TECHNICAL MANUAL

CTLA-4 Blockade Bioassay

Instructions for use of Products
JA3001 and JA3005

11/17
TM518

CTLA-4 Blockade Bioassay

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

The human immune system is comprised of a complex network of immune checkpoint molecules that facilitate the elimination of cells expressing foreign antigens while maintaining tolerance to self-antigen. Co-inhibitory immune checkpoint receptors have been shown to perform critical roles in the maintenance of immune homeostasis and are critical in cancer and regulating autoimmune diseases. Several co-inhibitory receptors such as Programmed Cell Death Protein 1 (PD-1), Cytotoxic T-Lymphocyte Associated protein 4 (CTLA-4), T cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (TIGIT), T-cell Immunoglobulin and Mucin-domain containing-3 (TIM-3) and Lymphocyte Activation Gene-3 (LAG-3) have been identifiedas targets for monoclonal antibodies to block co-inhibitory signals such that an active immunotherapy is launched against cancer. Immune checkpoint receptors are promising new immunotherapy targets for the treatment of a variety of diseases, including cancer and autoimmune-mediated disorders (1,2).

CTLA-4, also known as CD152, is an immune inhibitory receptor constitutively expressed on regulatory T cells (Tregs) and upregulated in activated T cells. CTLA-4 plays a critical role in regulating immune responses to tumor antigens and autoantigens. CTLA-4 is the counterpart of the co-stimulatory B7-CD28 pathway. When CTLA-4 expression is upregulated on the surface of T cells, the T cells bind B7 with a higher avidity, and thus out-compete the positive co-stimulatory signal from CD28. In addition, engagement of CTLA-4 by either of its ligands, CD80 (B7-1) or CD86 (B7-2) on an adjacent antigen presenting cell (APC) inhibits CD28 co-stimulation of T cell activation, cell proliferation and cytokine production. CTLA-4 has been a key target in drug discovery since the mid-1990s, when James Allison discovered the role of CTLA-4 in immune cells and observed that blocking it reduced tumor size. Following the success of CTLA-4 immunotherapy drug ipilimumab (marketed as YERVOY for metastatic melanoma), other new therapeutic antibodies and Fc fusion proteins designed to block the CTLA-4/CD80 and CD86 interaction have been launched as drugs or have shown promising results in clinical trials for the treatment of a variety of cancers (3–5).

Current methods used to measure the activity of potential biologic drugs targeting CTLA-4 rely on primary human T cells and measurement of functional endpoints such as cell proliferation, cell surface marker expression and interferon gamma (IFNγ) and interleukin-2 (IL-2) production. These assays are laborious and highly variable due to their reliance on donor primary cells and complex assay protocols. As a result, these assays are difficult to establish in drug development settings.

The CTLA-4 Blockade Bioassay[a–d] (Cat.# JA3001, JA3005), is a bioluminescent cell-based assay that overcomes the limitations of existing assays and can be used to measure the potency and stability of antibodies and other biologics targeting CTLA-4 (6,7). The assay consists of two genetically engineered cell lines:

- **CTLA-4 Effector Cells:** Jurkat T cells expressing human CTLA-4 and a luciferase reporter driven by a native promoter which responds to TCR/CD28 activation
- **aAPC/Raji Cells: Raji** cells expressing an engineered cell surface protein designed to activate cognate TCRs in an antigen-independent manner and endogenously expressing CTLA-4 ligands CD80 and CD86

The CTLA-4 Effector Cells and aAPC/Raji Cells are provided in thaw-and-use format as cryopreserved cells that can be thawed, plated and used in an assay without the need for cell propagation.

When the two cell types are co-cultured, CTLA-4 competes with CD28 for their shared ligands, CD80 and CD86, and thus inhibits CD28 pathway activation and promoter-mediated luminescence. Addition of an anti-CTLA-4 antibody blocks the interaction of CTLA-4 with its ligands CD80 and CD86 and results in promoter-mediated luminescence (Figure 1). The CTLA-4 Blockade Bioassay includes the necessary medium and serum to thaw, plate and assay the cells. The bioluminescent signal can be detected and quantified using the Bio-Glo™ Luciferase Assay System, also included in the kit, and a standard luminometer such as the GloMax® Discover System (see Section 7.B, Related Products).

In addition to the CTLA-4 Blockade Bioassay (Cat.# JA3001, JA3005), we offer TCR/CD3 Effector Cells (Cat.# J1651) for use as a negative control in the CTLA-4 Blockade Bioassay. When co-cultured with aAPC/Raji Cells, the CD28 signaling in the CTLA-4 Negative Cells is activated, however this response is not affected by anti-CTLA-4 antibodies (see Section 7.A, Representative Assay Results). We also offer Control Ab, Anti-CTLA-4 (Cat.# JA1020), a blocking antibody for use as a positive control.

The CTLA-4 Blockade Bioassay combines: 1) a simple, add-mix-read single-day workflow with 2) CTLA-4 Effector Cells and aAPC/Raji Cells provided in a frozen, thaw-and-use format, and 3) an optimized protocol, that together yield a quantitative bioassay that exhibits low variability and high accuracy. The thaw-and-use cells provided in the CTLA-4 Blockade Bioassay kits are manufactured under stringent quality control to provide high assay reproducibility with the convenience of an assay reagent that eliminates the need for continuous cell propagation.

