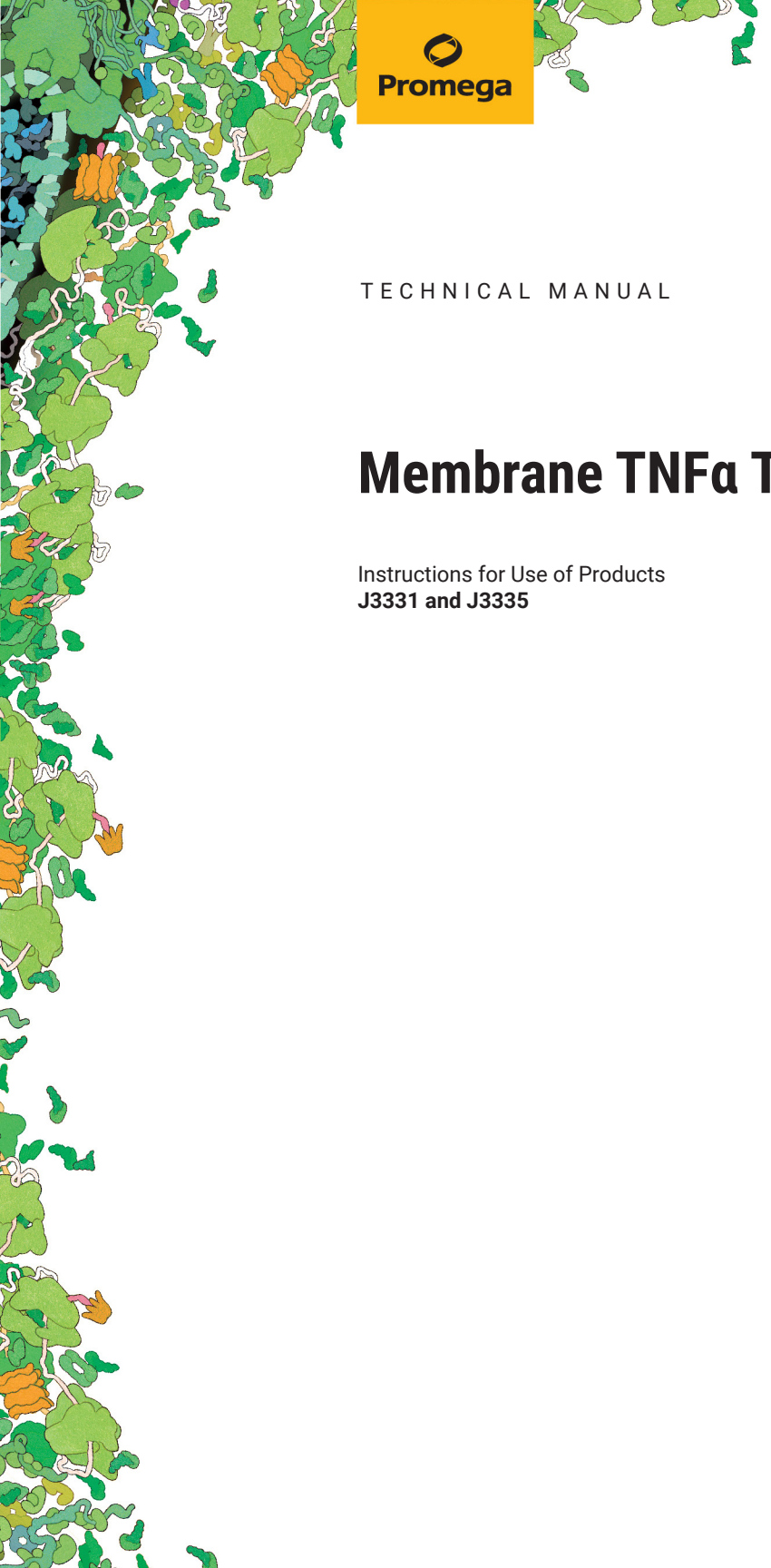

TECHNICAL MANUAL

Membrane TNFα Target Cells

Instructions for Use of Products
J3331 and J3335

Revised 5/25
TM675

Membrane TNFα Target Cells

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

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1. Description

Tumor necrosis factor alpha (TNFα) is a key cytokine involved in immune and inflammatory responses. TNFα is produced primarily by macrophages but can be generated by other leukocytes as well as endothelial cells, cardiomyocytes and other cell types. TNFα binding to its receptors, TNF receptor type 1 (TNFR1) and TNFR2, induces a myriad of cellular responses that are cell-context dependent. Typically, TNFα binding to TNFR1, TNFR2 or both, induces pro-inflammatory responses, such as immune cell activation, promotion of effector functions and cytokine production, as well as the acute phase response and hallmarks of inflammation: fever, swelling, redness and pain (1). In vivo, TNFα exists in both a trimeric membrane-bound form (mTNFα) and as a soluble protein. TNFα is produced as a type II membrane protein and cleaved by the metalloproteinase TNFα-converting enzyme (TACE, ADAM17; 2,3). Once cleaved, the soluble trimeric TNFα is biologically active, but a less potent activator of TNFR2 compared to mTNFα (4).

TNFα has a significant role in the pathology of several inflammatory and autoimmune disorders, including rheumatoid arthritis and ulcerative colitis (5,6). Blockade of TNFα/TNFR binding using neutralizing antibodies or engineered TNFR fusion proteins has proven to be a viable strategy for providing clinical benefit in inflammatory diseases. Furthermore, targeting TNFα-producing cells for destruction may provide additional advantages to reduce the inflammatory burden. Specifically, binding of TNFα-targeted antibodies to mTNFα-expressing cells can induce effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) to destroy the mTNFα-expressing inflammatory cells (7,8). Several FDA-approved TNFα antibodies have been confirmed to possess ADCC and CDC function. However, determining the ability of novel and biosimilar TNFα antibodies to induce cellular cytotoxicity is hampered by the lack of model cell lines naturally expressing mTNFα.

Membrane TNFα Target Cells[a] (Cat.# J3331, J3335), are genetically engineered cells stably expressing a cleavage-resistant form of mTNFα that enforces its surface expression. mTNFα Target Cells are provided in thaw-and-use format, which includes cryopreserved cells that are ready to be thawed for use as target cells in assays that measure the effector functions in ADCC and CDC for anti-TNFα blockers. In addition, they can be used to measure antibody binding affinity to mTNFα.

The Membrane TNFα Target Cells are also available in Cell Propagation Model (CPM) format (Cat.# J3322), which includes cryopreserved cells that can be thawed, propagated and banked for long-term use.

mTNFα Target Cells express TNFα on the cell surface, as demonstrated by flow cytometry (Figure 1). The assay signal is specific to anti-TNFα antibodies in both ADCC and CDC assays. When used in the ADCC Reporter Bioassay (Cat.# G7010), luminescence increases after adding anti-TNFα antibodies, but not after adding anti-VEGF or anti-CD20 antibodies (Figure 2). In a CDC assay, mTNFα Target Cell death is detected after adding anti-TNFα antibodies, but not after adding anti-VEGF or anti-CD20 antibodies (Figure 3). The ADCC Reporter Bioassay using mTNFα Target Cells is prequalified following International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines, and shows the precision, accuracy and linearity required for routine use in potency and stability studies (Table 1 and Figure 4). The bioassay can be performed in a two-day timeframe, and the workflow is simple, robust and compatible with both 96- and 384-well plate formats used for antibody screening in early drug discovery (Figure 5).

