TECHNICAL MANUAL

CD28 Blockade Bioassay, Propagation Model

Instructions for Use of Product
JA7072

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TM596

Promega

CD28 Blockade Bioassay, Propagation Model

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1. Description

The human immune system is regulated by a complex network of inhibitory and stimulatory receptors that facilitate the elimination of pathogens, while maintaining tolerance to self-antigens. T cells play a central role in cell-mediated immunity against pathogens; however, T cells also contribute to the pathogenesis and exacerbation of autoimmune disorders.

Optimal activation of naive T cells is initiated by engagement of the T cell antigen receptor (TCR)/CD3 complex and the co-stimulatory receptor CD28. CD28 binds to the B7 family members CD80 and CD86 (collectively referred to as B7 in this technical manual) on antigen presenting cells (APCs). Co-stimulation of T cells by CD28 activation initiates signaling cascades that result in AP-1 and NFκB transcription factor activation and nuclear translocation (1). These pathways significantly enhance T cell cytokine production—specifically, interleukin (IL)-2—which promotes T cell proliferation, differentiation and survival (2).

Blockade of CD28 has proven beneficial in preclinical and clinical studies to reduce autoimmunity and alloimmunity (3,4). Specifically, the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) - Fc fusion proteins, abatacept and belatacept are FDA-approved for the treatment of rheumatoid arthritis and renal transplantation, respectively. CTLA-4-Fc proteins work by binding B7 on APCs thereby inhibiting CD28 activation of T cells. However, these proteins similarly block intrinsic inhibitory signaling functions of CTLA-4, which may inadvertently boost effector responses in some settings. Therefore, specifically targeting CD28 may be more beneficial than targeting B7, especially in the setting of allograft rejection (5,6,7).

Activation of CD28 by agonist antibodies is a separate immunotherapy strategy to re-activate the immune system in settings of chronic infection or cancer. The CD28 Blockade Bioassay is not designed to detect agonistic activity of CD28 antibodies. The CD28 Bioassay (Cat.# JA6701) for screening and potency testing of CD28 agonist antibodies is available separately.

There are no easy-to-use functional bioassays available to measure the in vitro potency of potential biologic drugs that block the interaction between CD28/B7. Quantitative bioassays are needed in the development of biologic drugs designed to block CD28. Current methods rely on primary human T cells and APCs, and measurement of functional endpoints such as cell proliferation, cell surface marker expression and cytokine production. These assays are laborious and highly variable due to their reliance on donor cells, complex assay protocols and unqualified assay reagents. As a result, these assays are difficult to establish in a potential quality-controlled drug development setting.

The CD28 Blockade Bioassay, Propagation Model[a–e] (Cat.# JA7072), is a bioluminescent reporter cell-based assay that overcomes the limitations of existing assays. It can be used to measure the potency and stability of antibodies and other potential biologics that block CD28/B7 (6). The assay consists of two genetically engineered cell lines:

- **CD28 Effector Cells**: Jurkat T cells expressing endogenous TCR/CD3 and CD28, and a luciferase reporter driven by TCR/CD3 and CD28 pathway-dependent response elements
- **aAPC/Raji Cells**: Raji cells expressing an engineered cell surface protein designed to activate TCR/CD3 in an antigen-independent manner, and endogenously expressing the B7 ligands

The CD28 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, the aAPC/Raji Cells activate TCR/CD3 and CD28 on the Effector Cells to induce maximum promoter-mediated luminescence. Addition of a biologic that blocks CD28/B7 inhibits co-stimulation by CD28 and results in decreased promoter-mediated luminescence (Figure 1). The bioluminescent signal is quantified using the Bio-Glo™ Luciferase Assay System, and a standard luminometer such as the GloMax® Discover System (see Related Products, Section 9.C).

In addition to the CD28 Blockade Bioassay, we offer Control Ab, Anti-CD28 (Cat.# K1231) for use as a positive control.

