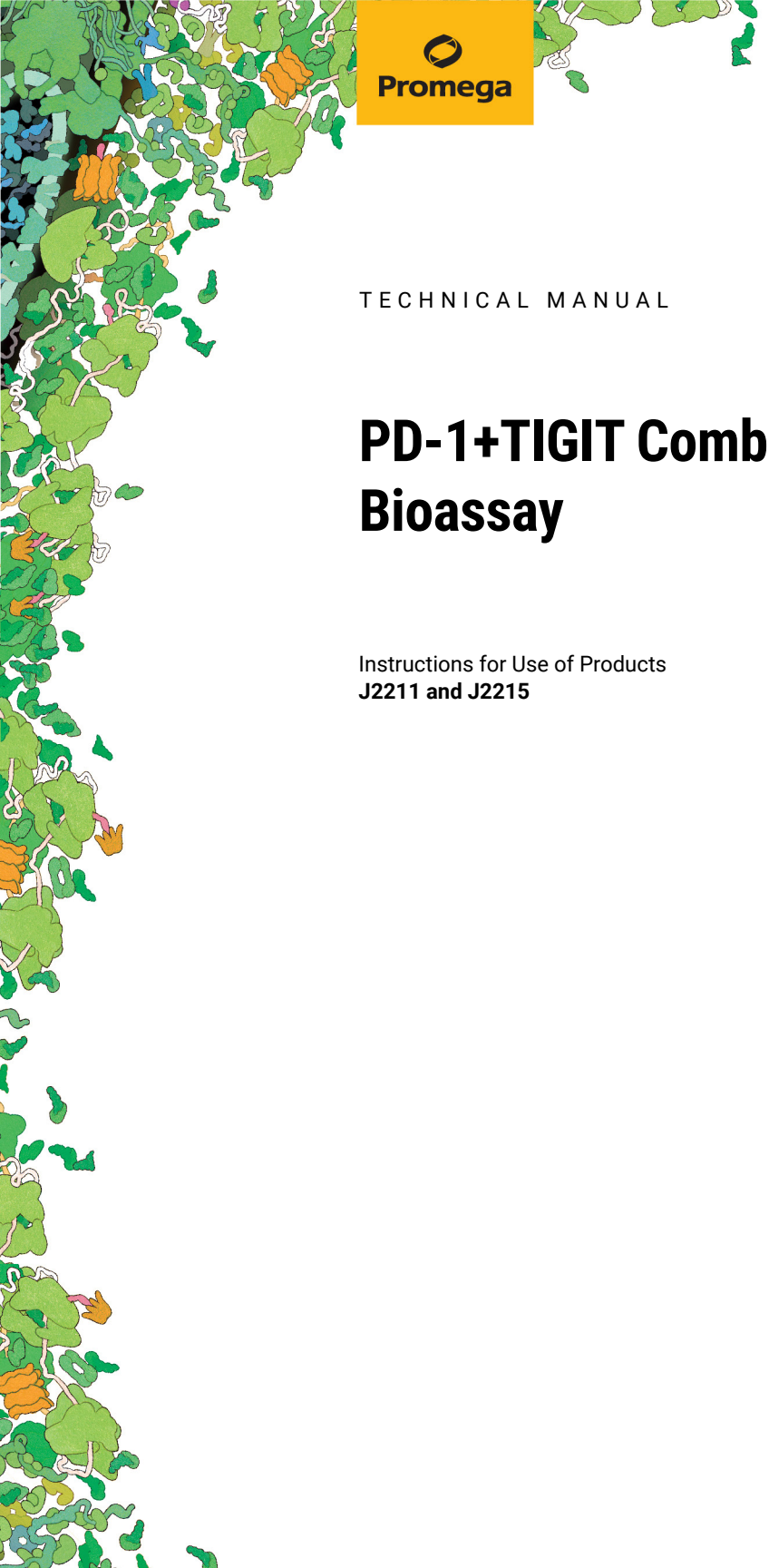

TECHNICAL MANUAL

PD-1+TIGIT Combination Bioassay

Instructions for Use of Products
J2211 and J2215

Revised 5/26
TM534

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PD-1+TIGIT Combination 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 regulated by a complex network of inhibitory and stimulatory receptors that facilitate the elimination of pathogens while maintaining tolerance to self-antigens. Inhibitory immune checkpoint receptors have been shown to perform critical roles in the maintenance of immune homeostasis but also have a significant role in cancer progression and autoimmune disease. Several immune checkpoint receptors have been identified, including Programmed Cell Death Protein 1 (PD-1), Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA-4), T cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (TIGIT) and Lymphocyte Activation Gene-3 (LAG-3). Blocking these receptors with monoclonal antibodies has proven to be an effective strategy to enhance anti-tumor immune responses and promote immune-mediated tumor rejection (1,2).

