TECHNICAL MANUAL

CD28 Bioassay, Propagation Model

Instructions for Use of Product
JA1072

Revised 9/21
TM622

Promega

CD28 Bioassay, Propagation Model

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 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 costimulatory 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). Costimulation of T cells by CD28 activation initiates signaling cascades that result in AP-1 and NFκB transcription factor activation (1). These pathways significantly enhance T cell cytokine production—specifically, interleukin-2 (IL-2)—which promotes T cell proliferation, differentiation and survival (2).

Antibodies that agonize CD28 have the potential to enhance immune responses against cancer and chronic infection. Unfortunately, disastrous phase 1 clinical results have stalled the development of these molecules (3). Therefore, the use of CD28 agonists for clinical use has been limited. However, CD28 agonist antibodies may be important in the development of alternative therapeutic modalities.

In adoptive cell transfer immunotherapy, T cells are extracted from patients, modified and expanded ex vivo, and re-infused into the patient. The most common method of ex vivo expansion is through the use of CD3- and CD28-targeted agonistic antibodies. Therefore, these agonist antibodies are considered critical reagents and must undergo appropriate quality controls (4,5).

Quantitative functional bioassays are needed in the development of biologic drugs designed to activate CD28. Current methods rely on primary human T cells with 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 quality-controlled drug development setting.

The CD28 Bioassay, Propagation Model[a–e] (Cat.# JA1072), 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 activate CD28 (6). The bioassay consists of two genetically engineered cell lines:

- **CD28 (TCR/CD3) Effector Cells:** Jurkat T cells endogenously expressing TCR, CD3 and CD28 receptors and engineered with a luciferase reporter driven by a CD28 pathway-dependent promoter.
- **FcγRIIb aAPC/CHO-K1 Cells[b]:** CHO-K1 cells engineered to express FcγRIIb that serves as a crosslinking receptor, which binds to antibody Fc domains and clusters agonist antibodies for maximal activity, and an engineered cell-surface protein designed to activate the T cell receptor (TCR) complex in an antigen-independent manner.

  Note: Alternatively, aAPC/CHO-K1 Cells (available separately, Cat.# J3312), engineered with the cell-surface protein designed to activate the TCR complex in an antigen-independent manner, can be substituted for use with FcγRIIb-independent antibodies or other biologics that do not require crosslinking for optimal activity.

The CD28 Effector Cells and FcγRIIb aAPC/CHO-K1 Cells are provided in Cell Propagation Model (CPM) format, which includes cryopreserved cells that can be thawed, propagated and banked for long-term use.

In addition to the CD28 Bioassay, we offer Control Ab, Anti-CD28 (Cat.# K1231) for use as a positive control for assay optimization and routine quality control. When screening for agonist antibodies of CD28, we recommend performing the assay with the provided FcγRIIb aAPC/CHO-K1 Cells in parallel with aAPC/CHO-K1 Cells in order to determine the role of FcγRIIb-mediated crosslinking in enhancing the effect of agonist antibodies.

Inducing the CD28 Effector Cells with a CD28 agonist antibody results in increased 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 10.C).

Blockade of CD28 has proven beneficial as a separate immunotherapy strategy in preclinical and clinical studies to reduce autoimmunity and alloimmunity (7,8). The CD28 Bioassay is not designed to detect blocking activity of CD28 antibodies. We offer separately the CD28 Blockade Bioassay (Cat.# JA6101) for screening and potency testing of CD28 antagonists.

The CD28 Bioassay reflects the mechanism of action (MOA) of biologics designed to activate CD28. Specifically, CD28 activation-mediated luminescence is increased following the addition of a CD28 activating antibody but not following addition of anti-ICOS or anti-4-1BB agonist antibodies (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 two-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 performed in the presence of human serum (in antibody samples), indicating potential for further development into a neutralizing antibody bioassay (Figure 5).