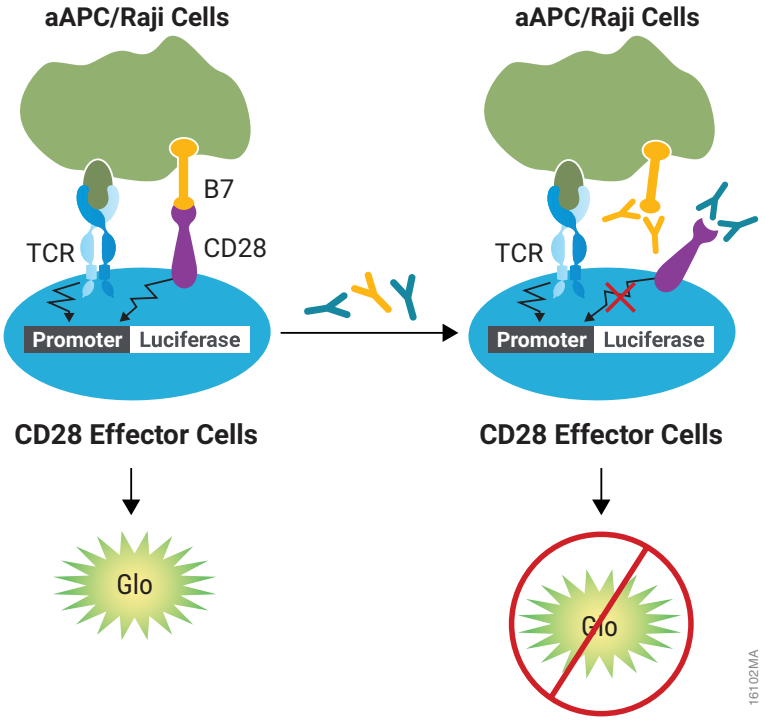

Figure 1. Representation of the CD28 Blockade Bioassay. The bioassay consists of two cell lines, CD28 Effector Cells and aAPC/Raji Cells. When co-cultured, the aAPC/Raji Cells activate TCR/CD3 and CD28 on the Effector Cells to induce maximum promoter-mediated luminescence. Addition of a biologic that blocks CD28/B7 inhibits T cell co-stimulation by CD28 and results in decreased promoter-mediated luminescence, which can be detected in a dose-dependent manner by addition of Bio-Glo™ Reagent and quantitation with a luminometer.

1. Description (continued)

The CD28 Blockade Bioassay reflects the mechanism of action (MOA) of biologics designed to block CD28/B7 interactions. Specifically, CD28 activation-mediated luminescence is reduced following the addition of a CD28 blocking biologic but not following addition of anti-ICOS or anti-PD-1 blocking Abs (Figure 2). The bioassay is prequalified following International Council for Homogenization 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 3). The bioassay can be performed in a one-day timeframe, and the workflow is simple, robust and compatible with both 96-well and 384-well plate formats used for antibody screening in early drug discovery (Figure 4). In addition, the bioassay can be used with up to 10% human serum (in antibody samples) (Figure 5), indicating potential for further development into a neutralizing antibody bioassay.

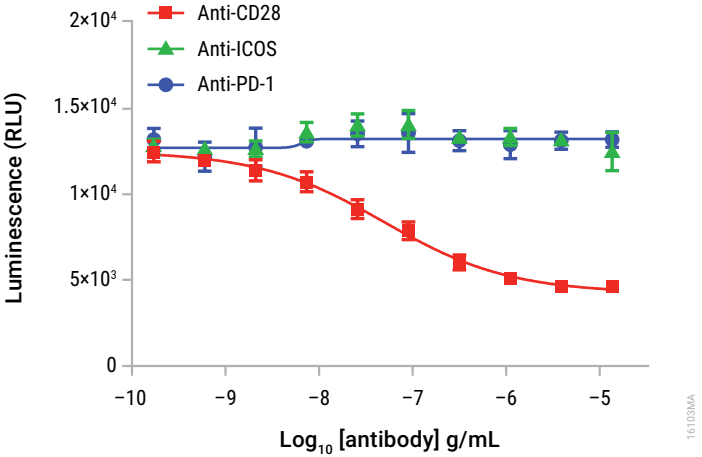

Figure 2. The CD28 Blockade Bioassay reflects the mechanism of action (MOA) and specificity of biologics designed to block the CD28/B7 interaction. CD28 Effector Cells were incubated with aAPC/Raji Cells in the presence of serial titrations of blocking Abs as indicated. After a 5-hour induction, Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover Detection System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

Table 1. The CD28 Bioassay Shows Precision, Accuracy and Linearity.

