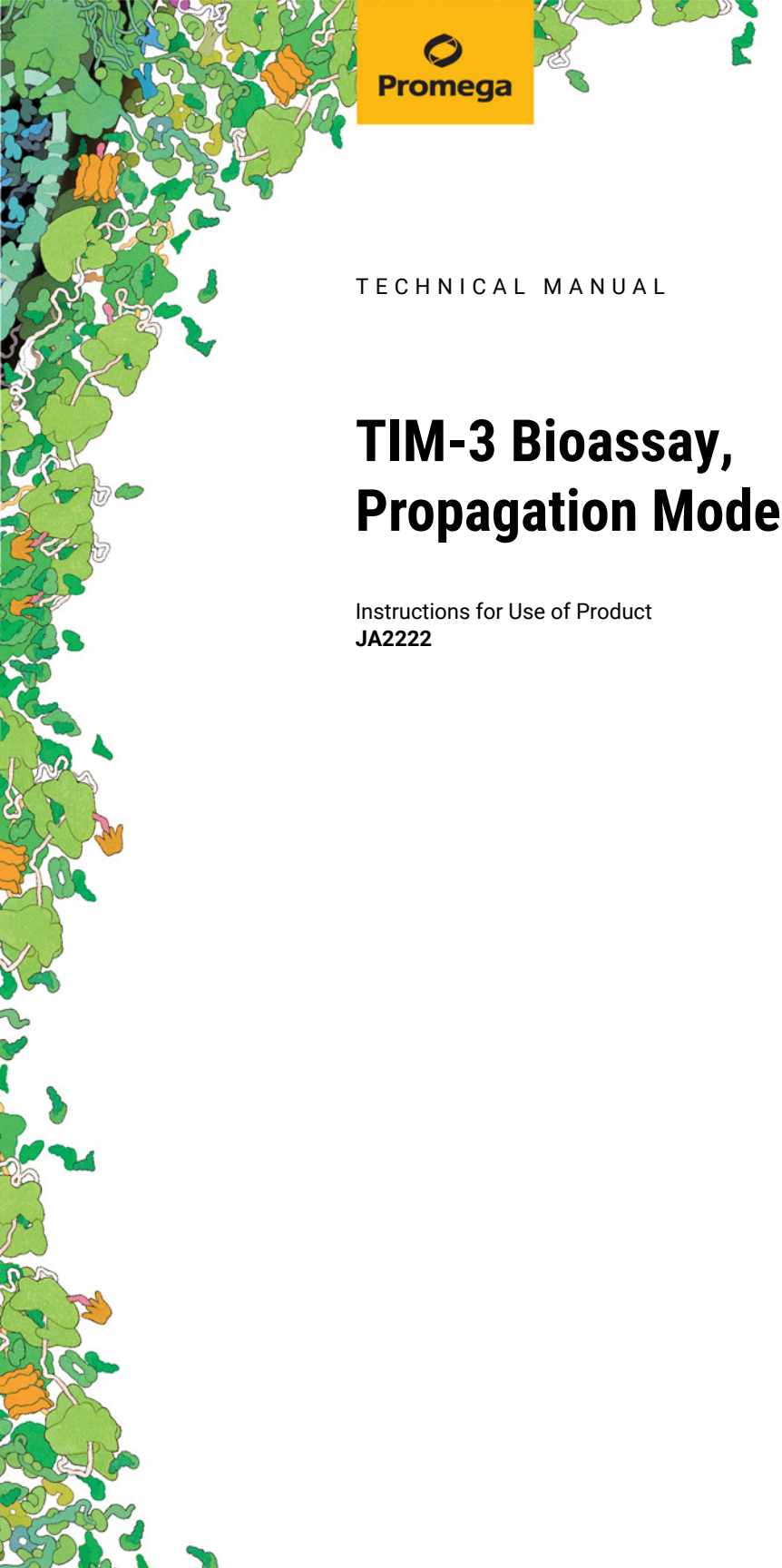

TECHNICAL MANUAL

TIM-3 Bioassay, Propagation Model

Instructions for Use of Product
JA2222

Revised 8/22
TM587

TIM-3 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 perform critical roles in the maintenance of immune homeostasis, but they also have a significant role in cancer progression and autoimmune disease. Multiple immune checkpoint receptors such as 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), lymphocyte activation gene-3 (LAG-3) and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) have been identified. Blocking these receptors with monoclonal antibodies is an effective strategy to enhance anti-tumor immune responses and promote immune-mediated tumor rejection (1,2).

TIM-3 (CD366, HAVCR2) is an immune checkpoint receptor expressed on a subset of activated T cells, regulatory T cells (Tregs), macrophages and dendritic cells. Preclinical studies have shown that TIM-3 signaling regulates Th1 cytokine responses, and that expression of TIM-3 is associated with resistance to autoimmunity. Consistent with these observations, blockade of TIM-3 leads to tumor rejection in mouse models of colorectal and ovarian cancer (3,4). While the in vivo effects of TIM-3 are clear, the cellular events leading to these outcomes are not understood. There is a growing body of literature suggesting a context- and cell type-dependent role for TIM-3 in T-cell activation that is more complex than reported previously. It is now evident that TIM-3 has the capacity to inhibit or costimulate T-cell receptor (TCR) signaling in different in vitro systems (5,6). Moreover, the identities and functional relevance of ligands for TIM-3 have long been the subject of intense scientific debate. Phosphatidylserine (PS), galectin-9, CEACAM-1 and others have been proposed as TIM-3 ligands (7–10), adding another layer of complexity to TIM-3 signaling.

