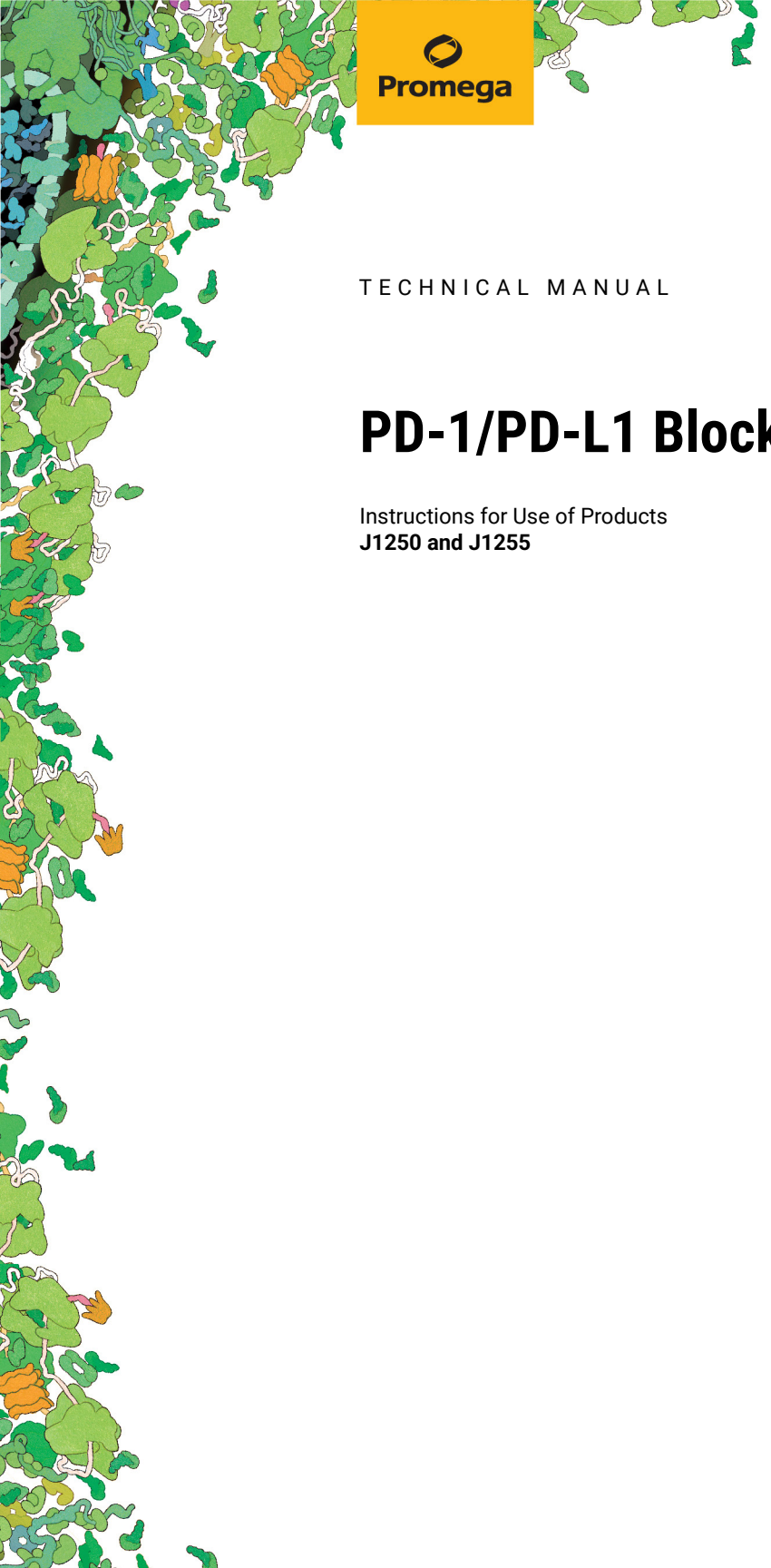


TECHNICAL MANUAL

PD-1/PD-L1 Blockade Bioassay

Instructions for Use of Products
J1250 and J1255

Revised 8/26
TM468

Promega

PD-1/PD-L1 Blockade Bioassay

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 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 autoimmune-mediated disorders (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).

