



TECHNICAL MANUAL

ADCC Reporter Bioassay, Complete Kit (Raji)

Instructions for Use of Product
G7015

ADCC Reporter Bioassay, Complete Kit (Raji)

All technical literature is available on the Internet at: www.promega.com/protocols/
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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^(a-d) 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, effector cells provided in frozen, thaw-and-use 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 applications across antibody drug research, development and manufactured lot release. The thaw-and-use cells provided in the ADCC Reporter Bioassay 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 and prepare cells each time.

1. Description (continued)

The ADCC Reporter Bioassay, Complete Kit (Raji), contains all the components and reagents necessary to perform an anti-CD20-based ADCC Reporter Bioassay. The kit contains Control Ab, Anti-CD20 (Cat.# GA1130), and is an ideal way for a new user to become acquainted with the bioassay. If your test antibody recognizes a different target antigen expressed on Raji cells, the kit can still be used to perform the ADCC Reporter Assay on your antibody. Because the ADCC Reporter Bioassay is different from a classic ADCC bioassay, we recommend evaluation of best E:T ratio and dose-range for your specific antibody/target antigen combination.

Related available products include ADCC Reporter Bioassay, Core Kit (Cat.# G7010 and G7018), which provides ADCC Bioassay Effector Cells in a thaw-and-use format; the ADCC Reporter Bioassay, Complete Kit (WIL2-S; Cat.# G7014), which includes the ADCC Bioassay Effector Cells and the ADCC Bioassay Target Cells (WIL2-S); and the ADCC Bioassay Effector Cells, Propagation Model (Cat.# G7102), through which the cells are available for banking and propagation under a unique purchase agreement.

ADCC is a desirable mechanism for killing target cancer cells using antibody-based drugs. 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 cross-linking of the two cell types occurs, leading to pathway activation of ADCC MOA (1). 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 (PBMCs) or the natural killer (NK) cell subpopulation as effector cells (2). These cells can be highly variable in response, are tedious to prepare and can result in high background readings.

The ADCC Reporter Bioassay uses an alternative readout at an earlier point in ADCC MOA pathway activation: 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 uses engineered Jurkat cells stably expressing the FcγRIIIa receptor, V158 (high 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.

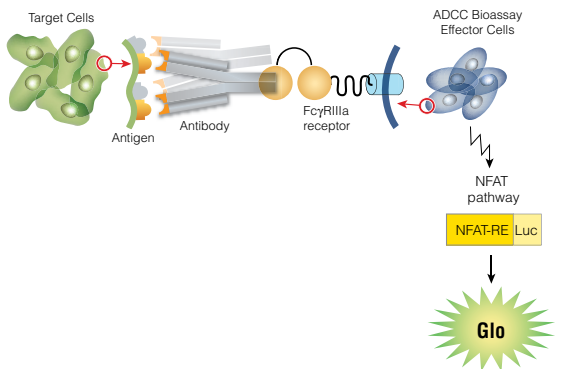


Figure 1. Representation of the ADCC Reporter Bioassay. Readout is luminescence signal from NFAT response element driving expression of firefly luciferase.

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

- Target cells, effector cells and specific antibody**
- WIL2-S, Jurkat/NFAT-*luc* + FcγRIIIa, rituximab
 - NO WIL2-S, Jurkat/NFAT-*luc* + FcγRIIIa, rituximab
 - ▲ WIL2-S, Jurkat-NFAT-*luc* (NO FcγRIIIa), rituximab
 - ▼ WIL2-S, NO Jurkat/NFAT-*luc* + FcγRIIIa, rituximab
 - WIL2-S, Jurkat/NFAT-*luc* + FcγRIIIa, NO rituximab
 - ▼ WIL2-S, Jurkat/NFAT-*luc* + FcγRIIIa, trastuzumab