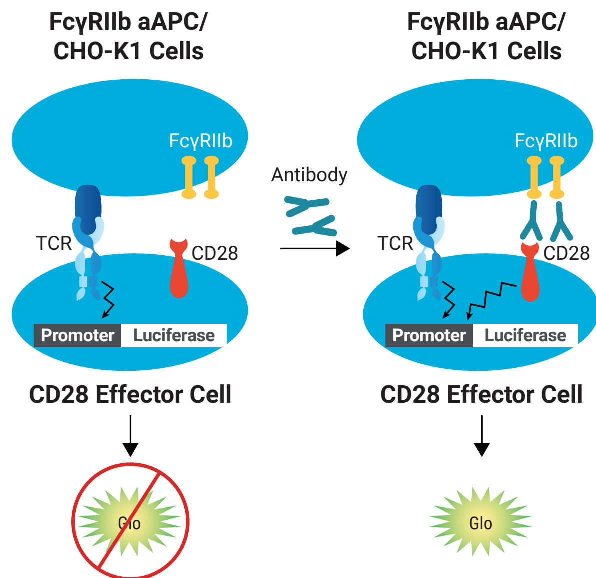

Figure 1. Representation of the CD28 Bioassay. The CD28 Bioassay for FcγRIIb-dependent agonist antibodies consists of two cell lines, CD28 Effector Cells and FcγRIIb aAPC/CHO-K1 Cells. In the absence of agonist antibody, CD28 is not activated and the luminescence signal is low. In the presence of FcγRIIb aAPC/CHO-K1 Cells, anti-CD28 antibody can be crosslinked, thereby enhancing induction of CD28 pathway-activating luminescence in a dose-dependent manner. CD28 Effector Cells can also be used for antibodies that are FcγRIIb-independent by substituting aAPC/CHO-K1 Cells.

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1. Description (continued)

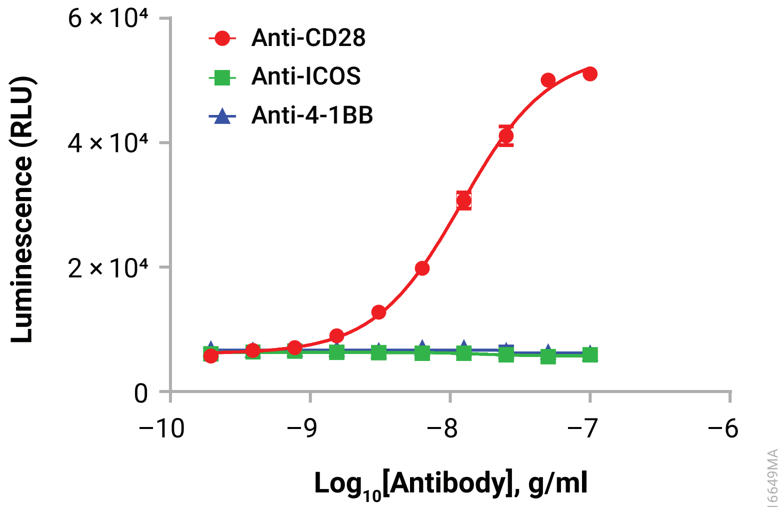

Figure 2. The CD28 Bioassay reflects the mechanism of action (MOA) and specificity of biologics designed to activate CD28. CD28 Effector Cells were incubated with FcγRIIb aAPC/CHO-K1 Cells in the presence of serial titrations of agonist antibodies as indicated. After a 5-hour incubation, Bio-Glo™ Reagent 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	43.6
	70	67.2
	100	93.5
	140	139.9
	200	205.5
Repeatability (% CV)	100% (Reference)	4.9
Intermediate Precision (% CV)		6.3
Linearity (r^2)		0.998
Linearity (y = mx + b)		y = 1.075x – 10.48

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 using the CD28 Bioassay for FcγRIIb-dependent antibodies. 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.

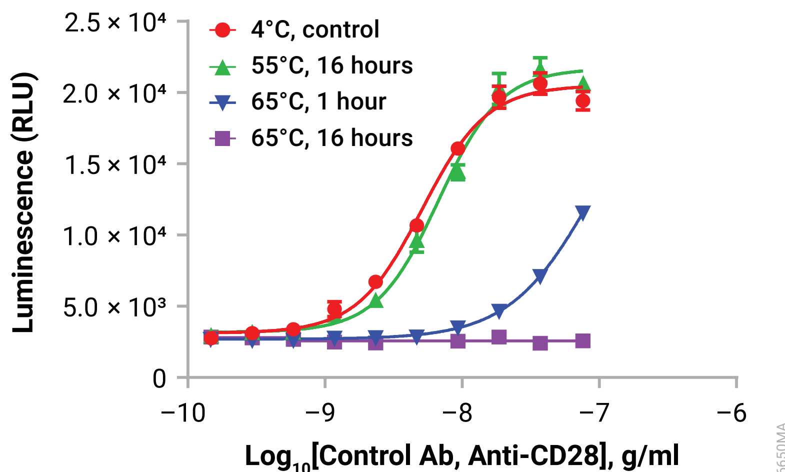

Figure 3. The CD28 Bioassay is stability-indicating. Samples of Control Ab, Anti-CD28 were maintained at 4°C (control) or heat-treated at the indicated times and temperatures, then analyzed using the CD28 Bioassay protocol for FcγRIIb-dependent antibodies. Bio-Glo™ Reagent was added and luminescence 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.