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1. Description (continued)

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[a,b] (Cat.# J1250, J1255) 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: CHO-K1 cells expressing human PD-L1 and an engineered cell surface protein designed to activate cognate TCRs in an antigen-independent manner

The PD-1 Effector Cells and PD-L1 aAPC/CHO-K1 Cells are provided in thaw-and-use format as cryopreserved cells that can be thawed, plated and used in an assay without the need for cell propagation.

When the two cell types are cocultured, 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 PD-1/PD-L1 Blockade Bioassay includes the necessary medium and serum to thaw, plate and assay the cells. The bioluminescent signal can be detected and quantified using the Bio-Glo™ Luciferase Assay System, also included in the kit, and a standard luminometer such as the GloMax® Discover System.

In addition to the PD-1/PD-L1 Blockade Bioassay (Cat.# J1250, J1255), we offer aAPC/CHO-K1 Cells (PD-L1 Negative Cells, Cat.# J1191, J1195) for use as a negative control in the PD-1/PD-L1 Blockade Bioassay. When cocultured with PD-1 Effector Cells, the PD-L1 Negative Cells activate TCR signaling, and this response is not affected by anti-PD-1 or anti-PD-L1 antibodies (see Section 7.A, Representative Assay Results). We also offer Control Ab, Anti-PD-1 (Cat.# J1201), a blocking antibody for use as a positive control.

The PD-1/PD-L1 Blockade Bioassay combines (1) a simple, add-mix-read single-day workflow with (2) PD-1 Effector Cells and PD-L1 aAPC/CHO-K1 Cells provided in a frozen, thaw-and-use format, and (3) an optimized protocol, that together yield a quantitative bioassay that exhibits low variability and high accuracy. The thaw-and-use cells provided in the PD-1/PD-L1 Blockade Bioassay kits are manufactured under stringent quality control to provide high assay reproducibility with the convenience of an assay reagent that eliminates the need for continuous cell propagation.

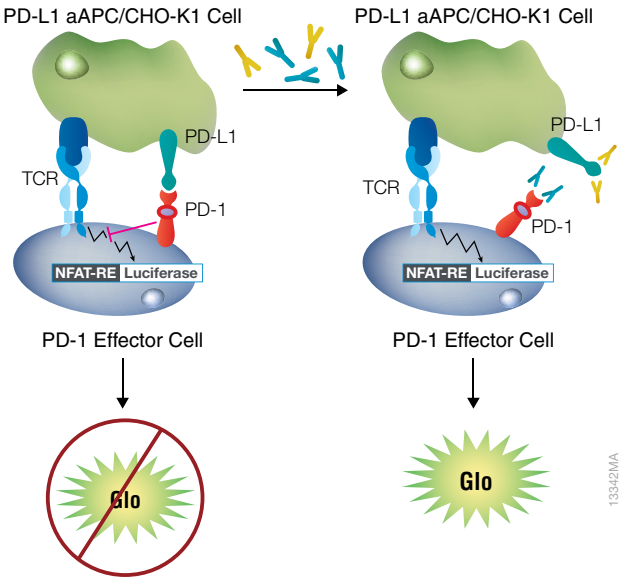


Figure 1. Representation of the PD-1/PD-L1 Blockade Bioassay. The bioassay consists of two genetically engineered cell lines, PD-1 Effector Cells and PD-L1 aAPC/CHO-K1 Cells. When cocultured, the PD-1/PD-L1 interaction inhibits TCR-mediated luminescence. When the PD-1/PD-L1 interaction is disrupted,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

The PD-1/PD-L1 Blockade Bioassay 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- and 384-well plate formats used for antibody screening in early drug discovery (Figure 4). Finally, the bioassay can be used with up 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.