Current methods used to measure the activity of drugs targeting TIM-3 rely on primary human T cells and measurement of functional endpoints such as cell proliferation, cell surface marker expression and cytokine production. These assays are laborious and highly variable due to their reliance on donor primary cells, complex assay protocols and unqualified assay reagents. As a result, these assays are difficult to establish in a quality-controlled drug development setting.

The TIM-3 Bioassay, Propagation Model (Cat.# JA2222)[a–e], is a bioluminescent reporter 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 TIM-3 (11). The assay consists of two cell lines:

- **TIM-3 Effector Cells:** Human T cells genetically engineered to express human TIM-3 and a NanoLuc® luciferase reporter driven by T cell activation pathway-dependent response elements
- **TIM-3 Target Cells:** MHCII-positive human cell line

The TIM-3 Effector and TIM-3 Target 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 cocultured, TIM-3 Target Cells provide low-level stimulation to the TIM-3 Effector Cells through the TCR. TIM-3 is engaged and activated by its ligands, including PS, which is present on both Effector and Target cells. The combined stimulation of the TCR and TIM-3 induces promoter-mediated luciferase activation and luminescence. Adding a TIM-3 blocking antibody prevents TIM-3 signaling and reduces promoter-mediated luminescence (Figure 1). The luminescent signal is quantified using the Bio-Glo-NL™ Luciferase Assay System, and a standard luminometer such as the GloMax® Discover System (see Related Products, Section 7.C). In the TIM-3 Effector Cells, we find increased IL-2 reporter activity and secretion compared to the parental cell line (data not shown). This elevated IL-2 reporter activity can be suppressed to parental cell line levels by adding TIM-3 antibody. The assay is therefore in agreement with the literature suggesting a costimulatory function for TIM-3.

In addition to the TIM-3 Bioassay, we offer the Control Ab, Anti-TIM-3 (Cat.# K1210) blocking antibody for use as a positive control.

The TIM-3 Bioassay reflects the mechanism of action (MOA) of biologics designed to block TIM-3 signaling. Specifically, TCR activation-mediated luminescence is reduced following the addition of anti-TIM-3 blocking Ab but not following the addition of anti-PD-1 (nivolumab), anti-CTLA-4 (ipilimumab), anti-TIGIT or anti-LAG3 blocking antibodies (Figure 2).

PS and galectin-9 are both endogenously present on the cell surface of TIM-3 Effector and Target Cells (data not shown). Studies with anti-PS or anti-galectin-9 blocking antibodies demonstrated that anti-PS, but not anti-galectin-9 specifically blocked TIM-3 signaling and reduced luciferase reporter activity in TIM-3 Effector cells (Figure 3).

The TIM-3 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 4). The bioassay can be performed in a two-day time frame. 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 5). In addition, the bioassay can be used in the presence of human serum, indicating potential for further development into a neutralizing antibody bioassay. Because there is some loss of light output in the presence of high concentrations of human serum, we recommend that samples be diluted for this application (Figure 6).

1. Description (continued)

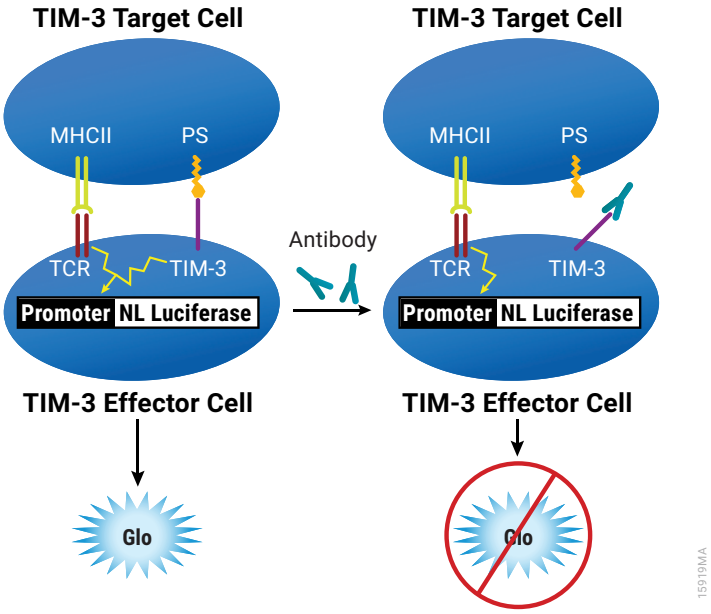

Figure 1. Representation of the TIM-3 Bioassay. The bioassay consists of two cell lines, TIM-3 Effector Cells and TIM-3 Target Cells. When cocultured, TIM-3 costimulates TCR pathway-activated luminescence of NanoLuc® luciferase (Nluc). Adding Anti-TIM-3 antibody blocks TIM-3 binding to ligands, including PS, resulting in reduced TCR pathway activation, which can be detected in a dose-dependent manner by adding Bio-Glo-NL™ Reagent and quantitated with a luminometer.

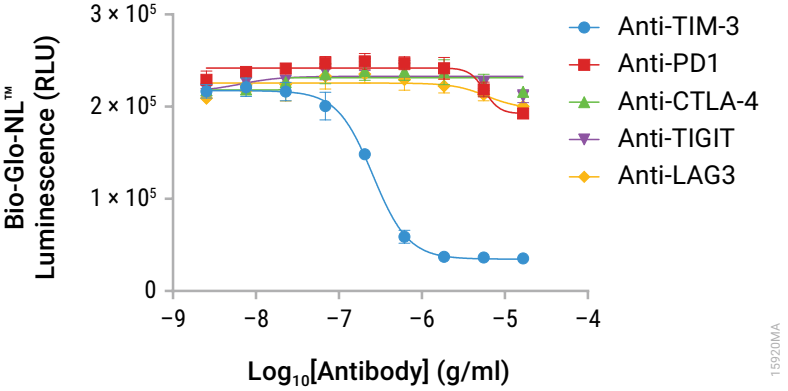

Figure 2. The TIM-3 Bioassay reflects the mechanism of action (MOA) and specificity of biologics designed to block TIM-3 signaling. TIM-3 Effector Cells were incubated with TIM-3 Target Cells in the presence of serial titrations of blocking Abs as indicated: Control Ab, Anti-TIM-3 (Cat.# K1210), anti-LAG-3 (Cat.# K1150), Control Ab, Anti-TIGIT (Cat.# J2051), anti-PD-1 (nivolumab) or anti-CTLA-4 (ipilimumab) blocking antibodies. After a 20-hour induction, Bio-Glo-NL™ Reagent was added, and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

