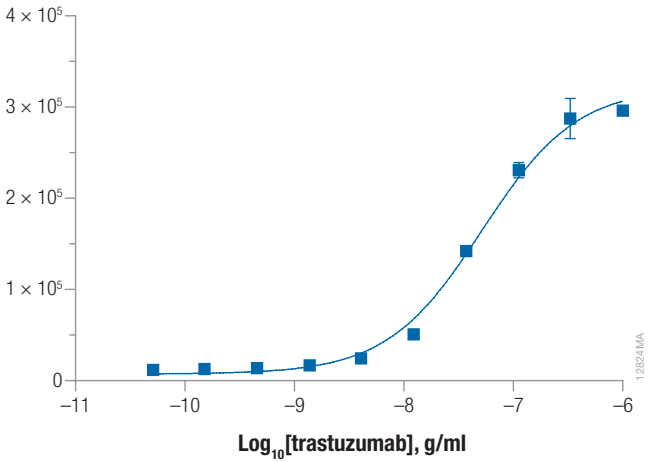

Figure 11. ADCC Reporter Bioassay response to trastuzumab (trade name: HERCEPTIN®) using ADCC Bioassay Effector Cells, F Variant and human breast cancer cell line SK-BR-3 as target cells. Cultured SK-BR-3 cells were plated at the density of 5,000 cells per well in complete culture medium overnight before bioassay. On the day of bioassay, the medium was removed carefully, and then the series of concentrations of trastuzumab were added to the cells, followed by addition of ADCC Bioassay Effector Cells, F Variant. The E:T ratio was 15:1. After 6 hours of induction at 37°C, Bio-Glo™ Luciferase Assay Reagent was added and luminescence determined using a GloMax®-Multi+ Luminometer. The data were fitted to a 4PL curve using GraphPad Prism® software. The EC_{50} of response was 51.7ng/ml for trastuzumab.

8. Related Products

T Cell Activation Bioassays

Product	Size	Cat.#
T Cell Activation Bioassay (NFAT)	1 each	J1621
T Cell Activation Bioassay (NFAT) 5X	5 each	J1625
T Cell Activation Bioassay (NFAT), Propagation Model	1 each	J1601
T Cell Activation Bioassay (IL-2)	1 each	J1651
T Cell Activation Bioassay (IL-2) 5X	5 each	J1655
T Cell Activation Bioassay (IL-2), Propagation Model	1 each	J1631

Not for Medical Diagnostic Use.

Immune Checkpoint Bioassays

Product	Size	Cat.#
4-1BB Bioassay	1 each	JA2351
4-1BB Bioassay 5X	5 each	JA2355
4-1BB Bioassay, Propagation Model	1 each	J2332
FcγRIIb CHO-K1 Cells	1 each	JA2251
FcγRIIb CHO-K1 Cells 5X	5 each	JA2255
FcγRIIb CHO-K1 Cells, Propagation Model	1 each	J2232
PD-1/PD-L1 Blockade Bioassay	1 each	J1250
PD-1/PD-L1 Blockade Bioassay 5X	5 each	J1255
PD-L1 Negative Cells	1 each	J1191
CTLA-4 Blockade Bioassay	1 each	JA3001
CTLA-4 Blockade Bioassay 5X	5 each	JA3005
TIGIT Negative Cells	1 each	J1921
PD-1+TIGIT Combination Bioassay	1 each	J2211
PD-1+TIGIT Combination Bioassay, 5X	5 each	J2215
Control Ab, Anti-CTLA-4	100µg	JA1020
Control Ab, Anti-PD-1	100µg	J1201
Control Ab, Anti-TIGIT	100µg	J2051
Control Ab, Anti-4-1BB	50µg	K1161
Control Ab, Anti-CD20	5µg	GA1130
Control Ab, Anti-LAG-3	100µg	K1150
LAG-3/MHCII Blockade Bioassay	1 each	JA1111
LAG-3/MHCII Blockade Bioassay 5X	5 each	JA1115
LAG-3/MHCII Blockade Bioassay, Propagation Model	1 each	JA2355

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Additional kit formats are available.

8. Related Products (continued)

Cytokine and Growth Factor Bioassays

Product	Size	Cat.#
IL-2 Bioassay	1 each	JA2201
IL-2 Bioassay 5X	5 each	JA2205
IL-2 Bioassay, Propagation Model	1 each	J2952
IL-15 Bioassay	1 each	JA2011
IL-15 Bioassay 5X	5 each	JA2015
IL-15 Bioassay, Propagation Model	1 each	J2962

Fc Effector Bioassays

Product	Size	Cat.#
ADCC Reporter Bioassay, Complete Kit (Raji)*	1 each	G7015
ADCC Reporter Bioassay, Target Kit (Raji)*	1 each	G7016
ADCC Reporter Bioassay, Core Kit*	1 each	G7010
ADCC Reporter Bioassay, F Variant, Core Kit**	1 each	G9790
FcγRIIa-H ADCP Reporter Bioassay, Complete Kit**	1 each	G9901
FcγRIIa-H ADCP Reporter Bioassay, Core Kit**	1 each	G9991

*For Research Use Only. Not for use in diagnostic procedures.
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Additional kit formats are available.

Note: Additional Bioassays are available from Promega Custom Assay Services. To view and order products from Custom Assay Services, see the Early Access listings at:
www.promega.com/applications/biologics-drug-discovery/functional-bioassays/target-pathway-assays/
or email: **CAS@promega.com**

Detection Reagent

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

Not for Medical Diagnostic Use.

Luminometers

Product	Size	Cat.#
GloMax® Navigator System	1 each	GM2000
GloMax® Discover System	1 each	GM3000
GloMax® Explorer System	1 each	GM3500

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9. Summary of Changes

The following changes were made to the 8/19 revision of this document:

1. Figures 9 and 10 were updated.
2. Section 8. Related Products was updated.

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