PD-1, also known as CD279, is an immune checkpoint receptor expressed on T and B lymphocytes following activation. Engagement of PD-1 by either of its ligands, PD-L1 (B7-H1) or PD-L2 (B7-DC), directly inhibits T cell receptor (TCR) signaling, leading to reduced cellular proliferation and cytokine production (3).

TIGIT, also known as WUCAM and Vstm3, is an immune checkpoint receptor expressed on lymphocytes. Highest expression levels are observed on effector $CD4^+$ and $CD8^+$ T cells, regulatory T cells and NK cells (4). TIGIT has several distinct mechanisms of action that inhibit lymphocyte activation. First, it is an inhibitory counterpart of the co-stimulatory receptor, CD226. When TIGIT is present on the surface of lymphocytes, it binds with much higher affinity than CD226 to their common ligand, CD155 (poliovirus receptor, PVR) (4). Therefore, TIGIT will outcompete CD226 for CD155 binding and thus negate CD226 co-stimulation. Second, TIGIT inhibits CD226 homodimerization in *cis*, preventing CD226 signaling (5). Third, the cytoplasmic tail of TIGIT contains an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM), which could potentially lead to inhibitory signaling. However, there is limited evidence to suggest that this is a major mechanism of TIGIT-induced inhibition in human T cells (6).

The clinical efficacy of PD-1 or PD-L1 blockade has been established, with several PD-1/PD-L1-targeted antibodies gaining FDA approval for assorted cancers. Furthermore, preclinical studies suggest that even more robust anti-tumor responses can be elicited using anti-PD-1/PD-L1 blocking antibodies (Abs) in combination with anti-TIGIT blocking Abs (5,7). In addition, clinical trials have indicated increased survival of patients subjected to combination immunotherapy (anti-PD-1 and anti-CTLA-4 Abs) compared to either agent alone (8). These studies suggest that combination immunotherapy can have a synergistic effect on T cell activation, anti-tumor responses and patient survival.

Developing therapeutics that simultaneously inhibit two immune checkpoint receptors (e.g., PD-1 and TIGIT) carries challenges for in vitro testing. Current methods used to measure the activity of drugs targeting PD-1 and TIGIT 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 a drug development setting.

The PD-1+TIGIT Combination Bioassay[a–e] (Cat.# J2211, J2215) 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 targeting PD-1 and TIGIT (9,10). The assay consists of two genetically engineered cell lines:

PD-1+TIGIT Effector Cells: Jurkat T cells engineered to express human PD-1 and human TIGIT with a luciferase reporter driven by a native promoter that can respond to both TCR activation and CD226 co-stimulation

PD-L1+CD155 aAPC/CHO-K1 Cells: CHO-K1 cells engineered to express human PD-L1, human CD155 and an engineered cell-surface protein designed to activate the T cell receptor (TCR) complex in an antigen-independent manner

The PD-1+TIGIT Effector Cells and PD-L1+CD155 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 culture and propagation.

When the two cell types are co-cultured, PD-1 binds to PD-L1 and inhibits TCR-induced activation and promoter-mediated luminescence. In addition, TIGIT inhibits CD226 activation and promoter-mediated luminescence. Addition of an anti-PD-1 Ab or an anti-TIGIT Ab blocks the interaction of PD-1 with PD-L1 or TIGIT with CD155, respectively, and results in a modest enhancement in promoter-mediated luminescence (Figures 1 and 2). Addition of antibodies that block both PD-1/PD-L1 and TIGIT/CD155 will result in much higher promoter-mediated luminescence due to synergy between these pathways (Figures 1 and 2). The bioluminescent signal is quantified using Bio-Glo™ Luciferase Assay System[b] and a standard luminometer such as the GloMax® Discover System.

In addition to the PD-1+TIGIT Combination Bioassay, we offer TIGIT Negative Cells (Cat.# J1921), which are also negative for PD-1, for use as a negative control in the PD-1+TIGIT Combination Bioassay. When co-cultured with PD-L1+CD155 aAPC/CHO-K1 Cells, the TCR/CD3 and CD226 signaling pathways in the TIGIT Negative Cells are activated, and this response is not significantly affected by anti-PD-1 or anti-TIGIT antibodies. We also offer Control Ab, Anti-PD-1 (Cat.# J1201), and Control Ab, Anti-TIGIT (Cat.# J2051), blocking antibodies for use as positive controls.

The PD-1+TIGIT Combination Bioassay combines: 1) a simple, add-mix-read two-day workflow with 2) PD-1+TIGIT Effector Cells and PD-L1+CD155 aAPC/CHO-K1 Cells provided in a frozen, thaw-and-use format, and 3) an optimized protocol, which together yield a quantitative bioassay that exhibits low variability and high accuracy. The thaw-and-use cells provided in the PD-1+TIGIT Combination 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.