Parameter	Results	
Accuracy	% Expected Relative Potency	% Recovery
	50	54.2
	70	68.7
	100	103.1
	140	136.5
	200	202.8
Repeatability (% CV)	100% (Reference)	15.2
Intermediate Precision (% CV)		13.2
Linearity (r^2)		0.997
Linearity (y = mx + b)		y = 0.993x – 1.802
A 50–200% theoretical potency series of Control Ab, Anti-CD28, was analyzed in triplicate in three independent experiments performed on three days by two analysts. Bio-Glo™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were analyzed and relative potencies were calculated after parallelism determination using JMP® software. Data were generated using thaw-and-use cells.		

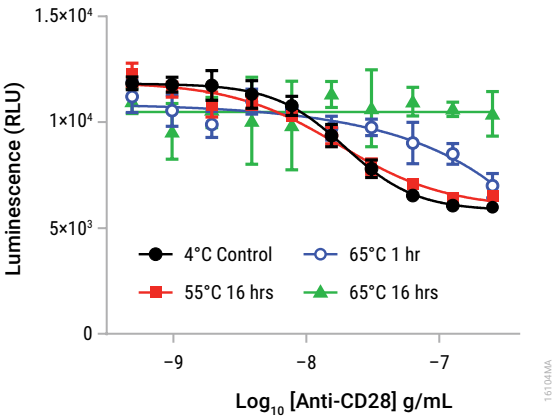

Figure 3. The CD28 Blockade Bioassay is stability-indicating. Samples of Control Ab, Anti-CD28 were maintained at 4°C (control) or heat-treated at the indicated times and temperatures, then analyzed using the CD28 Blockade Bioassay. Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover Detection 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)