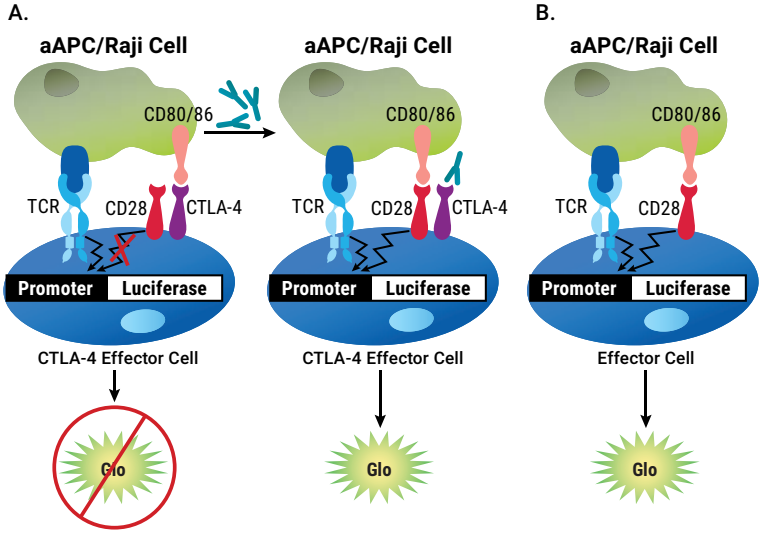

Figure 1. Representation of the CTLA-4 Blockade Bioassay. The bioassay consists of two genetically engineered cell lines, CTLA-4 Effector Cells and aAPC/Raji Cells. **Panel A.** When co-cultured, the CTLA-4/CD80 and CD86 interaction inhibits CD28 pathway activated luminescence. The addition of anti-CTLA-4 antibody blocks the CTLA-4/CD80 and CD86 interaction, thereby re-establishing CD28 pathway activated luminescence, which can be detected in a dose-dependent manner by addition of Bio-Glo™ Reagent and quantitation with a luminometer. **Panel B.** When co-cultured with non-CTLA-4-expressing Effector Cells (Cat.# J1631), activation also induces luminescence by activation of the CD28 pathway but in a manner independent of anti-CTLA-4 antibody.

1. Description (continued)

The CTLA-4 Blockade Bioassay reflects the mechanism of action (MOA) of biologics designed to block the interaction of CTLA-4 with its ligands, CD80 and CD86. Specifically, CD28-mediated luminescence is detected following the addition of anti-CTLA-4 blocking antibodies but not following addition of a non-specific anti-HER2, anti-PD-1 or anti-TIGIT blocking antibody (Figure 2). The bioassay is prequalified according to ICH guidelines and shows the precision, accuracy and linearity required for routine use in potency and stability studies (Table 1 and Figure 3). The assay can be performed in a single day (6-hour) or overnight (16-hour) timeframe. The 6-hour assay results in higher luminescence signal, while the 16-hour assay results in higher fold induction at the expense of sensitivity. The decision of assay time will depend in part on luminescence reader sensitivity and also on workflow preference (Figure 4). The bioassay workflow is simple and robust and compatible with both 96-well and 384-well plate formats used for antibody screening in early drug discovery (Figure 5). In addition, the bioassay can be used with up to 10% human serum with minimal impact on anti-CTLA-4 EC_{50} and fold induction (Figure 6), indicating potential for further development into a neutralizing antibody bioassay. Finally, the bioassay can also be used to measure activity of a Fab or F(ab´)2 fragment of anti-CTLA-4 blocking antibody (data not shown).

It is increasingly common during drug development to analyze potential therapeutic antibodies for Antibody Dependent Cell Cytoxicity (ADCC) activity. Another application of the CTLA-4 Blockade Bioassay is the ability to measure ADCC activity of anti-CTLA-4 blocking antibodies by combining ADCC Bioassay Effector cells, available separately (Cat.# G7010, G7018), with CTLA-4 Effector Cells (Figure 7). The Control Ab, Anti-CTLA-4 (Cat.# JA1020), a blocking antibody for use as a positive control for the CTLA-4 Blockade Bioassay, also serves as a positive control in the ADCC assay application.

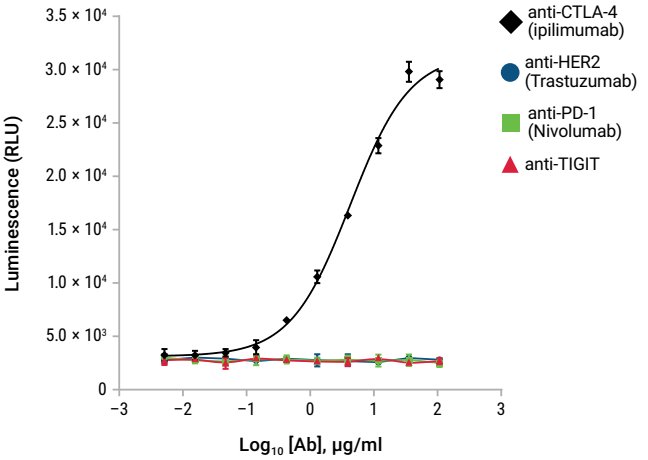

Figure 2. The CTLA-4 Blockade Bioassay reflects the mechanism of action (MOA) and specificity of biologics designed to block the CTLA-4/CD80 and CD86 interaction. CTLA-4 Effector Cells were incubated with aAPC/Raji Cells in the absence or presence of anti-CTLA-4, anti-PD-1, anti-TIGIT or anti-HER2 blocking antibodies, as indicated. Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover Detection System. Data were fitted to a 4-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

1. Description (continued)

Table 1. The CTLA-4 Blockade Bioassay Shows Precision, Accuracy and Linearity.