1. Description (continued)

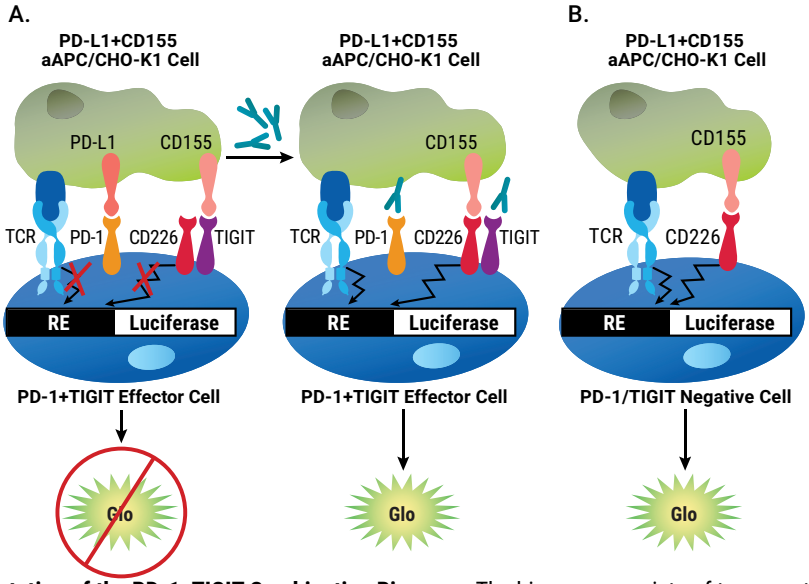

Figure 1. Representation of the PD-1+TIGIT Combination Bioassay. The bioassay consists of two genetically engineered cell lines, PD-1+TIGIT Effector Cells and PD-L1+CD155 aAPC/CHO-K1 Cells. **Panel A.** When co-cultured, PD-1 inhibits TCR pathway-activated luminescence, and TIGIT inhibits CD226 pathway-activated luminescence. The addition of anti-PD-1 Ab blocks PD-1 binding to PD-L1, resulting in full TCR pathway activation. The addition of anti-TIGIT Ab blocks the TIGIT/CD155 interaction, thereby re-establishing CD226 pathway-activated luminescence. Blocking of PD-1/PD-L1 and TIGIT/CD155 can be detected in a dose-dependent manner by addition of Bio-Glo™ Reagent and quantitation with a luminometer. **Panel B.** When cocultured with Effector Cells that do not express PD-1 or TIGIT (Cat.# J1920), TCR activation and CD226/CD155 induce luminescence.

The PD-1+TIGIT Combination Bioassay reflects the mechanism of action (MOA) of biologics designed to block the PD-1/PD-L1 and TIGIT/CD155 interactions. Specifically, TCR/CD3 and CD226-mediated luminescence is detected following the addition of anti-PD-1/PD-L1 or anti-TIGIT blocking Abs but not following the addition of anti-CTLA-4 blocking Abs (Figure 2). The bioassay is prequalified according to 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 assay can be performed in a two-day time frame. The bioassay workflow is simple, robust and compatible with both 96-well and 384-well plate formats used for antibody screening in early drug discovery (Figure 4). In addition, the bioassay can be used with up to 100% human serum (in antibody samples) with minimal impact on EC_{50} of antibody samples and fold induction (Figure 5), indicating potential for further development into a neutralizing antibody bioassay.