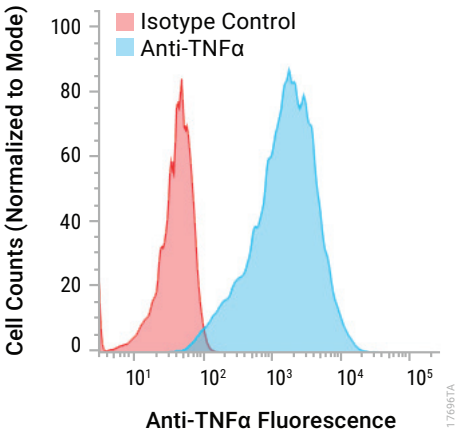

Figure 1. Surface expression of TNFα on mTNFα Target Cells. mTNFα Target Cells were labeled with isotype control or anti-TNFα (infliximab) followed by AlexaFluor® 488-conjugated anti-human IgG. Cells were analyzed on a BD LSRFortessa™ X-20 flow cytometer. Data analysis was performed with FlowJo™ software. Data were generated using thaw-and-use cells.

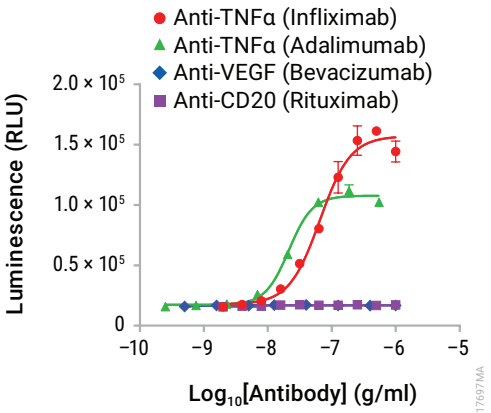

Figure 2. The ADCC Reporter Bioassay performed with mTNFα Target Cells reflects the mechanism of action (MOA) and shows specificity for antibodies designed to bind TNFα. ADCC Effector Cells were cocultured with mTNFα Target Cells in the presence of serial titrations of antibodies, as indicated. After a 6-hour induction, Bio-Glo™ Reagent was added, and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

1. Description (continued)

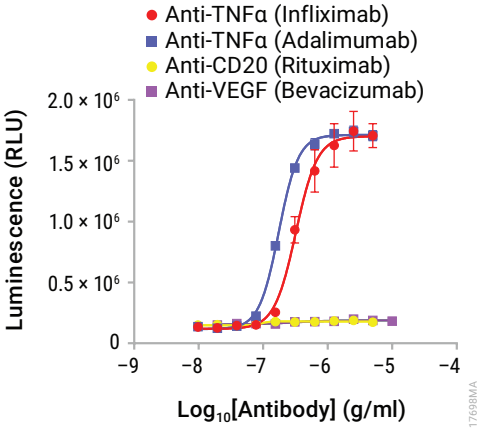

Figure 3. CDC assay with mTNFα Target Cells reflects the MOA and shows specificity for antibodies designed to bind TNFα. mTNFα Target Cells were incubated with 10% human serum in the presence of serial titrations of antibodies, as indicated. After a 6-hour incubation, CytoTox-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

Table 1. ADCC Reporter Bioassay using mTNFα Target Cells Shows Precision, Accuracy and Linearity.

Parameter	Results	
Accuracy	% Expected Relative Potency	% Recovery
	50	49.1
	70	72.0
	100	100.9
	140	150.1
	200	213.4
Repeatability (% CV)	100% (Reference)	2.1
Intermediate Precision (% CV)		9.4
Linearity (r^2)		0.9991
Linearity (y = mx + b)		y = 1.099x – 6.024

A 50–200% simulated potency series of infliximab was analyzed in triplicate in three independent experiments performed on three days by two analysts using mTNFα Target Cells in the ADCC Reporter Bioassay. Bio-Glo™ Reagent was added, and luminescence quantified using the GloMax® Discover System. Data were analyzed and relative potencies calculated after parallelism determination using JMP® software. Data were generated using thaw-and-use cells.

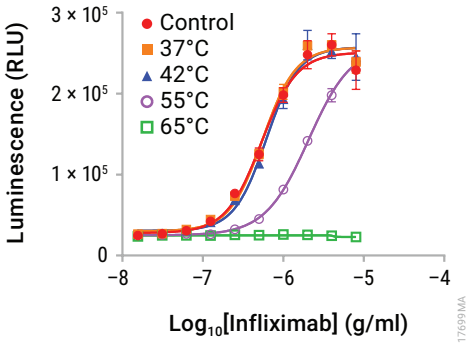

Figure 4. The ADCC Reporter Bioassay with mTNFα Target Cells is stability-indicating. Samples of anti-TNFα (infliximab) were maintained at 4°C (control) or heat-treated for 24 hours at the indicated temperatures, then analyzed using the ADCC Reporter Bioassay with mTNFα Target Cells. Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

1. Description (continued)

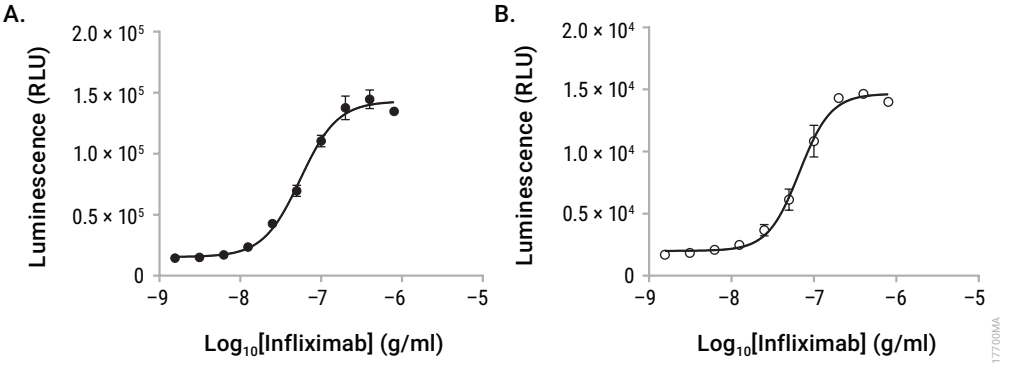

Figure 5. The ADCC Reporter Bioassay with mTNFα Target Cells is amenable to 384-well plate format. Panel A. The bioassay was performed in 96-well plates as described in this technical manual with a titration of anti-TNFα (infliximab). **Panel B.** The bioassay was performed with mTNFα Target Cells in 384-well plates, as described here. Thaw-and-use mTNFα Target Cells were thawed and plated at 2 × 10^3/15µl/well 20 hours prior to the assay, in a 384-well white assay plate. On the assay day, 5µl of a twofold serial dilution of 5X infliximab was added to the wells, followed by addition of 1.5 × 10^4/5µl/well of ADCC Effector Cells. After a 6-hour induction at 37°C, 5% CO_2, 25µl/well of Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were fitted to four-parameter logistic curves using GraphPad Prism® software. The EC_{50} values were 55 and 67ng/ml for 96- and 384-well plates, respectively. The fold induction was 9.3 and 7.3 for 96- and 384-well plates, respectively. Data were generated using thaw-and-use cells.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Membrane TNFα Target Cells	**1 each**	**J3331**