1. Description (continued)

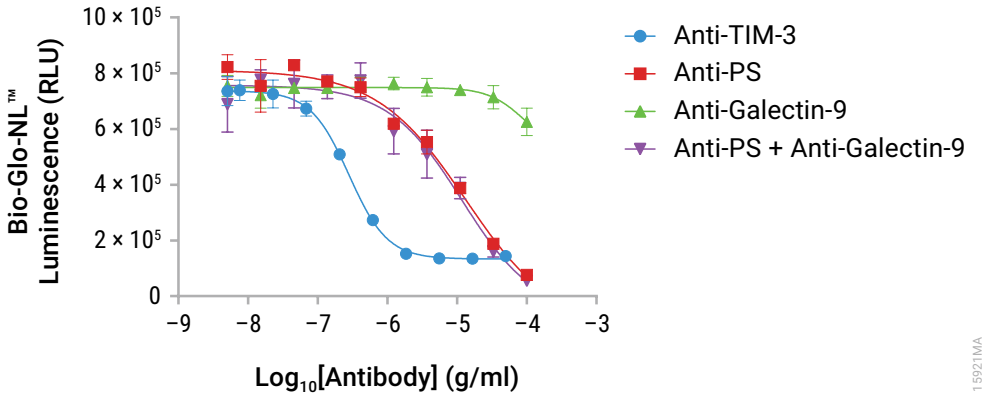

Figure 3. TIM-3 Bioassay is dependent on PS but not galectin-9. The TIM-3 Bioassay was performed as described in this technical manual, using Control Ab, Anti-TIM-3 (Cat.# K1210), or commercial antibodies against PS (Clone 1H6) and galectin-9 (Clone 9M1-3). Blockade of PS resulted in suppression of reporter activity similar to that seen with anti-TIM-3, while blockade of galectin-9 showed little effect, alone or in combination with anti-PS. Data were generated using thaw-and-use cells.

Table 1. The TIM-3 Bioassay Shows Precision, Accuracy and Linearity.

Parameter	Results	
Accuracy	Percent Expected Relative Potency	Percent Recovery
	50	102.6
	70	100.2
	100	97.7
	130	99.8
	150	107.7
Repeatability (% CV)	100% (Reference)	12.9
Intermediate Precision (% CV)		13.4
Linearity (r^2)		0.904
Linearity (y = mx + b)		y = 1.078x – 3.277

A 50–150% theoretical potency series of Control Ab, Anti-TIM-3, was analyzed in triplicate in three independent experiments performed on three days by two analysts. Bio-Glo-NL™ Reagent was added and luminescence from the NanoLuc® luciferase reporter 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.

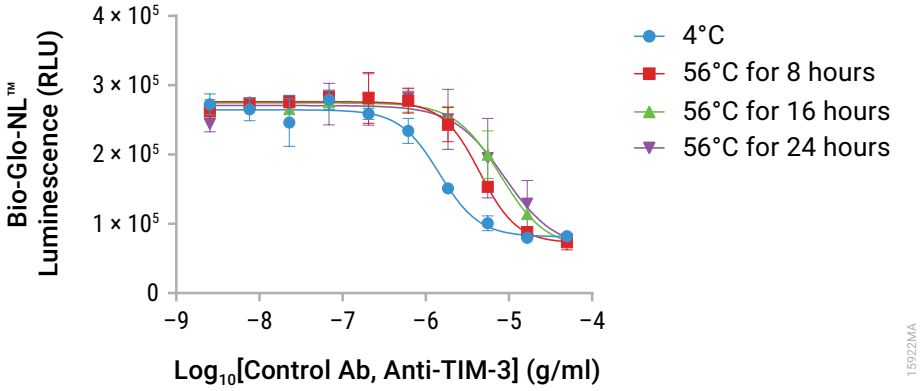

Figure 4. The TIM-3 Bioassay is stability-indicating. Samples of Control Ab, Anti-TIM-3, were maintained at 4°C (control) or heat-treated at 56°C for the indicated times, then analyzed using the TIM-3 Bioassay. Bio-Glo-NL™ Reagent was added, and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