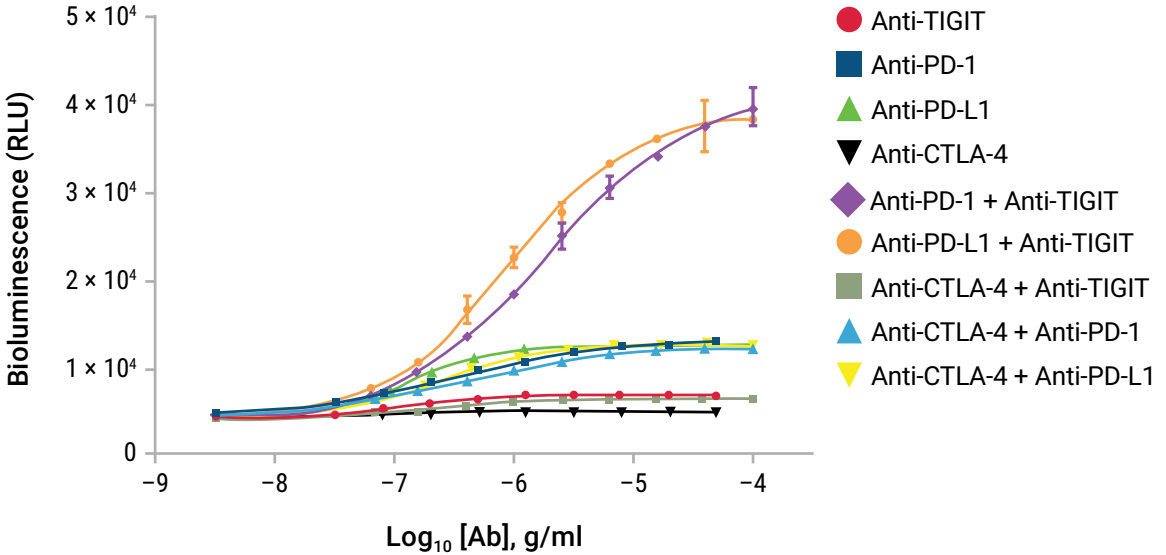

Figure 2. The PD-1+TIGIT Combination Bioassay reflects the mechanism of action (MOA) and specificity of biologics designed to block the PD-1/PD-L1 and TIGIT/CD155 interaction. PD-1+TIGIT Effector Cells were incubated with PD-L1+CD155 aAPC/CHO-K1 Cells in the presence of serial titrations of Control Ab, Anti-TIGIT (Cat.# J2051), Control Ab, Anti-PD-1 (Cat.# J1201), anti-PD-L1 Ab (research grade) or anti-CTLA-4 (ipilimumab) blocking Abs as indicated. Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using the GraphPad Prism® software. Data were generated using thaw-and-use cells.

1. Description (continued)

Table 1. The PD-1+TIGIT Combination Bioassay Shows Precision, Accuracy and Linearity.

Parameter	Results	
Accuracy	% Expected Relative Potency	% Recovery
	50	95.3
	70	105.5
	140	104.9
	200	109.9
Repeatability (% CV)	100% (Reference)	4.1
Intermediate Precision (% CV)		10.4
Linearity (r^2)		0.999
Linearity (y = mx + b)		y = 1.131x + 0.028

A 50–200% theoretical potency series of a 1:1 ratio of nivolumab (anti-PD-1 Ab) and Control Ab, Anti-TIGIT, was analyzed in triplicate in three independent experiments performed on three days by two analysts. Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were analyzed and relative potencies calculated after parallelism determination using JMP® software. Data were generated using thaw-and-use cells.

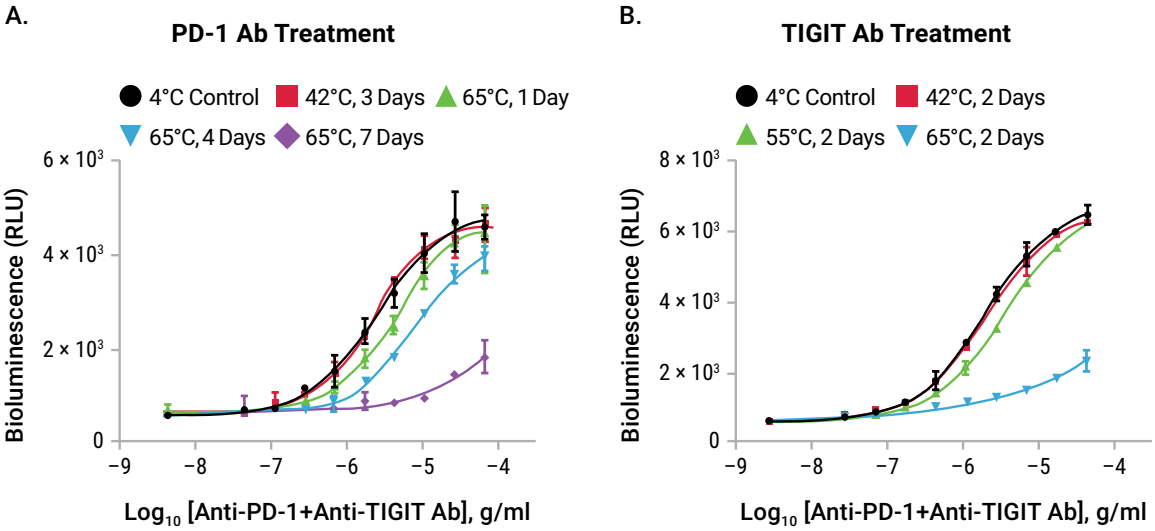

Figure 3. The PD-1+TIGIT Combination Bioassay is stability-indicating. Panel A. Samples of Control Ab, Anti-PD-1, were maintained at 4°C (control) or heat-treated at the indicated temperatures and times, mixed in a 1:1 ratio with non-heat-treated Control Ab, Anti-TIGIT, and then analyzed using the PD-1+TIGIT Combination Bioassay. **Panel B.** Samples of Control Ab, Anti-TIGIT, were maintained at 4°C (control) or heated at the indicated temperatures and times, mixed in a 1:1 ratio with non-heat-treated Control Ab, Anti-PD-1, and then analyzed using the PD-1+TIGIT Combination Bioassay. Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using the GraphPad Prism® software. Data were generated using thaw-and-use cells.

