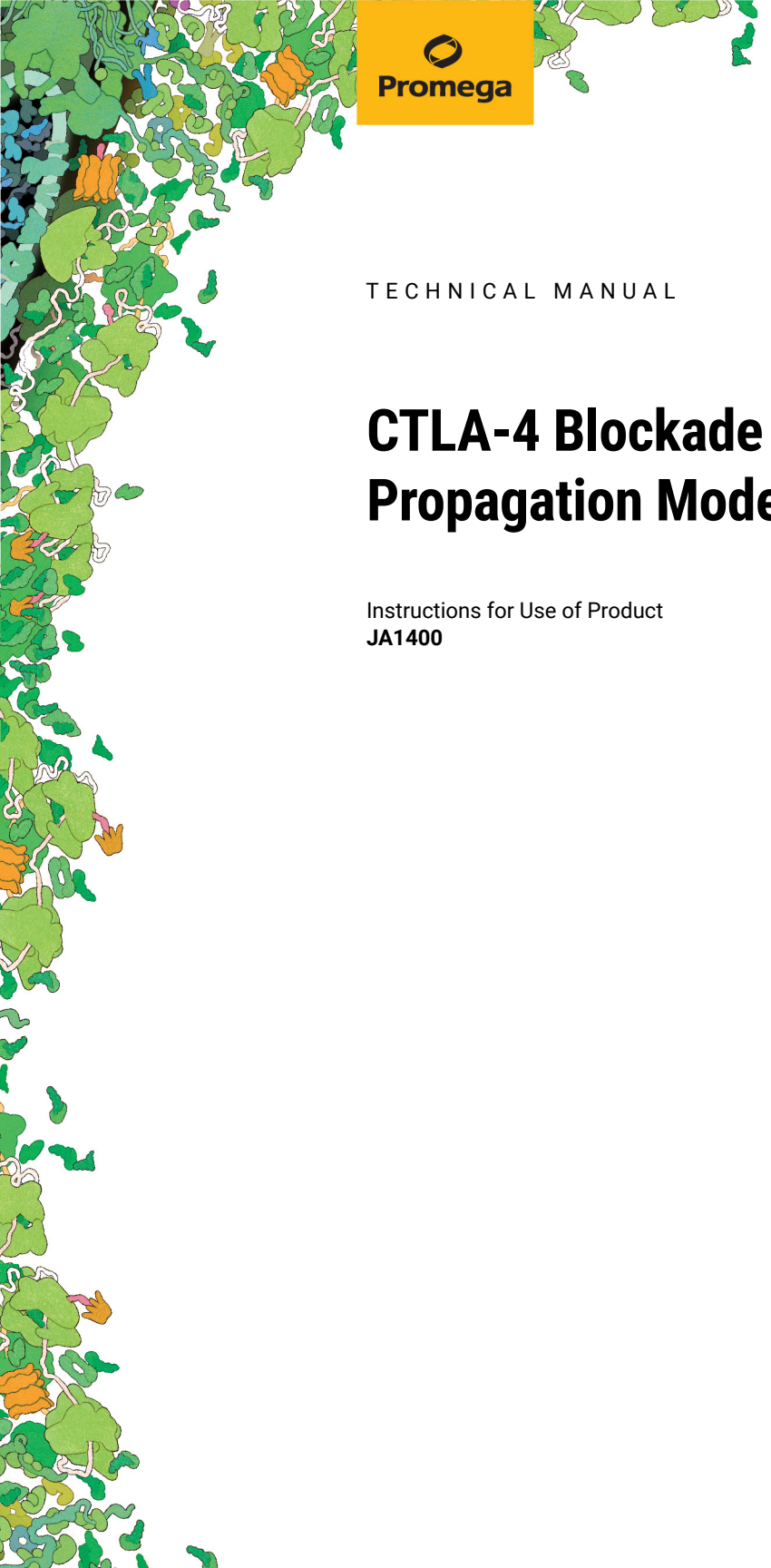


TECHNICAL MANUAL

CTLA-4 Blockade Bioassay, Propagation Model

Instructions for Use of Product
JA1400

Revised 8/26
TM517

Promega

CTLA-4 Blockade Bioassay, Propagation Model

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

1. Description 2
2. Product Components and Storage Conditions 12
3. Before You Begin 12
   - 3.A. Materials to Be Supplied by the User 13
   - 3.B. Preparing CTLA-4 Effector Cells 14
   - 3.C. Preparing aAPC/Raji Cells 15
4. Assay Protocol 17
   - 4.A. Preparing Assay Buffer, Bio-Glo™ Reagent and Antibody Samples 19
   - 4.B. Plate Layout Design 20
   - 4.C. Preparing and Plating CTLA-4 Effector Cells 21
   - 4.D. Preparing and Adding Antibody Serial Dilutions 22
   - 4.E. Preparing and Plating aAPC/Raji Cells 24
   - 4.F. Adding Bio-Glo™ Reagent 24
   - 4.G. Data Analysis 25
5. Troubleshooting 26
6. References 27
7. Appendix 28
   - 7.A. Representative Assay Results 28
8. Summary of Changes 30

Promega

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 identified as 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, Propagation Model[(a,b)] (Cat.# JA1400), 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 Cell Propagation Model (CPM) format, which includes cryopreserved cells that can be thawed, propagated and banked for long-term use.