Not for Medical Diagnostic Use. Includes:

- 1 vial Membrane TNFα Target Cells (0.5ml)

PRODUCT	SIZE	CAT.#
Membrane TNFα Target Cells 5X	**1 each**	**J3335**

Not for Medical Diagnostic Use. Includes:

- 5 vials Membrane TNFα Target Cells (0.5ml)

Storage Conditions: Upon arrival, immediately transfer the cell vials to below −140°C (freezer or liquid nitrogen vapor phase) for long-term storage. Do not store cell vials submerged in liquid nitrogen. **Do not** store cell vials at −80°C because this will decrease cell viability and cell performance.

3. Before You Begin

Please read through the entire protocol to become familiar with the components and the assay procedure before beginning.

Note the catalog number and lot number from the cell vial box label. This information can be used to download documents from the specified product from the website such as Certificate of Analysis.

Note: mTNFα Target Cells are intended to be used with user-provided antibodies or other biologics designed to bind to TNFα. Data generated using infliximab (Remicade®) and adalimumab (Humira®) are shown in Section 8.A Representative Assay Results.

To measure ADCC activity, mTNFα Target Cells can be used in conjunction with the thaw-and-use ADCC Reporter Bioassay (Cat.# G7010, G7018) or ADCC Reporter Bioassay, Propagation Model (Cat.# G7102) to detect ADCC function of anti-TNFα antibodies. To measure complement-dependent cytotoxicity (CDC) activity, we recommend using CytoTox-Glo™ Cytotoxicity Assay (Cat.# G9290).

mTNFα Target Cells are provided in frozen, thaw-and-use format and are ready to be used without any additional cell culture or propagation. When thawed and diluted as instructed, the cells will be at the appropriate concentration for the assay. The cells are sensitive, and care should be taken to follow cell thawing and plating procedures as described. Do not overmix or overwarm the cell reagents.

The recommended cell plating densities, induction time and assay buffer components described in Sections 4 and 5 were established using infliximab and ADCC Effector Cells (ADCC Reporter Bioassay) or in a CDC assay using CytoTox-Glo™ Reagent and complement-preserved human serum. You may need to adjust the parameters provided here and optimize assay conditions for your own assay readout and antibodies.

The ADCC Reporter Bioassay and the CDC assay with CytoTox-Glo™ Reagent produce a bioluminescent signal and require a sensitive luminescence plate reader. Bioassay development and performance data included in this Technical Manual were generated using the GloMax® Discover System (see Section 8.B, Related Products). An integration time of 0.5 seconds/well was used for all readings. These bioassays are compatible with most other plate-reading luminometers, though relative luminescence unit (RLU) readings will vary with the sensitivity and settings of each instrument. If your luminometer/plate reader requires gain adjustment for luminescence, use the well with the highest antibody concentration.

3. Before You Begin (continued)

Materials to Be Supplied by the User

Reagents

- user-defined anti-TNFα antibodies or other biologics samples (e.g., infliximab NDC 57894-030-01)
- Ham's F-12 medium with L-glutamine (e.g., GIBCO® Cat.# 11765062)
- fetal bovine serum (FBS; e.g., GIBCO® Cat.# 35-015-CV or HyClone Cat.# SH30071.03)
- **optional:** ADCC Reporter Bioassay (thaw-and-use; Cat.# G7010; for ADCC Bioassay)
- **optional:** Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941; for ADCC Bioassay)
- **optional:** normal human serum complement (Quidel Cat.# A113; for CDC assay)
- **optional:** CytoTox-Glo™ Cytotoxicity Assay (Cat.# G9290; for CDC assay)

Supplies and Equipment

- solid-white, flat-bottom 96-well (e.g., Corning® Cat.# 3917) or 384-well (e.g., Corning® Cat.# 3570) assay plates for plating and reading luminescence
- sterile clear V-bottom 96-well plate with lid (e.g., Costar® Cat.# 3896) for preparing antibody dilutions
- sterile dilution reservoirs with lid (e.g., Dilux™ Cat.# D-1002) for higher volume antibody dilutions
- pipettes (single-channel and 12-channel; for best results use both manual and electronic pipettes as needed)
- sterile 15ml and 50ml conical tubes
- sterile reagent reservoirs (e.g., Costar®/Corning® Cat.# 4870)
- 37°C, 5% CO_2 incubator
- 37°C water bath
- sensitive plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System or equivalent system; for ADCC Reporter Bioassay or CDC assay using CytoTox-Glo™ Cytotoxicity Assay)

4. Protocol for ADCC Reporter Bioassay using mTNFα Target Cells

This assay protocol requires two engineered cell lines: ADCC Bioassay Effector Cells (Cat.# G7010, G7018) and Membrane TNFα Target Cells (Cat.# J3331, J3335). ADCC Bioassay Effector Cells and Membrane TNFα Target Cells are also available in Propagation Model format, Cat.# G7102 and Cat.# J3322, respectively.

The procedure below illustrates the use of the mTNFα Target Cells in the ADCC Reporter Bioassay to test two anti-TNFα antibody samples against a reference sample, in a single assay run using the mTNFα Target Cells. Each test and reference antibody is run in triplicate, in a 10-point dilution series, in a single 96-well assay plate using the inner 60 wells. Other experimental and plate layouts are possible but may require further optimization.

Note: When preparing test and reference antibodies, choose an appropriate starting concentration and dilution scheme to achieve a complete dose-response curve with proper upper and lower asymptotes and sufficient points on the slope. For reference, we use 0.8µg/ml as a starting concentration (1X) and twofold serial dilutions when testing infliximab.

4.A. Preparing ADCC Bioassay Reagents

1. **mTNFα Target Cell Plating Medium:** On the day before the assay, prepare an appropriate amount of cell plating medium (90% Ham's F-12/10% FBS). Thaw the FBS overnight at 4°C or in a 37°C water bath on the day of use. Mix well and warm to 37°C before use. For reference, 30ml of cell plating medium is typically sufficient for 120 wells in a 96-well assay format using the inner 60 wells.

2. **ADCC Assay Buffer:** On the day of the assay, prepare an appropriate amount of ADCC assay buffer (96% RPMI 1640/4% Low IgG Serum). Mix well and warm to 37°C before use. For reference, 30ml of ADCC assay buffer is typically sufficient for 120 wells in a 96-well assay format using the inner 60 wells.

   Note: The recommended ADCC assay buffer contains 4% Low IgG Serum. This concentration and type of serum works well for the anti-TNFα antibody that we tested. If you experience assay performance issues when using this assay buffer, we recommend testing different serum concentrations in the range of 0.5–10%.