1. Description (continued)

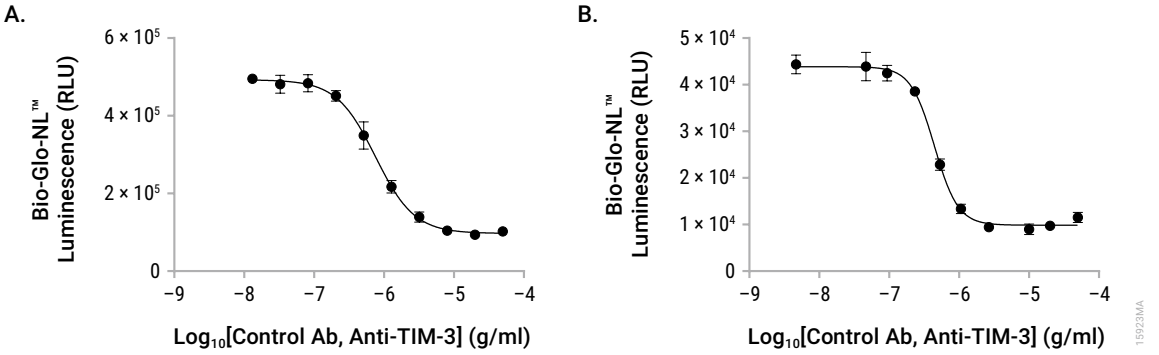

Figure 5. The TIM-3 Bioassay is amenable to 384-well plate format. Panel A. The TIM-3 Bioassay was performed in 96-well plates as described in this technical manual with a titration of Control Ab, Anti-TIM-3. **Panel B.** The TIM-3 Bioassay was performed in 384-well format using a MANTIS® liquid handler to dispense the cells. TIM-3 Effector Cells (10µl) were dispensed at 2 × 10^4 cells/well. Next, 10µl of TIM-3 Target Cells were added at 2 × 10^4 cells/well. Finally, 10µl of Control Ab, Anti-TIM-3, was added each well. The assay plate was incubated overnight in a 37°C, 5% CO_2 incubator. After a 20-hour incubation, 30µl of Bio-Glo-NL™ Reagent was added to each well, and luminescence quantified using the GloMax® Discover Detection System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. The IC_{50} values were 754ng/ml and 443ng/ml, and the maximum inhibition was 80% and 78% for the 96- and 384-well-plate format, respectively. Data were generated using thaw-and-use cells.

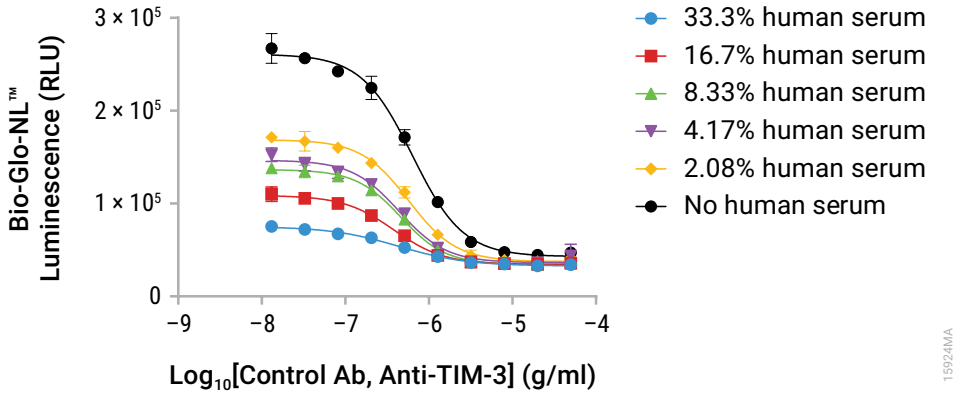

Figure 6. The TIM-3 Bioassay may be affected by human serum. Control Ab, Anti-TIM-3, was analyzed in the absence or presence of increasing concentrations of pooled normal human serum (0–100% in the antibody sample), resulting in final assay concentration of human serum (0–33.3%). Bio-Glo-NL™ Reagent was added, and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. The TIM-3 Bioassay showed reduced light output in the presence of 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.#
TIM-3 Bioassay, Propagation Model	**1 each**	**JA2222**

Not for Medical Diagnostic Use.

Includes:

- 2 vials TIM-3 Effector Cells (CPM), 2 × 10^7 cells/ml (1.0ml per vial)
- 2 vials TIM-3 Target Cells (CPM), 1 × 10^7 cells/ml (1.0ml per vial)

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

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

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 the Certificate of Analysis.

Note: The TIM-3 Bioassay is compatible only with the Bio-Glo-NL™ Luciferase Assay Reagent. **Do not** use the Bio-Glo™ Luciferase Assay Reagent (Cat.# G7940, G7941) with the TIM-3 Bioassay.

The TIM-3 Bioassay is intended to be used with user-provided antibodies or other biologics designed to block the interaction of TIM-3 with its ligands. Control Ab, Anti-TIM-3 (Cat.# K1210), is available separately for use in assay optimization and routine quality control. We strongly recommend including Control Ab, Anti-TIM-3, as a positive control in the first few assays to gain familiarity with the bioassay. Data generated using Control Ab, Anti-TIM-3, is shown in 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, incubation time and assay buffer components described in Section 4 were established using Control Ab, Anti-TIM-3. You may need to adjust the parameters provided here and optimize assay conditions for your own antibody or biologic.

The TIM-3 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 Related Products, Section 7.C). An integration time of 0.5 second/well was used for all readings. Relative luminescence unit readings will vary with the sensitivity and settings of each instrument. If using a reader with adjustable gain, we recommend a high-gain setting. The use of different instruments and gain adjustment will affect the magnitude of the raw data, but should not affect the measured relative potency of test samples.

Materials to Be Supplied by the User

Reagents

(Composition of Buffers and Solutions is provided in Section 9.B.)