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1. Description (continued)

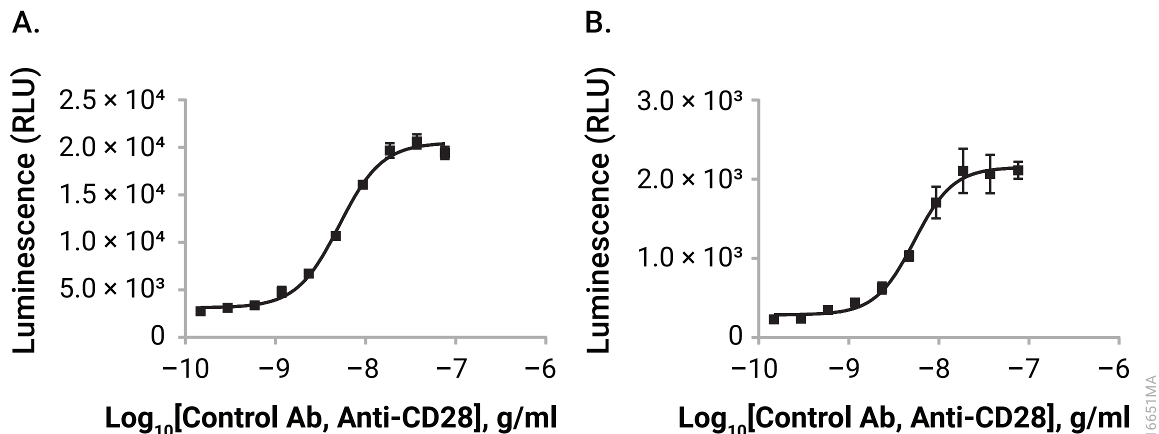

Figure 4. The CD28 Bioassay is amenable to 384-well plate format. Panel A. The CD28 Bioassay for FcγRIIb-dependent antibodies was performed in 96-well plates as described in this technical manual with a titration of Control Ab, Anti-CD28. **Panel B.** The CD28 Bioassay protocol for FcγRIIb-dependent antibodies was performed in 384-well format as briefly described here. FcγRIIb aAPC/CHO-K1 Cells were harvested and 8×10^3 cells/20µl/well plated 16–24 hours prior to assay, in a 384-well white assay plate (e.g., Corning® Cat.# 3570). On the day of the assay, spent media was removed from the 384-well plate immediately prior to plating and 8µl of 2X serially diluted Control Ab, Anti-CD28 was added, followed by the addition of 2×10^4 cells/8µl/well of CD28 Effector Cells. After the 5-hour assay induction at 37°C, 5% CO_2, 16µl/well of 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 EC_{50} values were 5.1 and 5.3ng/ml for the 96-well and 384-well formats, respectively, and the fold induction was 7.4 and 9.4 for the 96-well and 384-well formats, respectively. Data were generated using thaw-and-use cells.

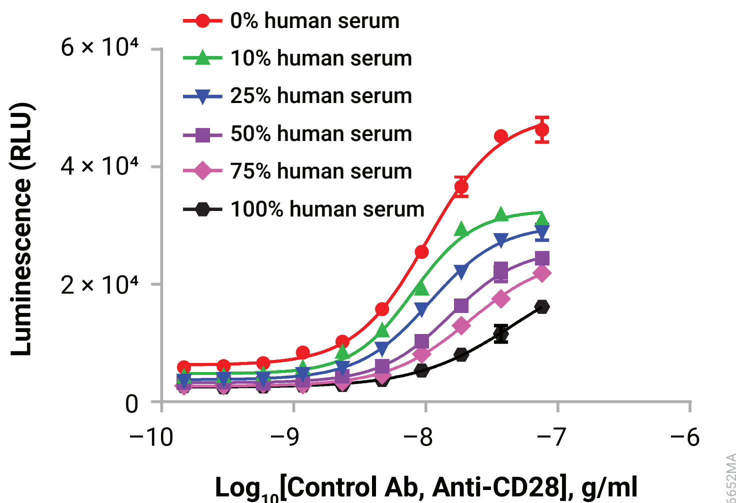

Figure 5. The CD28 Bioassay is tolerant to human serum. Following the CD28 Bioassay protocol for FcγRIIb-dependent antibodies, Control Ab, Anti-CD28 was analyzed in the absence or presence of pooled normal human serum (0–100% in the antibody sample), resulting in final assay concentration of human serum (0–50%). After the 5-hour assay induction at 37°C, 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. The CD28 Bioassay is tolerant to the presence of human serum with this serum pool. A different human serum pool showed similar effects on the assay (data not shown).