Parameter	Results	
Accuracy	% Expected Relative Potency	% Recovery
	50	105.3
	70	96.7
	130	108.4
	150	112.3
Repeatability (% CV)	100% (Reference)	6.7
Intermediate Precision (% CV)		10.2
Linearity (r^2)		0.991
Linearity (y = mx + b)		y = 1.175x – 11.45

A 50–150% theoretical potency series of ipilimumab (CTLA-4 blocking antibody) was analyzed in triplicate in three independent experiments performed on three days by two analysts. Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover Detection System. Data were analyzed and relative potencies calculated after parallelism determination using JMP® software. Data were generated using thaw-and-use cells.

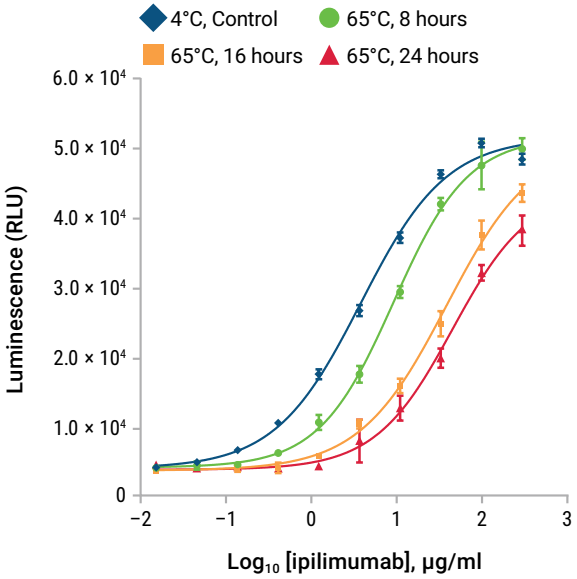

Figure 3. The CTLA-4 Blockade Bioassay is stability-indicating. Samples of anti-CTLA-4 antibody, ipilimumab, were maintained at 4°C (control) or heat-denatured at 65°C for the indicated times and analyzed using the CTLA-4 Blockade Bioassay. Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover Detection System. Data were fitted to a 4-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