3. **Bio-Glo™ Reagent:** For reference, 10ml of Bio-Glo™ Reagent is sufficient to assay 120 wells in a 96-well assay format. Thaw the Bio-Glo™ Luciferase Assay Buffer at 4°C overnight or in a room temperature water bath on the day of assay. Equilibrate the Bio-Glo™ Luciferase Assay Buffer to ambient temperature, protected from light. Transfer all of the Bio-Glo™ Luciferase Assay Buffer into the amber bottle containing the Bio-Glo™ Luciferase Assay Substrate and mix by inversion until the substrate is thoroughly dissolved. Equilibrate and store the reconstituted Bio-Glo™ Reagent at ambient temperature (22–25°C) protected from light before adding to assay plates. After reconstitution, the Bio-Glo™ Reagent can be stored at room temperature with ~18% loss in luminescence after 24 hours or at 4°C with ~12% loss of luminescence after 5 days.

Note: The ADCC Reporter Bioassay is compatible only with the Bio-Glo™ Luciferase Assay System. **Do not** use the Bio-Glo-NL™ Luciferase Assay System with the ADCC Reporter Bioassay.

4. **Test and Reference Samples:** Using ADCC assay buffer as the diluent, prepare stock starting dilutions (dilu1, 3X final concentration) of two test antibodies (200µl each) and one reference antibody (400µl) in 1.5ml microcentrifuge tubes. Store the tubes containing antibody starting dilutions appropriately before making antibody serial dilutions.

   Note: If you are using infliximab (10mg/ml stock) as a reference antibody in your assay, prepare a 200µg/ml working stock of anti-TNFα antibody infliximab by adding 4µl of infliximab stock (10mg/ml) to 196µl of ADCC assay buffer. Prepare 400µl starting dilution of 2.4µg/ml of infliximab (dilu1, 3X final concentration) by adding 4.8µl of infliximab working stock to 395.2µl of ADCC assay buffer.

4.A. Preparing ADCC Bioassay Reagents (continued)

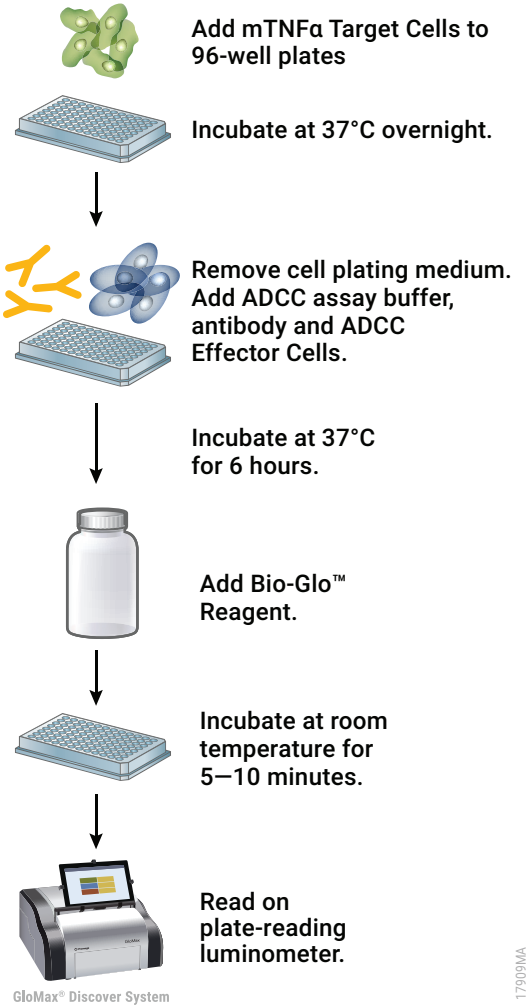

Figure 6. Schematic protocol for the ADCC Reporter Bioassay with mTNFα Target Cells.

4.B. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 7 as a guide. The protocol describes serial replicate dilutions (n = 3) of test and reference antibody to generate two 10-point dose-response curves for 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	Reference Ab
E	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
F	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	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 test antibody and reference antibody dilution series and wells containing ADCC assay buffer ("B") alone.

4.C. Plating mTNFα Target Cells

Thaw-and-use mTNFα Target Cells are sensitive and care should be taken to follow the cell thawing and plating procedures **exactly** as described. Do not overmix or overwarm the cell reagents. No additional cell culture or manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at any one time.

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

Note: Perform the following steps in a sterile cell culture hood.

1. On the day before performing the assay, prepare mTNFα Target Cell plating medium by combining 27ml of Ham's F-12 with 3ml of FBS to yield 90% Ham's F-12/10% FBS. Mix well and warm to 37°C prior to use.

2. Transfer 19.5ml of cell plating medium to a 50ml conical tube.
3. Remove one vial of mTNFα Target Cells from storage at –140°C and transfer to the bench on dry ice. Thaw the cells in a 37°C water bath until just thawed (about 2 minutes). While thawing, gently agitate and visually inspect the vial. Do not invert.
4. Gently mix the cell suspension by pipetting, then transfer 0.5ml cells to the tube containing 19.5ml of cell plating medium. Mix well by gently inverting the tube 1–2 times. Transfer the suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 100µl of the cell suspension to each of the inner 60 wells of a 96-well white flat-bottom assay plate.
5. Add 100µl of mTNFα Target Cell plating medium to each of the outside wells of the assay plates.
6. Place lids on the assay plates and incubate in a 37°C, 5% CO_2 incubator overnight (18–22 hours).

4.D. Preparing Antibody Serial Dilutions

The instructions described here are for preparing a single stock of twofold serial dilutions of a single antibody for analysis in triplicate (100µl of each dilution provides a sufficient volume for analysis in triplicate). Alternatively, you can prepare three independent stocks of serial dilutions to generate triplicate samples. To prepare twofold serial dilutions, 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 test antibody dose-response curves. For other dilution schemes, adjust the volumes accordingly.

Note: The instructions below use infliximab; follow the instructions below to prepare twofold serial dilutions. A twofold serial dilution for test antibodies is also listed as an example below.

Note: Perform the following steps in a sterile cell culture hood.

1. On the assay day, prepare ADCC Assay Buffer by combining by combining 1.2ml Low IgG Serum and 28.8ml of RPMI 1640 to yield 96% RPMI 1640/4% Low IgG Serum. Mix well and warm to 37°C prior to use.
2. To a sterile clear V-bottom 96-well plate, add 200µl of reference antibody starting dilution (dilu1, 3X final concentration) to wells A11 and B11 (see Figure 8).
3. Add 200µl of test antibodies 1 and 2 starting dilution (dilu1, 3X final concentration) to wells E11 and 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 100µl of the antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
6. Repeat equivalent twofold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.

   Note: Wells A2, B2, E2 and G2 contain 100µl of ADCC assay buffer without antibody as a negative control.
7. Cover the antibody dilution plate with a lid and keep at ambient temperature (22–25°C).

Recommended Plate Layout for Antibody Dilutions Prepared from a Single Antibody Stock													
	1	2	3	4	5	6	7	8	9	10	11	12	
A		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference Ab
B		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference Ab
C													
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 antibody serial dilutions.