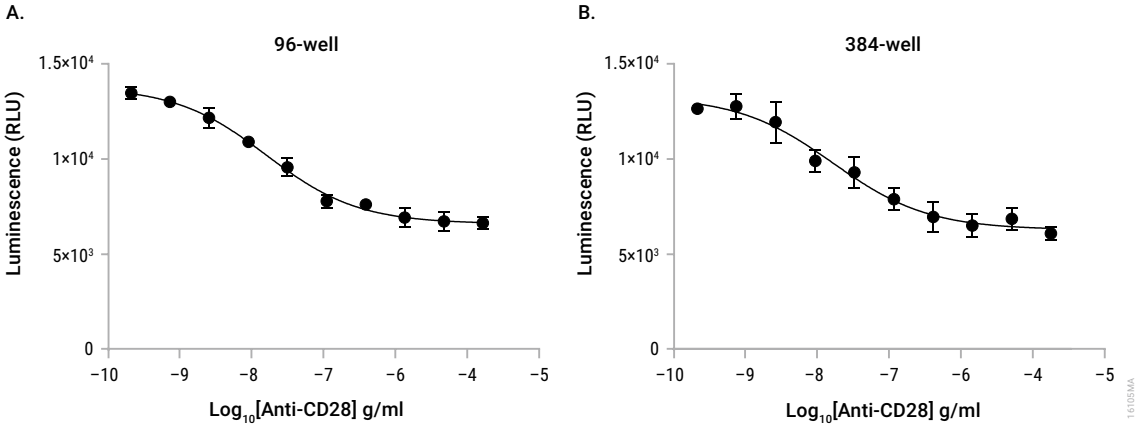

Figure 4. The CD28 Blockade Bioassay is amenable to 384-well plate format. Panel A. The CD28 Blockade Bioassay was performed in 96-well plates as described in this technical manual with a titration of Control Ab, Anti-CD28. **Panel B.** The CD28 Blockade Bioassay was performed in 384-well format as briefly described here. A titration of 3X concentrated Control Ab, Anti-CD28 (Cat.# K1231) was serially diluted and added to a 384-well white assay plate at 5µl/well. CD28 Effector Cells were added to the plate at 2×10^4 cells/5µl/well. The aAPC/Raji Cells at 2×10^4 cells/5µl/well were then added to the plate. After 5-hour assay incubation at 37°C, 5% CO_2, 15µl Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover Detection System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. The IC_{50} values were 16ng/ml for both formats and the percent maximal blocking was 51% and 50% for 96-well and 384-well format, respectively. Data were generated using thaw-and-use cells.

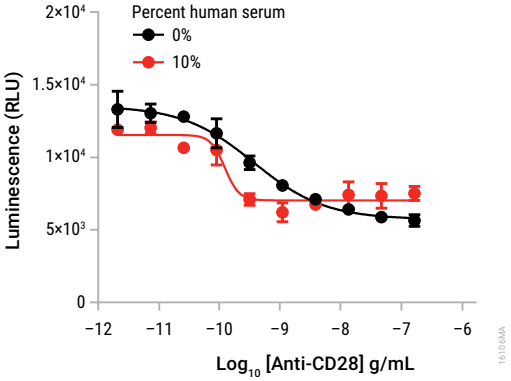

Figure 5. The CD28 Blockade Bioassay is tolerant to human serum. Control Ab, Anti-CD28 was analyzed in the absence or presence of pooled normal human serum (0% or 10% in the antibody sample). Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover Detection System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. The CD28 Blockade Bioassay was tolerant to ≤10% human serum. Higher concentrations of human serum may alter the bioassay performance. Data were generated using thaw-and-use cells.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
CD28 Blockade Bioassay, Propagation Model	**1 each**	**JA7072**

Not for Medical Diagnostic Use.

Includes:

- 2 vials TCR/CD3 Effector Cells (IL-2, CPM), (CD28 Effector Cells) 2.0 × 10^7 cells/ml (1.0ml per vial)
- 2 vials aAPC/Raji Cells (CPM), 2.0 × 10^7 cells/ml (1.0ml per vial)

Notes:

- CD28 Effector Cells are labeled TCR/CD3 Effector Cells (IL-2). Please note the vial label when placing the vials into storage.
- 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 because this will negatively impact cell viability and cell performance.

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3. Before You Begin

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

Remove the product label from the box containing vials with cells or note the catalog number and lot number from the label. This information can be used to download documents for the specified product from the website such as Certificate of Analysis.

Note: The CD28 Bioassy uses the Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941) for detection.

The CD28 Blockade Bioassay is intended to be used with user-provided antibodies or other biologics designed to block the interaction of CD28/B7. Control Ab, Anti-CD28 (Cat.# K1231) is available separately for use in assay optimization and routine quality control. We strongly recommend including Control Ab, Anti-CD28 as a positive control in the first few assays to gain familiarity with the assay. Data generated using Control Ab, Anti-CD28 is shown in Section 9.A, Representative Assay Results.

Cell thawing, propagation and banking should be performed exactly as described in Sections 4 and 5. 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. An accurate, reliable, and reproducible cell counting method is required for routine cell culturing and optimal bioassay performance.

The recommended cell plating densities, induction time and assay buffer components described in Section 6 were established using Control Ab, Anti-CD28. You may need to adjust the parameters provided here and optimize assay conditions for your own antibody or biologic samples.

The CD28 Blockade Bioassay produces a bioluminescent signal and requires a luminometer or sensitive luminescence plate reader. Bioassay development and performance data included in this Technical Manual were generated using the GloMax® Discover System (see Section 9.C, 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, though relative luminescence unit readings will vary with the sensitivity and settings of each instrument. If using a reader with adjustable gain, we recommend a high gain setting. The use of different instruments 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

(Composition of buffers and solutions is provided in Section 9.B.)