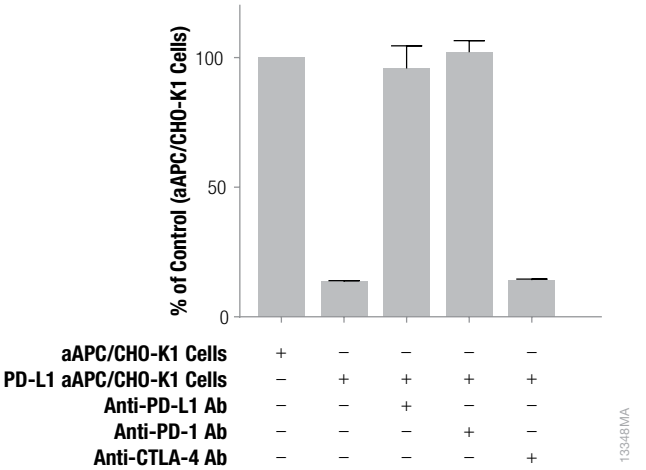


Figure 2. The PD-1/PD-L1 Blockade Bioassay reflects the mechanism of action (MOA) and specificity of biologics designed to block the PD-1/PD-L1 interaction. PD-1 Effector Cells were incubated with aAPC/CHO-K1 Cells (PD-L1 Negative Cells, Cat.# J1191) or with PD-L1 aAPC/CHO-K1 Cells in the absence or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

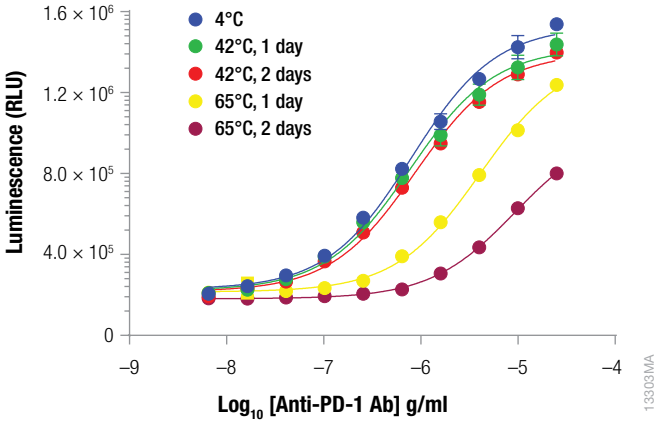


Figure 3. The PD-1/PD-L1 Blockade Bioassay is stability-indicating. Samples of Control Ab, Anti-PD-1(Cat.# J1201), were maintained at 4°C (control) or heat-denatured (42°C or 65°C) for the indicated times, then analyzed using the PD-1/PD-L1 Blockade Bioassay. Bio-Glo™ Reagent was added and luminescence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