- user-defined anti-TIM-3 antibodies or other biologics
- RPMI 1640 medium with L-glutamine and HEPES (e.g., Corning® Cat.# 10-041-CV or GIBCO Cat.# 22400)
- MEM non-essential amino acids, 100X (e.g., GIBCO Cat.# 11140050)
- sodium pyruvate, 100mM (e.g., GIBCO Cat.# 11360070)
- fetal bovine serum (e.g., VWR Cat.# 89510-194 or Gibco Cat.# 16000044)
- hygromycin B (e.g., GIBCO Cat.# 10687010)
- G-418 sulfate (Cat.# V8091)
- DMSO (e.g., Sigma Cat.# D2650)
- Trypan blue solution (e.g., Sigma Cat.# T8154)

- Bio-Glo-NL™ Luciferase Assay System (Cat.# J3081, J3082, J3083)
- **Optional:** Control Ab, Anti-TIM-3 (Cat.# K1210)

Supplies and Equipment

- white, flat-bottom, tissue culture-treated 96-well assay plates (e.g., Corning Cat.# 3917)
- sterile, clear V-bottom 96-well plate with lid (e.g., Costar Cat.# 3896) for preparing antibody dilutions
- pipettes (single-channel and 12-channel); for best results use both manual and electronic pipettes as needed
- sterile 15ml and 50ml conical tubes
- sterile reagent reservoirs (e.g., Corning 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)

4. Preparing TIM-3 Effector Cells

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

4.A. Effector Cell Thawing and Initial Cell Culture

1. Prepare 40ml of Initial Cell Culture Medium as described in Section 9.B and warm to 37°C. This initial antibiotic-free 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 TIM-3 Effector Cells from storage at –140°C and thaw in a 37°C water bath with gentle agitation (no inversion) until just thawed (typically 2–3 minutes).
4. Transfer all of the cells (approximately 1ml) to the 50ml conical tube containing 9ml of prewarmed initial cell culture medium.
5. Centrifuge at 90 × *g* for 10 minutes.
6. Carefully aspirate the medium, and resuspend the cell pellet in 25ml of prewarmed initial cell culture medium.
7. Transfer the cell suspension to a T75 tissue culture flask and horizontally place the flask in a 37°C, 5% CO_2 incubator.
8. Incubate for approximately 24 hours before passaging the cells.

4.B. Effector Cell Maintenance and Propagation

Note: For cell maintenance and propagation starting from the second cell passage, use the cell growth medium containing antibiotics, and monitor cell viability and doubling rate during propagation. The cell growth rate will stabilize by 7–10 days after thawing, at which time cell viability is typically >90% and the average cell doubling rate is 25 hours. Passage number should be recorded for each passage. In our experience, cells maintain their functionality for at least 25 passages, or 55 cell doublings if passaging is performed on a Monday-Wednesday-Friday schedule.

Cell Passage Schedule	Cell Seeding Density
2 days	4.0×10^5 cells/ml
3 days	2.5×10^5 cells/ml

1. On the day of cell passage, measure cell viability and density by Trypan blue staining.
2. Seed the cells at a density of 4×10^5 cells/ml if passaging for two days (e.g., Monday-Wednesday or Wednesday-Friday) or 2.5×10^5 cells/ml if passaging for three days (e.g., Friday-Monday). Always maintain the flasks in a horizontal position in the incubator.
3. 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).
4. Place the flasks in a 37°C, 5% CO_2 incubator.

4.C. Effector Cell Freezing and Banking

1. On the day of cell freezing, make new cell freezing medium and keep on ice.
2. Gently mix the cells with a pipette to create a homogenous cell suspension.
3. Remove a sample for cell counting by Trypan blue staining. Calculate the volume of cell freezing medium needed based on desired cell freezing densities of 5×10^6–2×10^7 cells/ml.
4. Transfer the cell suspension to 50ml sterile conical tubes or larger sized centrifuge tubes and centrifuge at 130 × *g* for 10–15 minutes.
5. Gently aspirate the medium, avoiding the cell pellet.
6. Carefully resuspend the cell pellet in ice-cold cell freezing medium to a final cell density of 5×10^6–2×10^7 cells/ml. Combine the cell suspensions in a single tube and dispense into cryovials.
7. 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.

5. Preparing TIM-3 Target Cells

5.A. Target Cell Thawing

1. Prepare 40ml of initial cell culture medium as described in Section 9.B and warm to 37°C.
2. Transfer 9ml of prewarmed initial cell culture medium to a 50ml conical tube.
3. Remove one vial of TIM-3 Target Cells from storage at –140°C and thaw in a 37°C water bath with gentle agitation (no inversion) until just thawed (typically 2–3 minutes).
4. Transfer all the cells (approximately 1ml) to the 50ml conical tube containing 9ml of prewarmed initial cell culture medium.
5. Centrifuge at 180 × *g* for 8 minutes.
6. Aspirate the medium and resuspend the cell pellet in 25ml of prewarmed initial cell culture medium.
7. Transfer the cell suspension to a T75 flask, and horizontally place the flask in a 37°C, 5% CO_2 incubator.
8. Incubate the cells for approximately 48 hours before passaging them.

5.B. Target Cell Maintenance and Propagation

Note: For cell maintenance and propagation starting from the second cell passage, monitor cell viability and doubling rate during propagation. Maintain the cell density in the range of 2.0×10^5–2.0×10^6 cells/ml to ensure optimal performance. The cell growth rate will stabilize by 5–7 days after thawing, at which time cell viability is typically >95% and the average cell doubling rate is 22 hours when seeded at the densities listed in the table below. Passage number should be recorded for each passage.

Cell Passage Schedule	Cell Seeding Density
2 days	4.0×10^5 cells/ml
3 days	2.0×10^5 cells/ml

1. On the day of cell passage, measure cell viability and density by Trypan blue staining.
2. Seed the cells at a density of 4×10^5 cells/ml if passaging for two days (e.g., Monday-Wednesday, or Wednesday-Friday) or 2.0×10^5 cells/ml if passaging for three days (e.g., Friday-Monday). Always maintain the flasks in a horizontal position in the incubator.
3. 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).
4. Place the flasks in a 37°C, 5% CO_2 incubator.