Reagents

- user-defined anti-CD28/B7 antibodies or other biologics samples
- RPMI 1640 Medium with L-glutamine and HEPES (e.g., Corning® Cat.# 10-041-CV or Gibco Cat.# 22400-105)
- fetal bovine serum (e.g., VWR Cat.# 89510-194, Gibco Cat.# 35-015-CV or HyClone Cat.# SH30071.03)
- hygromycin B (e.g., Gibco Cat.# 10687-010)
- sodium pyruvate (e.g., Gibco Cat.# 11360-070)
- MEM nonessential amino acids, 100X (e.g., Gibco Cat.# 11140-050)
- DMSO (e.g., Sigma Cat.# D2650)
- Trypan blue solution (e.g., Sigma Cat.# T8154)
- Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941)
- **optional:** Control Ab, Anti-CD28 (Cat.# K1231)

Supplies and Equipment

- solid-white, flat-bottom 96-well assay plates (e.g., Corning® Cat.# 3917) or 384-well assay plates (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., 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. Preparing CD28 Effector Cells

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

4.A. 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 CD28 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 37°C, 5% CO_2 humidified incubator.
8. Incubate for approximately 48 hours before passaging the cells.

4.B. 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 after thawing, at which time cell viability is typically >90%, and the average cell doubling rate is 28–30 hours. Passage number should be recorded for each passage. In our experience, cells maintain their functionality for up to 25 passages, or 46 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 4.5×10^5 cells/ml if passaging every two days (e.g., Monday-Wednesday or Wednesday-Friday) or 2.5×10^5 cells/ml if passaging every three days (e.g., Friday-Monday). Always maintain the flasks in a horizontal position in the incubator. Do not allow the cells to grow to a density greater than 2×10^6 cells/ml.
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 humidified incubator.

4.C. Cell Freezing and Banking

13. On the day of cell freezing, make fresh cell freezing medium and keep on ice.
14. Gently mix the cells with a pipette to create a homogenous 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.

5. Preparing aAPC/Raji Cells

5.A. 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 37°C, 5% CO_2 humidified incubator.
8. Incubate for approximately 48 hours before passaging the cells.

5.B. 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 after thawing, 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 two days (e.g., Monday-Wednesday or Wednesday-Friday) or $1.5–2 \times 10^5$ cells/ml if passaging every three 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 humidified incubator.

5.C. Cell Freezing and Banking

13. On the day of cell freezing, make fresh cell freezing medium and keep on ice.
14. Gently mix the cells with a pipette to create a homogenous 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 $130–180 \times g$, 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.

6. Assay Protocol

The procedure below illustrates the use of the CD28 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.

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 13.3µg/ml as a starting concentration (1X) and 3.5-fold serial dilution when testing Control Ab, Anti-CD28.

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

1. **Assay Buffer:** On the day of the assay, prepare an appropriate amount of assay buffer (90% RPMI 1640/10% 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 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 the Control Ab, Anti-CD28, 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%.

2. **Test and Reference Samples:** Using assay buffer as the diluent, prepare starting dilutions (dilu1, 3X final concentration) of two test antibodies (140µl each) and one reference antibody (280µl) 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-CD28, as a reference antibody in your assay, prepare a 280µl starting dilution with 40µg/ml of anti-CD28 antibody (dilu1, 3X final concentration) by adding 11.2µl of anti-CD28 stock (1.0mg/ml) to 268.8µl of assay buffer.

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. When stored appropriately, Bio-Glo™ reagent will maintain at least 80% activity after 24 hours at ambient temperature.