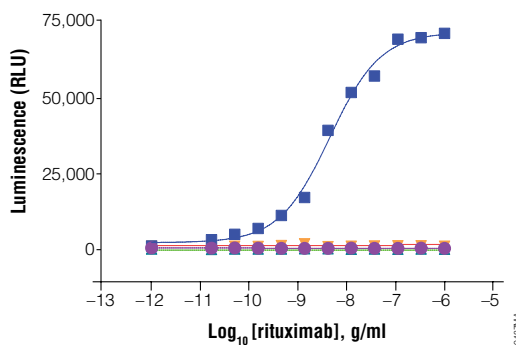


Figure 2. Specificity of the ADCC Reporter Bioassay. Serial dilutions of rituximab (anti-CD20 chimeric monoclonal antibody drug), trastuzumab (anti-Her2 humanized monoclonal antibody drug), or assay medium control (no antibody) were incubated for 6 hours of induction at 37°C with engineered Jurkat effector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

1. Description (continued)

The ADCC Reporter Bioassay has performance characteristics suitable for many applications of a bioassay used across antibody drug discovery, development and manufacture: It is stability-indicating and has the precision and accuracy suitable for a lot-release bioassay (Figure 3). Additionally the assay can be used to quantify effects of glycosylation differences on Fc effector function of antibodies in ADCC MOA (Figure 4), which would be useful for ADCC efficiency variant analysis, for example (5). Benchmarking studies demonstrate the ADCC Reporter Bioassay provides antibody activity ranking equivalent to a classic ADCC bioassay using PBMCs and LDH release as a measure of target cell death (Figure 5).

Parameter		Results	
		WIL2-S Target cells	Raji Target cells
Accuracy	% Expected Relative Potency	% Recovery	% Recovery
	50	97.7	101.0
	75	88.5	101.2
	125	98.4	93.8
	150	98.4	96.7
Repeatability (%CV)	(100% Reference)	5.0	9.1
Intermediate Precision (%CV)		7.3	3.0
Linearity (r ²)		0.995	0.997
Linearity (y = mx + b)		y = 1.016x - 0.052	y = 0.922x + 5

Figure 3. Bioassay characterization. The ADCC Reporter Bioassay was characterized in studies that evaluated accuracy, repeatability, intermediate precision and linearity across the 50–150% relative potency range. Dilution ranges for an anti-CD20 antibody were selected to ensure good coverage of upper and lower asymptotes and sufficient points in the intermediate dose-range for accurate slope and EC₅₀ determinations. A series of relative potency samples, of 50%, 75%, 125% and 150% 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 was characterized using ADCC Bioassay Target Cells (WIL2-S) and ADCC Bioassay Target Cells (Raji). Data were fitted to a 4PL curve using GraphPad Prism[®] software, and relative potencies were calculated after parallelism determination using SAS Institutes, Inc. JMP[®] software. Relative potencies were calculated using the 100% reference sample run as a triplicate dilution series in the same assay plate as the test sample.

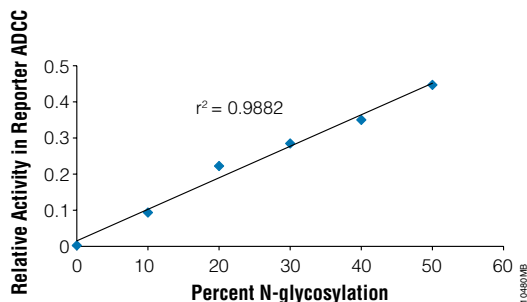


Figure 4. Detection of antibody glycosylation. Rituximab-blended samples containing mixtures of fully deglycosylated and fully N-glycosylated antibody were assayed in the ADCC Reporter Bioassay against a 100% relative activity reference sample of fully N-glycosylated rituximab in the same plate. Target cells were ADCC Bioassay Target Cells (WIL2-S), and the E:T ratio was 6:1. Biological activity was expressed relative to the 100% control run in the same assay plate and plotted against the percent of N-glycosylation present. Linear regression analysis was performed to determine correlation.