5.C. Target Cell Freezing and Banking

1. On the day of cell freezing, make new cell freezing medium and keep on ice.
2. Gently mix the cells with a pipette to create a homogenous cell suspension.
3. Remove a sample for cell counting by Trypan blue staining. Calculate the volume of cell freezing medium needed based on desired cell freezing densities of 5×10^6–2×10^7 cells/ml.
4. Transfer the cell suspension to 50ml sterile conical tubes or larger sized centrifuge tubes and centrifuge at 130 × *g* for 10–15 minutes.
5. Gently aspirate the medium, avoiding the cell pellet.
6. Carefully resuspend the cell pellet in ice-cold cell freezing medium to a final cell density of 5×10^6–2×10^7 cells/ml. Combine the cell suspensions in a single tube and dispense into cryovials.
7. 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.

6. Assay Protocol

This assay protocol illustrates the use of the TIM-3 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 for 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 upper and lower asymptotes and sufficient points on the slope. For reference, we use 50µg/ml of Control Ab, Anti-TIM-3, as a starting concentration (1X) and a 2.5-fold dilution series.

6.A. Preparing Assay Buffer and Antibody Samples

1. **Assay Buffer:** On the day of the assay, prepare an appropriate amount of assay buffer (95% RPMI 1640/5% 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 5% FBS. This concentration of FBS works well for the anti-TIM-3 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%.

2. **Bio-Glo-NL™ Reagent:** For reference, 10ml of reconstituted Bio-Glo-NL™ Reagent is sufficient to assay 120 wells in a 96-well assay format. The Bio-Glo-NL™ Luciferase Assay Substrate should always be stored at –20°C. Thaw the Bio-Glo-NL™ Luciferase Assay Buffer at room temperature (do not exceed 25°C) during the 16–24 hour induction period of the assay. We recommend preparing the reconstituted Bio-Glo-NL™ Luciferase Assay Reagent immediately before use.

Note: The TIM-3 Bioassay is compatible only with the Bio-Glo-NL™ Luciferase Assay Reagent. **Do not** use the Bio-Glo™ Luciferase Assay Reagent (Cat.# G7940, G7941) with the TIM-3 Bioassay.

3. **Test and Reference Samples:** Using assay buffer as the diluent, prepare starting dilutions (dilu1, 3X final concentration) of two test antibodies (200µl each) and one reference antibody sample (400µl) 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-TIM-3, as a reference in your assay, prepare 400µl at 150µg/ml for the starting dilution (dilu1, 3X final concentration) by adding 60µl of Control Ab, Anti-TIM-3 stock (1mg/ml), to 340µl of assay buffer.

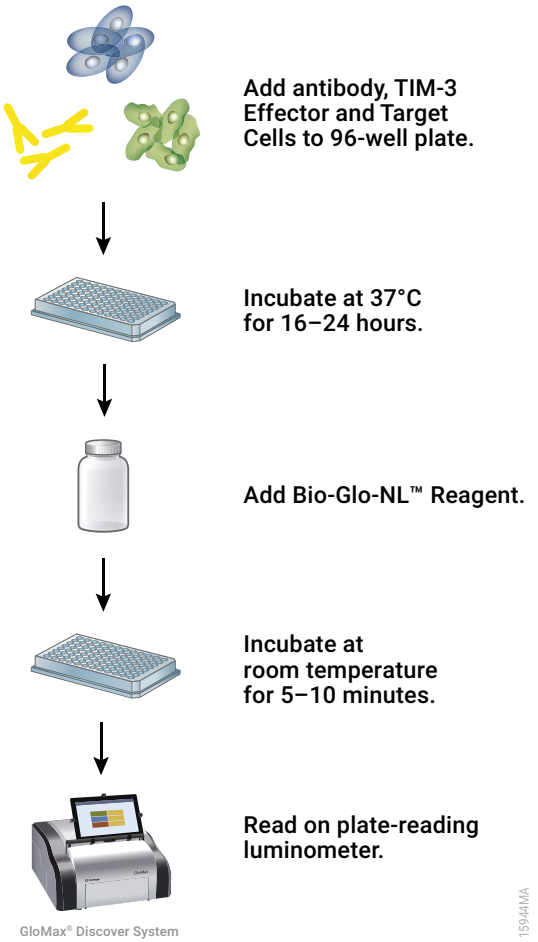

Figure 7. Schematic protocol for the TIM-3 Bioassay.

6.B. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 8 as a guide. The protocol describes serial replicate dilutions (n = 3) of test and reference antibodies to generate a ten point dose-response curves for each antibody.

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

Figure 8. Example plate layout showing non-clustered sample locations of test and reference antibody dilution series and wells containing assay buffer (denoted by "B") alone.

Day 1

6.C. 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 (100µl 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 200µl of each antibody at 3X the highest antibody concentration in your dose-response curve. For other dilution schemes, adjust the volumes accordingly.

1. On the day of 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 200µl of reference antibody starting dilution (dilu1, 3X final concentration) to wells A11 and B11 (see Figure 9).
3. Add 200µl of test antibodies 1 and 2 starting dilution (dilu1, 3X final concentration) to wells C11 and D11, respectively (see Figure 9).

4. Add 120µl 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.
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, C2, and D2 contain 120µl of assay buffer without antibody as a negative control.
7. Use a multichannel pipette to transfer 25µl antibody to each well of the assay plate as indicated in Figure 8.
8. Cover the plate with a lid and keep at ambient temperature while preparing TIM-3 Effector and Target Cells.

