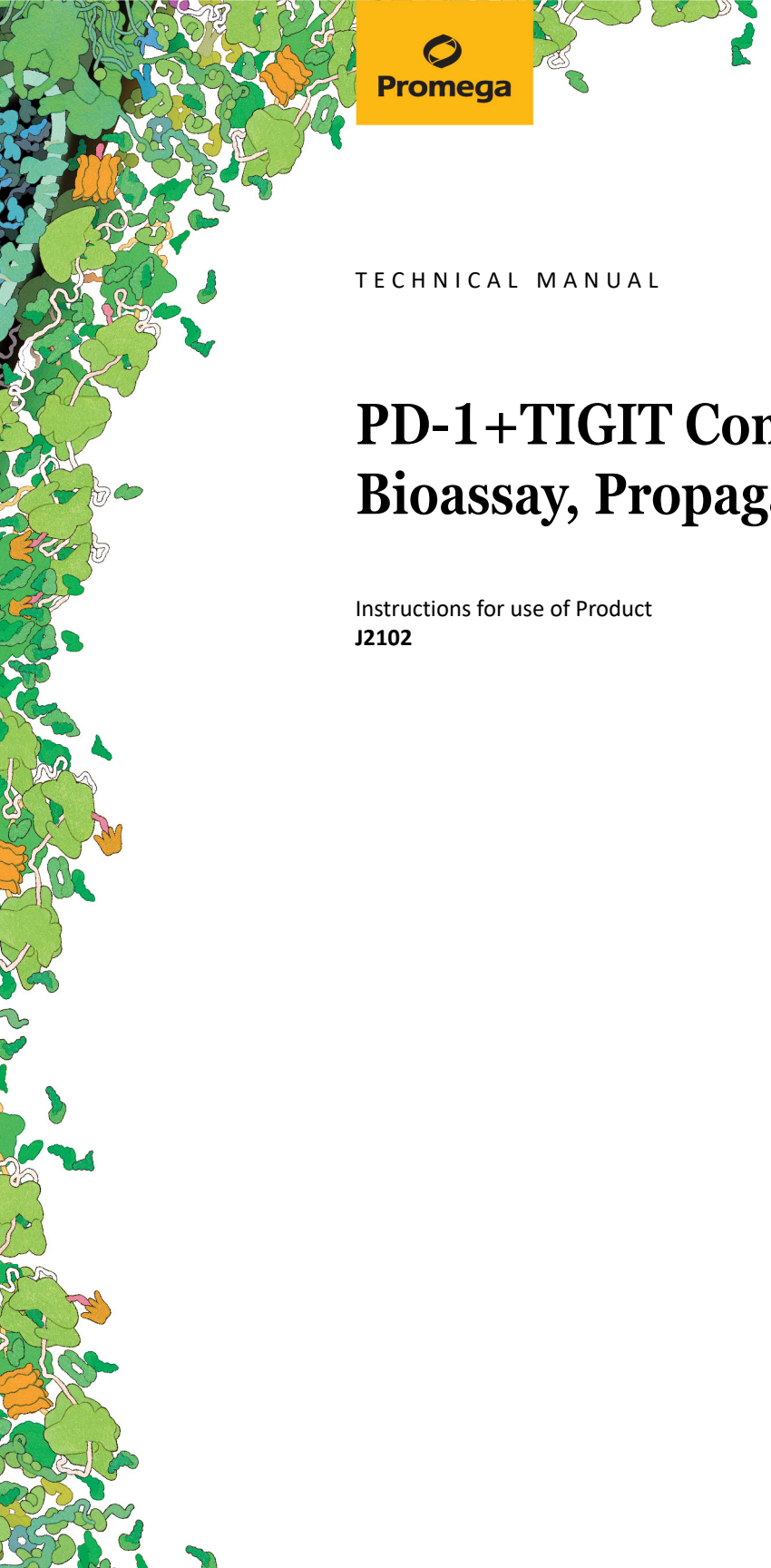

TECHNICAL MANUAL

PD-1+TIGIT Combination Bioassay, Propagation Model

Instructions for use of Product
J2102

Revised 2/22
TM532

Promega

PD-1+TIGIT Combination Bioassay, Propagation Model

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

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1. Description

The human immune system is regulated by a complex network of inhibitory and stimulatory receptors that facilitate the elimination of pathogens while maintaining tolerance to self-antigens. 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, Propagation Model[a–e] (Cat.# J2102), 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 and human CD155 with 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 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, 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 the Bio-Glo™ Luciferase Assay System and a standard luminometer such as the GloMax® Discover System (see Section 7.C, Related Products).