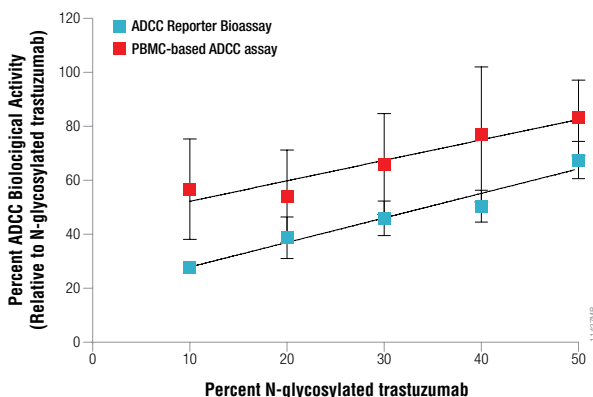


Figure 5. Antibody biological activity correlated with antibody glycosylation in an ADCC reporter assay and a classic PBMC-based ADCC assay. Blended antibody samples containing 10%, 20%, 30%, 40% or 50% of untreated trastuzumab (fully glycosylated) were prepared by mixing untreated and PNGase F-treated trastuzumab in appropriate proportions. Each of the blended trastuzumab samples was then assayed in triplicate against an untreated trastuzumab (reference sample) in the same plate using an ADCC reporter assay or a PBMC-based ADCC assay. Freshly cultured HER2⁺ SK-BR-3 cells were used as target cells in both assays. For the ADCC reporter assay, the E:T ratio was 15:1 and induction time was 6 hours. Results are mean \pm SD of three independent experiments using the same batch of frozen, thaw-and-use Jurkat effector cells. For the PBMC-based ADCC assay, the effector cells were PBMCs from the donors with Fc γ R11a V/V genotype for aa 158 and the E:T ratio was 50:1. After an overnight incubation, target cell lysis was measured by detecting the release of lactate dehydrogenase from lysed SK-BR-3 cells using CytoTox 96[®] Non-Radioactive Cytotoxicity Assay. Results are mean \pm SD of three independent experiments using PBMCs isolated from different donors. Percent ADCC biological activity is defined as the ratio of the EC₅₀ of untreated trastuzumab (N-glycosylated) to the EC₅₀ of the blended trastuzumab mixture in the same assay plate.

2. Product Components and Storage Conditions

Note: The ADCC Reporter Bioassay components are shipped separately because of temperature requirements. The ADCC Bioassay Effector Cells and Target Cells are shipped on dry ice. The Bio-Glo™ Luciferase Assay System^(d) and Low IgG Serum are shipped on dry ice, separately from the cells. The RPMI 1640 Medium is shipped at ambient temperature. Control Ab, Anti-CD20, is shipped on dry ice.

PRODUCT	SIZE	CAT.#
ADCC Reporter Bioassay, Complete Kit (Raji)	1 kit	G7015

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 (0.65ml)
- 1 vial ADCC Bioassay Target Cells (Raji) (0.55ml)
- 5µg/vial Control Ab, Anti-CD20 (0.5mg/ml)
- 4ml Low IgG Serum
- 10ml Bio-Glo™ Luciferase Assay Buffer
- 1 vial Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 36ml RPMI 1640 Medium

Storage Conditions: Upon arrival, immediately transfer the vials of ADCC Bioassay Effector Cells and Target Cells (Raji) 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 -30°C to -10°C . Avoid multiple freeze-thaw cycles of the serum. Store the Control Ab, Anti-CD20, at -30°C to -10°C . Bio-Glo™ Luciferase Assay Buffer and Bio-Glo™ Luciferase Assay Substrate should be stored at -30°C to -10°C . For optimal performance, reconstituted Bio-Glo™ Luciferase Assay Reagent should be used on the day of preparation. However, once reconstituted, Bio-Glo™ Luciferase Assay Reagent can be stored at -30°C to -10°C for up to 6 weeks. RPMI 1640 Medium should be stored at $+2^{\circ}\text{C}$ to $+10^{\circ}\text{C}$ 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 and Target Cells (Raji), when thawed and diluted as instructed, will be at the proper concentration for the bioassay. The effector-to-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.