6.D. Preparing TIM-3 Effector and Target Cells

While maintaining the TIM-3 Effector and Target Cells, follow the recommended cell seeding density. Changes in cell culture volume or seeding density can 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 TIM-3 Effector and Target Cells by Trypan blue staining and calculate the cell density and viability for each.
3. Transfer an appropriate amount of TIM-3 Effector Cells and Target Cells from the culture vessels to 50ml conical tubes or larger-sized centrifuge tubes. We recommend collecting at least 4 × 10^6 cells per assay plate of each cell type when using the recommended plate layout.
4. Pellet the cells at 130 × *g* for 10 minutes at ambient temperature and resuspend each assay buffer at 70% of the full volume needed to generate the targeted final cell density of 2.0 × 10^6 cells/ml.
5. Count the cells again, and adjust the assay buffer volume to achieve a final cell density for each of 2.0 × 10^6 cells/ml. You will need at least 4.0ml of TIM-3 Effector Cells and 4.0ml of TIM-3 Target Cells to fill 120 assay wells, or the inner 60 wells of two assay plates.
6. Transfer the TIM-3 Effector Cells to a sterile reagent reservoir. To avoid cells settling to the bottom of the reservoir, immediately use a multichannel pipette to transfer 25µl of Effector Cells to each well of the assay plates, according to the plate layout in Figure 9.
7. Transfer the TIM-3 Target Cells to a sterile reagent reservoir. To avoid cells settling to the bottom of the reservoir, immediately use a multichannel pipet to transfer 25µl of Target Cells to each well of the assay plates, according to the plate layout in Figure 9.
8. Add 75µl of assay buffer to each of the unused wells of the assay plates.

6.D. Preparing TIM-3 Effector and Target Cells (continued)

9. Cover the assay plates with a lid and place in a 37°C, 5% CO_2 incubator for 16–24 hours.

   Note: We have found that shorter incubation times (16–18 hours) result in the greatest light output (RLU) while longer incubations times (22–24 hours) minimize day-to-day variability. Incubation time can be optimized to fit your needs.

10. Thaw the Bio-Glo-NL™ Luciferase Assay Buffer at room temperature (do not exceed 25°C) during the 16–24 hour induction period of the assay.

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		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test Ab 1
D		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test Ab 2
E													
F													
G													
H													

Figure 9. Example plate layout showing antibody serial dilutions.

Day 2

6.E. Preparing and Adding Bio-Glo-NL™ Reagent

We recommend preparing the Bio-Glo-NL™ Luciferase Assay Reagent immediately before use. Ensure that the Bio-Glo-NL™ Luciferase Assay Buffer is equilibrated to room temperature (do not exceed 25°C) before reconstituting the reagent. Do not store the reconstituted reagent. Once reconstituted, the reagent will lose 10% activity in approximately 8 hours at room temperature.

Note: The TIM-3 Bioassay is compatible only with the Bio-Glo-NL™ Luciferase Assay Reagent. **Do not** use the Bio-Glo™ Luciferase Assay Reagent (Cat.# G7940, G7941) with the TIM-3 Bioassay.

1. Remove the Bio-Glo-NL™ Luciferase Assay Substrate from –20°C storage and mix by pipetting. Briefly centrifuge the tubes if the substrate has collected in the cap or on the sides of the tubes.

2. Prepare the desired amount of reconstituted Bio-Glo-NL™ Luciferase Assay Reagent by combining one volume of substrate with 50 volumes of buffer. For example, if the experiment requires 10ml of reagent, add 200µl of substrate to 10ml of buffer. Ten milliliters of Bio-Glo-NL™ Reagent is sufficient for 120 wells (two assay plates, using the inner 60 wells of each plate).
3. Remove assay plates from the incubator after the incubation period and equilibrate to room temperature for 10–15 minutes.
4. Using a manual multichannel pipette, add 75µl of Bio-Glo-NL™ Reagent to the inner 60 wells of the assay plates, taking care not to create bubbles.
5. Add 75µl of Bio-Glo-NL™ Luciferase Assay Reagent to wells B1, C1 and D1 of each assay plate to measure background signal.
6. Wait 5–10 minutes, then measure the luminescence in a GloMax® Discover System or a plate reader with glow-type luminescence reading capabilities. The luminescence intensity will decay gradually, with a signal half-life of approximately 120 minutes at room temperature.

   Note: Varying the Bio-Glo-NL™ incubation time will affect the raw RLU values but should not significantly change the IC_{50} value and fold maximum inhibition.

6.F. Data Analysis

1. Determine plate background by calculating the average relative light units (RLU) from wells B1, C1 and D1.
2. Calculate percent inhibition = $1 - \frac{\text{RLU (induced–background)}}{\text{RLU (no antibody control–background)}} \times 100$
3. Graph data as RLU versus Log_{10} [antibody] and percent inhibition versus Log_{10} [antibody]. Fit curves and determine the IC_{50} value of antibody response using appropriate curve fitting software (such as GraphPad Prism® software).

7. Troubleshooting

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

Symptoms	Possible Causes and Comments
Low luminescence measurements (RLU readout)	Ensure that you are using Bio-Glo-NL™ Reagent in the assay. The TIM-3 Bioassay is **not compatible** with Bio-Glo™ Reagent.
	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-NL™ Reagent leads to low RLU. Store the Bio-Glo-NL™ Substrate and Bio-Glo-NL™ Buffer as directed, and handle the Bio-Glo-NL™ Reagent according to the instructions. For best results, prepare immediately before use.
Poor viability or slow growth rate of Target Cells	Ensure that medium formulation is appropriate. Target Cells must be grown in RPMI 1640 containing HEPES, L-glutamate, NEAA, sodium pyruvate and 10% FBS.
Weak assay response (low percent inhibition)	Optimize the concentration range of your test sample(s) to achieve a full dose response with complete upper and lower asymptotes. The IC_{50} value obtained in the TIM-3 Bioassay may vary from the IC_{50} value obtained using other methods such as primary T cell-based assays.
	Ensure that TIM-3 Target and Effector Cells are growing well and have a viability of at least 90% on the day of the assay.
	TIM-3 Effector cells are sensitive to shear forces and should be handled gently after thawing or during resuspension. Avoid excessive trituration and never mix the cells by vortexing.
Nonspecific inhibition of reporter activity with control antibodies	For best results, use only purified antibodies. Components present in unpurified supernatants may inhibit the assay.