1. Description (continued)

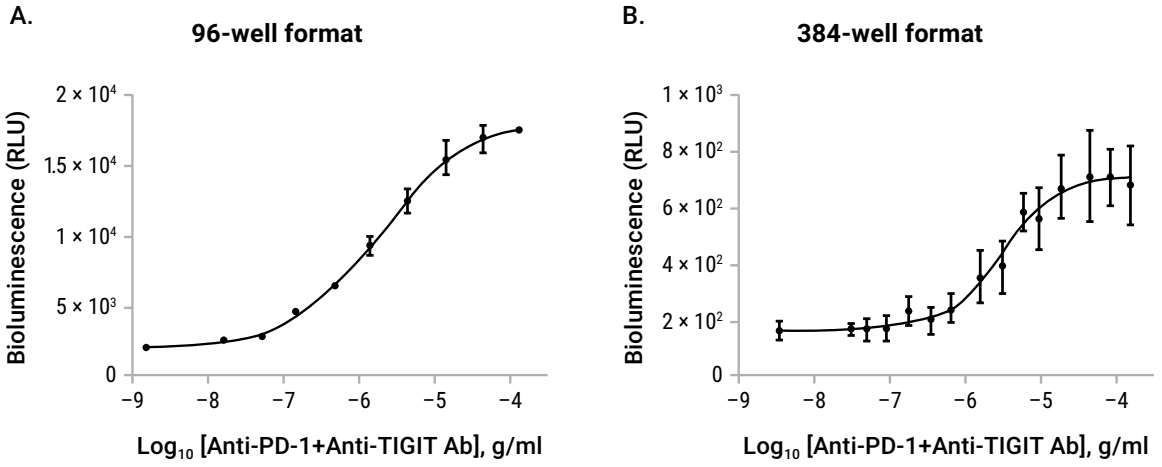

Figure 4. The assay is amenable to 384-well plate format and compatible with laboratory automation. Panel A. The PD-1+TIGIT Combination Bioassay was performed in 96-well plates as described in this technical manual using a 1:1 ratio of anti-PD-1 Ab (nivolumab) and Control Ab, Anti-TIGIT. **Panel B.** The PD-1+TIGIT Combination Bioassay was performed in 384-well format using a Multidrop™ Combi nL (Thermo Scientific) dispenser. PD-L1+CD155 aAPC/CHO-K1 Cells were plated at 6×10^3 cells/10µl/well in 384-well white assay plates and incubated overnight at 37°C, 5% CO_2. A 1:1 ratio of anti-PD-1 Ab (nivolumab) and Control Ab, Anti-TIGIT, was serially diluted and added to the plate at 5µl/well. PD-1+TIGIT Effector Cells were then added to the plate at 3×10^4 cells/5µl/well. After a 6-hour assay incubation, 20µl 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 1.6µg/ml and 1.5µg/ml, and the fold inductions were 9.7 and 5.2 for 96-well and 384-well format, respectively. Data were generated using thaw-and-use cells.