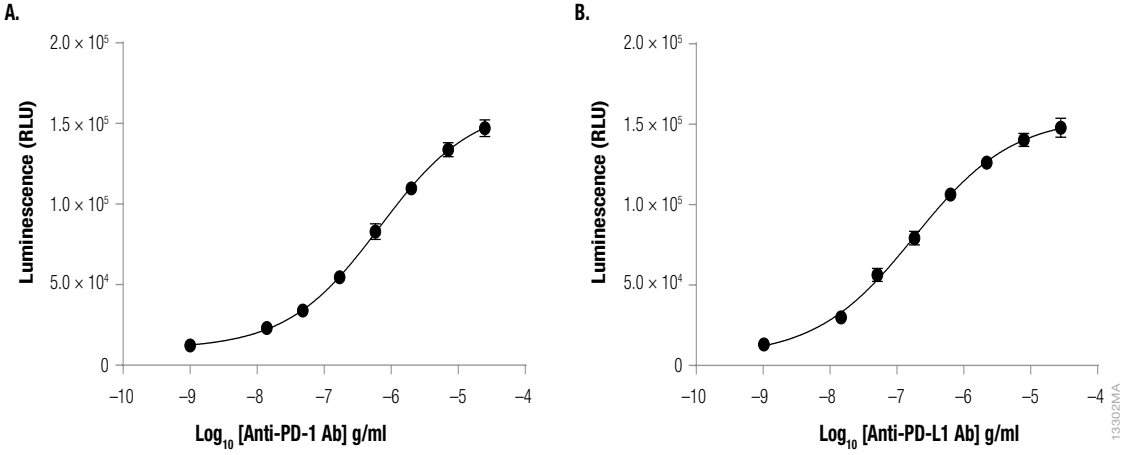


Figure 4. The assay is amenable to 384-well plate format and compatible with laboratory automation. Control Ab, Anti-PD-1 (Cat.# J1201; **Panel A**), or anti-PD-L1 Ab (**Panel B**) was tested in the PD-1/PD-L1 Blockade Bioassay with a Multidrop™ Combi nL (Thermo Scientific) and Tecan Freedom EVO® 200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

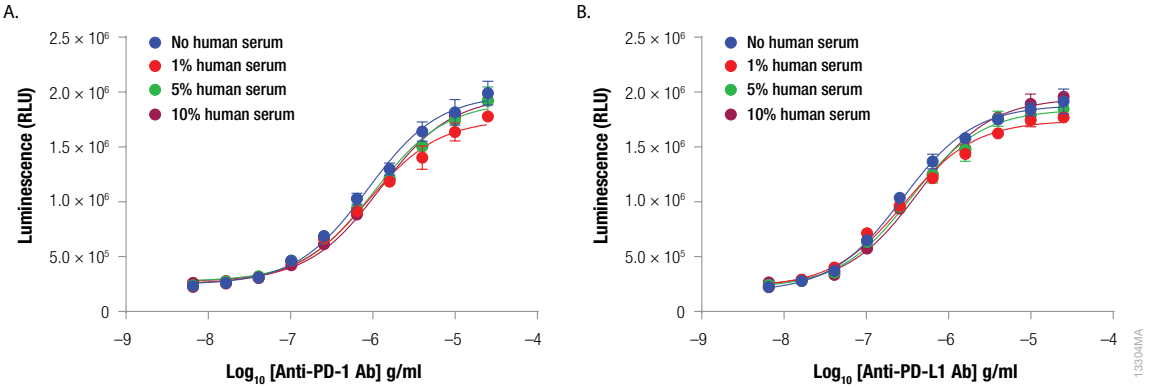


Figure 5. The PD-1/PD-L1 Blockade Bioassay is tolerant to human serum. Anti-PD-1 (**Panel A**) or anti-PD-L1 (**Panel B**) blocking antibody was analyzed in the absence or presence of increasing concentrations of pooled normal human serum, as indicated. Bio-Glo™ Reagent was added and luminescence quantified 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	**1 each**	**J1250**

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 PD-1 Effector Cells (0.5ml)
- 1 vial PD-L1 aAPC/CHO-K1 Cells (0.5ml)
- 36ml RPMI 1640 Medium
- 25ml Ham's F-12 Medium
- 4ml Fetal Bovine Serum
- 1 vial Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 10ml Bio-Glo™ Luciferase Assay Buffer

PRODUCT	SIZE	CAT.#
PD-1/PD-L1 Blockade Bioassay 5X	**5 each**	**J1255**

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 PD-1 Effector Cells (0.5ml)
- 5 vials PD-L1 aAPC/CHO-K1 Cells (0.5ml)
- 5 × 36ml RPMI 1640 Medium
- 5 × 25ml Ham's F-12 Medium
- 5 × 4ml Fetal Bovine Serum
- 5 vials Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 5 × 10ml Bio-Glo™ Luciferase Assay Buffer

Note: The PD-1/PD-L1 Blockade Bioassay components are shipped separately because of differing temperature requirements. The PD-1 Effector Cells and PD-L1 aAPC/CHO-K1 Cells are shipped on dry ice. The Bio-Glo™ Luciferase Assay System and Fetal Bovine Serum are shipped on dry ice, separately from the cells. The RPMI 1640 Medium and Ham's F-12 Medium are shipped at ambient temperature.