1. Description (continued)

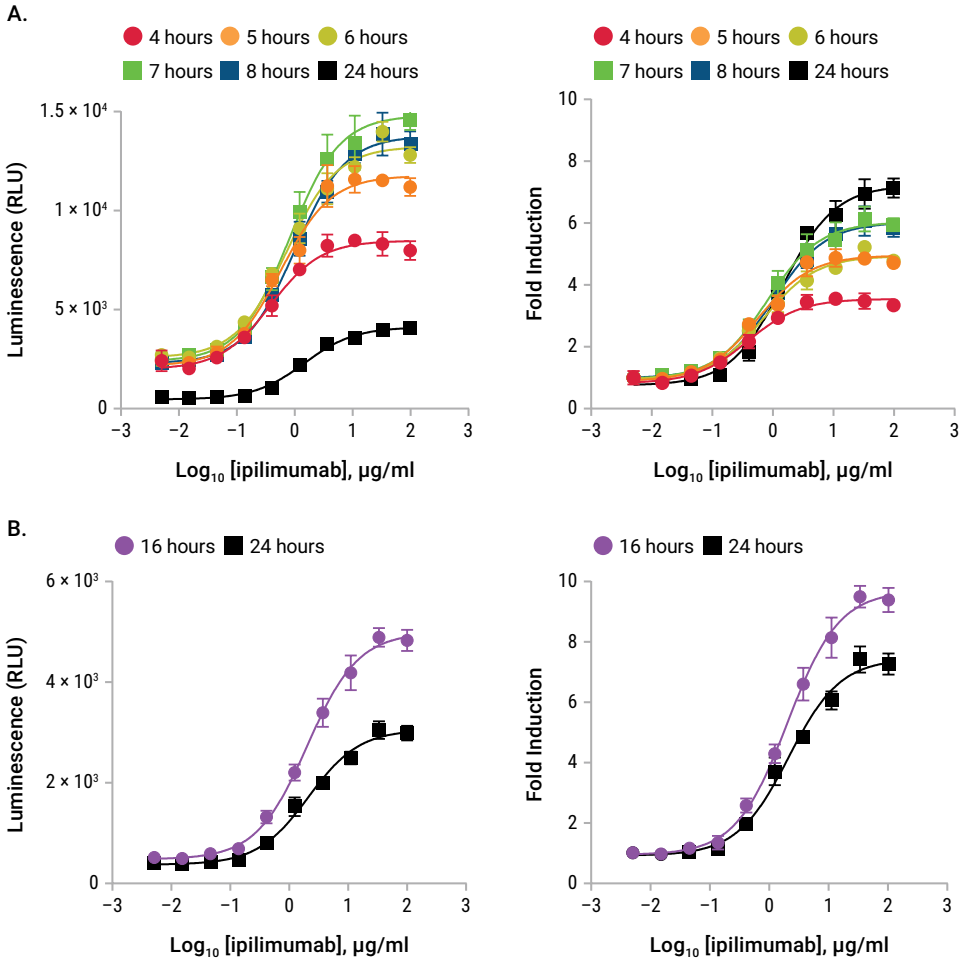

Figure 4. The CTLA-4 Blockade Bioassay can be run in one day (6 hours) or overnight (16 hours). Anti-CTLA-4 antibody, ipilimumab, was tested in the CTLA-4 Blockade Bioassay over a 4- to 24-hour timeframe. Bio-Glo™ Reagent was added, followed by quantification of luminescence (left panels) and fold induction (right panels) using the GloMax® Discover Detection System. Data were fitted to a 4-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells. **Panel A.** Assays were set up in the morning. The 6-hour assay time resulted in optimal luminescence signal with a manageable 1-day workflow. **Panel B.** Assays were set up in the evening. The 16-hour assay time resulted in optimal fold induction with a convenient 16-hour overnight workflow.

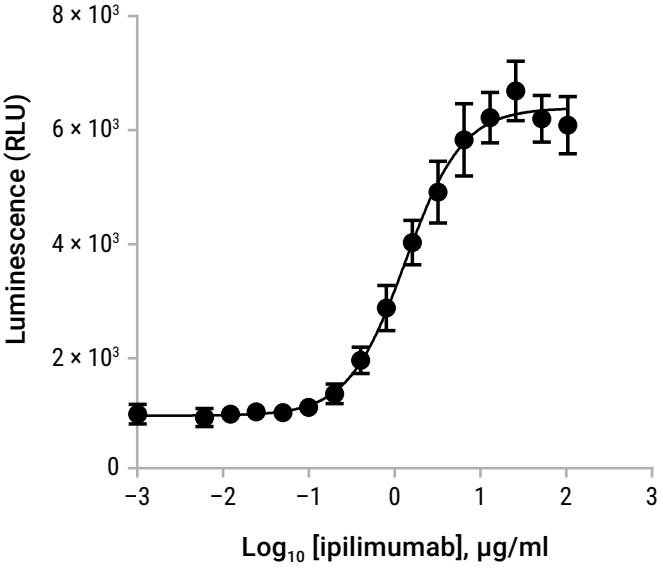

Figure 5. The assay is amenable to 384-well plate format and compatible with laboratory automation. Anti-CTLA-4 antibody, ipilimumab, was tested in the CTLA-4 Blockade Bioassay using a Multidrop™ Combi nL (Thermo Fisher Scientific) dispenser. Cells were plated at 4×10^4/well (CTLA-4 Effector Cells) and 2×10^4/well (aAPC/Raji Cells) in 5µl each/well. A two-fold serial dilution of ipilimumab was plated in 10µl/well. After 16-hour assay incubation, 20µl Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover Detection System. Data were fitted to a 4-parameter logistic curve using GraphPad Prism® software. The EC_{50} value was 1.3µg/ml. Data were generated using thaw-and-use cells.

1. Description (continued)

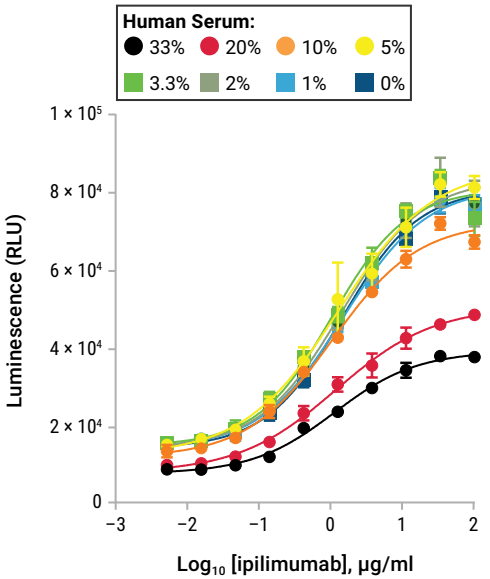

Figure 6. The CTLA-4 Blockade Bioassay is tolerant to human serum. Anti-CTLA-4 blocking antibody, ipilimumab, was analyzed in the absence or presence of increasing concentrations of pooled normal human serum (0–100% in the antibody sample), resulting in final assay concentration of human serum (0–33%). Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover Detection System. Data were fitted to a 4-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells. The CTLA-4 blockade assay is tolerant up to 10% serum with this human serum pool. A different human serum pool showed no effect on the assay (data not shown).