4.E. Adding Antibody to mTNFα Target Cells in Assay Plates

1. Take the 96-well assay plates containing mTNFα Target Cells out of the incubator. Invert the assay plate to remove the medium. Then, place the inverted plate on paper towels for 5–10 seconds to drain any remaining medium. Alternatively, remove 95µl of medium from each of the wells using a manual multichannel pipette.
2. Using a multichannel pipette, add 25µl of ADCC assay buffer to the inner 60 wells of both 96-well assay plates.
3. Using a multichannel pipette, add 25µl of the appropriate antibody dilution (Figure 8) to the assay plates according to the plate layout in Figure 7.
4. Add 75µl of ADCC assay buffer to each of the outside wells of the assay plates.
5. Cover the assay plates with lids and keep the plates on the bench before adding ADCC Bioassay Effector Cells at the next step.

4.F. Plating ADCC Bioassay Effector Cells

The thaw-and-use ADCC Effector Cells in the ADCC Reporter Bioassay (Cat.# G7010) are sensitive and care should be taken to follow the cell thawing and plating procedures **exactly** as described. Do not overmix or overwarm the cell reagents. No additional cell culture or manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at any one time.

1. Label a sterile 15ml conical tube “Effector Cells”. Add 3.6ml of prewarmed (37°C) ADCC assay buffer to the 15ml conical tube.

2. Remove one vial of ADCC Effector Cells from storage at –140°C and transfer to the bench on dry ice. Thaw the cells in a 37°C water bath until just thawed (about 2 minutes). While thawing, gently agitate and visually inspect the vial. Do not invert the vial.
3. Gently mix the cell suspension by pipetting, then transfer 630µl of cells to the 15ml conical tube containing 3.6ml of ADCC Assay Buffer. Mix well by gently inverting the tube 2 times.
4. Transfer the suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 25µl of the cell suspension to each of the inner 60 wells of the assay plates.
5. Cover the assay plates with lids and incubate in a 37°C, 5% CO_2 incubator for 6 hours.

   Note: The 6-hour assay time was optimized using infliximab. We recommend optimizing assay time (5–24 hours) with your own antibody or other biologic samples.

4.G. Adding Bio-Glo™ Reagent

Note: The ADCC Reporter Bioassay is compatible only with the Bio-Glo™ Luciferase Assay System. **Do not** use the Bio-Glo-NL™ Luciferase Assay System with the ADCC Reporter Bioassay.

Note: Bio-Glo™ Reagent should be at ambient temperature when added to assay plates.

1. Remove assay plates from the 37°C incubator and equilibrate to ambient temperature (22–25°C) on the bench for 15 minutes.
2. Add 75µl of Bio-Glo™ Reagent to the inner 60 wells of both assay plates using a manual multichannel pipette. Avoid creating any bubbles.
3. Add 75µl of Bio-Glo™ Reagent to wells B1, D1 and F1 in each assay plate to determine plate background.
4. Incubate at ambient temperature for 5–20 minutes.
5. Measure luminescence using a plate reader with glow-type luminescence reading capabilities.

4.H. Data Analysis

1. Determine the plate background by calculating the average RLU from wells B1, D1 and F1.
2. Calculate fold induction:

$$\text{Fold Induction} = \frac{\text{RLU (induced – background)}}{\text{RLU (no antibody control – background)}}$$

3. Graph data as RLU versus Log_{10}[antibody] and fold induction versus Log_{10}[antibody]. Fit curves and determine the EC_{50} value of antibody response using appropriate curve fitting software (such as GraphPad Prism® software).

5. Assay Protocol for CDC Assay using mTNFα Target Cells

The procedure below illustrates the use of the mTNFα Target Cells in a CDC assay (using CytoTox-Glo™ Cytotoxicity Assay, Cat.# G9290) to test two anti-TNFα antibody samples against a reference sample, in a single assay run using Membrane TNFα Target Cells. Each test and reference antibody is run in triplicate, in a 10-point dilution series, in a single 96-well assay plate. Other experimental and plate layouts are possible but may require further optimization.

Note: When preparing test and reference antibodies, choose an appropriate starting concentration and dilution scheme to achieve a complete dose-response curve with proper upper and lower asymptotes and sufficient points on the slope. For reference, we use 6.67µg/ml as a starting concentration (1X) and twofold serial dilution when testing infliximab.

5.A. Preparing CDC Assay Reagents

1. **mTNFα Target Cell Plating Medium/CDC Assay Buffer:** On the day before the assay, prepare an appropriate amount of cell plating medium/CDC assay buffer (90% Ham's F-12/10% FBS). Thaw the FBS overnight at 4°C or in a 37°C water bath on the day of use. Mix well and warm to 37°C before use. For reference, 50ml of cell plating medium/CDC assay buffer is typically sufficient for 132 wells in a 96-well assay format (Figure 10). After plating mTNFα Target Cells, remaining CDC assay buffer can be stored at 4°C overnight for use on the day of the assay.

2. **CytoTox-Glo™ Reagent:** Prepare an appropriate amount of CytoTox-Glo™ Reagent on the day of the assay. Thaw the CytoTox-Glo™ Assay Buffer in a room-temperature water bath, and equilibrate to ambient temperature, protected from light. Thirty minutes prior to the end of the assay, transfer 5ml of buffer into one amber bottle containing the AAF-Glo™ Substrate and mix by inversion, until the substrate is thoroughly dissolved. For reference, 10ml of CytoTox-Glo™ Reagent is enough for 132 assay wells in a 96-well assay format.

   For optimal results, use freshly prepared CytoTox-Glo™ Reagent. Use within 12 hours if stored at room temperature. CytoTox-Glo™ Reagent can be stored at 4°C for up to 7 days with no appreciable loss of performance. CytoTox-Glo™ Reagent can be stored in single-use aliquots for up to 4 months at –70°C. Freezing and thawing will damage the reagent and should be avoided.

3. **Normal Human Serum Complement:** Follow manufacturer's instructions for storage, preparation and handling.

   Note: The recommended assay conditions include 10% normal human serum complement. This concentration and type of complement works well for the anti-TNFα antibodies we tested. If you experience assay performance issues when using normal human serum complement, we recommend testing different concentrations in the range of 5–20%.

4. **Test and Reference Samples:** Using CDC assay buffer as the diluent, prepare stock starting dilutions (dilu1, 1.5X final concentration) of two test antibodies (400µl each) and one reference antibody (800µl) in 1.5ml tubes. Store the tubes containing antibody starting dilutions appropriately before making antibody serial dilutions.

   Note: If you are using infliximab (10mg/ml stock) as a reference antibody in your assay, prepare a 200µg/ml working stock of infliximab by adding 4µl of infliximab stock (10mg/ml) to 196µl of CDC assay buffer. Prepare 800µl starting dilution of 10µg/ml of infliximab (dilu1, 1.5X final concentration) by adding 40µl of infliximab working stock to 760µl of CDC assay buffer.