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.
- Store Bio-Glo™ Luciferase Assay Substrate and 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 Medium at +2°C to +10°C protected from fluorescent light. Store Ham's F-12 Medium at +2°C to +10°C. Minor variations in the color of Ham's F-12 Medium may be observed. The color change will not impact performance in the assay.

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3. Before You Begin

The PD-1/PD-L1 Blockade Bioassay is intended to be used with user-provided antibodies or other biologics designed to block the PD-1/PD-L1 interaction. Control Ab, Anti-PD-1 (Cat.# J1201), and PD-L1 Negative Cells (Cat.# J1191, J1195) are available separately for use in assay optimization and routine quality control. We strongly recommend including Control Ab, Anti-PD-1, as a positive control in the first few assays to gain familiarity with the assay. Data generated using these reagents is shown in Section 7.A, Representative Assay Results.

Please read through the entire protocol to become familiar with the components and the assay procedure before beginning. The PD-1 Effector Cells and PD-L1 aAPC/CHO-K1 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 exactly as described. Do not overmix or overwarm the cell reagents.

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 or GloMax® Multi+ System. An integration time of 0.5 second/well was used for all readings. The bioassay is compatible with most other plate-reading luminometers; 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

- user-defined anti-PD-1 or anti-PD-L1 blocking antibodies or other biologics samples
- sterile clear 96-well plate with lid (e.g., Costar® Cat. Cat.# 3370 or Linbro Cat.# 76-223-05)
- white, flat-bottom 96-well assay plates (e.g., Corning® Cat.# 3917)
- pipettes (single-channel and 12-channel; for best results both a manual and an electronic pipette are needed)
- sterile 15ml and 50ml conical tubes
- sterile reagent reservoirs (e.g., Corning® Cat.# 4870)
- 37°C, 5% CO_2 incubator
- 37°C water bath
- plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System)

4. Assay Protocol

This assay protocol illustrates the use of the PD-1/PD-L1 Blockade Bioassay to test two antibody samples against a reference sample in a single assay run. Each test and reference antibody is run in triplicate, in a ten-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 Control Ab, Anti-PD-1, and the anti-PD-1 antibodies pembrolizumab and nivolumab to achieve full dose curves.