Symptoms	Possible Causes and Comments
Variability in assay performance	Assay performance can be impacted 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. Handle the cells consistently according to the instructions in this manual. Ensure consisten[t] 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. Ensure you are using high-quality cell culture reagents (especially serum) and plasticware for maintaining cells in culture. Ensure consistent and accurate cell counting methods.
	Optimize assay incubation time. We have observed that shorter incubation times (16–18 hours) result in the greatest light output, while longer incubation times (22–24 hours) produce the least day-to-day variability. Ensure that incubation times are consistent between assays.

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9. Appendix

9.A. Representative Assay Results

The following data were generated using the TIM-3 Bioassay, Propagation Model, using Control Ab, Anti-TIM-3 (Figure 11).

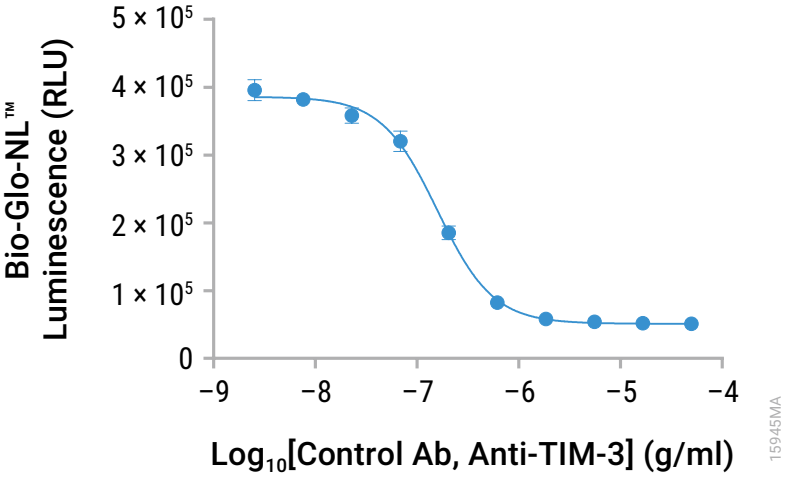

Figure 11. The TIM-3 Bioassay measures the activity of Control Ab, Anti-TIM-3. TIM-3 Target and Effector Cells, and a titration of Control Ab, Anti-TIM-3, were plated together and incubated overnight. The following day, Bio-Glo-NL™ Reagent was added and luminescence was measured using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. The IC_{50} was 157ng/ml and the maximum inhibition was 87%.

9.B. Composition of Buffers and Solutions

Initial Cell Culture Medium for TIM-3 Effector and Target Cells

90%	RPMI 1640 with L-glutamine and HEPES
10%	FBS
1mM	sodium pyruvate
0.1mM	MEM nonessential amino acids

Cell Growth Medium for TIM-3 Effector Cells

90% RPMI 1640 with L-glutamine and HEPES

10%	FBS
1mM	sodium pyruvate
0.1mM	MEM nonessential amino acids
400µg/ml	hygromycin B
800µg/ml	G-418

Cell Freezing Medium for TIM-3 Effector and Target Cells

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

Cell Growth Medium for TIM-3 Target Cells

90%	RPMI 1640 with L-glutamine and HEPES
10%	FBS
1mM	sodium pyruvate
0.1mM	MEM nonessential amino acids

Assay Buffer

95%	RPMI 1640 with L-glutamine and HEPES
5%	FBS

9.C. Related Products

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

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

**Not for Medical Diagnostic Use.

Additional kit formats and sizes are available.

Fc Effector Immunoassay

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

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

Immune Checkpoint Bioassays

Product	Size	Cat.#
4-1BB Bioassay	1 each	JA2351
CD40 Bioassay	1 each	JA2151
CTLA-4 Blockade Bioassay	1 each	JA3001
GITR Bioassay	1 each	JA2291
ICOS Bioassay	1 each	JA6801
ICOS Blockade Bioassay	1 each	JA6001
LAG-3/MHCII Blockade Bioassay	1 each	JA1111
OX40 Bioassay	1 each	JA2191
PD-1/PD-L1 Blockade Bioassay	1 each	J1250
PD-1+TIGIT Combination Bioassay	1 each	J2211
PD-L1 Negative Cells	1 each	J1191
TIGIT/CD155 Blockade Bioassay	1 each	J2201
TIM-3 Bioassay	1 each	JA2211

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

T Cell Activation Bioassays

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

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

Cytokine and Growth Factor Bioassays

Product	Size	Cat.#
IL-2 Bioassay	1 each	JA2201
IL-6 Bioassay	1 each	JA2501
IL-12 Bioassay	1 each	JA2601
IL-15 Bioassay	1 each	JA2011
IL-23 Bioassay	1 each	JA2511
RANKL Bioassay	1 each	JA2701
VEGF Bioassay	1 each	GA2001

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9.C. Related Products (continued)

Control Antibodies and Proteins

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

Detection Reagents

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

Not for Medical Diagnostic Use.

Luminometers

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

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

Note: Additional Fc Effector, Immune Checkpoint, T Cell Activation and Cytokine Bioassays are available through Promega Elite Access. To view and order Promega Bioassay products visit:
www.promega.com/products/reporter-bioassays/ or email: **eliteaccess@promega.com**

10. Summary of Changes

The following changes were made to the 8/22 revision of this Technical Manual:

1. Corrected Cell Seeding Density for tables in Sections 4.B and 5.B.
2. Updated disclaimers.
3. Replaced cover image and updated document font.

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