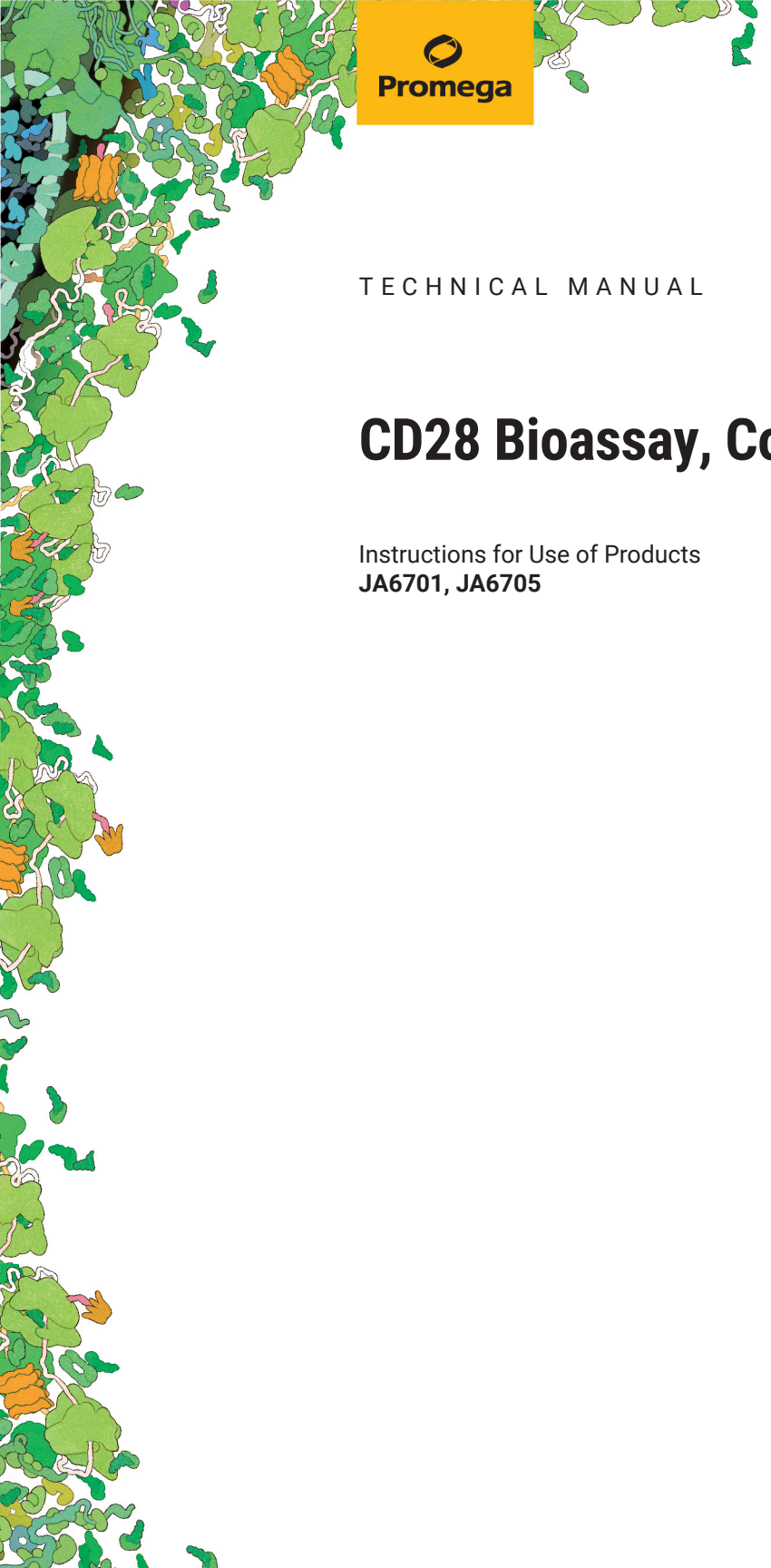

TECHNICAL MANUAL

CD28 Bioassay, Core Kit

Instructions for Use of Products
JA6701, JA6705

Revised 5/25
TM633

CD28 Bioassay, Core Kit

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

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1. Description

The human immune system is regulated by a complex network of inhibitory and stimulatory receptors that facilitate eliminating 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 CD28 agonists in the clinic 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 biologics 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, Core Kit[a–e] (Cat.# JA6701, JA6705), 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 the following 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.