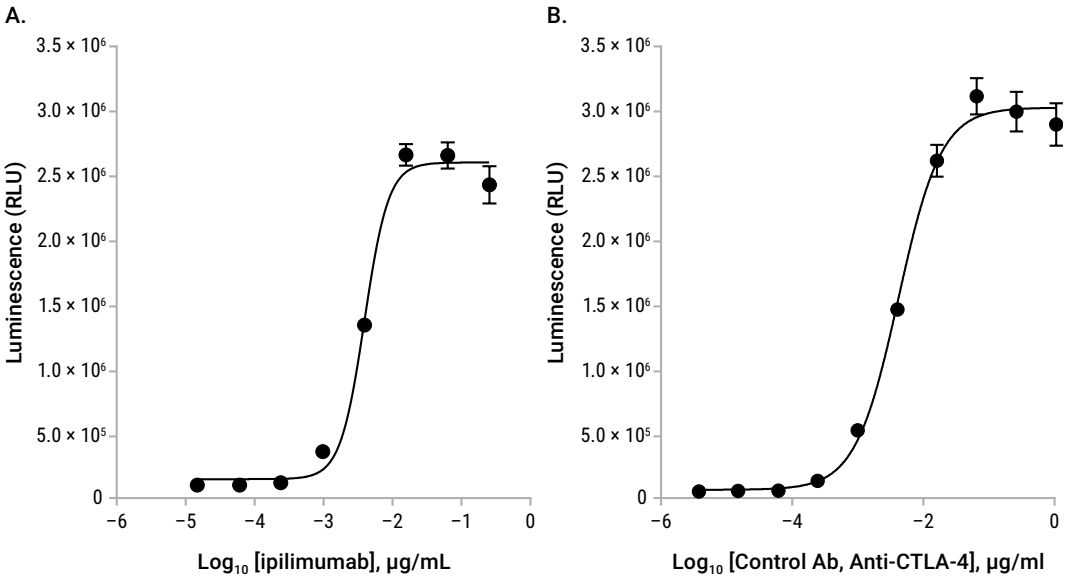

Figure 7. The CTLA-4 Blockade Bioassay can be used to measure ADCC activity. A 1:1 ratio of CTLA-4 Effector Cells (used as Target cells in this application) and ADCC Bioassay Effector Cells (Cat.# G7102) were incubated in the presence of anti-CTLA-4 blocking antibody, ipilimumab (**Panel A**), or Control Ab, Anti-CTLA-4 (Cat.# JA1020; **Panel B**), according to the instructions in ADCC Reporter Bioassay Core Kit Technical Manual TM383 (Cat.# G7010, G7018). Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover Detection System. Data were fitted to a 4-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
CTLA-4 Blockade Bioassay	**1 each**	**JA3001**

Not for Medical Diagnostic Use.

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

- 1 vial CTLA-4 Effector Cells (0.8ml)
- 1 vial aAPC/Raji Cells (0.8ml)
- 36ml RPMI 1640 Medium
- 4ml Fetal Bovine Serum
- 1 vial Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 10ml Bio-Glo™ Luciferase Assay Buffer

PRODUCT	SIZE	CAT.#
CTLA-4 Blockade Bioassay 5X	**5 each**	**JA3005**

Not for Medical Diagnostic Use.

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

- 5 vials CTLA-4 Effector Cells (0.8ml)
- 5 vials aAPC/Raji Cells (0.8ml)
- 5 × 36ml RPMI 1640 Medium
- 5 × 4ml Fetal Bovine Serum
- 5 vials Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 5 × 10ml Bio-Glo™ Luciferase Assay Buffer

Note: The CTLA-4Blockade Bioassay components are shipped separately because of differing temperature requirements. The CTLA-4 Effector Cells and aAPC/Raji Cells are shipped on dry ice. The Bio-Glo™ Luciferase Assay System and Fetal Bovine Serum are shipped on dry ice, separately from the cells. The RPMI 1640 Medium is shipped at ambient temperature.

Storage Conditions:

- Upon arrival, immediately transfer the cell vials to below −140°C (freezer or liquid nitrogen vapor phase) for long-term storage. Do not store cell vials submerged in liquid nitrogen. Do not store cell vials at −80°C because this will negatively impact cell viability and cell performance.
- Store Bio-Glo™ Luciferase Assay Substrate, Bio-Glo™ Luciferase Assay Buffer and Fetal Bovine Serum at −20°C. Avoid multiple freeze-thaw cycles of the serum.
- For optimal performance, use reconstituted Bio-Glo™ Reagent on the day of preparation. However, once reconstituted, Bio-Glo™ Reagent can be stored at −20°C for up to 6 weeks.
- Store RPMI 1640 Medium at 4°C, protected from fluorescent light.

3. Before You Begin

The CTLA-4 Blockade Bioassay is intended to be used with user-provided antibodies or other biologics designed to block the interaction of CTLA-4 with its ligands, CD80 and CD86. Control Ab, Anti-CTLA-4 (Cat.# JA1020), is available separately for use in assay optimization and routine quality control. CTLA-4 Negative Cells from the T Cell Activation Bioassay (IL-2) (Cat.# J1651, J1655) are also available separately and may be used for the negative control. We strongly recommend including Control Ab, Anti-CTLA-4, as a positive control in the first few assays to gain familiarity with the assay. Data generated using these reagents are shown in Section 7.A, Representative Assay Results.

Please read through the entire protocol to become familiar with the components and the assay procedure before beginning. The CTLA-4 Effector Cells and aAPC/Raji 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 exactly as described. Do not overmix or overwarm the cell reagents.

The CTLA-4 Blockade Bioassay produces a bioluminescent signal and requires a sensitive luminescence plate reader. Bioassay development and performance data included in this Technical Manual were generated using the GloMax® Discover or GloMax® Multi+ System (see Section 7.B, Related Products). An integration time of 0.5 second/well was used for all readings. The bioassay is compatible with most other plate-reading luminometers. Relative luminescence unit readings will vary with the sensitivity and settings of each instrument. We recommend a shorter assay time (6 hours) when using lower sensitivity readers to maximize the assay luminescence levels. Figure 4 above demonstrates the differences in assay luminescence signal and fold induction with varying assay times.

If you are using a reader with adjustable gain, we recommend a high-gain setting. The use of different instruments, shorter or longer assay times and gain adjustment will affect the magnitude of the raw data but should not affect the measured relative potency of test samples.