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 bioluminescent signal can be detected and quantified using the Bio-Glo™ Luciferase Assay System (Cat.# G7941) and a standard luminometer such as the GloMax® Discover System.

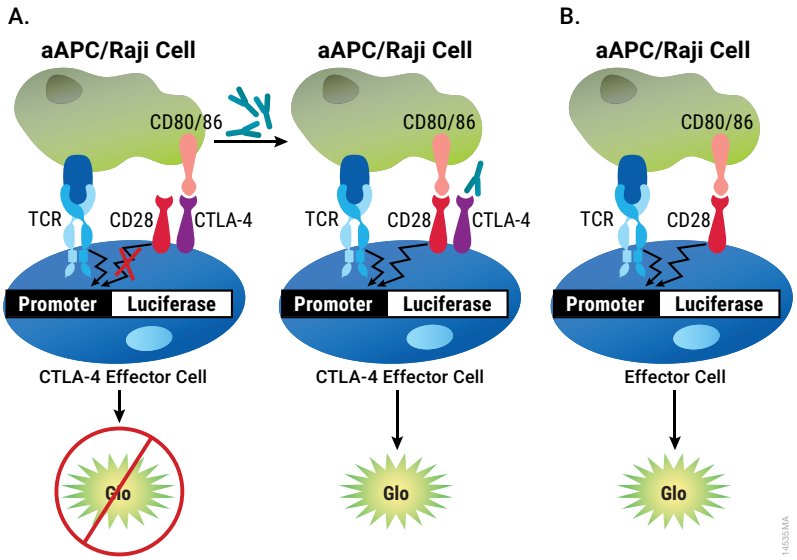


Figure 1. Representation of the CTLA-4 Blockade Bioassay. The bioassay consists of two genetically engineered cell lines, CTLA-4 Effector Cells and aAPC/Raji Cells. **Panel A.** When co-cultured, the CTLA-4/CD80 and CD86 interaction inhibits CD28 pathway activated luminescence. The addition of anti-CTLA-4 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.

Promega

1. Description (continued)

The CTLA-4 Blockade Bioassay, Propagation Model, 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 the 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- 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.# G7102), 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.

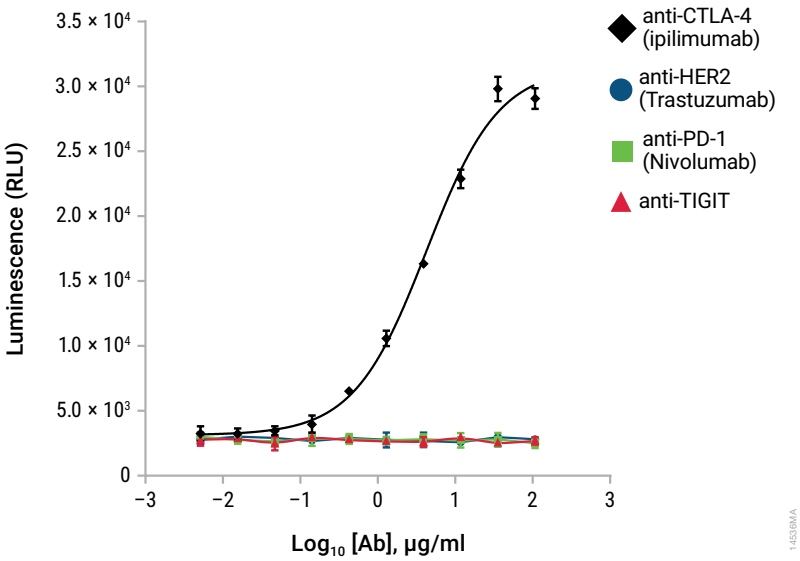


Figure 2. The CTLA-4 Blockade Bioassay reflects the mechanism of action (MOA) and specificity of biologics designed to block the CTLA-4/CD80 and CD86 interaction. CTLA-4 Effector Cells were incubated with aAPC/Raji Cells in the absence or presence of anti-CTLA-4, anti-PD-1, anti-TIGIT or anti-HER2 blocking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

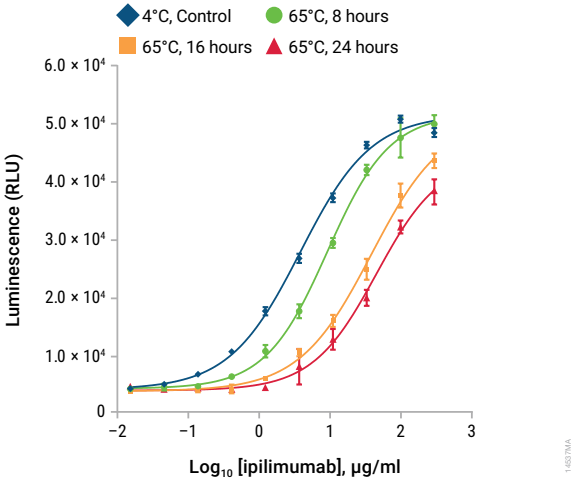


Figure 3. The CTLA-4 Blockade Bioassay indicates stability. Samples of anti-CTLA-4 antibody, ipilimumab, were maintained at 4°C (control) or heat-denatured at 65°C for the indicated times, and analyzed using the CTLA-4 Blockade Bioassay. Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover 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)