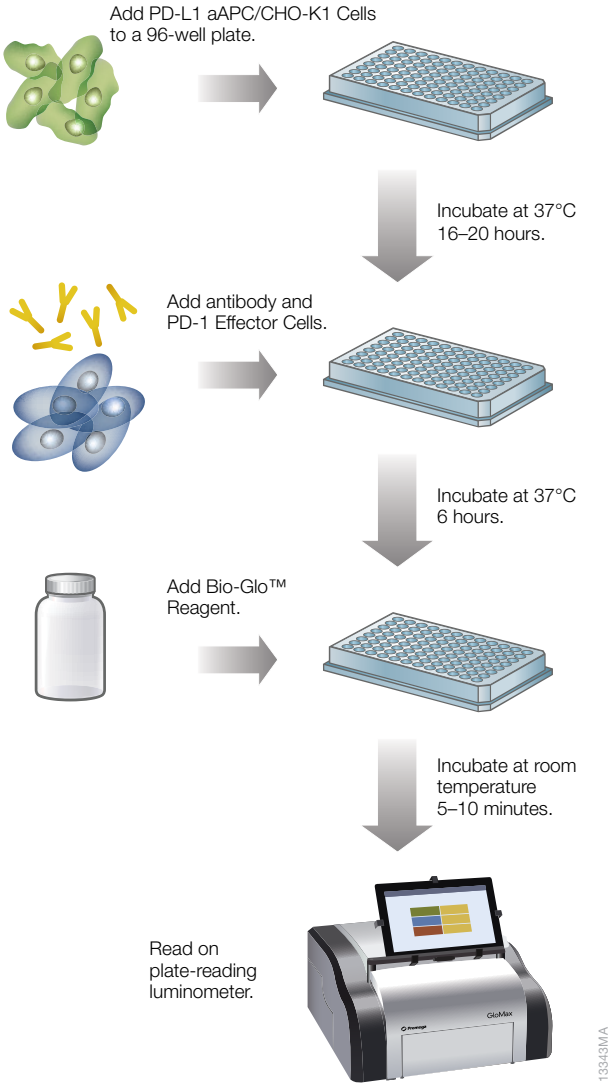


Figure 6. Schematic protocol for the PD-1/PD-L1 Blockade Bioassay.

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

1. **Cell Recovery Medium:** On the day before performing the assay, prepare 25ml of cell recovery medium (90% Ham's F-12/10% FBS) in a 50ml conical tube. Thaw the Fetal Bovine Serum (FBS) overnight at +2°C to +10°C or in a 37°C water bath on the day of use. Add 2.5ml of FBS to 22.5ml Ham's F-12 Medium to yield 90% Ham's F-12/10% FBS. Mix well and warm to 37°C prior to use. For reference, 25ml of cell recovery medium is sufficient to thaw 1 vial of PD-L1 aAPC/CHO-K1 cells. If multiple vials of PD-L1 aAPC/CHO-K1 cells will be used on the day of assay then scale the amount of cell recovery medium appropriately. Store the remaining FBS at +2°C to +10°C for use in preparing the assay buffer on the day of assay.

2. **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 in a refrigerator overnight or in a room temperature water bath on the day of assay. Equilibrate the Bio-Glo™ Luciferase Assay Buffer to ambient temperature, protected from light. Transfer all of the Bio-Glo™ Luciferase Assay Buffer into the amber bottle containing the Bio-Glo™ Luciferase Assay Substrate and mix by inversion until the Substrate is thoroughly dissolved. Equilibrate and store the reconstituted Bio-Glo™ Reagent at ambient temperature (22–25°C) protected from light before adding to assay plates. Approximate stability of Bio-Glo™ Reagent after reconstitution is 18% loss of luminescence after 24 hours at ambient temperature.

3. **Assay Buffer:** On the day of assay, add an appropriate amount of FBS to RPMI 1640 Medium to yield 99% RPMI 1640/1% FBS. For reference, 35ml of this assay buffer is typically sufficient for 120 wells in a 96-well assay format using the inner 60 wells. Mix well and warm to 37°C before use.

   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 different serum concentrations in the range of 0.5–10%.

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

   Note: If you are using Control Ab, Anti-PD-1 in your assay, prepare 260µl of 50µg/ml starting dilution (dilu1, 2X final concentration) by adding 6.5µl of Control Ab, Anti-PD-1 stock (2mg/ml) to 253.5µl 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 antibodies to generate two ten-point dose-response curves for each plate.

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

Figure 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

Notes:

Perform the following steps using aseptic technique in a sterile cell culture hood.

The thaw-and-use PD-L1 aAPC/CHO-K1 Cells 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.

If you are using the PD-L1 Negative Cells as a control in the assay, follow the instructions below to prepare and plate the cells.

1. On the day before performing the assay, add 2.5ml of FBS to 22.5ml of Ham's F12 Medium in a 50ml conical tube to make 25ml of cell recovery medium (90% Ham's F12/10% FBS) for thawing PD-L1 aAPC/CHO-K1 Cells.
2. Add 14.5ml of prewarmed (37°C) cell recovery medium to a 50ml conical tube.
3. Remove one vial of PD-L1 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 3–4 minutes). While thawing, gently agitate and visually inspect.
4. Gently mix the cell suspension by pipetting, then transfer the cells (0.5ml) to the 50ml conical tube containing 14.5ml cell recovery medium. Mix well by gently inverting 1–2 times.
5. 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 two 96-well, white, flat-bottom assay plates.
6. Add 100µl of cell recovery medium to each of the outside wells of the assay plates.
7. Cover the assay plates with a lid, and incubate the cells overnight (16–20 hours) in a 37°C, 5% CO_2 incubator.