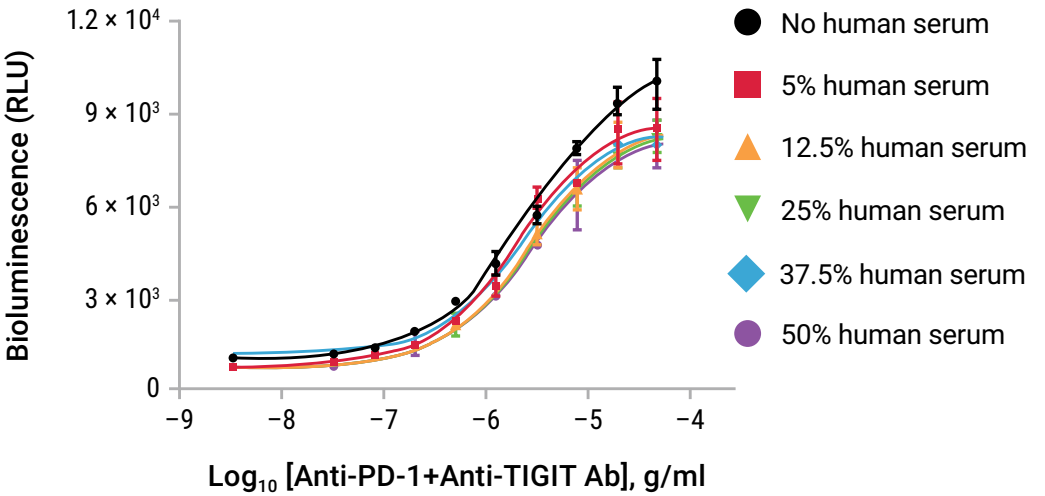

Figure 5. The PD-1+TIGIT Combination Bioassay is tolerant to human serum. A 1:1 ratio of Control Ab, Anti-PD-1, and Control Ab, Anti-TIGIT, was analyzed in the absence or presence of increasing concentrations of pooled normal human serum (0–100% in the antibody sample), resulting in final assay concentration of human serum (0–50%). Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using the GraphPad Prism® software. Data were generated using thaw-and-use cells. The PD-1+TIGIT Combination Bioassay is tolerant to serum with this human serum pool. A different human serum pool showed similar effects on the assay (data not shown).

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
PD-1+TIGIT Combination Bioassay	**1 each**	**J2211**

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+TIGIT Effector Cells (0.5ml)
- 1 vial PD-L1+CD155 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+TIGIT Combination Bioassay 5X	**5 each**	**J2215**

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+TIGIT Effector Cells (0.5ml)
- 5 vials PD-L1+CD155 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+TIGIT Combination Bioassay components are shipped separately because of differing temperature requirements. The PD-1+TIGIT Effector Cells and PD-L1+CD155 aAPC/CHO-K1 Cells are shipped on dry ice. The Bio-Glo™ Luciferase Assay Substrate and Buffer 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 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 Medium and Ham's F-12 Medium at +2°C to +10°C, protected from fluorescent light.

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 specific product from the website, such as Certificate of Analysis.

The PD-1+TIGIT Combination Bioassay is intended to be used with user-provided antibodies or other biologics designed to simultaneously block the interaction of PD-1 with PD-L1 and TIGIT with CD155. Control Ab, Anti-PD-1 (Cat.# J1201), and Control Ab, Anti-TIGIT (Cat.# J2051), as well as PD-1 and TIGIT Negative Cells (Cat.# J1921) are available separately for use in assay optimization and routine quality control. We strongly recommend including a 1:1 cocktail of Control Ab, Anti-PD-1, and Control Ab, Anti-TIGIT, as a positive control in the first few assays to gain familiarity with the assay. Data generated using these reagents is shown in Figures 2, 3 and 5 and Section 7.A, Representative Assay Results.

The PD-1+TIGIT Effector Cells and PD-L1+CD155 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 as described.

The PD-1+TIGIT Combination Bioassay produces a bioluminescent signal and requires a 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. An integration time of 0.5 seconds/well was used for all readings. Relative luminescence units will vary with the sensitivity and settings of the 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 robustness of the raw data but should not affect the measured relative potency of test samples.

3.A. Materials to be Supplied by the User

- user-defined anti-PD-1/anti-TIGIT blocking antibodies or anti-PD-1 × anti-TIGIT bispecific antibodies
- solid-white, flat-bottom 96-well assay plates (e.g., Corning® Cat.# 3917)
- sterile, clear V-bottom 96-well plate with lid (e.g., Costar® Cat.# 3896 or Linbro Cat.# 76-223-05) for preparing antibody dilutions
- pipettes (single-channel and 12-channel); for best results use both manual and electronic pipettes as needed
- sterile 15ml and 50ml conical tubes
- sterile reagent reservoirs (e.g., Corning® Cat.# 4870)
- 37°C, 5% CO_2 incubator
- 37°C water bath
- plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System or equivalent)

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4. Assay Protocol

This assay protocol illustrates the use of the PD-1+TIGIT Combination Bioassay to test two antibody samples against a reference sample in a single assay run. Each test and reference antibody is run in triplicate, in a 10-point dilution series, in a single 96-well assay plate using the inner 60 wells. Other experimental and plate layouts are possible but may require further optimization.

Note: When preparing test and reference antibodies, choose an appropriate starting concentration and dilution scheme to achieve a full dose-response curve with proper upper and lower asymptotes and sufficient points on the slope. For reference, we use a 1:1 ratio of Control Ab, Anti-PD-1, and Control Ab, Anti-TIGIT, at a starting concentration of 50µg/ml (1X) of each antibody and a 3-fold dilution series.

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

1. **Cell Recovery Medium:** On the day before the assay, prepare 14.5ml of cell recovery 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 to yield 90% Ham's F-12/10% FBS. Mix well and warm to 37°C before use. For reference, 14.5ml of cell recovery medium is sufficient to thaw 1 vial of PD-L1+CD155 aAPC/CHO-K1 Cells. If multiple vials of PD-L1+CD155 aAPC/CHO Cells will be used, then scale the amount of cell recovery medium appropriately. Warm the remaining Ham's F-12 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 in a 50ml conical tube.. Add 2ml of FBS to 18ml of RPMI 1640 Medium to yield 90% RPMI 1640/10% FBS. Mix well and warm to 37°C prior to use.