1. Description (continued)

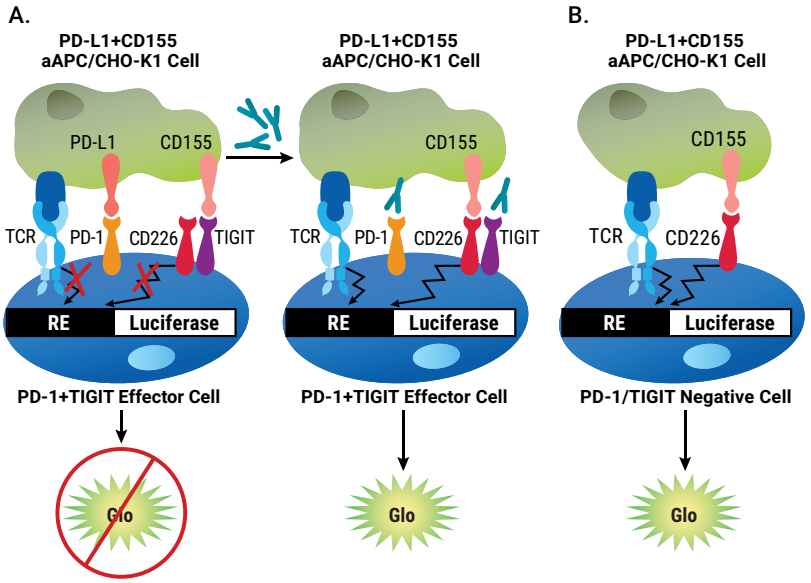

Figure 1. Representation of the PD-1+TIGIT Combination Bioassay. The bioassay consists of two genetically engineered cell lines, PD-1+TIGIT Effector Cells and PD-L1+CD155 aAPC/CHO-K1 Cells. **Panel A.** When co-cultured, PD-1 inhibits TCR pathway-activated luminescence, and TIGIT inhibits CD226 pathway-activated 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 co-cultured with Effector Cells that do not express PD-1 or TIGIT (Cat.# J1921), TCR activation and CD226/CD155 induce luminescence.

The PD-1+TIGIT Combination Bioassay, Propagation Model, reflects the mechanism of action (MOA) of biologics designed to block the PD-1/PD-L1 and TIGIT/CD155 interactions. Specifically, 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 Homogenization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines and shows the precision, accuracy and linearity required for routine use in potency and stability studies (Table 1 and Figure 3). The 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.

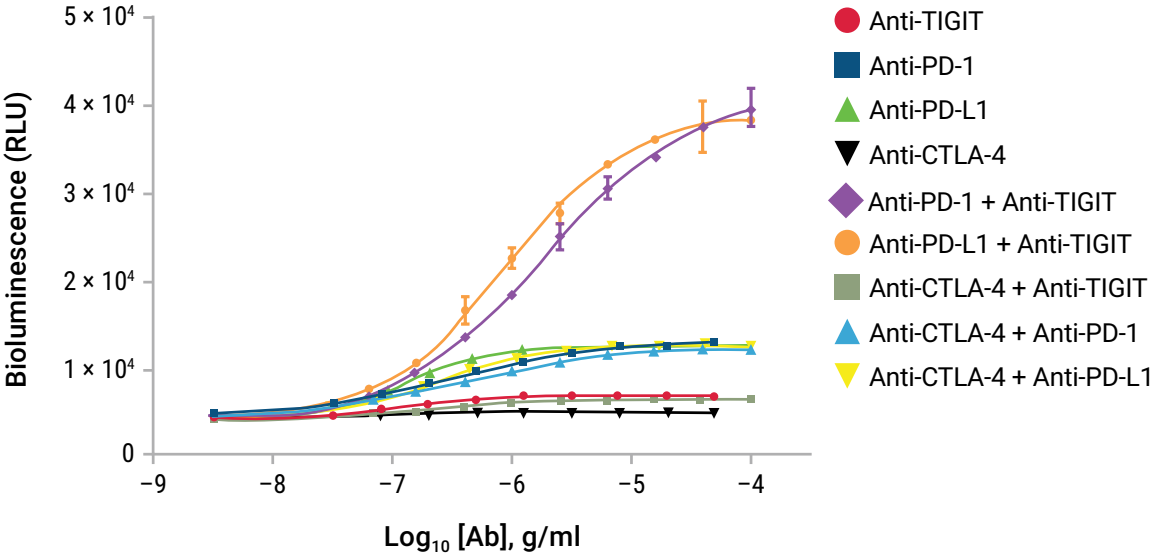

Figure 2. The PD-1+TIGIT Combination Bioassay reflects the mechanism of action (MOA) and specificity of biologics designed to block the PD-1/PD-L1 and TIGIT/CD155 interaction. PD-1+TIGIT Effector Cells were incubated with PD-L1+CD155 aAPC/CHO-K1 Cells in the presence of serial titrations of Control Ab, 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 Detection System. Data were fitted to a 4-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 Detection System. Data were analyzed and relative potencies calculated after parallelism determination using JMP® software. Data were generated using thaw-and-use cells.		

