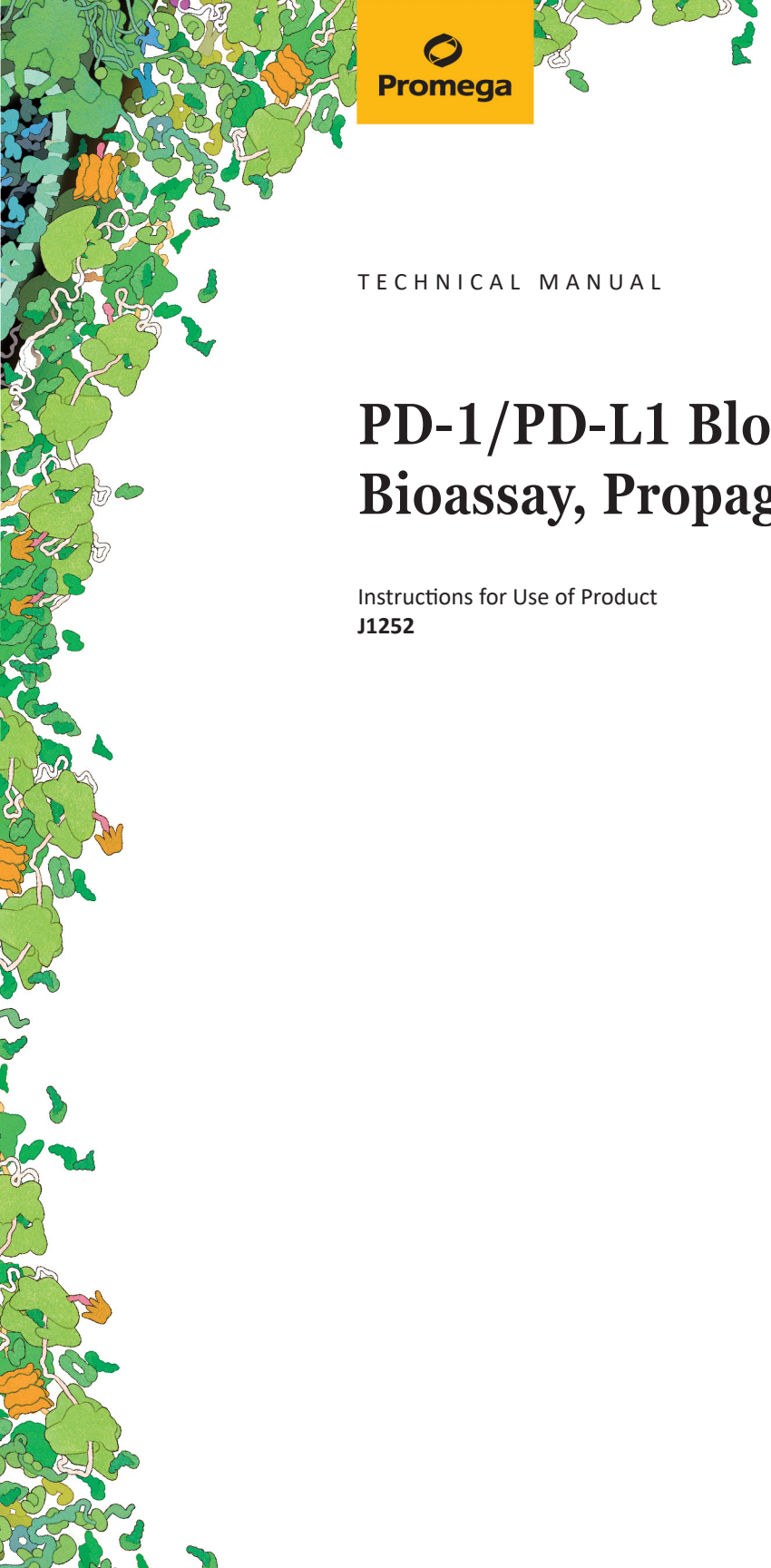

TECHNICAL MANUAL

PD-1/PD-L1 Blockade Bioassay, Propagation Model

Instructions for Use of Product
J1252

Revised 10/21
TM463

Promega

PD-1/PD-L1 Blockade 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

Promega

1. Description

The human immune system is comprised of a complex network of immune checkpoint molecules that facilitate the elimination of cells expressing foreign antigens while maintaining tolerance to self-antigen. Immune checkpoint receptors are promising new immunotherapy targets for the treatment of a variety of diseases, including cancer and autoimmunity (1,2).

Programmed cell death protein 1, also known as PD-1 and CD279, is an immune inhibitory receptor expressed on activated T cells and B cells and plays a critical role in regulating immune responses to tumor antigens and autoantigens (3). Engagement of PD-1 by either of its ligands, PD-L1 (B7-H1) or PD-L2 (B7-DC) on an adjacent cell inhibits TCR signaling and TCR-mediated proliferation, transcriptional activation and cytokine production. Therapeutic antibodies and Fc fusion proteins designed to block the PD-1/PD-L1 interaction show promising results in clinical trials for the treatment of a variety of cancers (4,5).

Current methods used to measure the activity of anti-PD-1 or anti-PD-L1 biologics rely on primary human T cells and measurement of functional endpoints such as cell proliferation, cell surface marker expression, and interferon gamma (IFNγ) and interleukin-2 (IL-2) production. These assays are laborious and highly variable due to their reliance on donor primary cells, complex assay protocols and unqualified assay reagents. As a result, these assays are difficult to establish in quality-controlled drug development settings.