5. **Digitonin:** Prior to completion of the assay, prepare 1mg/ml of digitonin in CytoTox-Glo™ Assay Buffer from 20mg/ml of stock provided in the CytoTox-Glo™ Cytotoxicity Assay (Cat.# G9290). Combine 5µl of digitonin stock with 95µl of CytoTox-Glo™ Assay Buffer to make 1mg/ml (10X) digitonin.

5.A. Preparing CDC Assay Reagents (continued)

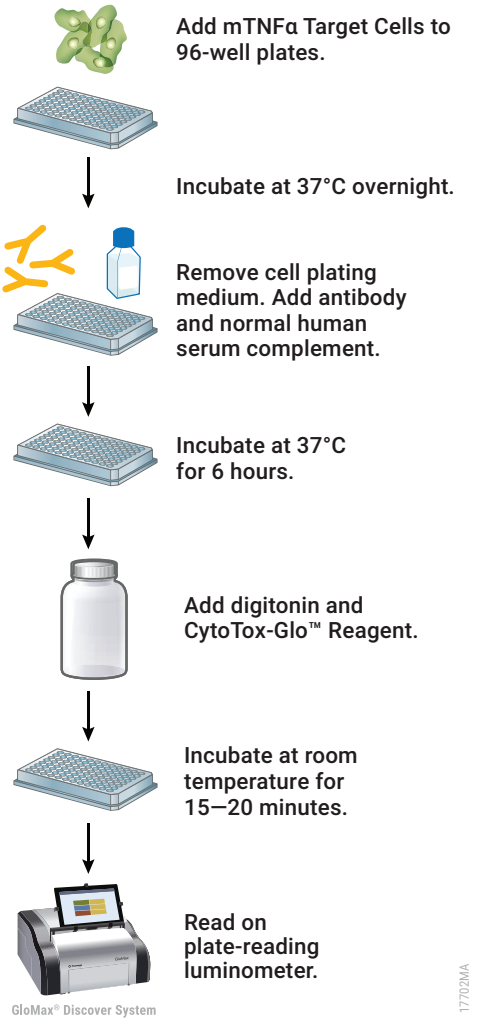

Figure 9. Schematic protocol for the CDC assay with mTNFα Target Cells.

5.B. Plate Layout Design

For the protocol described here, use the plate layout in Figure 10 as a guide. The protocol describes serial replicate dilutions (n = 3) of test and reference antibody to generate two 10-point dose-response curves for 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	D	Reference Ab
C	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	D	Test Ab
D	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	D	Reference Ab
E	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	D	Test Ab
F	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	D	Reference Ab
G	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	D	Test Ab
H	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)

Figure 10. Example plate layout showing non-clustered sample locations of test antibody and reference antibody dilution series and wells containing digitonin (D) or CDC assay buffer alone (B).

5.C. Plating mTNFα Target Cells

Thaw-and-use mTNFα Target Cells are sensitive and care should be taken to follow the cell thawing and plating procedures **exactly** as described. Do not overmix or overwarm the cell reagents. No additional cell culture or manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at any one time.

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

Note: Perform the following steps in a sterile cell culture hood.

1. On the day before the assay, prepare 50ml of mTNFα Target Cell plating medium by combining 45ml of Ham's F-12 with 5ml of FBS to yield 90% Ham's F-12/10% FBS. Mix well and warm to 37°C prior to use.
2. Transfer 19.5ml of mTNFα Target Cell plating medium to a 50ml conical tube.
3. Remove one vial of mTNFα Target Cells from storage at –140°C and transfer to the bench on dry ice. Thaw the cells in a 37°C water bath until just thawed (about 2 minutes). While thawing, gently agitate and visually inspect the vial. Do not invert.
4. Gently mix the cell suspension by pipetting, then transfer 0.5ml cells to the tube containing 19.5ml of cell plating medium. Mix well by gently inverting the tube 1–2 times. Transfer the suspension to a sterile reagent reservoir.
5. Using a multichannel pipette, immediately dispense 100µl of the cell suspension to B2 through G12 of a 96-well white flat-bottom assay plate (Figure 10).
6. Add 100µl of mTNFα Target Cell plating medium to each of the empty wells of the assay plates (Figure 10).
7. Place lids on the assay plates and incubate in a 37°C, 5% CO_2 incubator overnight (18–22 hours).

5.D. Preparing Antibody Serial Dilutions

The instructions described here are for preparing single stocks of twofold serial dilutions of a single antibody for analysis in triplicate (200µl of each dilution provides a sufficient volume for analysis in triplicate). Alternatively, you can prepare three independent stocks of serial dilutions to generate triplicate samples. To prepare twofold serial dilutions, you will need 800µl of reference antibody at 1.5X the highest antibody concentration in your dose-response curve. You will need 400µl of each test antibody at 1.5X the highest antibody concentration in each of the test antibody dose-response curves. For other dilution schemes, adjust the volumes accordingly.

Note: The instructions below use infliximab; follow the instructions below to prepare twofold serial dilutions. A twofold serial dilution for test antibodies is also listed as an example below.

1. On the day of the assay, warm CDC assay buffer prepared the day before to 37°C. Otherwise, prepare an appropriate amount of CDC assay buffer as described in Section 5.A.
2. To a sterile 12-well reservoir labeled reference, add 800µl of appropriate reference antibody starting dilution (dilu1, 1.5X final concentration) to well 11 (Figure 11).
3. To two additional sterile 12-well reservoirs labeled test 1 and test 2, add 400µl of test 1 and 2 antibody starting dilutions (dilu1, 1.5X final concentration) to well 11 (Figure 11).
4. For reference antibody, add 400µl of CDC assay buffer to wells 2 through 10 and 12 (well 1 is empty).
5. For test antibodies 1 and 2, add 200µl of CDC assay buffer to wells 2 through 10 and 12 (well 1 is empty).
6. Transfer 400µl of the reference antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
7. Repeat equivalent reference antibody twofold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.

   Note: Well 2 contains 400µl of CDC assay buffer without antibody, as a negative control. Well 12 contains 400µl of CDC assay buffer without antibody and will be used for digitonin addition at end of the assay.

8. Transfer 200µl of the test 1 and 2 antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.

9. Repeat equivalent test 1 and 2 antibody twofold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.

   Note: Well 2 contains 200µl of CDC assay buffer without antibody, as a negative control. Well 12 contains 200µl of CDC assay buffer without antibody and will be used for digitonin addition at end of the assay.

10. Cover the antibody dilution reservoirs with their lids and keep at ambient temperature (22–25°C) while preparing the normal human serum complement.

Recommended 12-well Reservoir Layouts for Antibody Dilutions Prepared from a Single Antibody Stock.

1	2	3	4	5	6	7	8	9	10	11	12	
	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	no Ab	Reference Ab

1	2	3	4	5	6	7	8	9	10	11	12	
	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	no Ab	Test Ab 1

1	2	3	4	5	6	7	8	9	10	11	12	
	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	no Ab	Test Ab 2

Figure 11. Example 12-well reservoir layouts showing antibody serial dilutions.

5.E. Preparing Normal Human Serum Complement

Normal human serum complement is heat labile and care must be taken to ensure it is fully intact when used in the assay. Follow manufacturer's instructions for storage and handling.