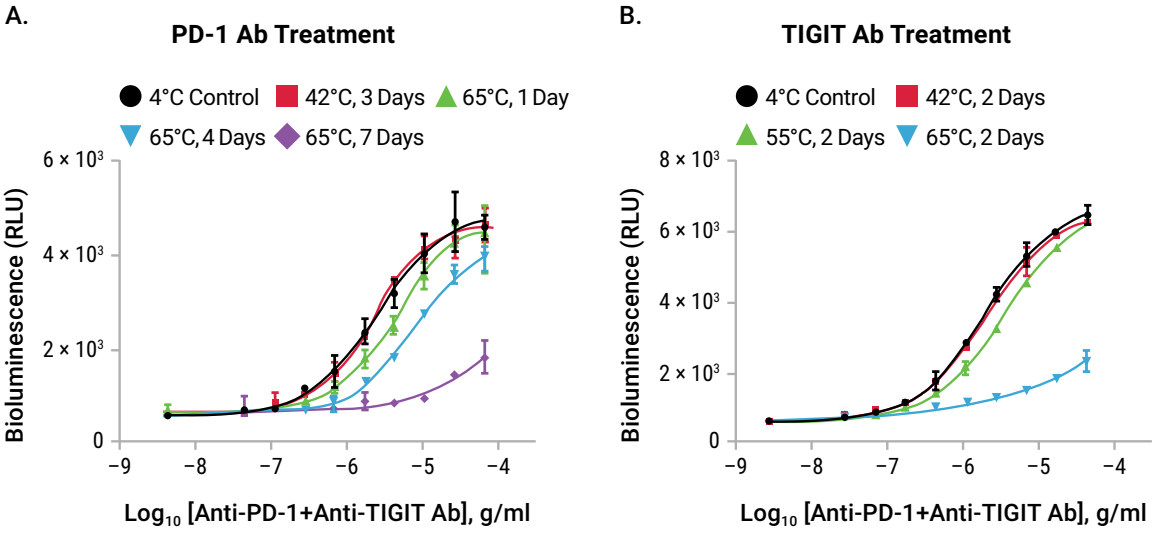

Figure 3. The PD-1+TIGIT Combination Bioassay is stability-indicating. Panel A. Samples of Control Ab, Anti-PD-1, were maintained at 4°C (control) or heat-treated at the indicated temperatures and times, mixed in a 1:1 ratio with non-heat-treated Control Ab, Anti-TIGIT, then analyzed using the PD-1+TIGIT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

1. Description (continued)

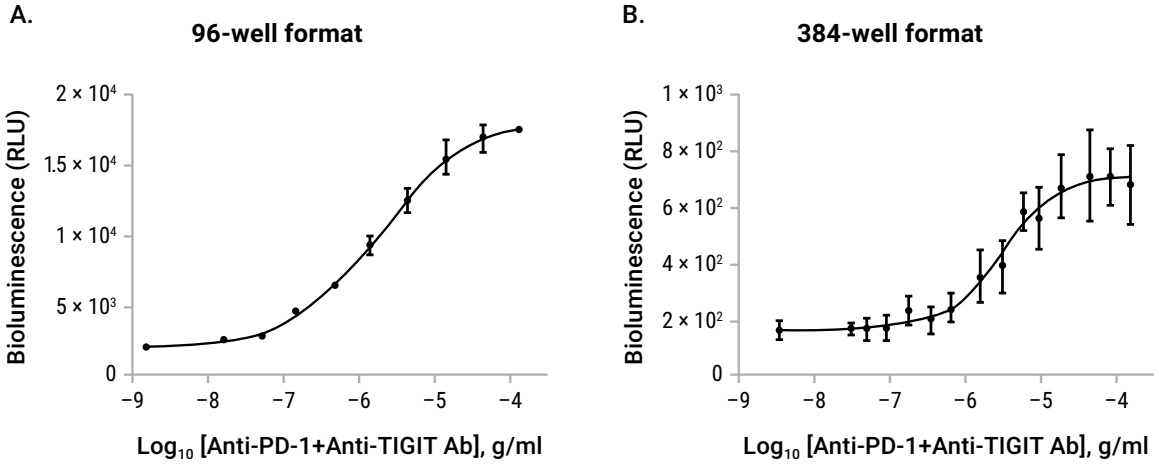

Figure 4. The assay is amenable to 384-well plate format and compatible with laboratory automation. **Panel A.** The PD-1+TIGIT Combination Bioassay was performed in 96-well plates as described in this technical manual using a 1:1 ratio of anti-PD-1 Ab (nivolumab) and Control Ab, Anti-TIGIT. **Panel 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 Detection System. Data were fitted to a 4-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.