The PD-1/PD-L1 Blockade Bioassay, Propagation Model[a–f] (Cat.# J1252), is a bioluminescent cell-based assay that overcomes the limitations of existing assays and can be used to measure the potency and stability of antibodies and other biologics designed to block the PD-1/PD-L1 interaction (6,7). The assay consists of two genetically engineered cell lines:

- PD-1 Effector Cells: Jurkat T cells expressing human PD-1 and a luciferase reporter driven by an NFAT-response element (NFAT-RE)
- PD-L1 aAPC/CHO-K1 Cells[b]: CHO-K1 cells expressing human PD-L1 and an engineered cell surface protein designed to activate cognate TCRs in an antigen-independent manner

Both cell lines 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 PD-1/PD-L1 interaction inhibits TCR signaling and NFAT-RE-mediated luminescence. Addition of either an anti-PD-1 or anti-PD-L1 antibody that blocks the PD-1/PD-L1 interaction releases the inhibitory signal and results in TCR activation and NFAT-RE-mediated luminescence (Figure 1). The bioluminescent signal is detected and quantified using Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941) and a standard luminometer, such as the GloMax® Discover System (see Related Products, Section 7.C).

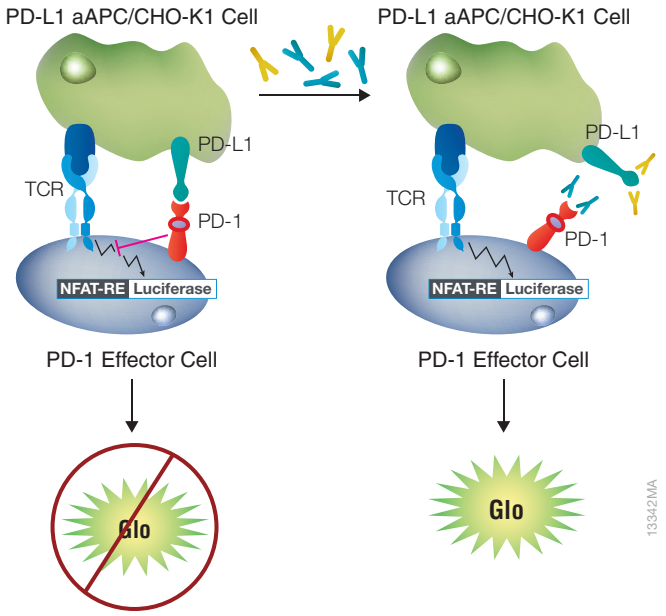

Figure 1. Representation of the PD-1/PD-L1 Blockade Bioassay. The bioassay consists of two genetically engineered cell lines, PD-1 Effector Cells and PD-L1 aAPC/CHO-K1 Cells. When co-cultured, the PD-1/PD-L1 interaction inhibits TCR-mediated luminescence. When the PD-1/PD-L1 interaction is disrupted, TCR activation induces luminescence (via activation of the NFAT pathway) that can be detected and quantified using Bio-Glo™ Reagent.

The PD-1/PD-L1 Blockade Bioassay, Propagation Model, reflects the mechanism of action (MOA) of biologics designed to block the PD-1/PD-L1 interaction. Specifically, TCR-mediated luminescence is detected following the addition of either anti-PD-1 or anti-PD-L1 blocking antibodies but not following addition of a non-specific anti-CTLA-4 blocking antibody (Figure 2). The bioassay is prequalified according to ICH guidelines and shows the precision, accuracy and linearity required for routine use in potency and stability studies (Table 1 and Figure 3). In addition, the bioassay workflow is simple and robust, and compatible with both 96-well and 384-well plate formats used for antibody screening in early drug discovery (Figure 4). Finally, the bioassay can be used with to 10% human serum with minimal impact on anti-PD-1 and anti-PD-L1 EC_{50} and fold induction (Figure 5), indicating potential for further development into a neutralizing antibody bioassay.

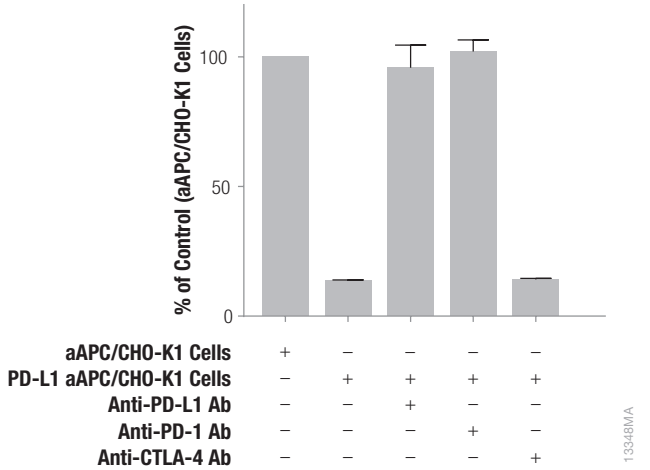

Figure 2. The PD-1/PD-L1 Blockade Bioassay reflects the mechanism of action (MOA) and specificity of biologics designed to block the PD-1/PD-L1 interaction. PD-1 Effector Cells were incubated with aAPC/CHO-K1 Cells (PD-L1 Negative Cells, Cat.# J1191) or with PD-L1 aAPC/CHO-K1 Cells in the absence or presence of anti-PD-1, anti-PD-L1 or anti-CTLA-4 blocking antibodies, as indicated. Bio-Glo™ Reagent was added, and luminescence was quantified. Data were analyzed using GraphPad Prism® software.

Table 1. The PD-1/PD-L1 Blockade Bioassay Shows Precision, Accuracy and Linearity.