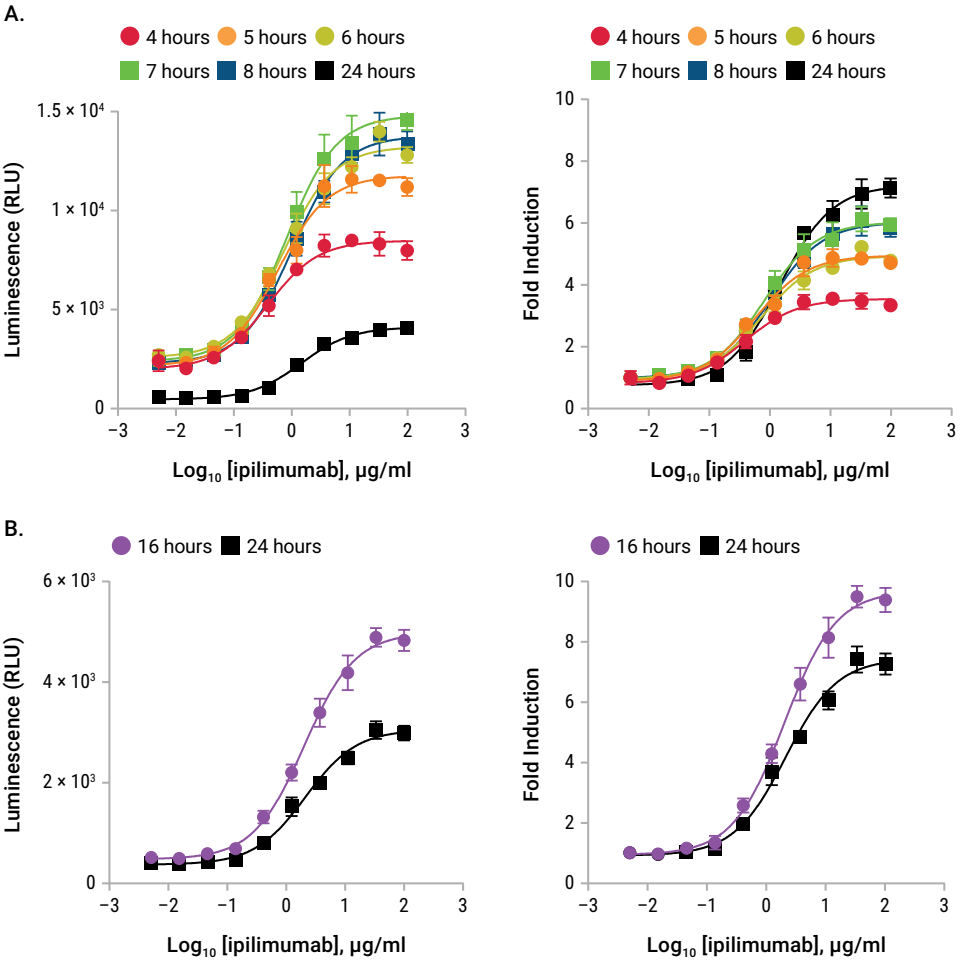


Figure 4. The CTLA-4 Blockade Bioassay can be run in one day (6 hours) or overnight (16 hours). Anti-CTLA-4 antibody, ipilimumab, was tested in the CTLA-4 Blockade Bioassay over a 4- to 24-hour timeframe. Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells. **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.

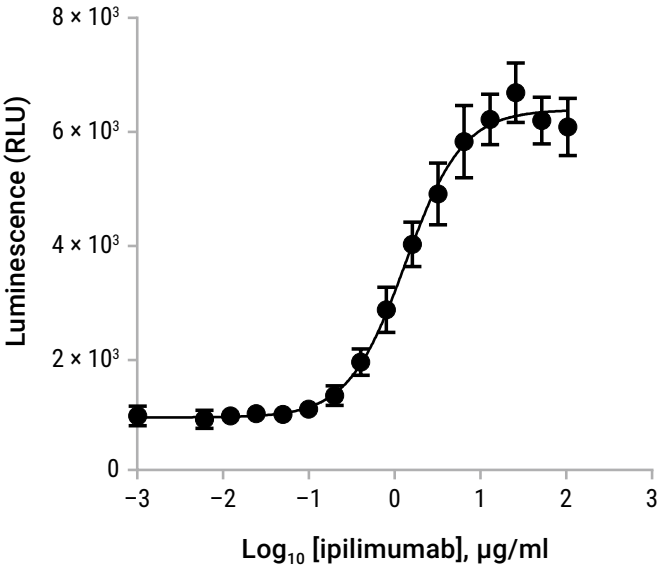


Figure 5. The assay is amenable to 384-well plate format and compatible with laboratory automation. Anti-CTLA-4 antibody, ipilimumab, was tested in the CTLA-4 Blockade Bioassay using a Multidrop™ Combi nL (Thermo Scientific) dispenser. Cells were plated at 4 × 10^4/well (Jurkat CTLA-4) and 2 × 10^4/well (aAPC/Raji) in 5µl each/well. A twofold serial dilution of ipilimumab was plated in 10µl/well. After a 16-hour assay incubation, 20µl of 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. The EC_{50} value was 1.3µg/ml. Data were generated using thaw-and-use cells.