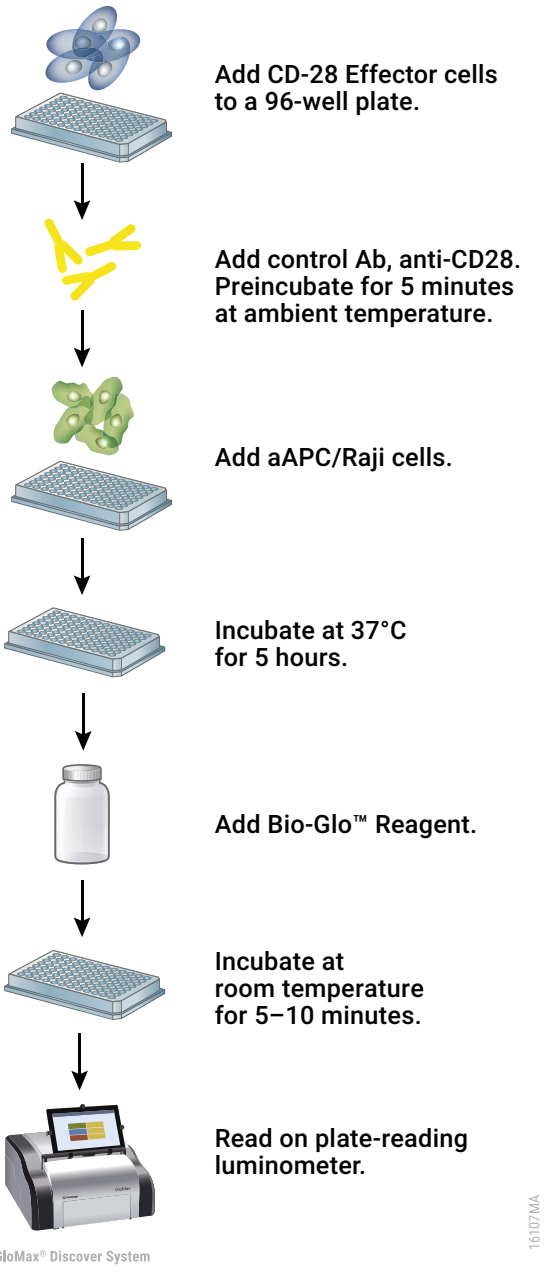

Figure 6. Schematic protocol for the CD28 Blockade Bioassay.

6.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 nonclustered sample locations of test antibody and reference antibody dilution series and wells containing assay buffer (denoted by "B") alone.

6.C. Preparing Antibody Serial Dilutions

The instructions described here are for preparation of a single stock of 3.5-fold 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 3.5-fold serial dilutions, you will need 280µl of reference antibody at 3X the highest antibody concentration in your dose-response curve. You will need 140µl 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-CD28 as a control in the assay, follow the instructions below to prepare a 3.5-fold serial dilution series.

1. On the day of the assay, prepare an appropriate amount of assay buffer as described in Section 6.A.
2. To a sterile clear V-bottom 96-well plate, add 140µl of reference antibody starting dilution (dilu1, 3X final concentration) to wells A11 and B11 (see Figure 8).
3. Add 140µl of test antibodies 1 and 2 starting dilution (dilu1, 3X final concentration) to wells E11 and G11, respectively (see Figure 8).

6.C. Preparing Antibody Serial Dilutions (continued)

4. Add 100µl of assay buffer to other wells in these four rows, from column 10 to column 2.
5. Transfer 40µl of the antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
6. Repeat equivalent 3.5-fold 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.
7. Cover the antibody dilution plate with a lid and keep at ambient temperature (22–25°C) while preparing aAPC/Raji Cells and CD28 Effector 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 8. Example plate layout showing antibody serial dilutions.

6.D. Preparing aAPC/Raji Cells

While maintaining the aAPC/Raji Cells, it is important to follow the recommended cell seeding density. 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 5.B.
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 × *g* for 10 minutes at ambient temperature, and resuspend the pellet in assay buffer at 70% of the full volume needed to generate the targeted final cell density of 4×10^6 cells/ml.
5. 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 4ml of aAPC/Raji Cells to fill 120 assay wells, or the inner 60 wells of two assay plates.