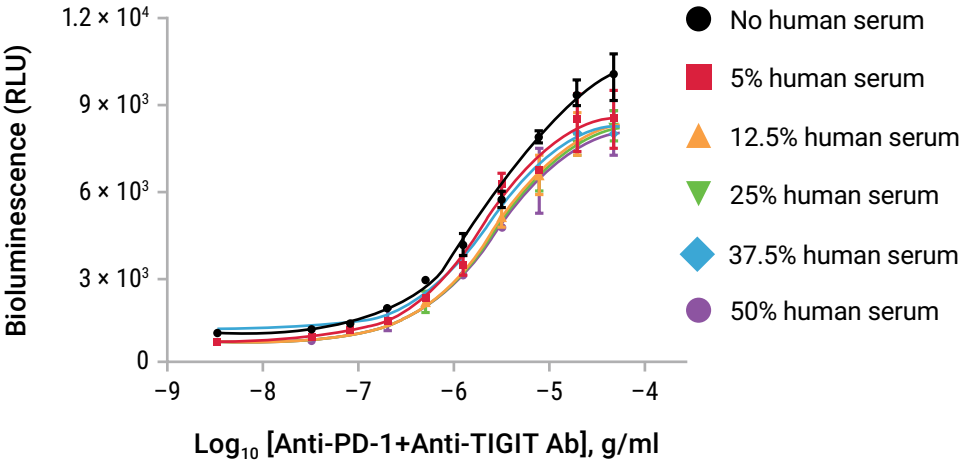

Figure 5. The PD-1+TIGIT Combination Bioassay is tolerant to human serum. A 1:1 ratio of Control Ab, Anti-PD-1, and Control Ab, Anti-TIGIT, was analyzed in the absence or presence of increasing concentrations of pooled normal human serum (0–100% in the antibody sample), resulting in final assay concentration of human serum (0–50%). Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover Detection System. Data were fitted to a 4-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). Data were generated using thaw-and-use cells.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
PD-1+TIGIT Combination Bioassay, Propagation Model	**1 each**	**J2102**

Not for Medical Diagnostic Use.

Includes:

- 2 vials PD-1+TIGIT Effector Cells (CPM), 2.3 × 10^7 cells/ml (1.0ml per vial)
- 2 vials PD-L1+CD155 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 because this will negatively impact cell viability and cell performance.

3. Before You Begin

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

Remove the product label from the box containing vials with cells or note the catalog number and lot number from the label. This information can be used to download documents for the specified product from the website such as Certificate of Analysis.

The PD-1+TIGIT Combination Bioassay, Propagation Model, 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), Control Ab, Anti-TIGIT (Cat.# J2051), and TIGIT Negative Cells (Cat.# J1621), which are also negative for PD-1, 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.

Cell thawing, propagation and banking should be performed exactly as described in Section 3. Cell seeding and propagation densities have been optimized to ensure stable cell growth, which is reflected in a steady cell doubling rate, to achieve optimal, consistent performance. **An accurate, reliable and reproducible cell counting method is required for routine cell culturing and optimal bioassay performance.** The recommended cell plating densities, induction time and assay buffer components described in Section 4 have been established. However, you may need to adjust the parameters provided here and optimize assay conditions for your own antibody or biologic.

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 (see Section 7.C, Related Products). An integration time of 0.5 seconds/well was used for all readings.

3.A. Materials to be Supplied by the User

(Composition of buffers and solutions is provided in Section 7.B).

Reagents

- user-defined anti-PD-1/anti-TIGIT blocking Abs or other biologics
- RPMI 1640 Medium with L-glutamine and HEPES (e.g., Corning Cat.# 10-041-CV or Gibco Cat.# 22400)
- Ham's F-12 Medium with L-glutamine (e.g., Gibco Cat.# 11765)
- fetal bovine serum (FBS; e.g., HyClone Cat.# SH30070.03 or Gibco Cat.# 16000044)
- hygromycin B (e.g., Gibco Cat.# 10687010)
- antibiotic G418 Sulfate Solution (e.g., Gibco Cat.# 10131035)
- sodium pyruvate (e.g., Gibco Cat.# 11360070)
- MEM nonessential amino acids (e.g., Gibco Cat.# 11140050)
- DMSO (e.g., Sigma Cat.# D2650)
- DPBS (e.g., Gibco Cat.# 14190)
- Accutase® solution (e.g., Sigma Cat.# A6964)
- 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 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)

3.B. Preparing PD-1+TIGIT Effector Cells

Cell Thawing and Initial Cell Culture

1. Prepare 40ml of initial cell culture medium by adding 4ml of FBS to 36ml of RPMI 1640 Medium prewarmed to 37°C. This initial cell culture medium will be used for culturing the cells immediately after thawing.
2. Transfer 9ml of prewarmed initial cell culture medium to a 50ml conical tube.
3. Remove one vial of PD-1+TIGIT 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 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 24 hours before passaging the cells.

Cell Maintenance and Propagation

Note: PD-1+TIGIT Effector Cells are different from TIGIT Effector Cells (Available with TIGIT/CD155 Blockade Bioassay, Propagation Model, Cat.# J2092) and PD-1 Effector Cells (Available with PD-1/PD-L1 Blockade Bioassay, Propagation Model, Cat.# J1252). Propagate the cells strictly according to the guidelines in this technical manual.