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
CD28 Bioassay, Propagation Model	**1 each**	**JA1072**

Not for Medical Diagnostic Use.

Includes:

- 2 vials TCR/CD3 Effector Cells (IL-2, CPM), (CD28 Effector Cells) 2×10^7 cells/ml (1.0ml per vial)
- 2 vials FcγRIIb aAPC/CHO-K1 Cells (CPM), 1.2×10^7 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.

The CD28 Effector Cells can also be paired with aAPC/CHO Cells for antibodies that are FcγRIIb-independent. The aAPC/CHO-K1 Cells, Propagation Model, is available separately as a standalone product.

PRODUCT AVAILABLE SEPARATELY	SIZE	CAT.#
aAPC/CHO-K1 Cells, Propagation Model	**1 each**	**J3312**

Not for Medical Diagnostic Use.

Includes:

- 2 vials aAPC/CHO-K1 Effector Cells (CPM), 1.2×10^7 cells/ml (1.0ml per vial)

Note:

1. 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.

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 Bioassay uses the Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941) for detection.

The CD28 Bioassay, Propagation Model, is intended to be used with user-provided antibodies or other biologics designed to activate CD28. Control Ab, Anti-CD28 (Cat.# K1231) and aAPC/CHO-K1 Cells (Cat.# J3312) are available separately for use in assay optimization and routine quality control. We strongly recommend including Control Ab, Anti-CD28 along with the provided FcγRIIb aAPC/CHO-K1 Cells as a positive control in the first few assays to gain familiarity with the assay. Data generated using Control Ab, Anti-CD28 are shown in Section 9.A, Representative Assay Results.

Cell thawing, propagation and banking should be performed exactly as described in Section 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 this technical manual were established using Control Ab, Anti-CD28 with FcγRIIb aAPC/CHO-K1 Cells or aAPC/CHO-K1 Cells. You may need to adjust the parameters provided here and optimize assay conditions for your own antibody or biologic samples.

The CD28 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 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 light unit (RLU) 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 antibodies or other biologics samples
- RPMI 1640 Medium with L-glutamine and HEPES (e.g., Corning® Cat.# 10-041-CV or GIBCO™ Cat.# 22400105)
- 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)
- Ham's F-12 Medium with L-glutamine (e.g., GIBCO™ Cat.# 11765062) for plating CHO-K1 Cells
- Accutase® solution (e.g., Sigma Cat.# A6964) for lifting CHO-K1 Cells
- DPBS (e.g., GIBCO™ Cat.# 14190) for washing CHO-K1 Cells
- **optional:** Control Ab, Anti-CD28 (Cat.# K1231). This antibody is FcγRIIb-dependent and requires the use of FcγRIIb aAPC/CHO-K1 Cells for optimal signal.
- **optional:** aAPC/CHO-K1 Cells if using FcγRIIb-independent antibodies (Cat.# J3312)

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) 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
- sensitive 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, labeled TCR/CD3 Effector Cells (IL-2), 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.

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 >95%, 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.

1. On the day of cell passage, measure cell viability and density by Trypan blue staining.
2. 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.
3. 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).
4. Place the flasks horizontally in a humidified 37°C, 5% CO_2 incubator.

4.C. Cell Freezing and Banking

1. On the day of cell freezing, prepare fresh cell freezing medium and keep on ice.
2. Gently mix the cells with a pipette to create a homogeneous cell suspension.
3. 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.
4. Transfer the cell suspension to 50ml sterile conical tubes or larger sized centrifuge tubes, and centrifuge at $130 \times g$, 4°C, for 10–15 minutes.
5. Gently aspirate the medium, taking care not to disturb the cell pellet.
6. 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.
7. 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 –140°C or below for long-term storage.

5. Preparing FcγRIIb aAPC/CHO-K1 Cells

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

Note: The same conditions listed below for the preparation of FcγRIIb aAPC/CHO-K1 Cells are also recommended for preparation of the aAPC/CHO-K1 Cells (Cat.# J3312).

5.A. Cell Thawing and Initial Cell Culture

1. Prepare 50ml of initial cell culture medium by adding 5ml of FBS to 45ml of Ham's F-12 medium prewarmed to 37°C. This inital 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 FcγRIIb aAPC/CHO-K1 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 concial tube containing 9ml of prewarmed initial cell culture medium.
5. Centrifuge at $90 \times g$ for 10 minutes.
6. Carefully aspirate the medium and resuspend the cell pellet in 40ml of prewarmed initial cell culture medium.
7. Transfer the cell suspension to a T150 tissue culture flasks and place the flasks horizontally in a humidified 37°C, 5% CO_2 incubator.
8. Incubate for approximately 24 hours before passaging the cells.