1. Dilute the normal human serum complement with CDC assay buffer, to achieve a 30% solution. Once diluted in the assay plate, this gives a 10% final concentration. You will need at least 4ml of 30% complement to fill 120 assay wells using the inner 60 wells of two assay plates (Figure 10).

5.F. Adding Antibody and Complement to Assay Plates

1. Remove the 96-well assay plates containing mTNFα Target Cells from the incubator. Invert the assay plate to remove the medium. Then, place the inverted plate on paper towels for 5–10 seconds to drain any remaining medium. Alternatively, remove 95µl of medium from each of the wells using a manual multichannel pipette.

2. Add 50µl of the appropriate antibody dilution to the assay plates using a multichannel pipette, according to the plate layout in Figure 10.

3. Add 25µl of the 30% normal human serum complement to each of the inner 60 wells of the assay plates using a multichannel pipette (Figure 10).
4. Add 25µl of CDC assay buffer to wells B12–G12 (digitonin wells) using a multichannel pipette.
5. Add 75µl of CDC assay buffer to each of the empty outside wells of the assay plates.
6. Cover the assay plates with lids and incubate in a 37°C, 5% CO_2 incubator for 6 hours.

   Note: The 6-hour assay time was optimized using infliximab. We recommend optimizing assay time (3–24 hours) with your own antibody or other biologic samples.

5.G. Adding Digitonin and CytoTox-Glo™ Reagent

1. During the 6-hour incubation, reconstitute the CytoTox-Glo™ Reagent according to the instructions in Section 5.A.
2. At the end of the 6-hour incubation, remove the assay plates from the incubator and immediately add 8µl of 1mg/ml digitonin (prepared in Section 5.A) to wells B12–G12, per the plate layout shown in Figure 10.
3. Gently shake the plate briefly to mix the digitonin in the well.
4. Allow plate to equilibrate to ambient temperature (15–20 minutes).
5. Add 40µl of CytoTox-Glo™ Reagent (at ambient temperature) to all wells containing mTNFα Target Cells (wells B2–G12).
6. Add 40µl of CytoTox-Glo™ Reagent to wells B1, D1 and F1 to determine plate background.
7. Incubate at ambient temperature for 15 minutes.
8. Measure luminescence using a plate reader with glow-type luminescence reading capabilities.

5.H. Data Analysis

1. Determine the plate background by calculating the average RLU from wells B1, D1 and F1.
2. Determine the maximum cytotoxicity by calculating the average RLU from wells B12–G12.
3. Calculate fold induction:

$$\text{Fold Induction} = \frac{\text{RLU (antibody} - \text{background)}}{\text{RLU (no antibody control} - \text{background)}}$$

4. Calculate percent specific lysis:

$$\text{Percent Specific Lysis} = \frac{\text{RLU (antibody} - \text{background)}}{\text{RLU (digitonin} - \text{background)}} \times 100$$

5. Graph data as RLU versus Log_{10}[antibody], fold induction versus Log_{10}[antibody] and percent specific lysis versus Log_{10}[antibody]. Fit curves and determine the EC_{50} value of antibody response using appropriate curve fitting software such as GraphPad Prism® software.

6. 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
Low luminescence measurements (RLU readout)	Choose a sensitive instrument designed for luminescence detection. Instruments designed primarily for fluorescence detection are not recommended. Luminometers measure and report luminescence as relative values, and actual numbers will vary between instruments.
	Some models of luminometers with low sensitivity should be avoided. If using a reader with an adjustable gain, we recommend a high gain setting.
	Insufficient cells per well can lead to low RLU. Handle and plate cells according to the instructions to ensure a sufficient number of viable cells per well.
	Low activity of Bio-Glo™ Reagent or CytoTox-Glo™ Reagent can lead to low RLU. Store and handle the reagents according to the instructions.
Weak assay response (low fold induction)	Optimize the concentration range of your test sample(s) to achieve a dose response with complete upper and lower asymptotes. The EC_{50} values obtained in the ADCC Reporter Bioassay or CDC assay with mTNFα Target Cells may vary from the EC_{50} value obtained using other methods.
	Optimize the ADCC Reporter Bioassay incubation time within a range of 5–24 hours or the CDC assay within a range of 3–24 hours, and choose the incubation time that gives optimal response.
	Optimize the normal human serum complement from 5–20% if CDC assay performance is not ideal. Complement must be kept on ice until ready to dilute.
	Optimize the Low IgG Serum concentration from 0.5–10% in ADCC assay buffer if ADCC Reporter Bioassay performance is not ideal.
Variability in assay performance	Ensure that incubation times are consistent between assays.
	Ensure that the Preparing and Plating protocols are strictly followed.

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7. References

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8. Appendix

8.A. Representative Assay Results

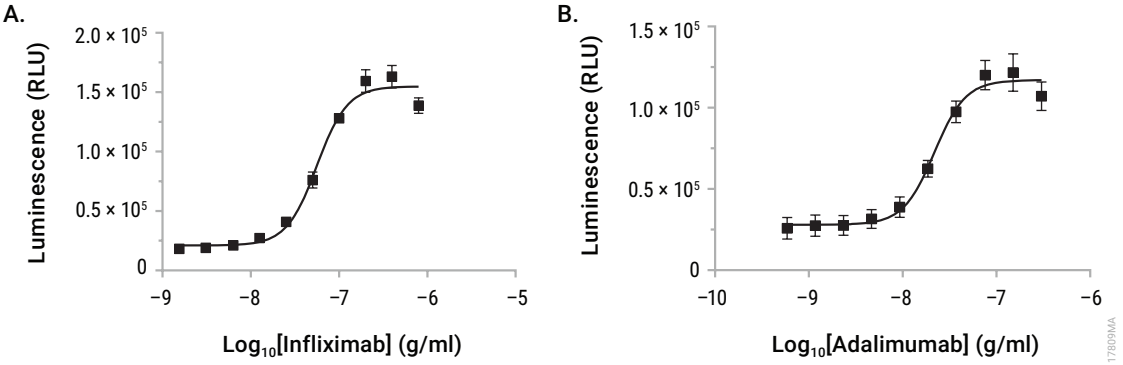

Figure 12. The ADCC Reporter Bioassay with mTNFα Target Cells measures the activity of the anti-TNFα antibody infliximab and adalimumab. mTNFα Target Cells were added to a 96-well assay plate 18-hours prior to the assay. On the day of assay, ADCC Effector Cells and a titration of infliximab (**Panel A)** or adalimumab (**Panel B**) were added. After a 6-hour induction at 37°C, Bio-Glo™ Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. The EC_{50} was 56ng/ml for infliximab and 22ng/ml for adalimumab. Fold induction was 8.5 and 4.7 for infliximab and adalimumab, respectively. Data were generated using thaw-and-use cells.