4.D. Preparing and Adding 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.

Note: If you are using Control Ab, Anti-PD-1, as a control in the assay, follow the instructions below to prepare 2.5-fold serial dilutions.

1. On the day of assay, prepare an appropriate amount of assay buffer as described in Section 4.A.
2. To a sterile clear 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 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. 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 wells. Alternatively, invert the assay plate over a sink to remove the medium. Then place the inverted plate on paper towels for 5–10 seconds to drain any remaining medium.
8. Using an electronic multichannel pipette, immediately add 40µl of the appropriate antibody dilution (see Figure 8) to the preplated PD-L1 aAPC/CHO-K1 Cells according to the plate layout in Figure 7.
9. Add 80µl of assay buffer to each of the outside wells of the assay plates.
10. Cover the assay plates with a lid and keep at ambient temperature (22°–25°C) while preparing the PD-1 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.

4.E. Preparing and Plating PD-1 Effector Cells

Note: The thaw-and-use PD-1 Effector Cells 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 a time.

1. Add 5.9ml of prewarmed (37°C) assay buffer to a 15ml conical tube.
2. Remove one vial of PD-1 Effector Cells from storage at –140°C and transfer to the bench on dry ice. Warm the cells in a 37°C water bath until just thawed (about 3–4 minutes). While thawing, gently agitate and visually inspect.
3. Gently mix the cell suspension by pipetting, then transfer the cells (0.5ml) to the 15ml conical tube containing 5.9ml of assay buffer. Mix well by gently inverting 1–2 times.
4. Transfer the cell 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.
5. Cover the assay plates with a lid and incubate the cells for 6 hours in a 37°C, 5% CO_2 incubator

4.F. Adding Bio-Glo™ Reagent

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

1. Remove the assay plates from the incubator and equilibrate to ambient temperature for 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} value or fold induction.
5. Measure luminescence using a luminometer or luminescence plate reader.

4.G. Data Analysis

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

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

   Note: When calculating fold induction, if sample RLUs are the same as or up to 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 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.
	Insufficient cells per well can 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 assay response (low fold induction)	Optimize the concentration range of your test sample(s) to achieve a full dose response with complete upper and lower asymptotes. The EC_{50} value obtained in the PD-1/PD-L1 Blockade 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 6–24 hours.