1. Description (continued)

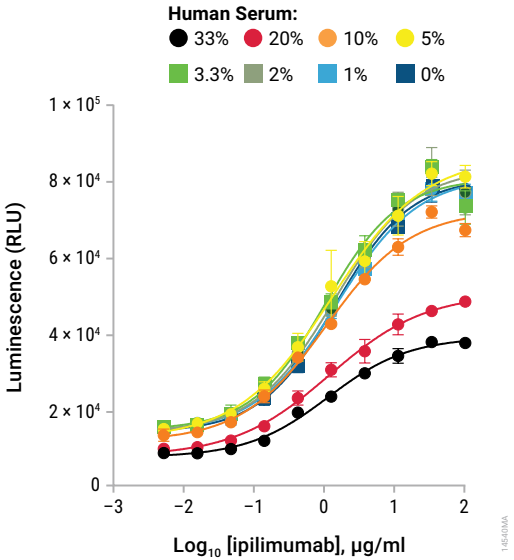


Figure 6. The CTLA-4 Blockade Bioassay tolerates human serum. Anti-CTLA-4 blocking antibody, ipilimumab, was analyzed in the absence or presence of increasing concentrations of pooled normal human serum (0–100% in the antibody sample), resulting in final assay concentration of human serum (0–33%). Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-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).

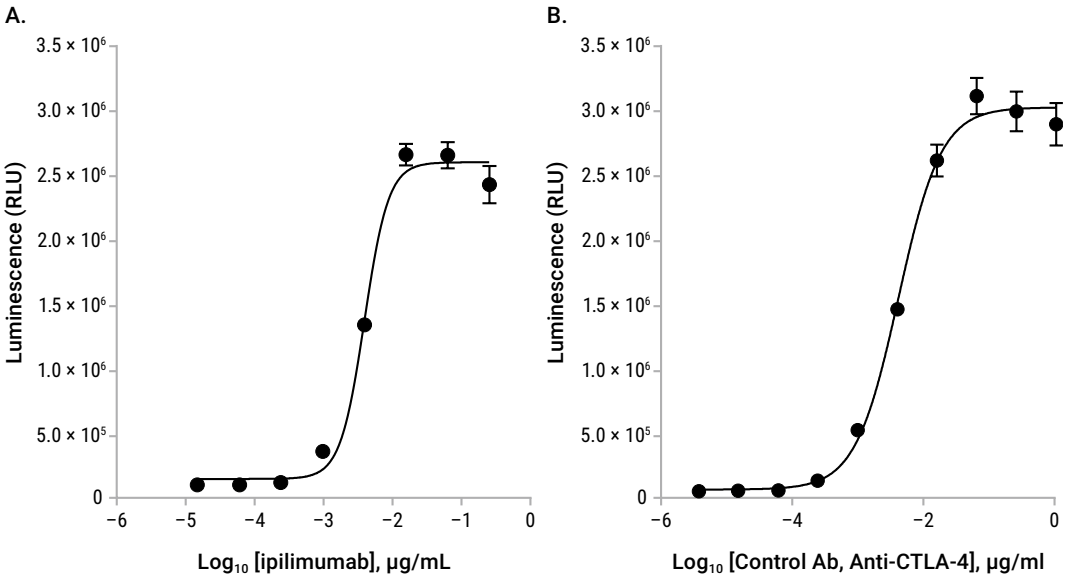


Figure 7. The CTLA-4 Blockade Bioassay can be used to measure ADCC activity. A 1:1 ratio of CTLA-4 Effector Cells (used as Target cells in this application) and ADCC Bioassay Effector Cells (Cat.# G7102) were incubated in the presence of anti-CTLA-4 blocking antibody, ipilimumab (**Panel A**), or Control 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 System. Data were fitted to a four-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, Propagation Model	**1 each**	**JA1400**

Not for Medical Diagnostic Use.

Includes:

- 2 vials CTLA-4 Effector Cells (CPM), 2 × 10^7 cells/ml (1.0ml per vial)
- 2 vials aAPC/Raji Cells (CPM) 2 × 10^7 cells/ml (1.0ml per vial)

Note: Thaw and propagate one vial to create frozen cell banks before use in an assay. The second vial should be reserved for future use.

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 as this will negatively impact cell viability and cell performance.

3. Before You Begin

The CTLA-4 Blockade Bioassay, Propagation Model, 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 is 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. Cell thawing, propagation and banking should be performed exactly as described in Section 3. Cell seeding and propagation densities have been optimized to ensure stable cell growth, which is reflected in a steady cell doubling rate, to achieve optimal, consistent performance. The recommended cell plating densities, induction time and assay buffer components described in Section 4 were established using the clinical-grade anti-CTLA-4 blocking antibody, ipilimumab. You may need to adjust the parameters provided here and optimize assay conditions for your own antibody or other biologic samples.

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. 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 RLU levels. Figure 4 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

Reagents

(Composition of buffers and solutions is provided in Section 7.B.)