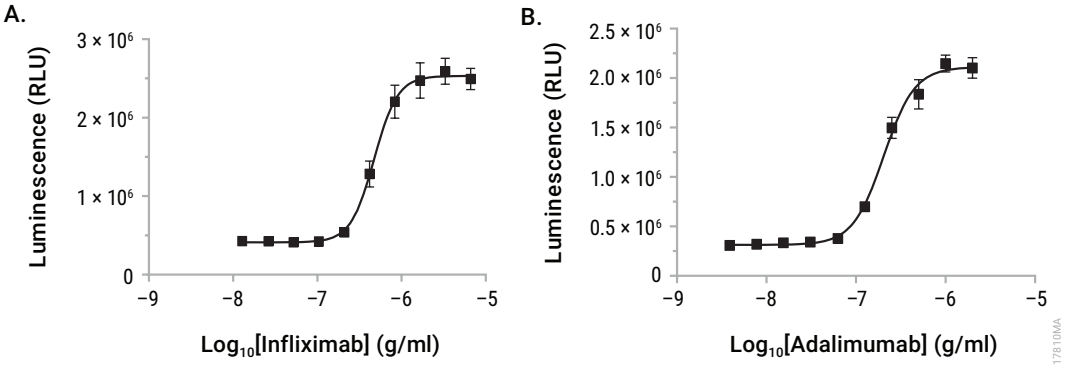

Figure 13. A CDC assay measures the activity of the anti-TNFα antibody infliximab and adalimumab. mTNFα Target Cells were added to a 96-well assay plate 18-hours prior to the assay. On the day of assay, 10% normal human serum complement and a titration of infliximab (**Panel A**) or adalimumab (**Panel B**) were added. After a 6-hour induction at 37°C, CytoTox-Glo™ Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. The EC_{50} for infliximab was 0.48µg/ml, the fold induction was 5.9 and the percent specific lysis was 39.5%. The EC_{50} for adalimumab was 0.2µg/ml, the fold induction 6.9 and the percent specific lysis 27.8%. Data were generated using thaw-and-use cells.

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8.B. Related Products

Fc Effector Bioassays

Product	Size	Cat.#
ADCC Reporter Bioassay, Complete Kit (Raji)*	1 each	G7015
ADCC Reporter Bioassay, Core Kit*	1 each	G7010
ADCC Reporter Bioassay, F Variant, Core Kit**	1 each	G9790
ADCC Reporter Bioassay, Target Kit (Raji)*	1 each	G7016
FcγRIIa-H ADCP Reporter Bioassay, Complete Kit**	1 each	G9901
FcγRIIa-H ADCP Reporter Bioassay, Core Kit**	1 each	G9991
Mouse FcγRIV ADCC Bioassay, Complete Kit	1 each	M1201
Mouse FcγRIV ADCC Bioassay, Core Kit	1 each	M1211
Membrane VEGF Target Cells	1 each	J3351
Membrane RANKL Target Cells	1 each	J3381

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

Fc Effector Immunoassay

Product	Size	Cat.#
Lumit® FcRn Binding Immunoassay	100 assays	W1151

Not for Medical Diagnostic Use. Additional kit formats and sizes are available.

Immune Checkpoint Bioassays

Product	Size	Cat.#
4-1BB Bioassay	1 each	JA2351
CD28 Bioassay	1 each	JA6701
CD28 Blockade Bioassay	1 each	JA6101
CD40 Bioassay	1 each	JA2151
CTLA-4 Blockade Bioassay	1 each	JA3001
GITR Bioassay	1 each	JA2291
ICOS Bioassay	1 each	JA6801
ICOS Blockade Bioassay	1 each	JA6001
LAG-3/MHCII Blockade Bioassay	1 each	JA1111
OX40 Bioassay	1 each	JA2191
PD-1/PD-L1 Blockade Bioassay	1 each	J1250
PD-1+TIGIT Combination Bioassay	1 each	J2211

Immune Checkpoint Bioassays (continued)

Product	Size	Cat.#
PD-L1 Negative Cells	1 each	J1191
TIGIT/CD155 Blockade Bioassay	1 each	J2201

Not for Medical Diagnostic Use. Additional kit formats are available.

T Cell Activation Bioassays

Product	Size	Cat.#
T Cell Activation Bioassay (IL-2)	1 each	J1651
T Cell Activation Bioassay (NFAT)	1 each	J1621
T Cell Activation Bioassay (TCRαβ-KO, CD4+)	1 each	GA1172
T Cell Activation Bioassay (TCRαβ-KO, CD8+)	1 each	GA1162
T Cell Activation Bioassay (TCRαβ-KO, CD4+, CD8+)	1 each	GA1182

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Cytokine and Growth Factor Bioassays

Product	Size	Cat.#
IL-2 Bioassay	1 each	JA2201
IL-6 Bioassay	1 each	JA2501
IL-12 Bioassay	1 each	JA2601
IL-15 Bioassay	1 each	JA2011
IL-23 Bioassay	1 each	JA2511
RANKL Bioassay	1 each	JA2701
VEGF Bioassay	1 each	GA2001

Not for Medical Diagnostic Use. Additional kit formats are available.

Macrophage-Directed Bioassays

Product	Size	Cat.#
SIRPα/CD47 Blockade Bioassay	1 each	JA6011
SIRPα/CD47 Blockade Bioassay, Fc-dependent	1 each	JA4801
TLR Bioassay	1 each	JA9011
ADCP Reporter Bioassay (THP-1)	1 each	JA9411

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8.B. Related Products (continued)

HiBiT Target Cell Killing (TCK) Bioassays

Product	Size	Cat.#
Raji (HT-HiBiT) TCK Bioassay	1 each	JA1211
Raji (LDH-HiBiT) TCK Bioassay	1 each	JA1311
Ramos (HiBiT) TCK Bioassay	1 each	JA1411
H929 (HiBiT) TCK Bioassay	1 each	JA1511

Not for Medical Diagnostic Use. Additional kit formats are available.

Control Antibodies and Proteins

Product	Size	Cat.#
Control Ab, Anti-4-1BB	50µg	K1161
Control Ab, Anti-CD20	5µg	GA1130
Control Ab, Anti-OX40	50µg	K1191
Control Ab, Anti-CD40	50µg	K1181
Control Ab, Anti-CTLA-4	100µg	JA1020
Control Ab, Anti-LAG-3	100µg	K1150
Control Ab, Anti-PD-1	100µg	J1201
Control Ab, Anti-TIGIT	100µg	J2051
Control Ab, Anti-TIM-3	100µg	K1210
Recombinant VEGF Ligand	10µg	J2371

Detection Reagent

Product	Size	Cat.#
Bio-Glo™ Luciferase Assay System	10ml	G7941
	100ml	G7940
Bio-Glo-NL™ Luciferase Assay System	10ml	J3081
	100ml	J3082
	1,000ml	J3083

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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Note: Additional Fc Effector, Immune Checkpoint, T Cell Activation, Cytokine, Macrophage, Primary Cell and Target Cell Killing Bioassays are available. To view and order Promega Bioassay products visit: **www.promega.com/products/reporter-bioassays/** or email: **EarlyAccess@promega.com**. For information on custom bioassay development and services visit the Promega Tailored R&D Solutions website: **www.promega.com/custom-solutions/tailored-solutions/**

9. Summary of Changes

The following changes were made to the 5/25 revision of this document:

1. Removed an expired patent statement.
2. Revised text about the label in Section 3.

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