The ADCC Bioassay Effector Cells and the ADCC Bioassay Target Cells (Raji) are provided in a frozen, thaw-and-use format and are ready to be used without any culturing procedures. Although they are ready-to-use, the cells are sensitive, and care should be taken not to overmix the cell reagents, as is commonly done with other cell preparations that tend to clump or settle. Follow the protocol instructions, warming the cells until just thawed (Sections 4.F and 4.H).

Because the ADCC Reporter Bioassay produces a bioluminescent readout, the assay requires a sensitive luminometer or luminescence plate reader for the detection of signal (e.g., 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 (Corning Cat.# 3917).

4. Assay Protocol for ADCC Reporter Bioassay, Complete Kit (Raji)

4.A. Before You Begin

Materials to Be Supplied by the User

- 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 CO₂ incubator
- 37°C water bath
- plate reader with glow luminescence read capability or luminometer (e.g., GloMax® Discover System)
- test antibody
- reference antibody (if desired)
- tissue culture-grade water

Overview

The kit should be used as a starter kit to gain familiarity with the ADCC Reporter Bioassay, and for this purpose you should use the provided Control Ab, Anti-CD20. If your target antigen is CD20 you may include test and/or reference samples of your antibody in assay runs. If your target antigen is a different antigen expressed on Raji cells, you may also use this kit to assay your antibody using thaw-and-use Raji cells. If you want to include other target cells in the ADCC Reporter Bioassay with assay runs of the Complete Kit (Raji), you may find instructions and guidelines in the *ADCC Reporter Bioassay, Core Kit Technical Manual*, #TM383, available from Promega.

4.A. Before You Begin (continued)

Our recommended protocol described here as an example protocol is designed to test two antibody samples in a single assay run. Samples are run as triplicate 10-point dilution series in a single 96-well assay plate for a total of two plates per kit. Other protocols and plate layouts are possible and may need to be optimized for your specific antibody.

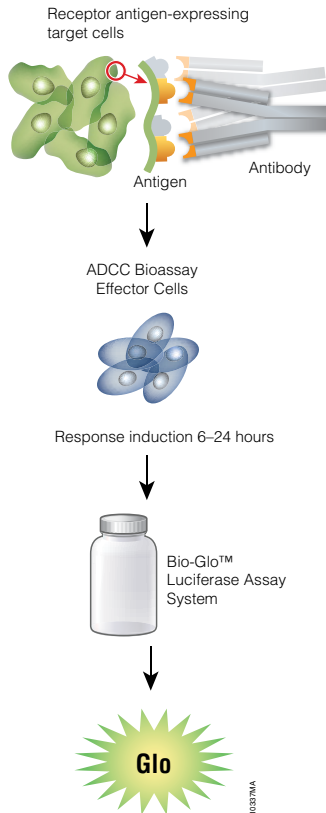


Figure 6. Schematic protocol for the ADCC Reporter Bioassay.

4.B. 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 IgG Serum in a 37°C water bath. In a 50ml conical tube, add 1.4ml of 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 dilution of Control Ab, Anti-CD20 (dilu1, 3X final concentration):** In a 1.5ml microcentrifuge tube, prepare 50µl of an intermediate dilution at 100µg/ml for the Control Ab, Anti-CD20 (0.5mg/ml), by adding 10µl of antibody stock solution to 40µl of ADCC Assay Buffer. Briefly vortex to mix. Further dilute the antibody to make a starting dilution of 3µg/ml by transferring 30µl of the 100µg/ml antibody solution to 970µl of ADCC Assay Buffer (dil1, 3µg/ml, 3X final concentration). Briefly vortex to mix and store on ice until ready to perform serial dilutions.
4. **Starting dilutions (dilu1, 3X final concentration) for two Test antibodies:** Decide the starting concentration (1X) for your particular 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 1µg/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.