Parameter	**Results**	
Accuracy	% Expected Relative Potency	% Recovery
	50	96.1
	75	99.9
	150	104.4
	200	104.9
Repeatability (% CV)	100% (Reference)	8.5
Intermediate Precision (% CV)		8.7
Linearity (r^2)		0.997
Linearity (y = mx + b)		y = 1.088x – 0.084

A 50–200% theoretical potency series of nivolumab (PD-1 blocking antibody) was analyzed in triplicate in three independent experiments performed on three days by two analysts. Bio-Glo™ Reagent was added and luminescence quantified. Data were analyzed and relative potencies calculated after parallelism determination using JMP® software. Data were generated using thaw-and-use cells.

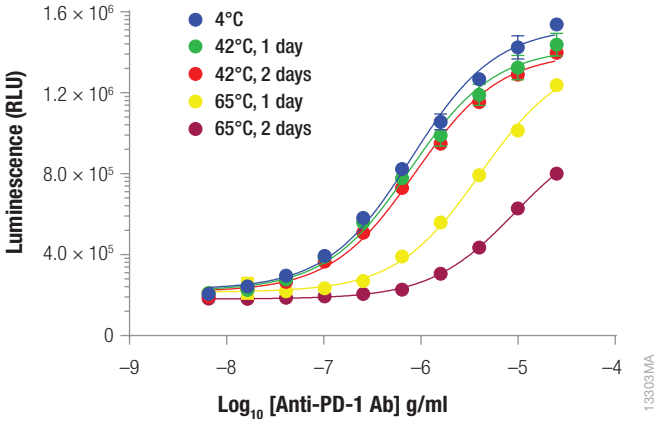

Figure 3. The PD-1/PD-L1 Blockade Bioassay indicates stability. Samples of Control Ab, Anti-PD-1 (Cat.# J1201), were maintained at 4°C (control) or heat-denatured (42°C or 65°C) for the indicated times, then analyzed using the PD-1/PD-L1 Blockade Bioassay. Bio-Glo™ Reagent was added and luminescence was quantified. Data were fitted to a 4PL curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

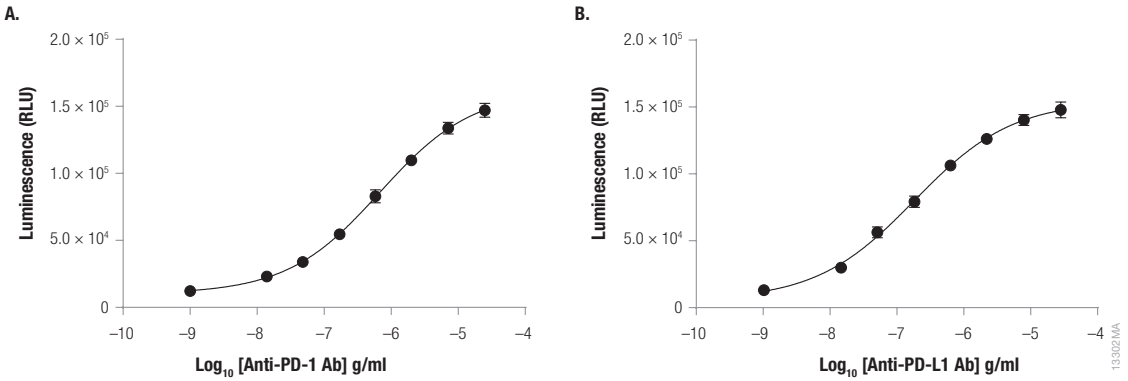

Figure 4. The assay is amenable to 384-well plate format and compatible with laboratory automation. Control Ab, Anti-PD-1 (Cat.# J1201; **Panel A**) or anti-PD-L1 Ab (**Panel B**) was tested in the PD-1/PD-L1 Blockade Bioassay with a Multidrop™ Combi nL (Thermo Scientific) and Tecan Freedom EVO® 200 with Multichannel Arm™ 384. Bio-Glo™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

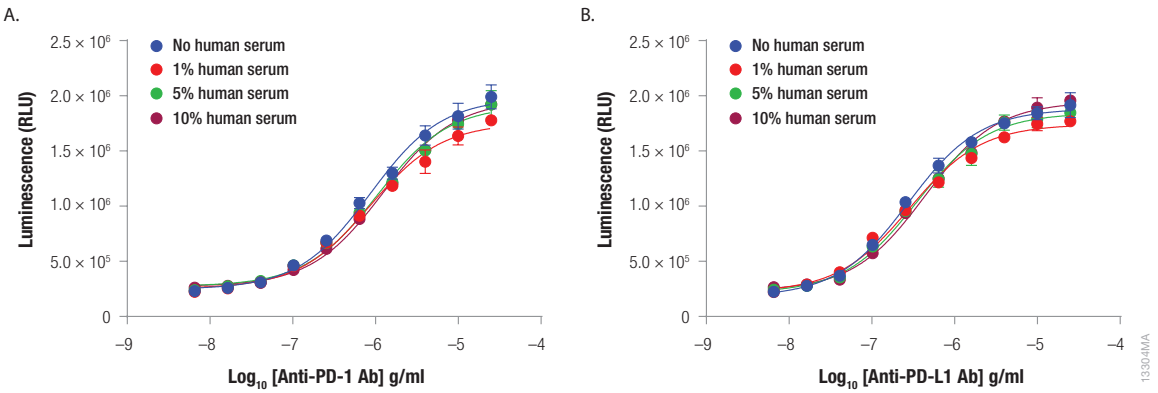

Figure 5. The PD-1/PD-L1 Blockade Bioassay tolerates human serum. Anti-PD-1 (**Panel A**) or anti-PD-L1 (**Panel B**) blocking antibody was analyzed in the absence or presence of increasing concentrations of pooled normal human serum, as indicated. Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Multi+ Detection System. Data were fitted to a 4PL curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
PD-1/PD-L1 Blockade Bioassay, Propagation Model	**1 each**	**J1252**