   Note: The recommended assay buffer contains 10% FBS. This concentration of FBS works well for the anti-PD-1 Abs and Control Ab, Anti-TIGIT, that 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%.

3. **Test and Reference Samples:** Using assay buffer as the diluent, prepare starting dilutions (dilu1, 2X final concentration) of two test antibodies (240µl each) and one reference antibody (480µl) 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, and Control Ab, Anti-TIGIT, as a reference in your assay, prepare 480µl of 100µg/ml starting dilution of each antibody for the starting dilution (dilu1, 2X final concentration) by adding 24µl of Control Ab, Anti-PD-1, stock (2mg/ml) and 48µl of Control Ab, Anti-TIGIT, stock (1mg/ml) to 408µl of assay buffer. Store the antibody starting dilution on ice until ready to use in the assay.

4. **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 room 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 at ambient temperature, Bio-Glo™ Reagent will lose approximately18% activity after 24 hours.

Note: The PD-1+TIGIT Combination Bioassay is compatible only with Bio-Glo™ Luciferase Assay Reagent. **Do not** use Bio-Glo-NL™ Luciferase Assay Reagent with the PD-1+TIGIT Combination Bioassay.

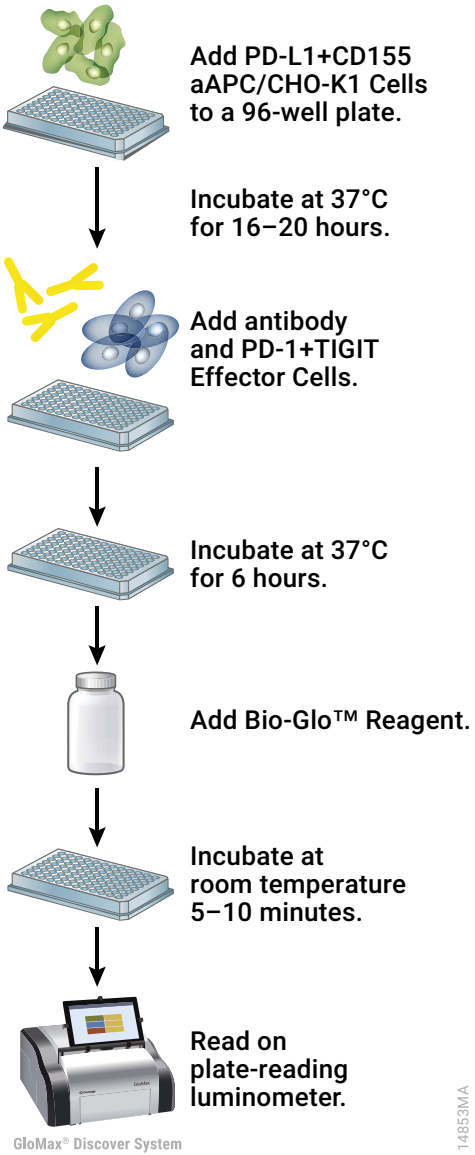

Figure 6. Schematic protocol for the PD-1+TIGIT Combination Bioassay.

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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 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 RPMI 1640 Medium (denoted by "B") alone.

4.C. Preparing and Plating PD-L1+CD155 aAPC/CHO-K1 Cells

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

The thaw-and-use PD-L1+CD155 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 a maximum of two vials of thaw-and-use cells at one time.

1. On the day before performing the assay, prepare new cell recovery medium (90% Ham's F-12/10% FBS) as described in Section 4.A.
2. Remove one vial of PD-L1+CD155 aAPC/CHO-K1 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 2–3 minutes). While thawing, gently agitate and visually inspect.
3. Gently mix the cell suspension by pipetting, and then transfer the cells (0.5ml) to the 50ml conical tube containing 14.5ml of prewarmed (37°C) cell recovery medium. Mix well by gently inverting.
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 a 96-well white flat-bottom assay plate. The final cell number in each well should be 4×10^4 cells/well.
5. Add 100µl of prewarmed (37°C) Ham's F-12 Medium to each of the outside wells of the assay plates.
6. 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 Antibody Serial Dilutions

The instructions described here are for preparations of a single stock of three-fold serial dilutions from a single antibody for analysis in triplicate (160µl of each dilution provides sufficient volume for analysis in triplicate). Alternatively, you can prepare three independent stocks of serial dilutions to generate triplicate samples. To prepare three-fold serial dilutions, you will need 480µl of reference antibody at 2X the highest antibody concentration in your dose-response curve. You will need 240µl 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, and Control Ab, Anti-TIGIT, as the reference in the assay, follow the instructions below to prepare 3-fold serial dilutions.