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6. References

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7. Appendix

7.A. Representative Assay Results

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

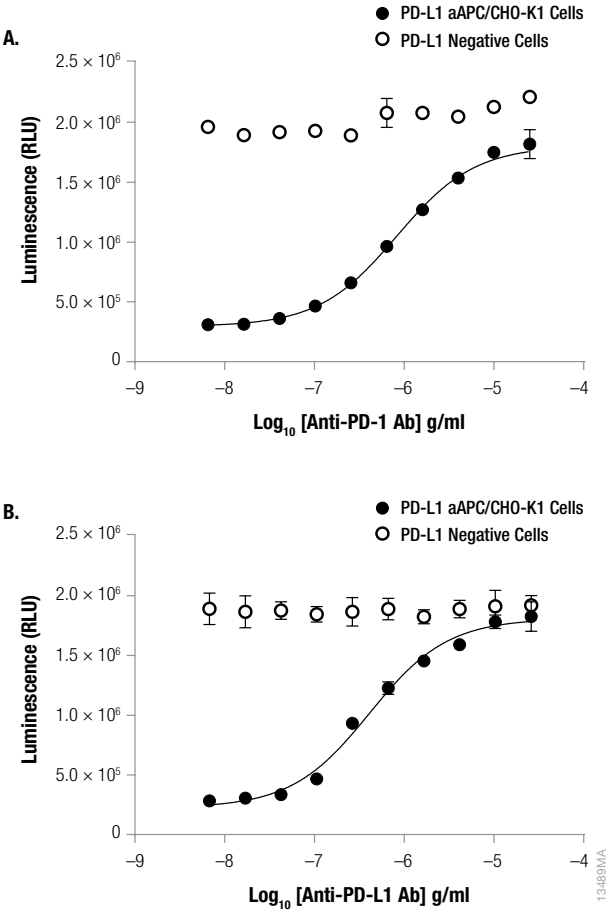


Figure 9. The PD-1/PD-L1 Blockade Bioassay measures the inhibitory activity of research-grade anti-PD-1 and anti-PD-L1 blocking antibodies. PD-L1 aAPC/CHO-K1 Cells were plated and incubated at 37°C for 16–20 hours prior to the addition of increasing concentrations of either anti-PD-1 (**Panel A**) or anti-PD-L1 (**Panel B**) antibodies and PD-1 Effector Cells. After 6 hours, Bio-Glo™ Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. The EC_{50} values were 0.83µg/ml (Anti-PD-1 Ab) and 0.40µg/ml (anti-PD-L1 Ab). Data were generated using thaw-and-use cells.

7.A. Representative Assay Results (continued)

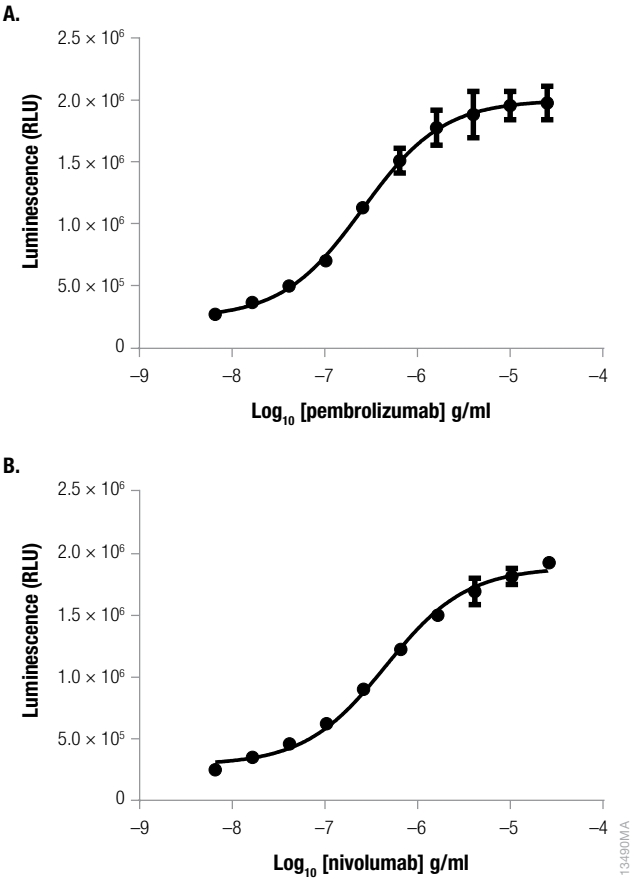


Figure 10. The PD-1/PD-L1 Blockade Bioassay measures potency of clinical-grade anti-PD-1 antibodies and the assay response to anti-PD-1 antibodies. PD-L1 aAPC/CHO-K1 Cells were plated and incubated for 16–20 hours prior to the addition of increasing concentrations of either pembrolizumab (**Panel A**) 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® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. EC_{50} values were 0.25µg/ml (pembrolizumab; **Panel A**) and 0.44µg/ml (nivolumab; **Panel B**). Data were generated using thaw-and-use cells.

8. Summary of Changes

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

1. Removed Section 7.B, Related Products.
2. Made minor text edits.
3. Removed old patent statements and licensing information.

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