Not for Medical Diagnostic Use. Includes:

- 2 vials PD-1 Effector Cells (CPM), 1.6 × 10^7 cells/ml (1.0ml per vial)
- 2 vials PD-L1 aAPC/CHO-K1 Cells (CPM) 1.2 × 10^7 cells/ml (1.0ml per vial)

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

Storage Conditions: Upon arrival, immediately transfer the cell vials to below –140°C (freezer or liquid nitrogen vapor phase) for long-term storage. Do not store cell vials submerged in liquid nitrogen. **Do not** store cell vials at –80°C as this will negatively impact cell viability and cell performance.

3. Before You Begin

Please read through the entire protocol to become familiar with the components and the assay procedure before beginning. Cell thawing, propagation, and banking should be performed ***exactly*** as described in Section 4. 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. By contrast, the recommended cell plating densities, induction time and assay buffer components described in Section 4 were established using two research-grade anti-PD-1 and anti-PD-L1 blocking antibodies. You may need to adjust the parameters provided here and optimize assay conditions for your own antibodies or other biologic samples.

The PD-1/PD-L1 Blockade Bioassay produces a bioluminescent signal and requires a sensitive luminometer or luminescence plate reader for the detection of luminescence. Bioassay development and performance data included in this Technical Manual were generated using the GloMax® Discover System and GloMax® Multi+ Detection System (see Related Products, Section 7.C). An integration time of 0.5 second/well was used for all readings. The bioassay is compatible with most other plate-reading luminometers; however, relative luminescence unit readings may vary due to the sensitivity and settings of each instrument. The use of different instruments 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 7.B.)

Reagents

- RPMI 1640 Medium with L-glutamine and HEPES (e.g., Corning Cat.# 10-041-CV or Gibco Cat.# 22400)
- Ham's F-12 Medium (e.g., Corning Cat.# 10-080-CV or Gibco Cat.# 11765-054)
- fetal bovine serum (e.g., HyClone Cat.# SH30070.03 or Corning Cat.# 35-015-CV)
- hygromycin B (e.g., Gibco Cat.# 10687-010)
- Antibiotic G-418 Sulfate Solution (Cat.# V8091)
- sodium pyruvate (e.g., Gibco Cat.# 11360)
- MEM nonessential amino acids (e.g., Gibco Cat.# 1114)
- DMSO (e.g., Sigma Cat.# D2650)
- DPBS (e.g., Gibco Cat.# 14190)
- 0.25% Trypsin (e.g., Gibco Cat.# 25200)
- Trypan blue solution (e.g., Sigma Cat.# T8154)
- Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941)

Supplies and Equipment

- white, flat-bottom 96-well assay plates (e.g., Corning Cat.# 3917)
- sterile clear 96-well plate with lid (e.g., Linbro Cat.# 76-223-05) for preparing antibody dilutions
- pipettes (single-channel and 12-channel)
- sterile 15ml and 50ml conical tubes
- sterile reagent reservoirs (e.g., Corning Cat.# 4870)
- 37°C, 5% CO_2 incubator
- 37°C water bath
- plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System)

3.B. Preparing PD-1 Effector Cells

Cell Thawing and Initial Cell Culture

1. Prepare 30ml of initial cell culture medium by adding 3ml of FBS to 27ml 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 PD-1 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 incubator.
8. Incubate for approximately 48 hours before passaging the cells.

Cell Maintenance and Propagation

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

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

Cell Freezing and Banking

13. On the day of cell freezing, make new cell freezing medium and keep on ice.
14. Gently mix the cells with a pipette to create a 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* 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 in a single tube and dispense into either 0.5ml or 1ml cryovials.
19. Freeze the cells using a controlled-rate freezer (preferred), or a Mr. Frosty® or a Styrofoam® rack in a –80°C freezer overnight. Transfer the vials to at or below –140°C for long-term storage.

3.C. Preparing PD-L1 aAPC/CHO-K1 Cells

Cell Thawing

1. Prepare new cell growth medium and prewarm to 37°C. This cell growth medium will be used for culturing the cells immediately post-thawing and for subsequent maintenance.
2. Transfer 9ml of prewarmed cell growth medium to a 50ml conical tube.
3. Remove one vial of PD-L1 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 the cells (approximately 1ml) to the 50ml conical tube containing 9ml of prewarmed cell growth medium.
5. Centrifuge at 230 × *g* for 10 minutes.
6. Aspirate the medium, and resuspend the cell pellet in 10ml of prewarmed cell growth medium.
7. Transfer the cell suspension to a T75 flask, and place the flask horizontally in a 37°C, 5% CO_2 incubator.
8. Incubate the cells for approximately 24–48 hours before passaging them.

3.C. Preparing PD-L1 aAPC/CHO-K1 Cells (continued)

Cell Maintenance and Propagation

Note: Maintain the cell density in the range of 0.75×10^4– 2.0×10^5 cells/cm^2 to ensure optimal performance. The average cell doubling rate is 22–25 hours when cells are seeded at the densities listed in the table below. Once established in steady culture, cell viability is typically >95%. Do not allow cells to become 100% confluent prior to passaging because this may affect assay performance. In our experience, cells maintain their functionality for up to 25 passages, or 60 cell doublings, if passaging is performed on a Monday-Wednesday-Friday schedule.