6.E. Preparing CD28 Effector Cells

While maintaining the CD28 Effector Cells, it is important to follow the recommended cell seeding density. 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 4.B.
2. Count the CD28 Effector Cells by Trypan blue staining, and calculate the cell density and viability.
3. Transfer an appropriate amount of CD28 Effector Cells from the culture vessel to a 50ml conical tube or larger sized centrifuge tube.
4. Pellet the cells at 130 × *g* for 10 minutes at ambient temperature, and resuspend the pellet in assay buffer at 70% of the full volume needed to generate the targeted final cell density of 4×10^6 cells/ml.
5. 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 4ml of CD28 Effector Cells to fill 120 assay wells, or the inner 60 wells of two assay plates.

6.F. Adding CD28 Effector Cells, Antibody and aAPC/Raji Cells to Assay Plates

1. Mix the CD28 Effector Cells by tube inversion and transfer 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.
2. 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. Gently swirl the assay plates to ensure mixing of the Effector Cells and antibody. Incubate the plate for 5 minutes at ambient temperature prior to adding the aAPC/Raji Cells.
3. Mix the aAPC/Raji Cells by tube inversion and 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. Gently swirl the assay plates to ensure mixing.
4. Add 75µl of assay buffer to each of the outside wells of the assay plates.
5. Cover the assay plates with lids and incubate in a 37°C, 5% CO_2 incubator for 5 hours.
   Note: The 5 hour assay time was optimized using the Control Ab, Anti-CD28. We recommend optimizing assay time (5–24 hours) with your own antibody or other biologic samples.

6.G. Adding Bio-Glo™ Reagent

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

1. After the 5-hour induction time, remove the assay plates from the incubator and equilibrate to ambient temperature for 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, D1 and F1 of each assay plate to measure the background signal.
4. Incubate at ambient temperature for 5–10 minutes.

Note: Varying the incubation time will affect the raw relative light unit (RLU) values but should not significantly change the IC_{50} value and percent inhibition.

5. Measure luminescence using a luminometer or luminescence plate reader.

6.H. Data Analysis

1. Determine the plate background by calculating the average RLU from wells B1, D1 and F1.
2. $$\text{Calculate percent inhibition} = \frac{1 - \text{RLU (antibody} - \text{background)}}{\text{RLU (no antibody control} - \text{background)}} \times 100$$
3. Graph data as RLU versus Log_{10} [antibody] and percent inhibition versus Log_{10} [antibody]. Fit curves and determine the IC_{50} value of antibody response using appropriate curve fitting software (such as GraphPad Prism® software).

7. 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	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 RLU 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 leads to low RLU. Store and handle the Bio-Glo™ Reagent according to the instructions.
Weak assay response (low percent inhibition)	Optimize the concentration range of your test sample(s) to achieve a full dose response with complete upper and lower asymptotes. The IC_{50} value obtained in the CD28 Blockade Bioassay may vary from the IC_{50} value obtained using other methods such as primary T cell-based assays.
	Optimize the assay incubation time within a range of 5–24 hours.
Variability in assay performance	Assay performance can be impacted by variations in cell growth conditions including plating, harvest density and viability, centrifuge times and speeds and freezing/DMSO exposure conditions during cell banking. Handle the cells consistently according to the instructions in this manual. Ensure consistent and accurate cell counting methods.
	Poor cell viability and variations in doubling time may affect assay performance. Ensure consistent cell growth by handling the cells exactly according to the instructions. Avoid one-day cell passages whenever possible, especially with the CD28 Effector Cells. Ensure you are using high quality cell culture reagents (especially serum) and plasticware for maintaining cells in culture. Ensure consistent and accurate cell counting methods.