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 >90% and the average cell doubling rate is 30–34 hours. Passage number should be recorded for each passage. In our experience, cells maintain their functionality for up to 25 passages, or 41 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 5×10^5 cells/ml if passaging every two days (e.g., Monday-Wednesday or Wednesday-Friday) or 3×10^5 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°, 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 homogeneous 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 5×10^6–5×10^7 cells/ml.
16. Transfer the cell suspension to 50ml sterile conical tubes or larger sized centrifuge tubes and centrifuge at 130 × *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 5×10^6–5×10^7 cells/ml. Combine the cell suspension in a single tube and dispense into 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 into –140°C or below for long-term storage.

3.C. Preparing PD-L1+CD155 aAPC/CHO-K1 Cells

Cell Thawing and Initial Cell Culture

1. Prepare 50ml of initial cell culture medium by adding 5ml of FBS to 45ml of Ham's F-12 Medium prewarmed to 37°C. This 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-L1+CD155 aAPC/CHO-K1 Cells from storage at –140°C and thaw in a 37°C water bath with gentle agitation (no inversion) until just thawed (typically 2–3 minutes).
4. Transfer all of the cells (approximately 1ml) to the 50ml tube containing 9ml of prewarmed initial cell culture medium.
5. Centrifuge at 230 × *g* for 10 minutes.
6. Carefully aspirate the medium, and resuspend the cell pellet in 40ml of prewarmed initial cell culture medium.
7. Transfer the cell suspension to a T150 tissue culture flask, and place the flask horizontally in a 37°C, 5% CO_2 incubator.
8. Incubate for approximately 24 hours before passaging the cells.

3.C Preparing PD-L1+CD155 aAPC/CHO-K1 Cells (continued)

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. Maintain the cell density in the range of 0.7×10^4–2.5×10^5 cells/cm^2 to ensure optimal performance. The cell growth rate will stabilize by 5–7 days post-thaw, at which time cell viability is typically >95% and the average cell doubling rate is 18–22 hours when seeded at the densities listed in the table below. Do not allow cells to become 100% confluent before passaging because this may affect performance in subsequent passages. Passage number should be recorded for each passage. In our experience, cells maintain their functionality for up to 25 passages, or 64 cell doublings if passaging is performed on a Monday-Wednesday-Friday schedule. We recommend Accutase® solution for routine cell culturing and cell preparation for the assay.

Cell Passage Schedule	Cell Seeding Density
Every 2 days	4×10^4 cells/cm^2
Every 3 days	1.8×10^4 cells/cm^2
Every 4 days	0.7×10^4 cells/cm^2

9. On the day of cell passage, aspirate the cell culture medium and wash the cells with DPBS.
10. Add 2ml of Accutase® solution to each T75 flask and place in a 37°C, 5% CO_2 incubator for 5–7 minutes or until the cells round up and detach from the bottom of the flask.
11. Add 8ml of cell culture medium to each flask. Transfer the cell suspension to a sterile 50ml (or larger) conical tube.
12. Count the cells by Trypan blue staining. Calculate the cell numbers needed for the next experiment based on the 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., 20ml volume per T75 flask or 40ml 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 achieve the desired cell seeding density per area.
15. Place the flasks in the 5% CO_2 incubator. Incubate the cells for 48–96 hours before passaging them.

Cell Freezing and Banking

16. On the day of cell freezing, make new cell freezing medium and keep on ice.
17. Aspirate the cell culture medium, and wash the cells with DPBS.
18. Add 2ml of Accutase® solution to each T75 flask and place in a 37°C, 5% CO_2 incubator for 5–7 minutes or until the cells round up and detach from the bottom of the flask.
19. Add 8ml of cell culture medium to each flask. Transfer the cell suspension to a 50ml (or larger) conical centrifuge tube.
20. Count the cells by Trypan blue staining.
21. Centrifuge at 230 × *g* for 10 minutes.
22. Gently resuspend the cell pellet in ice-cold freezing medium to a final cell density of 5×10^6–2×10^7 viable cells/ml. Combine the cell suspensions into a single tube and dispense into 1ml cryovials.
23. 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.

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 references, 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. **PD-L1+CD155 aAPC/CHO-K1 Cell Recovery Medium:** On the day before the assay, prepare 40ml 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 4ml of FBS to 36ml of Ham's F-12 Medium. Mix well and warm to 37°C before use.

2. **Assay Buffer:** On the day of the assay, prepare an appropriate amount of assay buffer (90% RPMI 1640/10% FBS). Thaw the FBS overnight at 4°C or in a 37°C water bath on the day of use. Mix well and warm to 37°C prior to use. For reference, 30ml of assay buffer is typically sufficient for 120 wells in a 96-well assay format using the inner 60 wells.

   Note: The recommended assay buffer contains 10% FBS. This concentration of FBS works well for the 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. **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.

   If you are using a large (100ml) size of Bio-Glo™ Luciferase Assay System, dispense the 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.