Cell Passage Schedule	Cell Seeding Density
every 2 days	$3–4 \times 10^4$ cells/cm^2
every 3 days	$1.5–2 \times 10^4$ cells/cm^2
every 4 days	$0.75–1 \times 10^4$ cells/cm^2,

9. On the day of cell passage, aspirate the cell culture medium and wash the cells with DPBS.
10. Add 1ml of trypsin to each T75 flask and place in a 37°C, 5% CO_2 incubator for 3–5 minutes or until the cells round up and detach from the bottom of the flask.
11. Neutralize the trypsin by adding 9ml of cell culture medium to each flask. Transfer the cell suspension to a sterile 50ml conical (or larger) centrifuge tube.
12. Count the cells by Trypan blue staining. Calculate the cell numbers needed for the next experiment based on desired cell seeding density per area and flask size. We suggest that you maintain a consistent ratio of culture volume to culture surface area (e.g., 10ml volume per T75 flask or 20ml volume per T150 flask).
13. Add an appropriate amount of cell growth medium to achieve the desired cell seeding density per area.
14. Transfer the appropriate volume of cell suspension to new flasks.
15. Place the flasks in the 5% CO_2 incubator. Incubate the cells for approximately 24–48 hours before passaging them.

Cell Freezing and Banking

17. On the day of cell freezing, make new cell freezing medium and keep on ice.
18. Aspirate the cell culture medium, and wash the cells with DPBS.
19. Add 1ml of trypsin to each T75 flask, and place in a 37°C, 5% CO_2 incubator for 3–5 minutes or until the cells round up and detach from the bottom of the flask.
20. Neutralize the trypsin by adding 9ml of cell culture medium to each flask. Transfer the cell suspension to a 50ml conical or larger sized centrifuge tube.
21. Count the cells by Trypan blue staining.
22. Centrifuge at $230 \times g$ for 10 minutes.
23. Gently resuspend the cell pellet in ice-cold cell freezing medium to a final cell density of 5×10^6–1×10^7 viable cells/ml. Combine the cell suspensions in a single tube and dispense into 0.5ml or 1ml cryovials.

24. Freeze the cells using a controlled-rate freezer (preferred), a Mr. Frosty® or a Styrofoam® rack in a –80°C freezer overnight. Transfer the vials to storage at –140°C or below for long-term storage.

4. Assay Protocol

This assay protocol illustrates the use of the PD-1/PD-L1 Blockade Bioassay, Propagation Model, 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 full dose-response curve with proper upper and lower asymptotes and sufficient points on the slope. For reference, we use 25µg/ml as a starting concentration (1X) and 2.5-fold dilution when testing the anti-PD-1 antibodies pembrolizumab and nivolumab to achieve full dose curves.

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

1. **Bio-Glo™ Reagent:** For reference, 10ml of Bio-Glo™ Reagent is sufficient for 120 wells in a 96-well assay format. Thaw the Bio-Glo™ Luciferase Assay Buffer in a refrigerator 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 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 the reconstituted Bio-Glo™ Reagent to ambient temperature before adding to assay plates.

   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 6 weeks. Avoid repeated freeze-thaw cycles. On the day of the assay, thaw the appropriate amount of reconstituted Bio-Glo™ Reagent in a room temperature water bath for at least 1–2 hours before use. Approximate stability of Bio-Glo™ Reagent after reconstitution is 18% loss of luminescence over 24 hours at ambient temperature.

2. **Assay Buffer:** Prepare an appropriate amount of assay buffer on the day of the assay. Thaw the FBS in a 37°C water bath taking care not to overheat it. Add an appropriate amount of FBS to RPMI 1640 medium to yield 99% RPMI 1640/1% FBS. Mix well and warm to 37°C prior to 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 1% FBS. This concentration of FBS works well for all of the anti-PD-1 and anti-PD-L1 antibodies we have tested. If you experience assay performance issues when using this assay buffer, we recommend testing serum concentrations in the range of 0.5–10%.

3. **Test and Reference Samples:** Prepare starting dilutions (dilu1, 2X final concentration) of two test antibodies and one reference antibody. Using assay buffer as the diluent, prepare 500µl of reference antibody starting dilution and 250µl of each test antibody starting dilution in 1.5ml Eppendorf tubes. Store the tubes containing antibody starting dilutions appropriately before making antibody serial dilutions.