Prepare 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.

Note: This set of starting dilutions is appropriate for running two assay plates in which each plate has triplicate 10-point dilutions of Control Ab, Anti-CD20, and triplicate 10-point dilutions for a test antibody; the two test antibodies are assumed to be different preparations, one on each plate. Different antibody layouts are possible. For example, you may later substitute a reference preparation of your anti-CD20 antibody rather than use the kit Control Ab, Anti-CD20.

4.C. Plate Layout Design

We recommend orienting samples within an assay plate in a nonclustered fashion to help minimize any well positional effects on the response. For the protocol we describe here, use the plate layout in Figure 7 as a guide for each of two plates. **The protocol uses serial replicate dilutions (n = 3) of the Control Ab, Anti-CD20, and each of two test antibodies to generate two 10-point dose response curves in each plate.** Other plate layouts and antibody samples may be substituted as appropriate.

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	B	Assay Buffer (B)
B	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Control 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	Control Ab	
F	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Control 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 nonclustered sample locations of Control Ab, Anti-CD20, antibody dilution series, a single test antibody dilution series, and “ADCC Assay Buffer” control, color coded for location.

4.D. Preparing Antibody Serial Dilutions Using Control Ab, Anti-CD20

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.

In order 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. For your reference, when tested in ADCC Reporter Bioassay, the starting concentrations and serial dilution schemes are 1×10^{-6} g/ml, fourfold serial dilution for rituximab, and 1×10^{-6} g/ml, threefold serial dilution for trastuzumab. These provide full dose-response curves in both cases.

The following set of serial dilutions described is appropriate for running two assay plates in which each plate has triplicate 10-point dilutions of Control Ab, Anti-CD20, and triplicate 10-point dilutions for a test antibody; the two test antibodies are assumed to be different preparations, one on each plate. **Threefold serial dilutions are assumed to be appropriate for test antibodies and 2.5-fold serial dilutions are recommended for Control Ab, Anti-CD20. For different serial dilution schemes, adjust volumes accordingly.**

1. Obtain a sterile clear V-bottom 96-well plate for preparing antibody serial dilutions for Control Ab, Anti-CD20, and two test antibodies.
2. Prepare 2.5-fold serial dilutions of Control Ab, Anti-CD20, in rows A and B. Add 200µl of Control Ab, Anti-CD20, starting dilution (dilu1, 3µg/ml, 3X final concentration) to well A11 and well B11, and dispense 120µl of ADCC Assay Buffer into wells 2–10 within rows A and B. Using a multichannel pipette, transfer 80µl from the antibody starting dilutions in column 11 into column 10. Mix well by pipetting. Avoid creating bubbles. Repeat equivalent 2.5-fold serial dilutions across columns from right to left until column 3 is reached. See Figure 8.
3. Prepare appropriate serial dilutions specific for each of your test antibodies in rows E and G, using individual serial dilution scheme for each test antibody based on previous testing results in a classic ADCC assay. Add appropriate volume (e.g., 150µl for threefold serial dilutions) of Test antibody 1 and Test antibody 2 starting dilution (dilu1, 3X final) to wells E11 and G11, respectively, and dispense appropriate volume (e.g., 100µl for threefold serial dilutions) of ADCC Assay Buffer into wells 2–10 within rows E and G. One by one, prepare appropriate serial dilutions for each of your test antibodies from column 11 to column 3 using similar steps as described above for the Control Ab, Anti-CD20, within row E and row G, respectively; a transfer volume of 50µl is suitable for threefold serial dilutions. See Figure 8.
Note: Wells A2, B2, E2 and G2 contain ADCC Assay Buffer as “no-antibody” control.
4. 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		Control Ab, Anti-CD20
B		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Control Ab, Anti-CD20
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. Control Ab, Anti-CD20, and test antibodies for serial dilutions from a single antibody stock to generate triplicates.