5.B. Cell Maintenance and Propagation

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 hours. Passage number should be recorded for each passage. In our experience, cells maintain their functionality for up to 25 passages. In our experience, cells maintain their functionality for up to 25 passages if passaging is performed on a Monday-Wednesday-Friday schedule.

1. On the day of cell passage, aspirate the cell culture medium and wash the cells with DPBS.
2. Add 2ml of Accutase® solution to each T75 flask and place the flasks in a humidified 37°C, 5% CO_2 incubator for 5–7 minutes or until the cells round up and detach from the bottom of the flasks.
3. Add 8ml of cell growth medium to each T75 flask. Transfer the cell suspension to a sterile 15ml or 50ml conical tube.
4. Count the cells by Trypan blue staining. We recommend seeding the cells at a density of 4×10^4 cells/cm^2 if passaging every two days (e.g., Monday-Wednesday or Wednesday-Friday) or 2×10^4 cells/cm^2 if passaging every three days (e.g., Friday-Monday).
5. Add an appropriate amount of cell growth media to a new flask.
6. Transfer the appropriate volume of cell suspension to achieve the desired cell seeding density per area.
7. Place the flasks horizontally in a humidified 37°C, 5% CO_2 incubator.

5.C. Cell Freezing and Banking

1. On the day of cell freezing, make fresh cell freezing medium and keep on ice.
2. Aspirate the cell culture medium and wash the cells with DPBS.
3. Add 2ml of Accutase® solution to each T75 flask and place the flasks in a humidified 37°C, 5% CO_2 incubator for 5–7 minutes or until the cells round up and detach from the bottom of the flasks.
4. Add 8ml of cell growth medium to each T75 flask. Transfer the cell suspension to a sterile 15ml or 50ml conical tube.
5. Remove a sample for cell couting by Trypan blue staining. Calculate the volume of cell freezing medium needed based on the desired cell freezing densities of 3×10^6–1×10^7 cells/ml.
6. Transfer the cell suspension to 50ml sterile conical tubes or larger-sized centrifuge tubes, and centrifuge at $180 \times g$, 4°C for 10 minutes.
7. Carefully aspirate the supernatant and avoid disturbing the cell pellet.
8. Gently resuspend the cell pellet in ice-cold cell freezing medium to a final cell density of 3×10^6–1.2×10^7 cells/ml. Combine the cell suspensions into a single tube and dispense into cryovials.
9. 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 –140°C or below for long-term storage.

6. Assay Protocol for FcγRIIb-Dependent or FcγRIIb-Independent Antibodies

This assay protocol requires two engineered cell lines: CD28 Effector Cells and FcγRIIb aAPC/CHO-K1 Cells for FcγRIIb-dependent antibodies or aAPC/CHO-K1 Cells available separately (Cat.# J3312) for FcγRIIb-independent antibodies. The FcγRIIb aAPC/CHO-K1 Cells and aAPC/CHO-K1 Cells are also available in Thaw-and-Use format (Cat.# JA9331, JA9335 and Cat.# JA9441, JA9445, respectively; see *CD28 Bioassay Technical Manual*, #TM633). Either CPM or Thaw-and-Use format can be used in this assay.

The procedure below illustrates the use of the CD28 Bioassay to test two FcγRIIb-dependent antibody samples against a reference sample in a single assay run using the FcγRIIb aAPC/CHO-K1 Cells, Propagation Model format (Figure 6). 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.

When preparing test and reference antibodies, choose an appropriate starting concentration and dilution scheme to achieve a complete dose-response curve with proper upper and lower asymptotes and sufficient points on the slope. For reference, we use 0.15µg/ml as a starting concentration (1X) and twofold serial dilution when testing Control Ab, Anti-CD28 using the FcγRIIb-dependent method. For the FcγRIIb-independent method, we used 1.5µg/ml as a starting concentration (1X) and a twofold serial dilution when testing Control Ab, Anti-CD28.

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

1. **FcγRIIb aAPC/CHO-K1 Cell Plating Medium:** On the day before the assay, prepare 40ml of cell plating medium (90% Ham's F-12/10% FBS) in a 50ml conical tube. Thaw the FBS overnight at 4°C or in a 37°C water bath on the day of use. Add 4ml of FBS to 46ml of Ham's F-12 medium. Mix well and warm to 37°C before use.
2. **Assay Buffer:** On the day of the assay, prepare an appropriate amount of assay buffer (90% RPMI 1640/10% FBS). Mix well and warm to 37°C before use. For reference, 30ml 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%.
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 the 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.

   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 an appropriate amount of reconstituted Bio-Glo™ Reagent in a room temperature water bath for at least 1–2 hours before use. The Bio-Glo™ Reagent can be stored at room temperature after reconstitution with ~18% loss in luminescence after 24 hours.