4. Assay Protocol (continued)

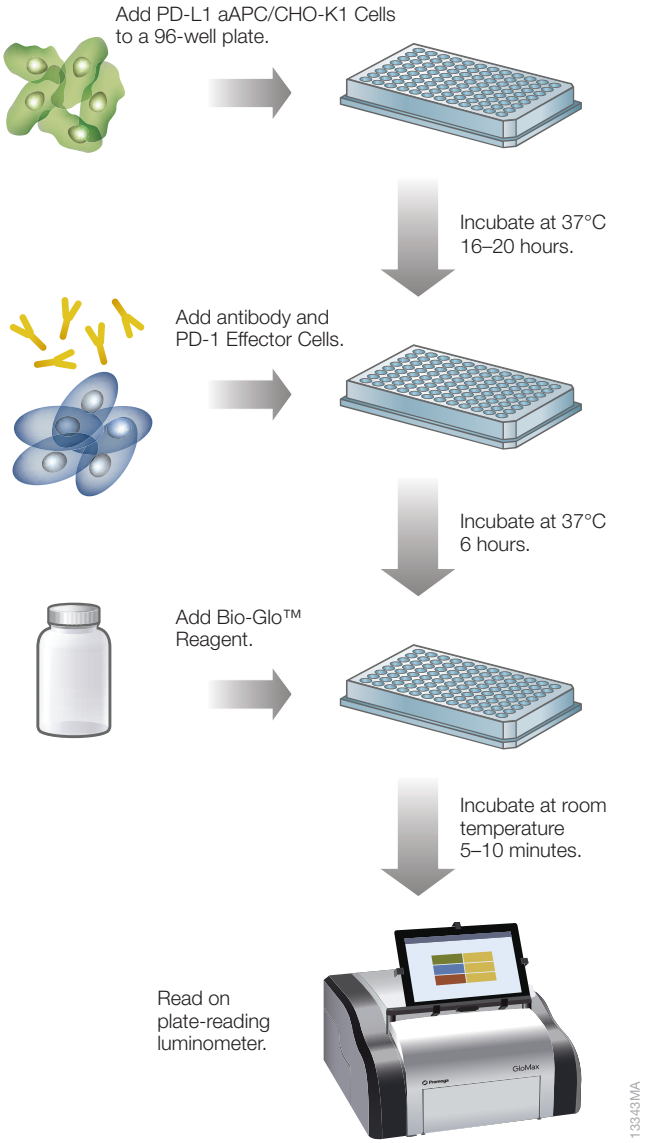

Figure 6. Schematic protocol for the PD-1/PD-L1 Blockade Bioassay.

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 antibodies to generate two 10-point dose-response curves in 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 and reference antibody dilution series and wells containing assay buffer (denoted by "B") alone.

4.C. Preparing and Plating PD-L1 aAPC/CHO-K1 Cells

While maintaining the PD-L1 aAPC/CHO-K1 Cells, it is important to follow the recommended cell seeding density because 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 an assay after the cell doubling rate has stabilized during propagation.

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

1. On the day before performing the assay, prepare new cell recovery medium (Ham's F-12/10% FBS) for the PD-L1 aAPC/CHO-K1 Cells.
2. Aspirate the cell culture medium from the PD-L1 aAPC/CHO-K1 Cells and wash with DPBS.
3. Add an appropriate amount of Trypsin to each T75 flask and place horizontally in a 37°C, 5% CO_2 incubator for 3–5 minutes or until the cells round up and detach from the bottom of the flask.
4. Neutralize the trypsin by adding a 4X volume of cell recovery medium to each flask. Transfer the cell suspension to a 50ml conical tube or larger size centrifuge tubes.

5. Gently mix the cell suspension and count the cells by Trypan blue staining.
6. Centrifuge at 230 × *g* for 10 minutes.
7. Gently resuspend the cell pellet in cell recovery medium to achieve a concentration of 4×10^5 viable cells/ml.
8. Transfer the cell 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 96-well flat, white-bottom assay plates. The final cell concentration in each well should be 40,000 cells/well.
9. Add 80µl of cell recovery medium to each of the outside wells of the assay plates.
10. Place the lid on the assay plates and incubate in a 37°C, 5% CO_2 incubator overnight.

4.D. Preparing Antibody Serial Dilutions

The instructions described here are for preparation of a single stock of 2.5-fold 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 2.5-fold serial dilutions, you will need 500µl of reference antibody at 2X the highest antibody concentration in your dose-response curve. You will need 250µl 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.

1. On the assay day, prepare an appropriate amount of assay buffer as described in Section 4.A.
2. To a sterile clear V-bottom 96-well plate, add 250µl of reference antibody starting dilution (dilu1, 2X final concentration) to wells A11 and B11.
3. Add 250µl 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 100µl of the antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
6. Repeat equivalent 2.5-fold serial dilutions across the columns from right to left until you reach column 3.

Note: Wells A2, B2, E2 and G2 contain 150µl of assay buffer without antibody as a negative control.

7. Cover the assay plate with a lid and keep at ambient temperature (22–25°C) while preparing the PD-1 Effector Cells.

Recommended Plate Layout: 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.

4.E. Preparing PD-1 Effector Cells

While maintaining the PD-1 Effector Cells, follow the recommended cell seeding density because changes in cell culture volume or seeding density will affect cell growth rate and assay performance. 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.

1. Passage the cells two days before performing the assay as described in Section 3.A. To ensure optimal and consistent assay performance, maintain the cell density, upon harvest, in the range of 1.4–2.0 × 10^6 cells/ml and cell viability at greater than 95%.
2. Count the PD-1 Effector Cells by Trypan blue staining, and calculate the cell density and viability.
3. Transfer an appropriate amount of PD-1 Effector Cells from the culture vessel to a 50ml conical tube or larger sized centrifuge tube.
4. Pellet the cells at 130–180 × *g* for 10 minutes at ambient temperature and resuspend in assay buffer at 70% of the full volume needed to generate the targeted final cell density of 1.25 × 10^6 cells/ml.
5. Count the cells again, and adjust the volume of assay buffer to achieve a final cell density of 1.25 × 10^6 cells/ml. You will need at least 6ml of PD-1 Effector Cells to fill 120 assay wells, or the inner 60 wells of two assay plates.