4.E. Optimization of Assay Conditions for Other Antibodies Directed Against Raji Target Cells


The ADCC Reporter Bioassay, Complete Kit (Raji), has undergone extensive development testing to establish robust and optimal assay parameters. Other antibodies recognizing alternative Raji antigens may require or benefit from some assay optimization in order to achieve ideal assay results. Examples of parameters that may benefit include: effector-to-target cell ratios (E:T), establishing a full antibody dose range, assay induction time, and alternative serum concentrations for preparation of the ADCC Assay Buffer. When diluted as directed, the use of the frozen cells provided in this kit will yield an E:T ratio of 6:1, with 75,000 effector cells/well plated. Other ratios across a range from 2.5:1 to 25:1 can be evaluated while ensuring sufficient assay response is obtained for precise and accurate assay results. This should include demonstration of full dose-response and appropriate serial dilutions for good coverage of the quasi-linear portion of dose-curve and achievement of good asymptotes. The ADCC Assay Buffer provided here contains 4% Low IgG Serum; other serum percentages (1–10%) may be optimal for alternative antibodies.

4.F. Plating ADCC Bioassay Target Cells (Raji)

Note: If you want to include other target cells in ADCC Reporter Bioassay with assay runs of the Complete Kit (Raji), you will find instructions and guidelines in the *ADCC Reporter Bioassay, Core Kit Technical Manual*, #TM383, available from Promega at: www.promega.com/protocols

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

1. Dispense 75µl of ADCC Assay Buffer into outermost wells, labeled “B” in Figure 7, of two white 96-well assay plates. Place the plates in a 37°C, CO₂ tissue culture incubator to pre-equilibrate (10–15 minutes).
2. Label a 15ml conical tube, “ADCC Bioassay Target Cells (Raji)”. Add 9.5ml of ADCC Assay Buffer (prewarmed to 37°C) to the tube.
3. Remove one vial of ADCC Bioassay Target Cells (Raji) from –140°C freezer storage or vapor phase of liquid nitrogen to dry ice for transport to the bench immediately before 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.

4. Gently mix the cell suspension by pipetting 1–2 times. Transfer 0.5ml of cells to the tube labeled “ADCC Bioassay Target Cells (Raji)” containing 9.5ml of ADCC Assay Buffer. Mix well by gently inverting the tube two times.
5. Transfer the cell suspension to a sterile reagent reservoir. Immediately, using a multichannel pipette, add 25µl of target cells to the inner 60 wells as defined in Figure 7. Cover plates with lids and keep the plates on the bench before adding antibody dilutions and ADCC Bioassay Effector 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 at the next step.

4.H. Plating ADCC Bioassay Effector Cells

1. Label a sterile 15ml conical tube, "ADCC Bioassay Effector Cells". Add 3.6ml of ADCC Assay Buffer (prewarmed to 37°C) to the tube.
2. Remove one vial of ADCC Bioassay Effector Cells 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 one or two times. Transfer 630µl of cells to the 15ml "ADCC Bioassay Effector Cells" tube containing 3.6ml of ADCC Assay Buffer. Mix well by gently inverting the tube two 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 CO₂ 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 all the inner 60 wells of the assay plates; avoid creating any bubbles.

Note: Bio-Glo™ Reagent should be at ambient temperature.

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 read 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 = $\text{RLU (induced-background)} / \text{RLU (no antibody control-background)}$

Note: When calculating Fold of Induction, if the sample RLU's 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 Log₁₀ [antibody] and Fold of Induction versus Log₁₀ [antibody]. Fit curves and determine EC₅₀ 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

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 crosstalk from neighboring wells due to use of unsuitable assay plates or improper settings for the detection instrument. See also the “matrix effect” comment 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. If you must use an instrument primarily designed for fluorescence detection, ensure no filters are used. Luminometers measure and report luminescence as relative values, and actual numbers will vary between instruments. See Section 3 for more recommendations on how to set up the luminometer.