The CD28 Bioassay should be performed with one of the following genetically engineered cell lines:

- **FcγRIIb aAPC/CHO-K1 Cells (Cat.# JA9331, JA9335):** CHO-K1 cells engineered to express FcγRIIb that serves as a crosslinking receptor, binding to antibody Fc domains and clustering 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.
- **aAPC/CHO-K1 Cells (Cat.# JA9441, JA9445):** Engineered to express 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, FcγRIIb aAPC/CHO-K1 Cells and aAPC/CHO-K1 are provided in thaw-and-use format, as cryopreserved cells that can be thawed, plated and used in assay without the need for cell culture and propagation.

In addition to the CD28 Bioassay FcγRIIb aAPC/CHO-K1 and aAPC/CHO-K1 Cells, 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 FcγRIIb aAPC/CHO-K1 Cells in parallel with aAPC/CHO-K1 Cells 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 7.B.).

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, Core Kit 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.

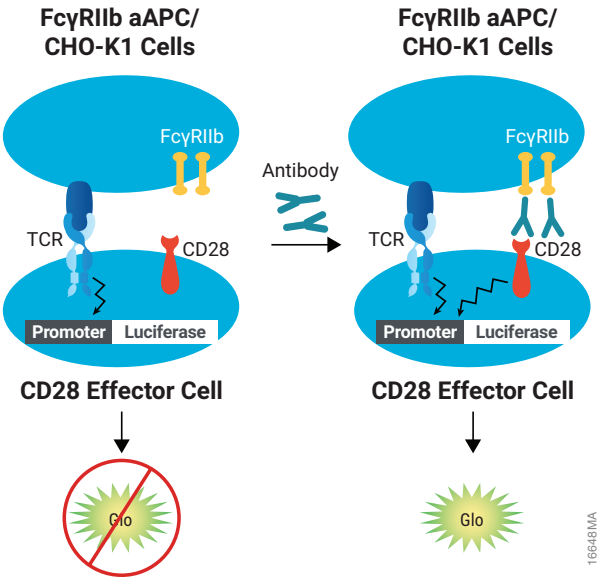

Figure 1. Representation of the CD28 Bioassay. The CD28 Bioassay for FcγRIIb-dependent agonist antibodies consists of two cell lines, CD28 Effector Cells and FcγRIIb aAPC/CHO-K1 Cells. In the absence of agonist antibody, CD28 is not activated and the luminescence signal is low. In the presence of FcγRIIb 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 with antibodies that are FcγRIIb-independent by substituting aAPC/CHO-K1 Cells.

1. Description (continued)

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 Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines and shows the precision, accuracy and linearity required for routine use in potency and stability studies (Table 1 and Figure 3). The bioassay can be performed in a two-day timeframe, and the workflow is simple, robust and compatible with both 96- and 384-well plate formats used for antibody screening in early drug discovery (Figure 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).

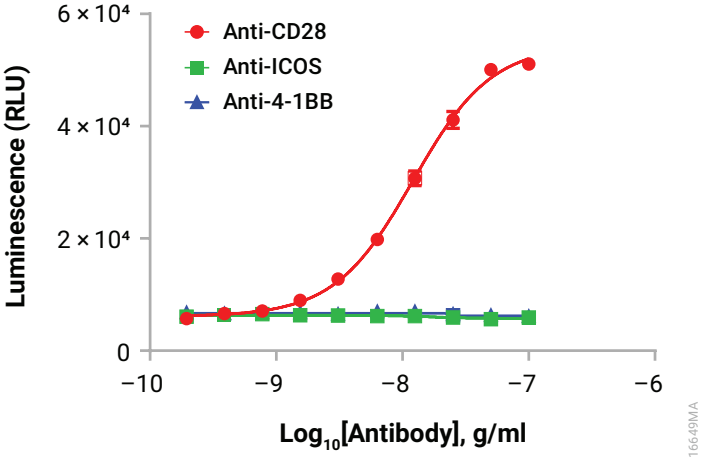

Figure 2. The CD28 Bioassay reflects the mechanism of action (MOA) and specificity of biologics designed to activate CD28. CD28 Effector Cells were incubated with FcγRIIb aAPC/CHO-K1 Cells in the presence of serial titrations of agonist antibodies as indicated. After a 5-hour incubation, Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover 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, Core Kit 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.