4.F. Adding Antibody Samples and PD-1 Effector Cells to Assay Plates

1. Remove the 96-well assay plates containing PD-L1 aAPC/CHO-K1 Cells from the incubator, and using a manual multichannel pipette, remove 95µl of medium from each of the inner 60 wells. Alternatively, 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.
2. Using an electronic multichannel pipette, immediately add 40µl of the appropriate antibody dilution to the pre-plated PD-L1 aAPC/CHO-K1 Cells (from Step 1) according to the plate layout in Figure 7. After you add antibody dilution and PD-1 Effector Cells to the inner 60 wells, add 80µl of assay buffer to the outside wells.
3. Transfer the PD-1 Effector Cells prepared in Section 4.E to a sterile reagent reservoir. Using the same multichannel pipette, dispense 40µl (50,000 cells) of PD-1 Effector Cells into the wells containing PD-L1 aAPC/CHO-K1 Cells and antibody.
4. Cover the assay plate with a lid and incubate in a 37°C, 5% CO_2 incubator for 6 hours.

4.G. Adding Bio-Glo™ Reagent

Note: Bio-Glo™ Reagent should be at ambient temperature when added to assay plates.

1. Remove the assay plates from the incubator and equilibrate to ambient temperature (22–25°C) for 5–10 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, C1 and D1 of each assay plate to measure the background signal.
4. Incubate at ambient temperature for 5–30 minutes.

Note: Varying the incubation time will impact the raw RLU values but should not significantly change the EC_{50} or fold induction.

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

4.H. Data Analysis

1. Measure plate background by calculating the average relative light units (RLU) from wells B1, C1 and D1.
2. Calculate fold induction:

$$\text{Fold Induction} = \frac{\text{RLU (induced} - \text{background)}}{\text{RLU (no antibody control} - \text{background)}}$$

Note: When calculating fold induction, if sample RLUs are equal to or as much as 100X the plate background RLU, there is no need to subtract plate background from sample RLU.

3. Graph data as RLU versus Log_{10} [antibody] and fold induction versus Log_{10} [antibody]. Fit curves to determine EC_{50} of antibody response using appropriate curve fitting software (such as GraphPad Prism® software).

5. Troubleshooting

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

Symptoms	Possible Causes and Comments
Cell viability is low during cell propagation	Seeding cells at a density that is too low or too high could lead to low cell viability. Handle and propagate the cells according to the instructions provided in Section 3. Do not let the cells overgrow.
	Centrifugation above the recommended speed could lead to low cell viability.
Assay performance is variable	If the cells were not grown under controlled and consistent conditions, assay performance will vary. Follow the cell culture guidelines and ensure accurate and consistent cell seeding density and culture volume. Monitor cell doubling rate for consistency.
Low luminescence measurements (RLU readout)	Choose an instrument designed for luminescence detection. Instruments primarily designed for fluorescence detection are not recommended. Luminometers measure and report luminescence as relative values, and actual RLU numbers will vary between instruments.
	Insufficient cells per well could lead to low RLU. Handle and plate the 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 reporter response (low fold induction)	Optimize the concentration range of your test sample(s) to achieve a full dose response with complete upper and lower asymptotes. The EC_{50} obtained in the PD-1/PD-L1 Blockade Bioassay may vary from the EC_{50} obtained using other methods such as primary T cell-based assays.
	Optimize the assay incubation time within a range of 6–24 hours.

Promega

6. References

1. Mahoney, K.M. *et al.* (2015) Combination cancer immunotherapy and new immunomodulatory targets. *Nature Rev. Drug Disc.* **14**, 561–84.
2. Melero, I. *et al.* (2015) Evolving synergistic combinations of targeted immunotherapies to combat cancer. *Nature Rev. Cancer* **15**, 457–72.
3. Herbst, R.S. *et al.* (2013) A study of MPDL3280A, an engineered PD-L1 antibody in patients with locally advanced or metastatic tumors. *J. Clin. Oncol.* **31(suppl.)**, 3000.
4. Robert, C. *et al.* (2015) Pembrolizumab versus ipilimumab in advanced melanoma. *N. Engl. J. Med.* **372**, 2521–32.
5. Larkin, J. *et al.* (2015) Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. *N. Engl. J. Med.* **373**, 23–34.
6. Cheng, Z.J. *et al.* (2015) Novel PD-1 blockade bioassay to assess therapeutic antibodies in PD-1 and PD-L1 immunotherapy programs. *American Association of Cancer Research (AACR) Annual Meeting*, Poster #5440.
7. Cong, M. *et al.* (2015) Novel bioassay to assess PD-1/PD-L1 therapeutic antibodies in development for immunotherapy. *Genetic Engineering News* **35,** 10.

7. Appendix

7.A. Representative Assay Results

The following data were generated using the PD-1/PD-L1 Blockade Bioassay, Propagation Model, using research grade anti-PD-1 or anti-PD-L1 blocking antibodies (Figure 9) or clinical grade anti-PD-1 blocking antibodies (Figure 10).