3.A. Materials to Be Supplied by the User

- user-defined anti-CTLA-4 blocking antibodies or other biologics samples
- solid-white, flat-bottom 96-well assay plates (e.g., Corning Cat.# 3917) or 384-well assay plate (e.g., Corning Cat.# 3570) for plating and reading luminescence
- sterile clear V-bottom 96-well plate with lid (e.g., Costar Cat. Cat.# 3896 or Linbro Cat.# 76-223-05) for preparing 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
- plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System or equivalent system)

4. Assay Protocol

This assay protocol illustrates the use of the CTLA-4 Blockade Bioassay to test two antibody samples against a reference sample in a single assay run. Each test and reference antibody is run in triplicate, in a 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.

For 384-well assay setup, we recommend preparing the cells and antibody as described in the protocol below and plating 1/5 the volume used in the 96-well assay as a starting point. Further optimization may be required for various automated platforms.

Note: When preparing test and reference antibodies, choose an appropriate starting concentration and dilution scheme to achieve a full dose-response curve with proper upper and lower asymptotes and sufficient points on the slope. For reference, we use 30µg/ml as a starting concentration (1X) and threefold serial dilutions when testing Control Ab, Anti-CTLA-4 (Cat.# JA1020), and 100µg/ml as a starting concentration (1X) and threefold serial dilutions when testing the anti-CTLA-4 antibody, ipilimumab, to achieve full dose curves.

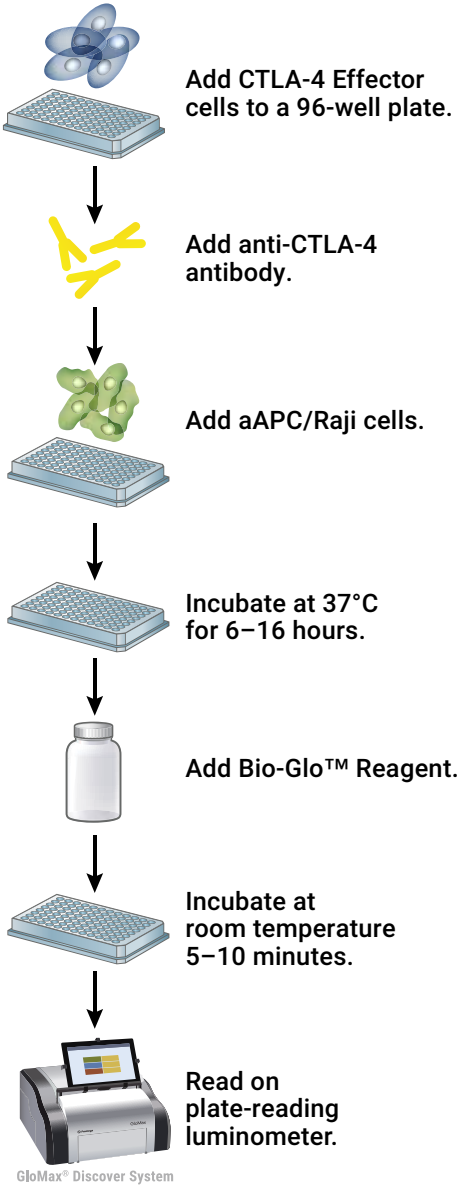

Figure 8. Schematic protocol for the CTLA-4 Blockade Bioassay.

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4.A. Preparing Assay Buffer, Bio-Glo™ Reagent and Antibody Samples

1. **Assay Buffer:** On the day of the assay, prepare 40ml of assay buffer (90% RPMI 1640/10% FBS) in a 50ml conical tube. Thaw the Fetal Bovine Serum (FBS) overnight at 4°C or in a 37°C water bath on the day of use. Add 4ml of FBS to 36ml of RPMI 1640 Medium to yield 90% RPMI 1640/10% FBS. Mix well and warm to 37°C prior to use. For reference, 40ml of assay buffer is typically sufficient for 120 wells in a 96-well assay format using the inner 60 wells.

   Note: The recommended assay buffer contains 10% FBS. This concentration of FBS works well for all of the anti-CTLA-4 antibodies we have 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%.

2. **Bio-Glo™ Reagent:** 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. When stored appropriately, Bio-Glo™ reagent will maintain at least 80% activity after 24 hours at ambient temperature. For reference, 10ml of Bio-Glo™ Reagent is sufficient to assay 120 wells in a 96-well assay format.

3. **Test and Reference Samples:** Using assay buffer as the diluent, prepare starting dilutions (dilu1, 3X final concentration) of two test antibodies (150µl each) and one reference antibody (300µl) in 1.5ml tubes. Store the tubes containing antibody starting dilutions appropriately before making antibody serial dilutions.

   Note: If you are using Control Ab, Anti-CTLA-4 (Cat.# JA1020), as a reference antibody in your assay, prepare 300µl of 90µg/ml starting dilution (dilu1, 3X final concentration) by adding 27µl of Control Ab, Anti-CTLA-4, stock (1mg/ml) to 273µl of assay buffer. Store the antibody starting dilution on ice until ready to use in the assay.

   Tip: To streamline assay setup, prepare antibody serial dilutions prior to thawing and plating cells.