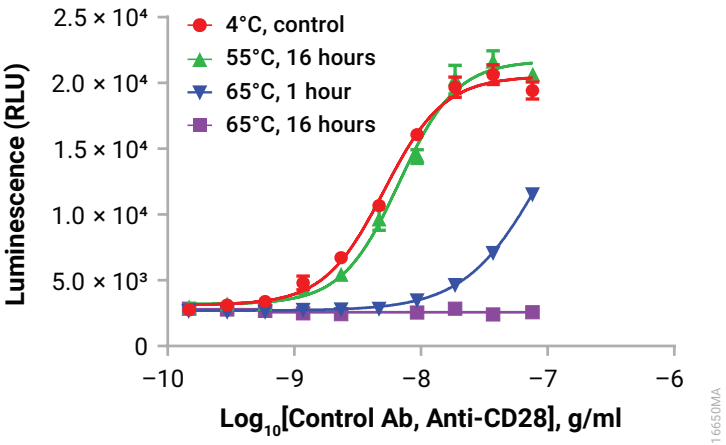

Figure 3. The CD28 Bioassay is stability-indicating. Samples of Control Ab, Anti-CD28, were maintained at 4°C (control) or heat-treated at the indicated times and temperatures, then analyzed using the CD28 Bioassay protocol for FcγRIIb-dependent antibodies. Bio-Glo™ Reagent was added and luminescence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

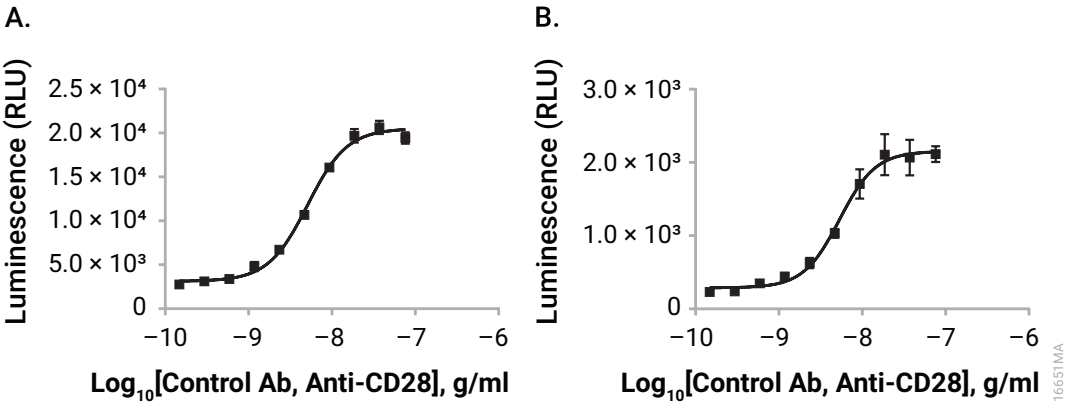

Figure 4. The CD28 Bioassay is amenable to 384-well plate format. Panel A. The CD28 Bioassay for FcγRIIb-dependent antibodies was performed in 96-well plates as described in this technical manual with a titration of Control Ab, Anti-CD28. **Panel B.** The CD28 Bioassay protocol for FcγRIIb-dependent 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 medium 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 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- and 384-well formats, respectively, and the fold induction was 7.4 and 9.4 for the 96- and 384-well formats, respectively. Data were generated using thaw-and-use cells.