4. **Test and Reference Samples:** Using assay buffer as the diluent, prepare starting dilutions (dilu1, 2X final concentration) of two test antibodies (225µl each) and one reference antibody (450µl) 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 450µl of 100µg/ml starting dilution of each antibody (dilu1, 2X final concentration) by adding 22.5µl of Control Ab, Anti-PD-1, stock (2mg/ml) and 45µl of Control Ab, Anti-TIGIT, stock (1mg/ml) to 382.5µl of assay buffer. Store the antibody starting dilution on ice until ready to use in the assay.

   Tip: To streamline assay setup, prepare antibody serial dilutions before harvesting and plating cells.

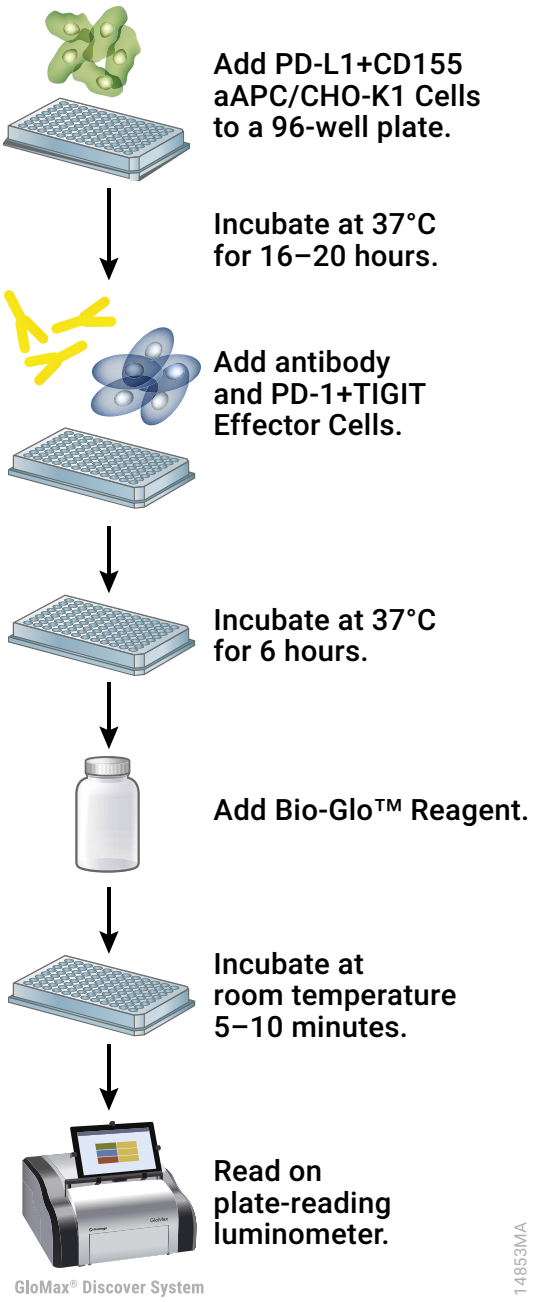

Figure 6. Schematic protocol for the PD-1+TIGIT Combination Bioassay

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 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+CD155 aAPC/CHO-K1 Cells

While maintaining the PD-L1+CD155 aAPC/CHO-K1 Cells, it is important to follow the recommended cell seeding density. Changes in cell culture volume or seeding density will affect growth rate and assay performance. Do not allow the cells to grow to 100% confluence. Only use the cells in the assay after the cell doubling rate has stabilized during propagation.

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

1. We recommend passaging the PD-L1+CD155 aAPC/CHO-K1 Cells two days before plating for the assay as described in Section 3.C to ensure optimal and consistent assay performance.
2. On the day before performing the assay, prepare new PD-L1+CD155 aAPC/CHO-K1 Cell Recovery Medium (90% Ham's F-12/10% FBS) for the PD-L1+CD155 aAPC/CHO-K1 Cells.
3. Aspirate the cell culture medium from the PD-L1+CD155 aAPC/CHO-K1 Cells and wash with DPBS.

4. Add 2ml of Accutase® solution to each T75 flask and place in a 37°C, 5% CO_2 incubator for 5–7 minutes or until the cells round up and detach from the bottom of the flask.
5. Add 8ml of PD-L1+CD155 aAPC/CHO-K1 Cell Recovery Medium to the flask. Transfer the cell suspension to a 50ml (or larger) conical centrifuge tube.
6. Gently mix and count the PD-L1+CD155 aAPC/CHO-K1 Cells by Trypan blue staining.
7. Centrifuge at 230 × *g* for 10 minutes.
8. Gently resuspend the cell pellet in cell recovery medium to achieve a concentration of 4×10^5 viable cells/ml.
9. Transfer the suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 100µl of the cell suspension to each of the inner 60 wells of a 96-well white flat-bottom assay plate. The final cell number in each well should be 4×10^4 cells/well.
10. Add 100µl of cell recovery medium to each of the outside wells of the assay plates.
11. Place lids on the assay plates and incubate in a 37°C, 5% CO_2 incubator overnight (18–22 hours).