An insufficient number of 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, complement (or other components from serum) supernatant of phage display or hybridoma culture could nonspecifically impact antibody binding to the FcγRIIIa receptor or affect the 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.

Symptoms

Weak ADCC response

Causes and Comments

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 any EC_{50} of antibody in the ADCC Reporter Bioassay will not necessarily be the same as those 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 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 Serum in a range of 1–10%, 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 bioassays 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 for both assays.

5. Troubleshooting (continued)

Symptoms

EC₅₀ for Ab varies between classic ADCC bioassay and Promega ADCC Reporter Bioassay

Possible Causes and Comments

EC₅₀ refers to the concentration of the substance (mAb in this assay) that gives 50% of the maximal biological response. The EC₅₀ 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 EC₅₀ value of any antibody can differ dramatically between different assays and is not an intrinsic property of the antibody. It is normal if the EC₅₀ value for an Ab differs between ADCC Reporter Bioassay and other ADCC bioassays.

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 (Sections 4.F and 4.H). 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.* (2012) Quantitative evaluation of fucose reducing effects in a humanized antibody on Fcγ receptor binding and antibody-dependent cell-mediated cytotoxicity activities. *mAbs* **4**, 326–40.
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. 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.
5. 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 (Figure 9) were generated using the ADCC Bioassay Effector Cells with the ADCC Bioassay Target Cells (Raji) as provided in the ADCC Reporter Bioassay, Complete Kit (Raji).

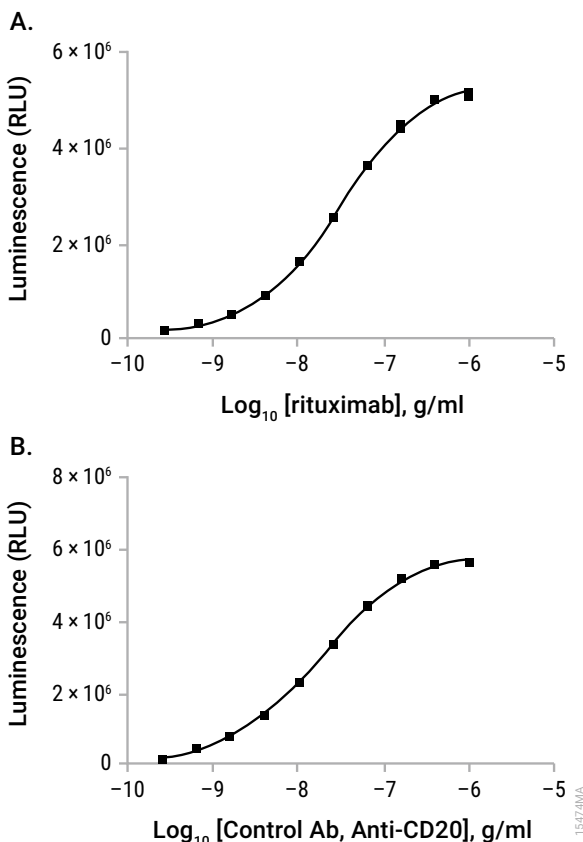


Figure 9. ADCC Reporter Bioassay response to rituximab (Panel A; trade name: RITUXAN®), or Control Ab, Anti-CD20 (Panel B). ADCC Bioassay Target Cells (Raji) were incubated with a series of concentrations of rituximab or Control Ab, Anti-CD20, followed by addition of ADCC Bioassay Effector Cells. The 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. The data were fitted to a 4PL curve using GraphPad Prism® software. The EC₅₀ response determined was 30.4ng/ml for rituximab and 18.3ng/ml for Control Ab, Anti-CD20.



8. Summary of Changes

The following changes were made to the 4/26 revision of this document:

1. Removed original Section 8, Related Products and renumbered Summary of Changes to Section 8.
2. Deleted old patent statements.
3. Made minor text updates.

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