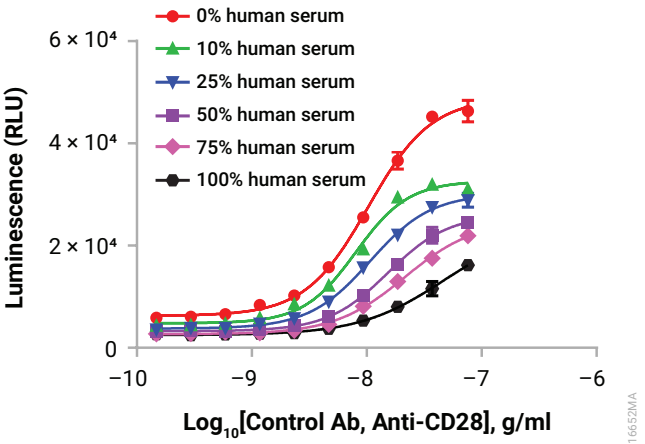

Figure 5. The CD28 Bioassay is tolerant to human serum. Following the CD28 Bioassay protocol for FcγRIIb-dependent antibodies, Control Ab, Anti-CD28, was analyzed in the absence or presence of pooled normal human serum (0–100% in the antibody sample), resulting in final assay concentration of human serum (0–50%). After a 5-hour induction at 37°C, Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells. The 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).

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2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
CD28 Bioassay, Core Kit	**1 each**	**JA6701**

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 120 assays using the inner 60 wells of two 96-well plates. Includes:

- 1 vial TCR/CD3 Effector Cells (IL-2), (CD28 Effector Cells, 0.8ml)
- 36ml RPMI 1640 Medium
- 4ml Fetal Bovine Serum
- 1 vial Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 10ml Bio-Glo™ Luciferase Assay Buffer

PRODUCT	SIZE	CAT.#
CD28 Bioassay, Core Kit 5X	**1 each**	**JA6705**

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 600 assays using the inner 60 wells of ten 96-well plates. Includes:

- 5 vials TCR/CD3 Effector Cells (IL-2), (CD28 Effector Cells, 0.8ml)
- 5 × 36ml RPMI 1640 Medium
- 5 × 4ml Fetal Bovine Serum
- 5 vials Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 5 × 10ml Bio-Glo™ Luciferase Assay Buffer

Storage Conditions:

- Upon arrival, immediately transfer the cell vials to below –140°C (freezer or liquid nitrogen vapor phase) for long-term storage. Do not store cell vials submerged in liquid nitrogen. Do not store cell vials at –80°C because this will negatively impact cell viability and cell performance.
- Store Bio-Glo™ Luciferase Assay Substrate, Bio-Glo™ Luciferase Assay Buffer and Fetal Bovine Serum at –30°C to –10°C. Avoid multiple freeze-thaw cycles of the serum. For optimal performance, use reconstituted Bio-Glo™ Reagent on the day of preparation. However, once reconstituted, Bio-Glo™ Reagent can be stored at –30°C to –10°C for up to 6 weeks.
- Store RPMI 1640 at 4°C, protected from fluorescent light.

Available Separately

PRODUCT	SIZE	CAT.#
FcγRIIb aAPC/CHO-K1 Cells	**1 each**	**JA9331**
FcγRIIb aAPC/CHO-K1 Cells 5X	**1 each**	**JA9335**
aAPC/CHO-K1 Cells	**1 each**	**JA9441**
aAPC/CHO-K1 Cells 5X	**1 each**	**JA9445**

Not for Medical Diagnostic Use.

Notes:

- CD28 Effector Cells are labeled as follows: TCR/CD3 Effector Cells (IL-2). Please note the vial label when placing the vials into storage.
- CD28 Effector Cells can be paired with either FcγRIIb aAPC/CHO-K1 Cells or aAPC/CHO-K1 Cells for antibodies that are FcγRIIb-dependent and -independent, respectively. Both FcγRIIb aAPC/CHO-K1 Cells and aAPC/CHO-K1 Cells are available separately in thaw-and-use format as standalone products.
- CD28 Bioassay components, FcγRIIb aAPC/CHO-K1 Cells, and aAPC/CHO-K1 Cells are shipped separately because of different temperature requirements. The CD28 Effector Cells, FcγRIIb aAPC/CHO-K1 Cells, and aAPC/CHO-K1 Cells are shipped on dry ice. The Bio-Glo™ Luciferase Assay Substrate, Bio-Glo™ Luciferase Assay Buffer and Fetal Bovine Serum are shipped on dry ice, separately from the cells. The RPMI 1640 Medium and Ham's F12 Medium are shipped at ambient temperature.