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8. References

1. Rudd, C.E. *et al*. (2009) CD28 and CTLA-4 coreceptor expression and signal transduction. *Immunol. Rev.* **229**, 12–26.
2. Thompson, C.B. *et al.* (1989) CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. *Proc. Natl. Acad. Sci. USA*. **86**, 1333–7.
3. Lubrano, E. *et al*. (2018) Abatacept for the treatment of psoriatic arthritis. *Exper. Rev. Clin. Immunol.* **14**, 899–905.
4. Vincenti, F. *et al*. (2016) Belatacept and long-term outcomes in kidney transplantation. *N. Engl. J. Med*. **374**, 333–343.
5. Zhang, T. *et al.* (2011) Selective CD28 blockade attenuates acute and chronic rejection of murine cardiac allografts in a CTLA-4-dependent manner. *Am. J. Transplant*. **11**, 1599–609.
6. Watkins, B.K. *et al*. (2018) CD28 blockade controls T cell activation to prevent graft-versus-host disease in primates. *J. Clin. Invest*. **128**, 3991–4007.
7. Liu, D., *et al*. (2018) Selective CD28 blockade attenuates CTLA-4-dependent CD8+ memory T cell effector function and prolongs graft survival. *JCI Insight*. **3**, eCollection 2018 Jan 11.
8. Wang, J. *et al*. (2018) Cell-based reporter bioassays to evaluate the Fc gamma receptor-dependent agonistic activities for therapeutic antibodies targeting immune costimulatory receptors. *Cancer Res*. **78**, 2732.

9. Appendix

9.A. Representative Assay Results

The following data were generated using the CD28 Blockade Bioassay, Propagation Model, using Control Ab, Anti-CD28 (Figure 9).

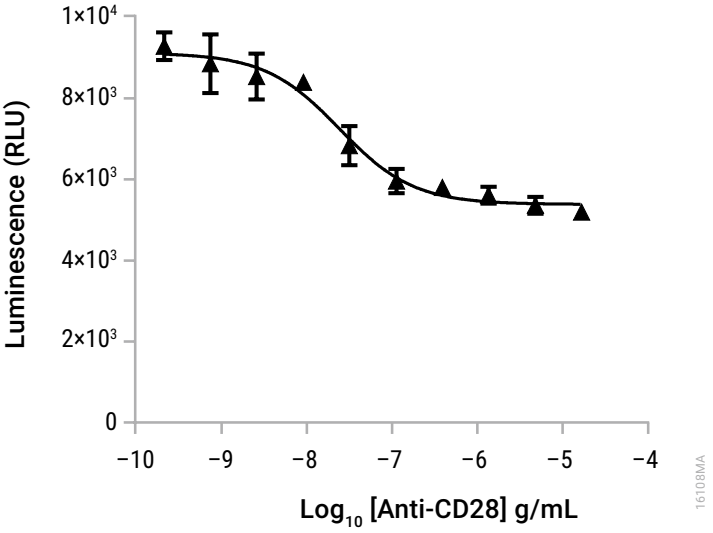

Figure 9. The CD28 Blockade Bioassay measures the blocking activity of Control Ab, Anti-CD28. CD28 Effector Cells, a titration of Control Ab, Anti-CD28 (Cat.# K1231) and aAPC/Raji Cells were added to a 96-well assay plate. After a 5-hour induction at 37°C, Bio-Glo™ Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. The IC_{50} was 24ng/ml and the percent maximal blocking was 42%.

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9.B. Composition of Buffers and Solutions

Initial Cell Culture Medium for CD28 Effector Cells

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

Cell Growth Medium for CD28 Effector 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 CD28 Effector Cells

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

Initial Cell Culture Medium 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

9.C. 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

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

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

Fc Effector Immunoassays

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

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

Immune Checkpoint Bioassays

Product	Size	Cat.#
4-1BB Bioassay	1 each	J2351
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
PD-L1 Negative Cells	1 each	J1191
TIGIT/CD155 Blockade Bioassay	1 each	J2201
TIM-3 Bioassay	1 each	JA2211

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

9.C. Related Products (continued)

T Cell Activation Bioassays

Product	Size	Cat.#
T Cell Activation Bioassay (NFAT)	1 each	J1621
T Cell Activation Bioassay (IL-2)	1 each	J1651

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

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 and sizes 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 Reagents

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

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

Note: Additional Fc Effector, Immune Checkpoint, T Cell Activation and Cytokine Bioassays are available through Promega Elite Access. To view and order Promega Bioassay products visit: **www.promega.com/products/reporter-bioassays** or email: **eliteaccess@promega.com**

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