4. **Test and Reference Samples:** Using assay buffer as the diluent, prepare starting dilutions (dilu1, 2X final concentration) of two test antibodies (300µl each) and one reference antibody (600µl) in 1.5ml tubes. Store the tubes containing antibody starting dilutions appropriately before making antibody serial dilutions.

   Notes:
   For **FcγRIIb dependent assay**, if you are using FcγRIIb aAPC/CHO-K1 Cells and Control Ab, Anti-CD28 as a reference antibody in your assay, prepare a 10µg/ml **working stock** of Control Ab, Anti-CD28 by adding 2µl of Control Ab, Anti-CD28 **stock** (1.0mg/ml) to 198µl of assay buffer. Prepare a 600µl starting dilution of 0.3µg/ml of Control Ab, Anti-CD28 (dilu1, 2X final concentration) by adding 18µl of Control Ab, Anti-CD28 **working stock** (10µg/ml) to 582µl of assay buffer. Store the antibody starting dilution on ice until ready to use in the assay.

   For **FcγRIIb independent assay**, if you are using aAPC/CHO-K1 Cells and Control Ab, Anti-CD28 as a reference antibody in your assay, prepare a 10µg/ml **working stock** of Control Ab, Anti-CD28 by adding 2µl of Control Ab, Anti-CD28 **stock** (1.0mg/ml) to 198µl of assay buffer. Prepare a 600µl starting dilution of 3µg/ml of Control Ab, Anti-CD28 (dilu1, 2X final concentration) by adding 180µl of Control Ab, Anti-CD28 **working stock** (10µg/ml) to 420µl of assay buffer. Store the antibody starting dilution on ice until ready to use in the assay.

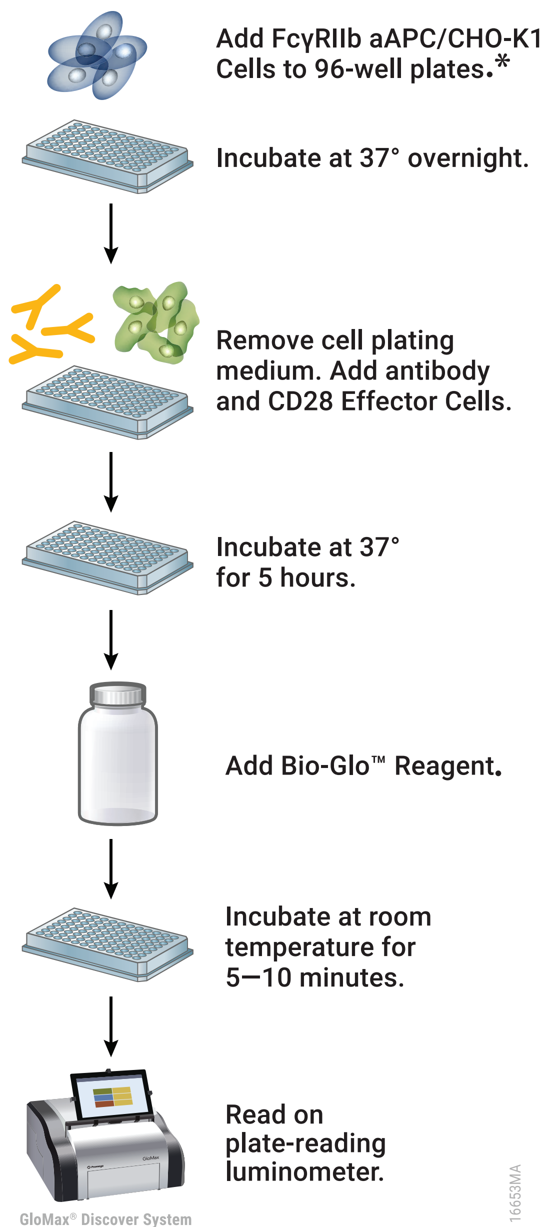

Figure 6. Schematic protocol for the CD28 Bioassay, Propagation Model for FcγRIIb-dependent antibodies. *For FcγRIIb-independent antibodies substitute aAPC/CHO-K1 Cells (Cat.# J3312).

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.

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6.C. Preparing and Plating FcγRIIb aAPC/CHO-K1 Cells

While maintaining the FcγRIIb aAPC/CHO-K1 Cells, follow the recommended cell seeding density (refer to Section 5 for culture instructions for FcγRIIb aAPC/CHO-K1 Cells, Propagation Model). Changes in cell culture volume or seeding density will affect cell growth rate and assay performance. Do not allow the cells to grow to 100% confluence. Only use the cells in the assay after the cell doubling rate has stabilized during propagation.