4.D. Preparing Antibody Serial Dilutions

The instructions described here are for preparations of a single stock of 3-fold serial dilutions from a single antibody for analysis in triplicate (225µl 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 3-fold serial dilutions, you will need 450µl of reference antibody at 2X the highest antibody concentration in your dose-response curve. You will need 225µl 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 225µl of reference antibody starting dilution (dilu1, 2X final concentration) to wells A11 and B11 (see Figure 8).
3. Add 225µl of test antibodies 1 and 2 starting diluton (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 75µl of the antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
6. Repeat equivalent 3-fold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.
7. Cover the assay plate with a lid and keep at ambient temperature (22–25°C) while preparing the PD-1+TIGIT Effector Cells

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

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 PD-1+TIGIT Effector Cells

While maintaining the PD-1+TIGIT Effector Cells, it is important to follow the recommended cell seeding density. Changes in cell culture volume or seeding density will affect cell growth rate and assay performance. Only use the cells in the assay after the cell doubling rate has stabilized during propagation.

1. Passage the cells two days before performing the assay as described in Section 3.B. To ensure optimal and consistent assay performance, maintain the cell density, upon harvest, in the range of 1.2–1.8 × 10^6 cells/ml and cell viability at greater than 90%.
2. Count the PD-1+TIGIT Effector Cells by Trypan blue staining, and calculate the cell density and viability.
3. Transfer an appropriate amount of PD-1+TIGIT Effector Cells from the culture vessel to a 50ml conical tube or larger-sized centrifuge tube.
4. Pellet the cells at 130 × *g* for 10 minutes at ambient temperature and resuspend in assay buffer at 70% of the full volume needed to generate the targeted final cell density of 2.5 × 10^6 cells/ml.
5. Count the cells again, and adjust the volume of assay buffer to achieve a final cell density of 2.5 × 10^6 cells/ml. You will need at least 6ml of PD-1+TIGIT Effector Cells to fill 120 assay wells, or the inner 60 wells of two assay plates.

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

1. 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.
2. Using an electronic multichannel pipette, immediately add 40µl of the appropriate antibody titration to the pre-plated PD-L1+CD155 aAPC/CHO-K1 Cells (from Step 1) according to the plate layout in Figure 7.
3. Add 80µl of assay buffer to the outside wells of the 96-well assay plates.
4. Transfer the PD-1+TIGIT Effector Cells prepared in Section 4.E to a sterile reagent reservoir. Using a multichannel pipette, dispense 40µl (1×10^5 cells) of PD-1+TIGIT Effector Cells into the wells containing PD-L1+CD155 aAPC/CHO-K1 Cells and antibody.
5. Cover the assay plates with lids 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 (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.H. Data Analysis

1. Determine plate background by calculating the average RLU from wells B1, C1 and D1.
2. Calculate fold induction = RLU (induced–background)/RLU (no antibody control–background)
3. Graph data as RLU versus Log_{10} [antibody] and fold induction versus Log_{10} [antibody]. Fit curves and determine the EC_{50} value of antibody response using appropriate curve fitting software (such as GraphPad Prism® software).

5. Troubleshooting

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

Symptoms	Possible Causes and Comments
Low luminescence measurements (RLU readout)	Choose an instrument designed for plate-reading luminescence detection. Instruments designed primarily for fluorescence detection are not recommended. Luminometers measure and report luminescence as relative values, and actual RLU numbers will vary between instruments. Some models of luminometers with low sensitivity should be avoided. If using a reader with an adjustable gain, we recommend a high gain setting.
	Insufficient cells per well 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.
Variability in assay performance	Assay performance can be affected by variations in cell growth conditions, including plating and harvest density and viability, centrifuge times and speeds, and freezing/DMSO exposure conditions during cell banking. Ensure consistent and accurate cell counting methods.
	Poor cell viability and variations in doubling time may affect assay performance. Ensure consistent cell growth by handling the cells exactly according to the instructions. Avoid 1-day cell passages whenever possible, especially with the PD-1+TIGIT Effector Cells. Ensure you are using high-quality plasticware and cell culture reagents (especially serum) for maintaining cells in culture. Ensure consistent and accurate cell counting methods.
	If untreated control RLU is less than 100-fold above plate reader background RLU, subtract background RLU from all samples before calculating fold induction.
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.
	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.