3. Before You Begin

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

Note the catalog number and lot number from the cell vial box label. This information can be used to download documents 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 is intended for use with user-provided antibodies or other biologics designed to activate CD28. Control Ab, Anti-CD28 (Cat.# K1231), FcγRIIb aAPC/CHO-K1 Cells (Cat.# JA9331, JA9335), and aAPC/CHO-K1 Cells (Cat.# JA9441, JA9445) are available separately for use in assay optimization and routine quality control. We strongly recommend using FcγRIIb aAPC/CHO-K1 Cells and Control Ab, Anti-CD28 in the first few assays to gain familiarity with the assay. Data generated using FcγRIIb aAPC/CHO-K1 Cells with Control Ab, Anti-CD28, are shown in Figures 2–5 and Section 7.A, Representative Assay Results.

The CD28 Effector Cells are provided in frozen, thaw-and-use format and are ready to be used without any additional cell culture or propagation. When thawed and diluted as instructed, the cells will be at the appropriate concentration for the assay. The cells are sensitive, and care should be taken to follow cell thawing and plating procedures as described. Do not overmix or overwarm the cell reagents.

The 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 7.B., Related Products). An integration time of 0.5 second/well was used for all readings. The bioassay is compatible with most other plate-reading luminometers, 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.

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3.A. Materials to Be Supplied by the User

- user-defined anti-CD28 antibodies or other biologics samples
- 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.,Corning®/Costar® 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 (Cat.# GM3000) or equivalent)
- **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:** FcγRIIb aAPC/CHO-K1 Cells if using FcγRIIb-dependent antibodies (Cat.# JA9331, JA9335)
- **optional:** aAPC/CHO-K1 Cells if using FcγRIIb-independent antibodies (Cat.# JA9441, JA9445)

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

This assay protocol requires two engineered cell lines. First, the CD28 Effector Cells, which are included with this assay. Second, either FcγRIIb aAPC/CHO-K1 Cells (Cat.# JA9331) for FcγRIIb-dependent antibodies, or aAPC/CHO-K1 Cells (Cat.# JA9441) for FcγRIIb-independent antibodies, both of which are available separately.

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. Each test and reference antibody is run in triplicate, in a tenf-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 testing antibodies not dependent on FcγRIIb-mediated crosslinking, use aAPC/CHO-K1 Cells instead of FcγRIIb aAPC/CHO-K1 Cells in this protocol. The aAPC/CHO-K1 Cells and FcγRIIb aAPC/CHO-K1 Cells are thawed and plated using the same protocol. When using the aAPC/CHO-K1 Cells, we use 1.5µg/ml as a starting concentration (1X) of Control Ab, Anti-CD28, and a twofold serial dilution series.