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

1. We recommend passaging the FcγRIIb aAPC/CHO-K1 Cells two days before plating for the assay (as described in Section 5) to ensure optimal and consistent assay performance.
2. On the day before performing the assay, prepare FcγRIIb aAPC/CHO-K1 cell plating medium (Ham's F-12/10% FBS) for the FcγRIIb aAPC/CHO-K1 Cells.
3. Aspirate the cell culture medium from the FcγRIIb aAPC/CHO-K1 Cells and wash with DPBS.
4. Add 3ml of Accutase® solution to each T75 flask, and place the flasks in a 37°C, 5% CO_2 incubator for 5–7 minutes or until the cells round up and detach from the bottom of the flasks.
5. Add 7ml of FcγRIIb aAPC/CHO-K1 cell plating medium to the flasks. Transfer the cell suspension to a 50ml (or larger) conical centrifuge tube.
6. Gently mix and count the FcγRIIb aAPC/CHO-K1 Cells by Trypan blue staining.
7. Centrifuge at 230 × *g* for 10 minutes.
8. Gently resuspend the cell pellet in cell plating medium to achieve a concentration of 4×10^5 viable cells/ml.
9. Transfer the suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 100µl of the cell suspension to each of the inner 60 wells of a 96-well white flat-bottom assay plate. The final cell number in each well should be 4×10^4 cells/well.
10. Add 100µl of cell plating medium to each of the outside wells of the assay plates.
11. Place lids on the assay plates and incubate in a 37°C, 5% CO_2 incubator overnight (18–22 hours).

6.D. Preparing Antibody Serial Dilutions

The instructions described here are for the preparation of a single stock of twofold 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 twofold serial dilutions, you will need 600µl of reference antibody at 2X the highest antibody concentration in your dose-response curve. You will need 300µl of each test antibody at 2X 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 twofold serial dilutions. A twofold serial dilution for test antibodies is listed as an example below as well.

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 300µl of reference antibody starting dilution (dilu1, 2X final concentration) to wells A11 and B11 (see Figure 8).

3. Add 300µl of test antibodies 1 and 2 starting dilution (dilu1, 2X final concentration) to wells E11 and G11, respectively (see Figure 8).
4. Add 150µl of assay buffer to other wells in these four rows, from column 10 to column 2.
5. Transfer 150µl of the antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
6. Repeat equivalent twofold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.

   Note: Wells A2, B2, E2 and G2 contain 150µl 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 the 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.

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6.E. Preparing CD28 Effector Cells

While maintaining the CD28 Effector Cells (TCR/CD3 (IL-2)), 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 and cell viability is greater than 95%.

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 2.5×10^6 cells/ml.
5. Count the cells again and adjust the volume of assay buffer to achieve a final cell density of 2.5×10^6 cells/ml. You will need at least 6ml of CD28 Effector Cells to fill 120 assay wells, or the inner 60 wells of two assay plates.

6.F. Adding CD28 Effector Cells and Antibody to Assay Plates

1. Take the 96-well assay plates containing FcγRIIb aAPC/CHO-K1 Cells out of the incubator. Invert the assay plate above a sink to remove the medium. Then, place the inverted plate on paper towels for 5–10 seconds to drain any remaining medium. Alternatively, remove 95µl of medium from each of the wells using a manual multichannel pipette.
2. Using a multichannel pipette, add 40µl of the appropriate antibody dilution (Figure 8) to the assay plates according to the plate layout in Figure 7.
3. Mix the CD28 Effector Cells by tube inversion and transfer the suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 40µl of the cell suspension to each of the inner 60 wells of the assay plates. Gently swirl the assay plates to ensure mixing of the Effector Cells and antibody.
4. Add 80µl 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 80µl of Bio-Glo™ Reagent to the inner 60 wells of the assay plates, taking care not to create bubbles.
3. Add 80µl 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 RLU values but should not significantly change the EC_{50} value and fold induction.
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. Calculate fold induction = $$\frac{\text{RLU (induced – background)}}{\text{RLU (no antibody control – background)}}$$
3. Graph data as RLU versus Log_{10} [antibody] and fold induction versus Log_{10} [antibody]. Fit curves and determine the EC_{50} value of antibody response using appropriate curve fitting software (such as GraphPad Prism® software).