- user-defined anti-CTLA-4 blocking antibodies or other biologics samples
- RPMI 1640 Medium with L-glutamine and HEPES (e.g., Corning® Cat.# 10-041-CV or GIBCO® Cat.# 22400)
- fetal bovine serum (e.g., HyClone Cat.# SH30070.03 or GIBCO® Cat.# 35-015-CV)
- hygromycin B (e.g., GIBCO® Cat.# 10687-010)
- Antibiotic G-418 Sulfate Solution (Cat.# V8091)
- sodium pyruvate (e.g., GIBCO® Cat.# 11360)
- MEM nonessential amino acids (e.g., GIBCO® Cat.# 11140)
- DMSO (e.g., Sigma Cat.# D2650)
- trypan blue solution (e.g., Sigma Cat.# T8154)
- Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941)

Supplies and Equipment

- 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.# 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., 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)

3.B. Preparing CTLA-4 Effector Cells

Cell Thawing and Initial Cell Culture

1. Prepare 40ml of initial cell culture medium by adding 4ml of FBS to 36ml of RPMI 1640 medium prewarmed to 37°C. This initial cell culture medium will be used for culturing the cells immediately after thawing.
2. Transfer 9ml of prewarmed initial cell culture medium to a 50ml conical tube.
3. Remove one vial of CTLA-4 Effector Cells from storage at –140°C and thaw in a 37°C water bath with gentle agitation (no inversion) until just thawed (typically 2–3 minutes).
4. Transfer all of the cells (approximately 1ml) to the 50ml conical tube containing 9ml of prewarmed initial cell culture medium.
5. Centrifuge at 90 × *g* for 10 minutes.
6. Carefully aspirate the medium, and resuspend the cell pellet in 25ml of prewarmed initial cell culture medium.
7. Transfer the cell suspension to a T75 tissue culture flask, and place the flask horizontally in a humidified 37°C, 5% CO_2 incubator.
8. Incubate for approximately 48 hours before passaging the cells.

Cell Maintenance and Propagation

Note: For cell maintenance and propagation starting from the second cell passage, use the cell growth medium containing antibiotics, and monitor cell viability and doubling rate during propagation. The cell growth rate will stabilize by 7–10 days post-thaw, at which time cell viability is typically >95%, and the average cell doubling rate is 24–26 hours. Passage number should be recorded for each passage. In our experience, cells maintain their functionality for up to 20 passages or 45 cell doublings if passaging is performed on a Monday-Wednesday-Friday schedule.

9. On the day of cell passage, measure cell viability and density by Trypan blue staining.
10. Seed the cells at a density of 4×10^5 cells/ml if passaging every 2 days (e.g., Monday-Wednesday or Wednesday-Friday) or 2.5×10^5 cells/ml if passaging every 3 days (e.g., Friday-Monday). Always maintain the flasks in a horizontal position in the incubator.
11. Maintain the cell culture by adding fresh cell growth medium to the cell suspension in the original flask or by transferring the cells to a new flask while maintaining a consistent ratio of culture volume to flask surface area (e.g., 25ml volume per T75 flask or 50ml volume per T150 flask).
12. Place the flasks horizontally in a 37°C, 5% CO_2 incubator.

Cell Freezing and Banking

13. On the day of cell freezing, make new cell freezing medium and keep on ice.
14. Gently mix the cells with a pipette to create a homogeneous cell suspension.
15. Remove a sample for cell counting by Trypan blue staining. Calculate the volume of cell freezing medium needed based on desired cell freezing densities of 5×10^6–2×10^7 cells/ml.
16. Transfer the cell suspension to 50ml sterile conical tubes or larger sized centrifuge tubes and centrifuge at 130–180 × *g*, 4°C, for 10–15 minutes.
17. Gently aspirate the medium, taking care not to disturb the cell pellet.
18. Carefully resuspend the cell pellet in ice-cold cell freezing medium to a final cell density of 5×10^6–2×10^7 cells/ml. Combine the cell suspensions into a single tube and dispense into cryovials.
19. Freeze the cells using a controlled-rate freezer (preferred) or a Mr. Frosty® or a Styrofoam® rack in a –80°C freezer overnight. Transfer the vials to at or below –140°C for long-term storage.

3.C. Preparing aAPC/Raji Cells

Cell Thawing and Initial Cell Culture

1. Prepare 40ml of initial cell culture medium by adding 4ml of FBS to 36ml of RPMI 1640 medium prewarmed to 37°C. This initial cell culture medium will be used for culturing the cells immediately after thawing.
2. Transfer 9ml of prewarmed initial cell culture medium to a 50ml conical tube.
3. Remove one vial of aAPC/Raji Cells from storage at –140°C and thaw in a 37°C water bath with gentle agitation (no inversion) until just thawed (typically 2–3 minutes).
4. Transfer all of the cells (approximately 1ml) to the 50ml conical tube containing 9ml of prewarmed initial cell culture medium.
5. Centrifuge at 90 × *g* for 10 minutes.
6. Carefully aspirate the medium, and resuspend the cell pellet in 25ml of prewarmed initial cell culture medium.
7. Transfer the cell suspension to a T75 tissue culture flask and place the flask horizontally in a humidified 37°C, 5% CO_2 incubator.
8. Incubate for approximately 48 hours before passaging the cells.