6. References

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7. Appendix

7.A. Representative Assay Results

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

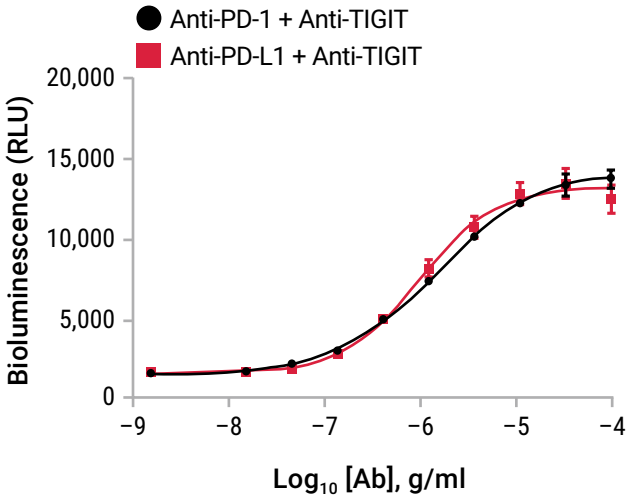

Figure 9. The PD-1+TIGIT Combination Bioassay measures the activity of Control Ab, Anti-PD-1, and Control Ab, Anti-TIGIT. PD-1+CD155 aAPC/CHO-K1 Cells were plated overnight. The following day, a titration of a 1:1 ratio of Control Ab, Anti-PD-1, and Control Ab, Anti-TIGIT, was added, followed by PD-1+TIGIT 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 EC_{50} values were 1.5 and 1.0 µg/ml and the fold inductions were 9.4 and 8.4 for anti-PD-1 Ab + anti-TIGIT Ab and anti-PD-L1 Ab + anti-TIGIT Ab, respectively.

7.B. Composition of Buffers and Solutions

initial cell culture medium for PD-1+TIGIT Effector Cells

90%	RPMI 1640 Medium with L-Glutamine and HEPES
10%	FBS

cell growth medium for PD-1+TIGIT Effector Cells

90%	RPMI 1640 Medium with L-Glutamine and HEPES
10%	FBS
200µg/ml	hygromycin B
1mg/ml	G-418 Sulfate Solution
1mM	sodium pyruvate
0.1mM	MEM nonessential amino acids

cell freezing medium for PD-1+TIGIT Effector Cells

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

initial cell culture medium for PD-L1+CD155 aAPC/CHO-K1 Cells

90%	Ham's F-12 Medium with L-Glutamine
10%	FBS

cell growth medium for PD-L1+CD155 aAPC/CHO-K1 Cells

90%	Ham's F-12 Medium with L-Glutamine
10%	FBS
200µg/ml	hygromycin B
500µg/ml	G-418 Sulfate Solution

cell freezing medium for PD-L1+CD155 aAPC/CHO-K1 Cells

85%	Ham's F-12 Medium with L-Glutamine
10%	FBS
5%	DMSO

cell recovery medium for PD-L1+CD155 aAPC/CHO-K1 Cells

90%	Ham's F-12 Medium with L-Glutamine
10%	FBS

assay buffer

90%	RPMI 1640 Medium with L-Glutamine and HEPES
10%	FBS

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7.C. Related Products

Immunotherapy Bioassays

Product	Size	Cat.#
PD-1+TIGIT Combination Bioassay	1 each	J2211
PD-1+TIGIT Combination Bioassay 5X	1 each	J2215
TIGIT Negative Cells	1 each	J1921
Control Ab, Anti-PD-1	1 each	J1201
Control Ab, Anti-TIGIT	1 each	J2051

Not for Medical Diagnostic Use.
Additional kit formats are available

Immune Checkpoint Bioassays

Product	Size	Cat.#
PD-1/PD-L1 Blockade Bioassay	1 each	J1250
PD-1/PD-L1 Blockade Bioassay 5X	1 each	J1255
PD-L1 Negative Cells	1 each	J1191
CTLA-4 Blockade Bioassay	1 each	JA3001
CTLA-4 Blockade Bioassay 5X	1 each	JA3005
Control Ab, Anti-CTLA-4	1 each	JA1020
TIGIT/CD155 Blockade Bioassay	1 each	J2201
TIGIT/CD155 Blockade Bioassay 5X	1 each	J2205
TIGIT/CD155 Blockade Bioassay, Propagation Model	1 each	J2092

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

T Cell Activation Bioassays

Product	Size	Cat.#
T Cell Activation Bioassay (NFAT)	1 each	J1621
T Cell Activation Bioassay (NFAT) 5X	1 each	J1625
T Cell Activation Bioassay (IL-2)	1 each	J1651
T Cell Activation Bioassay (IL-2) 5X	1 each	J1655

Not for Medical Diagnostic Use.

Fc Effector Bioassays

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

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Additional kit formats are available.

Detection Reagents

Product	Size	Cat.#
Bio-Glo™ Luciferase Assay System	10ml	G7941

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

Luminometers

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

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Note: Additional Immunotherapy and Fc Effector Bioassays are available from Promega Custom Assay Services. To view and order products from Custom Assay Services visit: **www.promega.com/CAS**
or email: **CAS@promega.com**

7.D. Summary of Changes

The following changes were made to the 2/22 revision of this document:

1. Updated Figure 6.
2. Edited Section 3.A, Materials to Be Supplied by the User.
3. Updated cover page.

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