4.B. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 9 as a guide. The protocol describes serial replicate dilutions (n = 3) of test and reference antibodies 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	Test Ab
E	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ab
F	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ab
G	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
H	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)

Figure 9. Example plate layout showing non-clustered sample locations of test and reference antibody dilution series and wells containing assay buffer (denoted by "B") alone.

4.C. Preparing and Plating CTLA-4 Effector Cells

The following steps should be performed using aseptic technique in a sterile cell culture hood if setting up a 16-hour assay. For a 6-hour assay, the setup may be performed on the bench.

Note: The thaw-and-use CTLA-4 Effector Cells included in this kit 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 a time.

1. On the day of assay, prepare an appropriate amount of assay buffer as described in Section 4.A.
2. Add 3.2ml of prewarmed (37°C) assay buffer to a 15ml conical tube.
3. Remove one vial of CTLA-4 Effector Cells from storage at −140°C and transfer to the bench on dry ice. Warm the cells in a 37°C water bath until just thawed (about 2–3 minutes). While thawing, gently agitate and visually inspect.
4. Gently mix the cell suspension by pipetting, and transfer the cells (0.8ml) to the 15ml conical tube containing 3.2ml of assay buffer. Mix well by gently inverting or pipetting 1–2 times.
5. Transfer the cell 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 two 96-well, solid, white, flat-bottom assay plates.
6. Add 75µl of assay buffer to each of the outside wells of the assay plates.
7. Cover the assay plates with a lid and keep at ambient temperature (22–25°C). Proceed to Section 4.D.

4.D. Preparing and Adding Antibody Serial Dilutions

The instructions described here are for preparation of a single stock of threefold serial dilutions of a single antibody for analysis in triplicate (150µl of each dilution provides a sufficient volume for analysis in triplicate). Alternatively, you can prepare three independent stocks of serial dilutions to generate triplicate samples. To prepare threefold serial dilutions, you will need 300µl of reference antibody at 3X the highest antibody concentration in your dose-response curve. You will need 150µl 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: If you are using Control Ab, Anti-CTLA-4 (Cat.# JA1020), as a control in the assay, follow the instructions below to prepare threefold serial dilutions.

1. To a sterile clear V-bottom 96-well plate, add 150µl of reference antibody starting dilution (dilu1, 3X final concentration) to wells A11 and B11 (see Figure 10).
2. Add 150µl of test antibodies 1 and 2 starting dilution (dilu1, 3X final concentration) to wells E11 and G11, respectively (see Figure 10).
3. Add 100µl of assay buffer to other wells in these four rows, from column 10 to column 2.

4. Transfer 50µl of the antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
5. Repeat equivalent threefold 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 assay buffer without antibody as a negative control.

   Steps 1–5 can be completed prior to plating CTLA-4 Effector Cells in Section 4.C.
6. Remove the lid from the 96-well assay plates containing CTLA-4 Effector Cells.
7. Using an electronic multichannel pipette, add 25µl of the appropriate antibody dilution (see Figure 10) to the plated CTLA-4 Effector Cells according to the plate layout in Figure 9.
8. Cover the assay plates with a lid and keep at ambient temperature (22°–25°C) while preparing the aAPC/Raji Cells.

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 10. Example plate layout showing antibody serial dilutions.

4.E. Preparing and Plating aAPC/Raji Cells

Note: The thaw-and-use aAPC/Raji Cells included in this kit 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. Add 7.2ml of prewarmed (37°C) assay buffer to a 15ml conical tube.
2. Remove one vial of aAPC/Raji 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 2–3 minutes). While thawing, gently agitate and visually inspect.
3. Gently mix the cell suspension by pipetting, and transfer the cells (0.8ml) to the 15ml conical tube containing 7.2ml assay buffer. Mix well by gently inverting or pipetting 1–2 times.
4. Transfer the cell suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 25µl of the cell suspension to to the pre-plated CTLA-4 Effector Cells and Anti-CTLA-4 Control Antibody. The final assay volume is 75µl.
5. Cover the assay plates with a lid and incubate for 6 hours in a 37°C, 5% CO_2 incubator.

Note: The 6-hour assay time was optimized for maximum luminescence signal. We recommend optimizing the assay time (6–16 hours) for optimal assay response (see Figure 4).

Plating CTLA-4 Effector Cells and aAPC/Raji Cells as indicated will result in a 2:1 ratio of Effector:Target cells. If higher luminescence signals are desired, the dilution volume of the aAPC/Raji Cells may be halved to attain a 1:1 ratio of Effector:Target cells.

4.F. Adding Bio-Glo™ Reagent

Note: Bio-Glo™ Reagent should be at ambient temperature (22–25°C) when added to assay plates.

1. Remove the assay plates from the incubator and equilibrate to ambient temperature for 10–15 minutes.
2. Using a manual multichannel pipette, add 75µl of Bio-Glo™ Reagent to the inner 60 wells of the assay plates, taking care not to create bubbles.
3. Add 75µl of Bio-Glo™ Reagent to wells B1, C1 and D1 of each assay plate to measure the background signal.
4. Incubate at ambient temperature for 5–15 minutes.

Note: Varying the incubation time will affect the raw luminescence values but should not significantly change the EC_{50} value and fold induction.

5. Measure luminescence using a luminometer luminescence plate reader.

4.G. Data Analysis

1. Measure plate background by calculating the average relative light units (RLU) from wells B1, C1 and D1.
2. Calculate fold induction = RLU (induced–background)/RLU (no antibody control–background).