3.C. Preparing aAPC/Raji Cells (continued)

Cell Maintenance and Propagation

Note: For cell maintenance and propagation starting from the second cell passage, use the cell growth medium containing antibiotics, and monitor cell viability and doubling rate during propagation. The cell growth rate will stabilize by 5–7 days post-thaw, at which time cell viability is typically >95% and the average cell doubling rate is 22–26 hours. Passage number should be recorded for each passage. In our experience, cells maintain their functionality for up to 20 passages or 45 cell doublings if passaging is performed on a Monday-Wednesday-Friday schedule.

9. On the day of cell passage, measure cell viability and density by Trypan blue staining.
10. Seed the cells at a density of 3×10^5 cells/ml if passaging every 2 days (e.g., Monday-Wednesday or Wednesday-Friday) or $1.5–2 \times 10^5$ cells/ml if passaging every 3 days (e.g., Friday-Monday). Always maintain the flasks in a horizontal position in the incubator.
11. Maintain the cell culture by adding fresh cell growth medium to the cell suspension in the original flask or by transferring the cells to a new flask while maintaining a consistent ratio of culture volume to flask surface area (e.g., 25ml volume per T75 flask or 50ml volume per T150 flask).
12. Place the flasks horizontally in a 37°C, 5% CO_2 incubator.

Cell Freezing and Banking

13. On the day of cell freezing, make new cell freezing medium and keep on ice.
14. Gently mix the cells with a pipette to create a homogeneous cell suspension.
15. Remove a sample for cell counting by Trypan blue staining. Calculate the volume of cell freezing medium needed based on desired cell freezing densities of $5 \times 10^6–2 \times 10^7$ cells/ml.
16. Transfer the cell suspension to 50ml sterile conical tubes or larger sized centrifuge tubes and centrifuge at 130–180 × *g*, 4°C, for 10–15 minutes.
17. Gently aspirate the medium taking care not to disturb the cell pellet.
18. Carefully resuspend the cell pellet in ice-cold cell freezing medium to a final cell density of $5 \times 10^6–2 \times 10^7$ cells/ml. Combine the cell suspensions into a single tube and dispense into cryovials.
19. Freeze the cells using a controlled-rate freezer (preferred) or a Mr. Frosty® or a Styrofoam® rack in a –80°C freezer overnight. Transfer the vials to at or below –140°C for long-term storage.

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 1/5th 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.

4. Assay Protocol (continued)

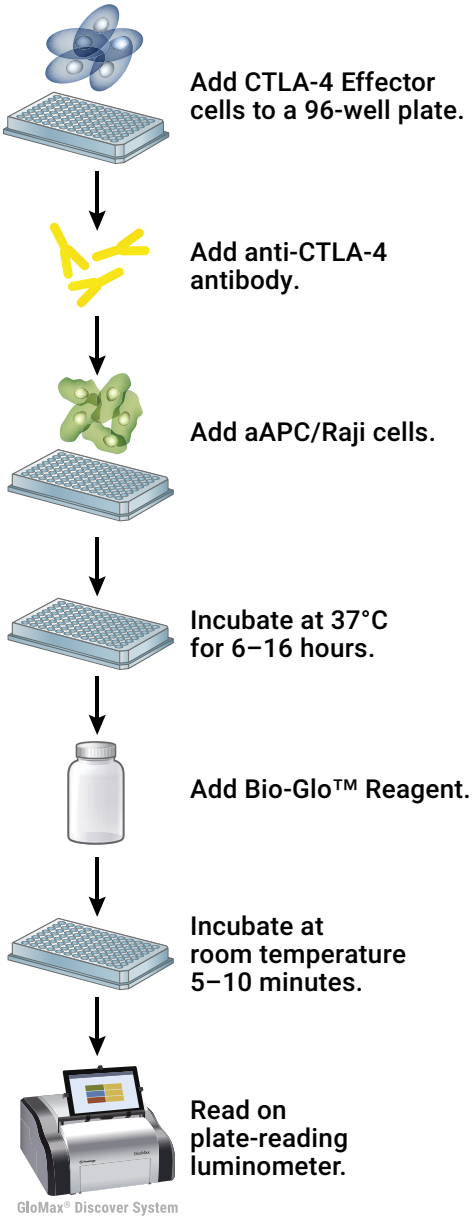


Figure 8. Schematic protocol for the CTLA-4 Blockade Bioassay.

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.

   If you are using a large (100ml) size of Bio-Glo™ Luciferase Assay System, dispense the reconstituted Bio-Glo™ Reagent into 10ml aliquots and store at –20°C for up to six weeks. Avoid repeated freeze-thaw cycles. On the day of the assay, thaw the appropriate amount of reconstituted Bio-Glo™ Reagent in a room temperature water bath for at least 1–2 hours before use. When stored appropriately, Bio-Glo™ reagent will maintain at least 80% activity after 24 hours at ambient temperature.

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 harvesting 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 ten-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

While maintaining the CTLA-4 Effector Cells, follow the recommended cell seeding density because changes in cell culture volume or seeding density will affect cell growth rate and assay performance. Only use the cells in the assay after the cell doubling rate has stabilized during propagation.