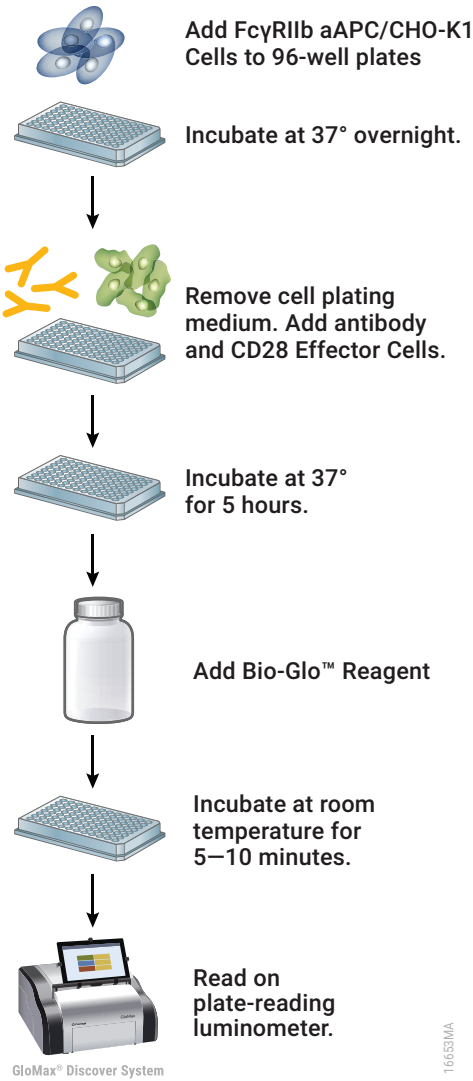

Figure 6. Schematic protocol for the CD28 Bioassay for FcγRIIb-dependent antibodies.

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4.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 14.5ml 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 1.5ml of FBS to 13ml of Ham's F-12 medium. Mix well and warm to 37°C before use. For reference, 14.5ml of Cell Plating Medium is sufficient to thaw and plate 1 vial of FcγRIIb aAPC/CHO-K1 Cells. If multiple vials will be thawed, adjust the amount of Cell Plating Medium appropriately. Warm the remaining Ham's F12 Medium to 37°C. Store the remaining FBS at 4°C for use in preparing the assay buffer on the day of the assay.
2. **Assay Buffer:** On the day of the assay, prepare 20ml of assay buffer (90% RPMI 1640/10% FBS). Add 2ml of FBS to 18ml RPMI 1640 Medium. Mix well and warm to 37°C before use. Warm the remaining RPMI 1640 Medium to 37°C.

   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 Antibody 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:

   a. 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.

   b. 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.

4.B. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 7 as a guide. The protocol describes serial replicate dilutions (n = 3) of test and reference antibody to generate two ten-point dose-response curves for each plate.

Recommended Plate Layout Design													
	1	2	3	4	5	6	7	8	9	10	11	12	
A	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)
B	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ab
C	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
D	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ab
E	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
F	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ab
G	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
H	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)

Figure 7. Example plate layout showing non-clustered sample locations of test antibody and reference antibody dilution series and wells containing assay buffer (denoted by "B") alone.

4.C. Plating FcγRIIb aAPC/CHO-K1 Cells

Notes:

- Perform the following steps in a sterile cell culture hood.
- Thaw-and-use FcγRIIb aAPC/CHO-K1 Cells are sensitive and care should be taken to follow the cell thawing and plating procedures exactly as described. Do not overmix or overwarm the cell reagents. No additional cell culture or manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at any one time.
- Follow the instructions below when using aAPC/CHO-K1 Cells for FcγRIIb-independent antibodies.

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

1. On the day before performing the assay, prepare 14.5ml of Cell Plating Medium (90% Ham's F12/10% FBS) as described in Section 4.A.
2. Remove one vial of FcγRIIb aAPC/CHO-K1 Cells from storage at –140°C and transfer to the bench on dry ice. Thaw the cells in a 37°C water bath until just thawed (about 2 minutes). While thawing, gently agitate and visually inspect the vial. Do not invert.
3. Gently mix the cell suspension by pipetting, then transfer 0.5ml cells to the tube containing 14.5ml of Cell Plating Medium. Mix well by gently inverting the tube 1–2 times.
4. 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 two 96-well white flat-bottom assay plates.
5. Add 100µl of prewarmed (37°C) Ham's F12 Medium to each of the outside wells of the assay plates.
6. Place lids on the assay plates and incubate in a 37°C, 5% CO_2 incubator overnight (18–22 hours).

4.D. Preparing Antibody Serial Dilutions

The instructions described here are for 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 4.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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4.E. Preparing CD28 Effector Cells

The thaw-and-use CD28 Effector Cells (TCR/CD3 (IL-2)) included in this kit are sensitive and care should be taken to follow the cell thawing and plating procedures exactly as described. Do not overmix or overwarm the cell reagents. No additional cell culture or manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at any one time.