Note: When calculating fold induction, if the no-antibody control sample RLUs are at least 100X the plate background RLUs, there is no need to subtract plate background from sample RLU.

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. 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
Low luminescence measurements (RLU readout)	Choose an instrument designed for plate-reading luminescence detection. Instruments designed primarily for fluorescence detection are not recommended. Luminometers measure and report luminescence as relative values, and actual Relative Light Units numbers will vary between instruments.
	Choice of assay time will affect assay luminescence signal. Shorter assay time (6 hours) will increase luminescence compared to longer assay time (16 hours). See Figure 4.
	Insufficient cells per well can lead to low luminescence signal. Handle and plate cells according to the instructions to ensure a sufficient number of viable cells per well.
	Low cell viability can lead to low luminescence readout and variability in assay performance.
	Low activity of Bio-Glo™ Reagent leads to low luminescence signal. Store and handle the Bio-Glo™ Reagent according to the instructions.
Weak assay response (low fold induction)	Optimize the concentration range of your test sample(s) to achieve a full dose response with complete upper and lower asymptotes. The EC_{50} value obtained in the CTLA-4 Blockade Bioassay may vary from the EC_{50} obtained using other methods such as primary T cell-based assays.
	Optimize the assay incubation time within a range of 6–16 hours.
	If untreated control RLU is less than 100X above plate reader background RLU, subtract plate reader background RLU from all samples prior to calculating fold induction.

6. References

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7. Appendix

7.A. Representative Assay Results

The following data were generated using the CTLA-4 Blockade Bioassay using research or clinical grade anti-CTLA-4 blocking antibodies (Figure 11).

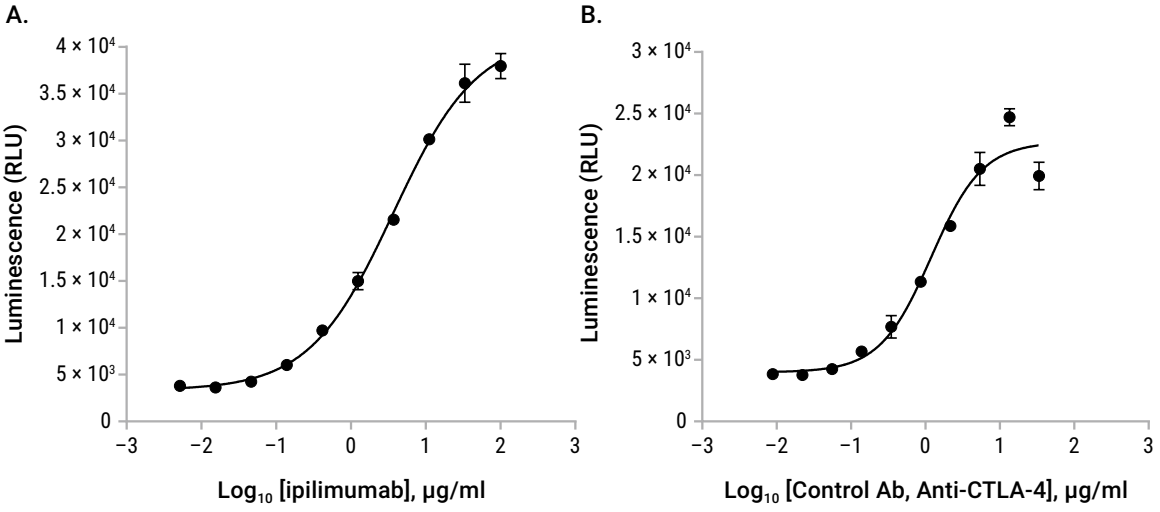

Figure 11. The CTLA-4 Blockade Bioassay measures the inhibitory activity of clinical grade anti-CTLA-4 blocking antibodies. CTLA-4 Effector Cells and aAPC/Raji Cells were incubated with increasing concentrations of anti-CTLA-4 antibody, ipilimumab (**Panel A**), or Control Ab, Anti-CTLA-4 (Cat.# JA1020; **Panel B**). After 6 hours, Bio-Glo™ Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a 4-parameter logistic curve using using GraphPad Prism® software. The EC_{50} values were 3.6µg/ml (ipilimumab) and 1.2µg/ml (Control Ab). Data were generated using thaw-and-use cells.

7.B. Related Products

CTLA-4 Blockade Bioassays

Product	Size	Cat.#
CTLA-4 Blockade Bioassay, Cell Propagation Model	1 each	JA1400
Control Ab, Anti-CTLA-4	1 each	JA1020

Not for Medical Diagnostic Use.

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

T Cell Activation Bioassays

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

Not for Medical Diagnostic Use.

Promega

Immune Checkpoint Bioassays

Product	Size	Cat.#
PD-1/PD-L1 Blockade Bioassay	1 each	J1250
PD-1/PD-L1 Blockade Bioassay 5X	1 each	J1255
PD-L1 Negative Cells	1 each	J1191
Control Ab, Anti-PD-1	1 each	J1201

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

Detection Reagents

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

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

Luminometers

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

For Research Use Only. Not for use in diagnostic procedures.

Note: Additional Fc Effector, Immune Checkpoint and Cytokine bioassays are available from Promega Custom Assay Services. To view and order products from Custom Assay Services visit: **www.promega.com/CAS** or email: **CAS@promega.com**

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