1. Passage the cells two days before performing the assay as described in Section 3.B. To ensure optimal and consistent assay performance, maintain the cell density, upon harvest, in the range of 1.4–2.0 × 10^6 cells/ml and cell viability at greater than 95%.
2. Count the CTLA-4 Effector Cells by Trypan blue staining, and calculate the cell density and viability.
3. Transfer an appropriate amount of CTLA-4 Effector Cells from the culture vessel to a 50ml conical tube or larger sized centrifuge tube.
4. On the day of the assay, prepare an appropriate amount of assay buffer as described in Section 4.A.
5. Pellet the cells at 130–180 × *g* for 10 minutes at ambient temperature and resuspend in assay buffer at 70% of the full volume needed to generate the targeted final cell density of 4 × 10^6 cells/ml.
6. Count the cells again, and adjust the volume of assay buffer to achieve a final cell density of 4 × 10^6 cells/ml. You will need at least 5ml of CTLA-4 Effector Cells to fill 120 assay wells, or the inner 60 wells of two assay plates, including dead volume.
7. 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.
8. Add 75µl of assay buffer to each of the outside wells of the assay plates.
9. 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 of Section 4.D 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

While maintaining the aAPC/Raji Cells, follow the recommended cell seeding density because changes in cell culture volume or seeding density will affect cell growth rate and assay performance. Only use the cells in the assay after the cell doubling rate has stabilized during propagation.

1. Passage the cells two days before performing the assay as described in Section 3.C. To ensure optimal and consistent assay performance, maintain the cell density, upon harvest, in the range of 1.2–1.8 × 10^6 cells/ml and cell viability at greater than 95%.
2. Count the aAPC/Raji Cells by Trypan blue staining, and calculate the cell density and viability.
3. Transfer an appropriate amount of aAPC/Raji Cells from the culture vessel to a 50ml conical tube or larger sized centrifuge tube.
4. Pellet the cells at 130–180 × *g* for 10 minutes at ambient temperature and resuspend in assay buffer at 70% of the full volume needed to generate the targeted final cell density of 2 × 10^6 cells/ml.
5. Count the cells again, and adjust the volume of assay buffer to achieve a final cell density of 2 × 10^6 cells/ml. You will need at least 5ml of aAPC/Raji Cells to fill 120 assay wells, or the inner 60 wells of two assay plates.
6. Transfer the cell suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 25µl of the cell suspension to the plated CTLA-4 Effector Cells and anti-CTLA-4 antibody. The final assay volume is 75µl.
7. 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 concentration of the aAPC/Raji Cells may be doubled 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 or 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**. Email: **techserv@promega.com**

Symptoms	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. Shorter assay time (6 hours) will increase luminescence signal 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 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

1. Mahoney, K.M., Rennert, P.D. and Freeman, G.J. (2015) Combination cancer immunotherapy and new immunomodulatory targets. *Nat. Rev. Drug Discov.* **14**, 561–84.
2. Melero, I. *et al.* (2015) Evolving synergistic combinations of targeted immunotherapies to combat cancer. *Nat. Rev. Cancer* **15,** 457–72.
3. Robert, C. *et al.* (2015) Pembrolizumab versus ipilimumab in advanced melanoma. *N. Engl. J. Med.* **372,** 2521–32.
4. Larkin, J. *et al.* (2015) Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. *N. Engl. J. Med.* **373,** 23–34.
5. Blank, C.U. and Enk, A. (2014) Therapeutic use of anti-CTLA-4 antibodies. *Int. Immunol.* **27,** 3–10.
6. Leach, D. R., Krummel, M.F. and Allison, J.P. (1996) Enhancement of antitumor immunity by CTLA-4 blockade. *Science.* **271,** 1734–6.
7. Grailer, J. *et al.* (2017) Quantitative cell-based bioassays to advance individual or combination immune checkpoint immunotherapy. *American Association of Cancer Research (AACR) Annual Meeting.* Poster# 5610.