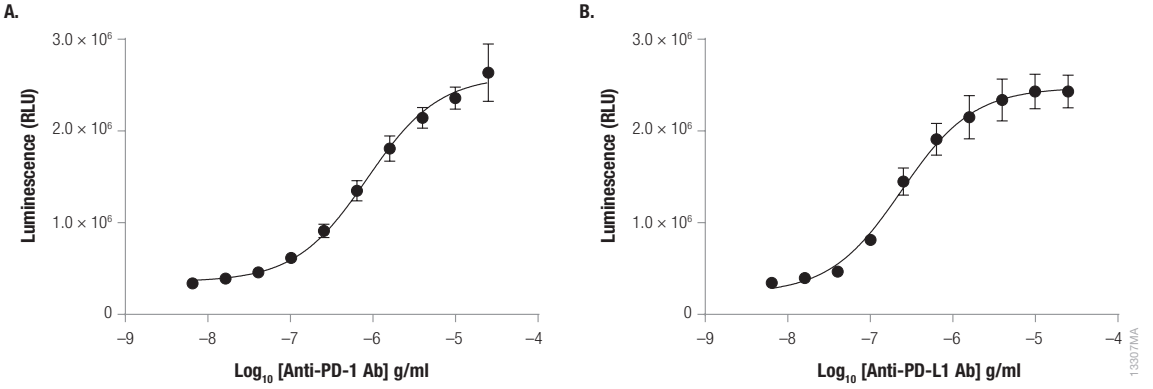

Figure 9. The PD-1/PD-L1 Blockade Bioassay measures the inhibitory activity of research grade anti-PD-1 and anti-PD-L1 blocking antibodies. PD-L1 aAPC/CHO-K1 Cells were plated and incubated at 37°C for 16–20 hours prior to the addition of increasing concentrations of either anti-PD-1 (**Panel A**) or anti-PD-L1 (**Panel B**) antibodies and PD-1 Effector Cells. After 6 hours of incubation, Bio-Glo™ Reagent was added and luminescence measured using the GloMax® Multi+ Detection System. Data were fitted to a 4PL curve using GraphPad Prism® software. The EC_{50} values were 0.85µg/ml (Anti-PD-1 Ab) and 0.24µg/ml (anti-PD-L1 Ab).

7.A. Representative Assay Results (continued)

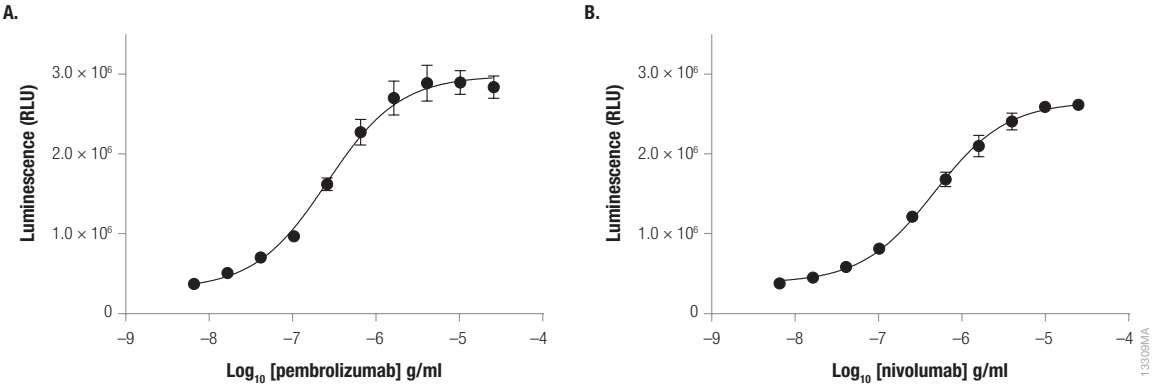

Figure 10. The PD-1/PD-L1 Blockade Bioassay measures potency of clinical grade anti-PD-1 antibodies and the assay response to anti-PD-1 antibodies. PD-L1 aAPC/CHO-K1 Cells were plated and incubated for 16–20 hours prior to the addition of increasing concentrations of either pembrolizumab (**Panel A**) or nivolumab (**Panel B**) and PD-1 Effector Cells. After 6 hours of incubation at 37°C, Bio-Glo™ Reagent was added and luminescence measured using the GloMax® Multi+ Detection System. Data were fitted to a 4PL curve using GraphPad Prism® software. EC_{50} values were 0.25µg/ml (pembrolizumab; **Panel A**) and 0.47µg/ml (nivolumab; **Panel B**).

7.B. Composition of Buffers and Solutions

Initial Cell Culture Medium for PD-1 Effector Cells

90%	RPMI 1640 with L-glutamine
10%	FBS

Cell Growth Medium for PD-1 Effector Cells

90%	RPMI 1640 with L-glutamine
10%	FBS
200µg/ml	hygromycin B
500µg/ml	Antibiotic G-418 Sulfate Solution
1mM	sodium pyruvate
0.1mM	MEM nonessential amino acids

Cell Freezing Medium for PD-1 Effector Cells

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

Cell Growth Medium for PD-L1 aAPC/CHO-K1 Cells

90%	Ham's F-12 medium
10%	FBS
200µg/ml	hygromycin B
250µg/ml	Antibiotic G-418 Sulfate Solution

Cell Freezing Medium for PD-L1 aAPC/CHO-K1 Cells

85%	Ham's F-12 medium
10%	FBS
5%	DMSO

Cell Recovery Medium for PD-L1 aAPC/CHO-K1 Cells

90%	Ham's F-12 medium
10%	FBS

Assay Buffer

99%	RPMI 1640 with L-glutamine
1%	FBS

Promega

7.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 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

Immune Checkpoint Bioassays (continued)

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

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

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	5 µg	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

7.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

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 e-mail: **eliteaccess@promega.com**

8. Summary of Changes

The following changes were made to the 10/21 revision of this document:

1. Added a new Limited Use Label License disclaimer on pages 2 and 25.
2. Updated formatting of the equation on p. 16.

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

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

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

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

[c]U.S. Pat. No. 8,008,006 and European Pat. No. 1341808.

[d]Patent Pending.

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

[f]Product cannot be used for proficiency testing.

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

Freedom EVO is a registered trademark of Tecan AG Corporation. GraphPad Prism is a registered trademark of GraphPad Software, Inc. JMP is a registered trademark of SAS Institute, Inc. Mr. Frosty is a registered trademark of Nalge Nunc International Corporation. Multichannel Arm is a trademark of Tecan Group Ltd. Multidrop is a trademark of Thermo Fisher Scientific, Ltd. Styrofoam is a registered trademark of Dow Chemical Company.

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

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

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