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 240µl of reference antibody starting dilution (dilu1, 2X final concentration) to wells A11 and B11 (see Figure 8).
3. Add 240µl of test antibodies 1 and 2 starting dilution (dilu1, 2X final concentration) to wells E11 and G11, respectively (see Figure 8).
4. Add 160µl of assay buffer to other wells in these four rows, from column 10 to column 2.
5. Transfer 80µl of the antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.

4.D. Preparing Antibody Serial Dilutions (continued)

6. Repeat equivalent 3-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 160µl of assay buffer without antibody as a negative control.

7. Remove the 96-well assay plates containing PD-L1+CD155 aAPC/CHO-K1 Cells from the incubator. Using a manual multichannel pipette, remove 95µl of medium from each of the 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.

8. Using an electronic multichannel pipette, add 40µl of the appropriate antibody dilution (see Figure 8) to the pre-plated PD-L1+CD155 aAPC/CHO-K1 Cells according to the plate layout in Figure 7.

9. Add 80µl of RPMI 1640 Medium 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+TIGIT 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+TIGIT Effector Cells

Note: The thaw-and-use PD-1+TIGIT 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 a maximum of two vials of thaw-and-use cells at one time.

1. Add 6ml of prewarmed (37°C) assay buffer to a 15ml conical tube.
2. Remove one vial of PD-1+TIGIT Effector 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 2–3 minutes). While thawing, gently agitate and visually inspect.
3. Gently mix the cell suspension by pipetting, and then transfer the cells (0.5ml) to the 15ml conical tube containing 6ml assay buffer. Mix well by gently inverting the tube.
4. Transfer the cell suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 40µl of the cell suspension to the inner 60 wells of the assay plates.
5. Cover the assay plates with a lid and incubate 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 10–15 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, C1 and D1 of each assay plate to measure the background signal.
4. Incubate at ambient temperature for 5–15 minutes.

   Note: Varying the incubation time will affect the raw relative light unit (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.G. Data Analysis

1. Determine plate background by calculating the average RLU from wells B1, C1 and D1.
2. Calculate fold induction:

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

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 or low cell viability 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+TIGIT Combination Bioassay may vary from the EC_{50} value obtained using other methods such as primary T cell-based assays, as well as the PD-1 Assay and the TIGIT/CD155 Assay.
	If untreated control RLU is less than 100-fold above plate reader background RLU, subtract plate background RLU from all samples before calculating fold induction.

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7. Appendix

7.A. Representative Assay Results

The following data were generated with the PD-1+TIGIT Combination Bioassay using Control Ab, Anti-PD-1, and Control Ab, Anti-TIGIT (Figure 9).

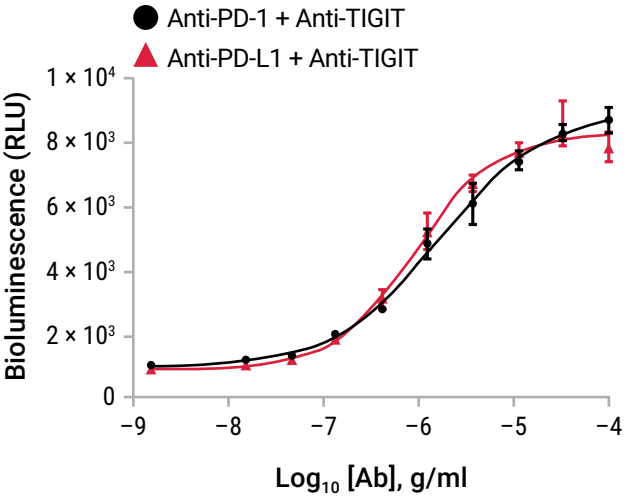

Figure 9. The PD-1+TIGIT Combination Bioassay measures the activity of Control Ab, Anti-PD-1, and Control Ab, Anti-TIGIT. PD-L1+CD155 aAPC/CHO-K1 Cells were plated overnight. The following day, a titration of a 1:1 ratio of either Control Ab, Anti-PD-1, and Control Ab, Anti-TIGIT, or a research-grade anti-PD-L1 Ab and Control Ab, Anti-TIGIT, was added, followed by PD-1+TIGIT Effector Cells. After 6 hours, Bio-Glo™ Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a 4-parameter logistic curve using GraphPad Prism® software. The fold inductions were 9.1 for both Anti-PD-1+Anti-TIGIT and Anti-PD-L1+Anti-TIGIT. The EC_{50} values were 1.6µg/ml for Anti-PD-1+Anti-TIGIT and 0.93µg/ml for Anti-PD-L1+Anti-TIGIT.

8. Summary of Changes

The following changes were made to the 5/26 revision of this document:

1. Removed Section 7.B, Related Products.
2. Made minor text and formatting updates.

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[e]Patent Pending.

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