7. Appendix

7.A. Representative Assay Results

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

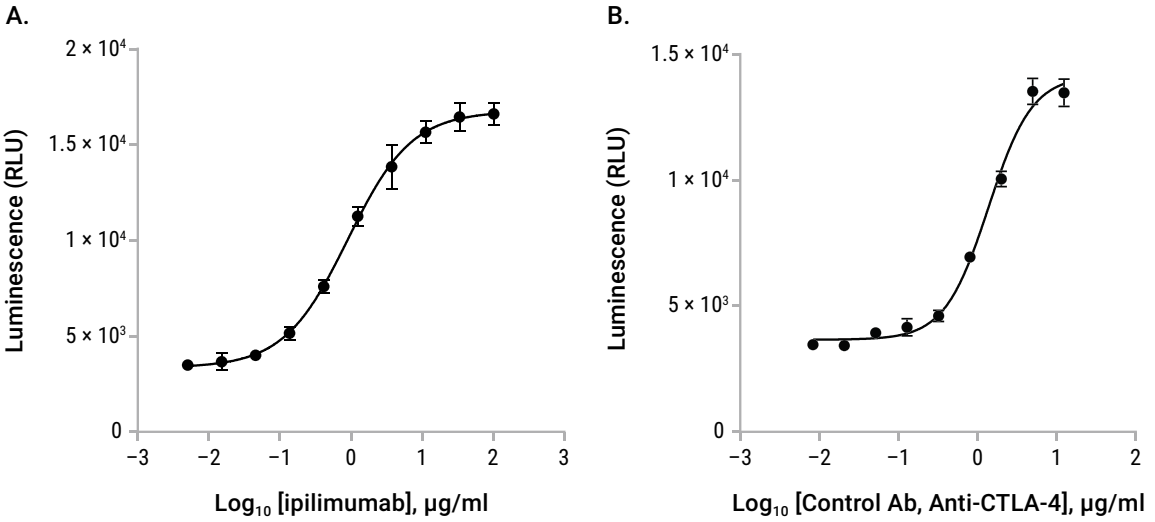


Figure 11. The CTLA-4 Blockade Bioassay measures the inhibitory activity of research- or clinical-grade anti-CTLA-4 blocking antibodies. CTLA-4 Effector Cells and aAPC/Raji Cells were incubated with increasing concentrations of anti-CTLA-4 antibody, ipilimumab (**Panel A**), or Control Ab, Anti-CTLA-4 (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 four-parameter logistic curve using using GraphPad Prism® software. The EC_{50} values were 0.9µg/ml (ipilimumab) and 1.5µg/ml (Control Ab, Anti-CTLA-4). Data were generated using propagated cells.

7.B. Composition of Buffers and Solutions

Initial Cell Culture Medium for CTLA-4 Effector Cells

90%	RPMI 1640 with L-glutamine and HEPES
10%	FBS

Cell Growth Medium for CTLA-4 Effector Cells

90%	RPMI 1640 with L-glutamine and HEPES
10%	FBS
200µg/ml	hygromycin B
500µg/ml	Antibiotic G-418 Sulfate Solution (Cat.# V8091)
1mM	sodium pyruvate
0.1mM	MEM nonessential amino acids

Cell Freezing Medium for CTLA-4 Effector Cells

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

Initial Cell Culture Medium for aAPC/Raji Cells

90%	RPMI 1640 with L-glutamine and HEPES
10%	FBS

Cell Growth Medium for aAPC/Raji Cells

90%	RPMI 1640 with L-glutamine and HEPES
10%	FBS
200µg/ml	hygromycin B
1mM	sodium pyruvate
0.1mM	MEM nonessential amino acids

Cell Freezing Medium for aAPC/Raji Cells

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

Assay Buffer

90%	RPMI 1640 with L-glutamine and HEPES
10%	FBS

8. Summary of Changes

The following changes were made to the 8/26 revision of this document:

1. Removed Section 7.C, Related Products.
2. Made minor text edits.
3. Removed old patent statements.

[a]NOT FOR MEDICAL DIAGNOSTIC USE. FOR IN VITRO USE ONLY. BY USE OF THIS PRODUCT, RECIPIENT AGREES TO BE BOUND BY THE TERMS OF THIS LIMITED USE STATEMENT. If the recipient is not willing to accept the conditions of this limited use statement, and the product is unused, Promega will accept return of the unused product and provide the recipient with a full refund.

This product may not be further sold or transferred by the recipient and may be used only by the recipient, and then only for (1) research use, (2) discovery, development and monitoring of biologic drugs and vaccines, (3) quality assurance testing of biologic drugs and vaccines, and (4) product release assays for biologic drugs and vaccines. No other commercial use is allowed. "Commercial use" means any and all uses of this product by recipient for monetary or other consideration, including providing a service, information or data to unaffiliated third parties, and resale of this product for any use. Recipient has no right to modify, derivatize, genetically engineer or otherwise create variations of the cells or genes stably transfected within the cells except that recipient may propagate and store the cells for long-term use. In addition, recipient must use Bio-Glo™ Luciferase Assay System purchased from Promega Corporation for all luminescence assays using this product or contact Promega to obtain a license for use of this product with reagents other than Promega's. PROMEGA MAKES NO REPRESENTATIONS OR WARRANTIES OF ANY KIND, EITHER EXPRESSED OR IMPLIED, INCLUDING AS TO MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE WITH REGARDS TO THIS PRODUCT. The terms of this agreement shall be governed under the laws of the State of Wisconsin, USA.

[b]Licensed from Lonza Cologne GmbH under U.S. Pat. Nos. 7,700,357, 8,192,990 and 8,003,389, European Pat. Nos. 1297119, 1522587, 1607484 and 1741778 and other pending and issued patents.

© 2017–2026 Promega Corporation. All Rights Reserved.

GloMax is a registered trademark of Promega Corporation. Bio-Glo is a trademark of Promega Corporation.

Corning and Costar are registered trademarks of Corning, Inc. GIBCO is a registered trademark of Life Technologies Corporation. GraphPad Prism is a registered trademark of GraphPad Software, Inc. JMP is a registered trademark of SAS Institute, Inc. Mr. Frosty is a registered trademark of Nalge Nunc International Corporation. Multidrop is a trademark of Thermo Fisher Scientific, Ltd. Styrofoam is a registered trademark of Dow Chemical Company. Yervoy is a registered trademark of Bristol Myers Squibb.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our website for more information.

All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.