1. Label a sterile 15ml conical tube "Effector Cells". Add 6.75ml of prewarmed (37°C) assay buffer to the 15ml conical tube.
2. Remove one vial of CD28 Effector Cells (TCR/CD3 (IL-2)) from storage at –140°C and transfer to the bench on dry ice. Thaw the cells in a 37°C water bath until just thawed (about 2 minutes). While thawing, gently agitate and visually inspect the vial.
3. Gently mix the cell suspension by pipetting, then transfer 0.75ml cells to the 15ml conical tube containing 6.75ml of assay buffer. Mix well by gently inverting the tube.

4.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 prewarmed (37°C) RPMI 1640 Medium 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.

4.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.

4.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).

5. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com** Email: **techserv@promega.com**

Symptoms	Causes and Comments
Low luminescence measurements (RLU readout)	Choose an instrument designed for plate-reading luminescence detection. Instruments designed primarily for fluorescence detection are not recommended. Luminometers measure and report luminescence as relative values, and actual 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.

6. References

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7. Appendix

7.A. Representative Assay Results

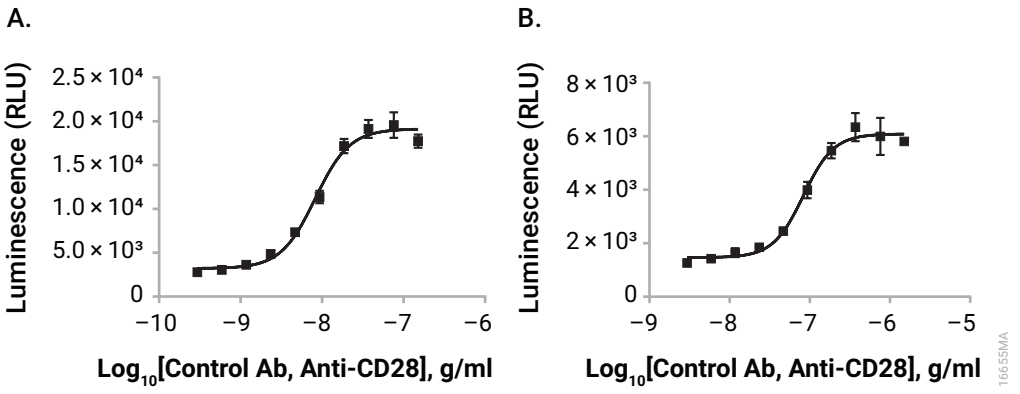

Figure 9. The CD28 Bioassay measures the activity of Control Ab, Anti-CD28. Panel A. FcγRIIb aAPC/CHO-K1 Cells were added to a 96-well assay plate 18 hours prior to the assay. On the day of the assay, a titration of Control Ab, Anti-CD28 (Cat.# K1231) and CD28 Effector Cells was added. After a 5-hour 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 8.2ng/ml and the fold induction was ~6.9. **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 was 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 83.5ng/ml and the fold induction was ~4.5.

7.B. Related Products

Fc Effector Bioassays

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

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

Fc Effector Immunoassay

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

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

Immune Checkpoint Bioassays

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

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Immune Checkpoint Bioassays (continued)

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

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T Cell Activation Bioassays

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

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

Control Antibodies and Proteins

Product	Size	Cat.#
Control Ab, Anti-4-1BB	50µg	K1161
Control Ab, Anti-CD-20	5µg	GA1130
Control Ab, Anti-OX40	50µg	K1191
Control Ab, Anti-CD40	50µg	K1181
Control Ab, Anti-CTLA-4	100µg	JA1020
Control Ab, Anti-LAG-3	100µg	K1150
Control Ab, Anti-PD-1	100µg	J1201
Control Ab, Anti-TIGIT	100µg	J2051
Control Ab, Anti-TIM-3	100µg	K1210
Recombinant VEGF ligand	10µg	J2371

Detection Reagent

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

Not for Medical Diagnostic Use.

Luminometers

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

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

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

8. Summary of Changes

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

1. Updated text about the label in Section 3.

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