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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 fold induction)	Optimize the concentration range of your test sample(s) to achieve a complete dose response with complete upper and lower asymptotes. The EC_{50} value obtained in the CD28 Bioassay may vary from the EC_{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. If performing the assay for the first time, we recommend that you try using the FcγRIIb aAPC/CHO-K1 Cells since your antibody of interest may be dependent on crosslinking by FcγRIIb.
	Optimize the FBS concentration from 0.5–10% in assay buffer if assay performance is not ideal.
	If untreated control RLU is less than 100-fold above the plate reader background RLU, subtract the plate background RLU from all samples before calculating fold induction.
Variability in assay performance	Variations in cell growth conditions including cell plating, harvest density, cell viability and cell doubling time may cause low assay performance and high assay variation. Avoid one-day cell passages whenever possible, especially with the CD28 Effector Cells. Use high-quality cell culture reagents (especially serum) and plasticware for maintaining cells in culture. Ensure consistent cell growth by handling the cells exctly according to the instructions.

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Symptoms	Possible Causes and Comments
Variability in assay performance	Inappropriate cell handling during cell harvest, including long centrifuge times and high centrifuge speeds may cause low assay performance and high assay variation. Centrifuge the cells exactly according to the instructions.
	Inappropriate cell freezing/DMSO exposure may cause low assay performance and high assay variation. Freeze the cells exactly according to the instructions.
	Inappropriate cell counting methods may lead to variation in cell numbers in culture and assays and cause high assay variation. Ensure consistent and accurate cell counting methods.

8. References

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9. Appendix

9.A. Representative Assay Results

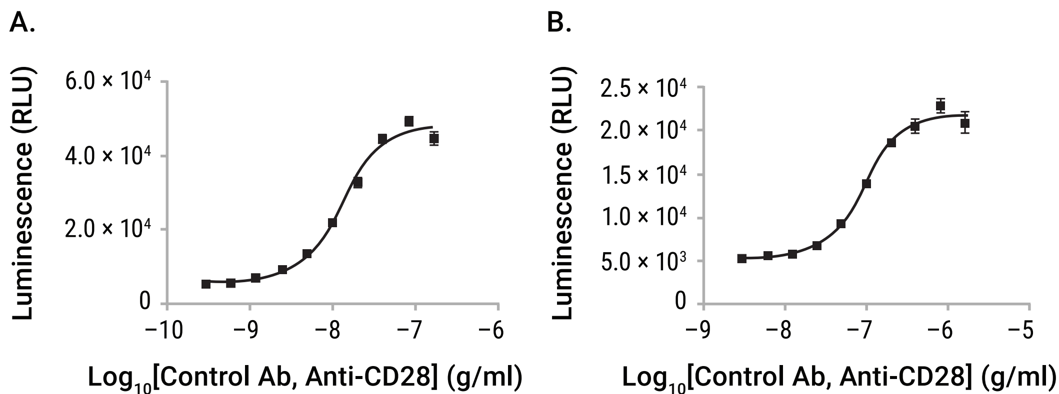

Figure 9. The CD28 Bioassay measures the activity of Control Ab, Anti-CD28. Panel A. FcγRIIb aAPC/ CHO-K1 Cells were added to a 96-well assay plate 18 hours prior to the assay. On the day of the assay, a titration of Control Ab, Anti-CD28 (Cat.# K1231) and CD28 Effector Cells were added. After a 5-hour 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 EC_{50} was 12ng/ml and the fold induction was 9.6. **Panel B**. aAPC/ CHO-K1 Cells were added to a 96-well assay plate 18 hours prior to the assay. On the day of the assay, a titration of Control Ab, Anti-CD28 (Cat.# K1231) and CD28 Effector Cells were added. 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 EC_{50} was 90ng/ml and the fold induction was 4.0.

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

Assay Buffer

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

Initial Cell Culture Medium for FcγRIIb aAPC/CHO-K1 Cells and aAPC/CHO-K1 Cells

90%	Ham's F-12
10%	FBS

Cell Growth Medium for FcγRIIb aAPC/CHO-K1 Cells

90%	Ham's F-12
10%	FBS
400µg/ml	hygromycin B
10µg/ml	Blasticidin

Cell Growth Medium for aAPC/CHO-K1 Cells

90%	Ham's F-12
10%	FBS
200µg/ml	Hygromycin B

Cell Plating Medium for FcγRIIb aAPC/CHO-K1 Cells, Propagation Model and aAPC/CHO-K1 Cells, Propagation Model

90%	Ham's F-12
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

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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

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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

9.C. Related Products (continued)

Detection Reagent

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

Not for Medical Diagnostic Use.

Luminometers

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

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Note: Additional Fc Effector, Immune Checkpoint, T Cell Activation 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**

10. Summary of Changes

The following changes were made to the 9/21 revision of this document:

1. Figure 9 and the related figure legend have been updated.
2. A new Limited Use Statement has been added on page 29.
3. The cover image has been updated.

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