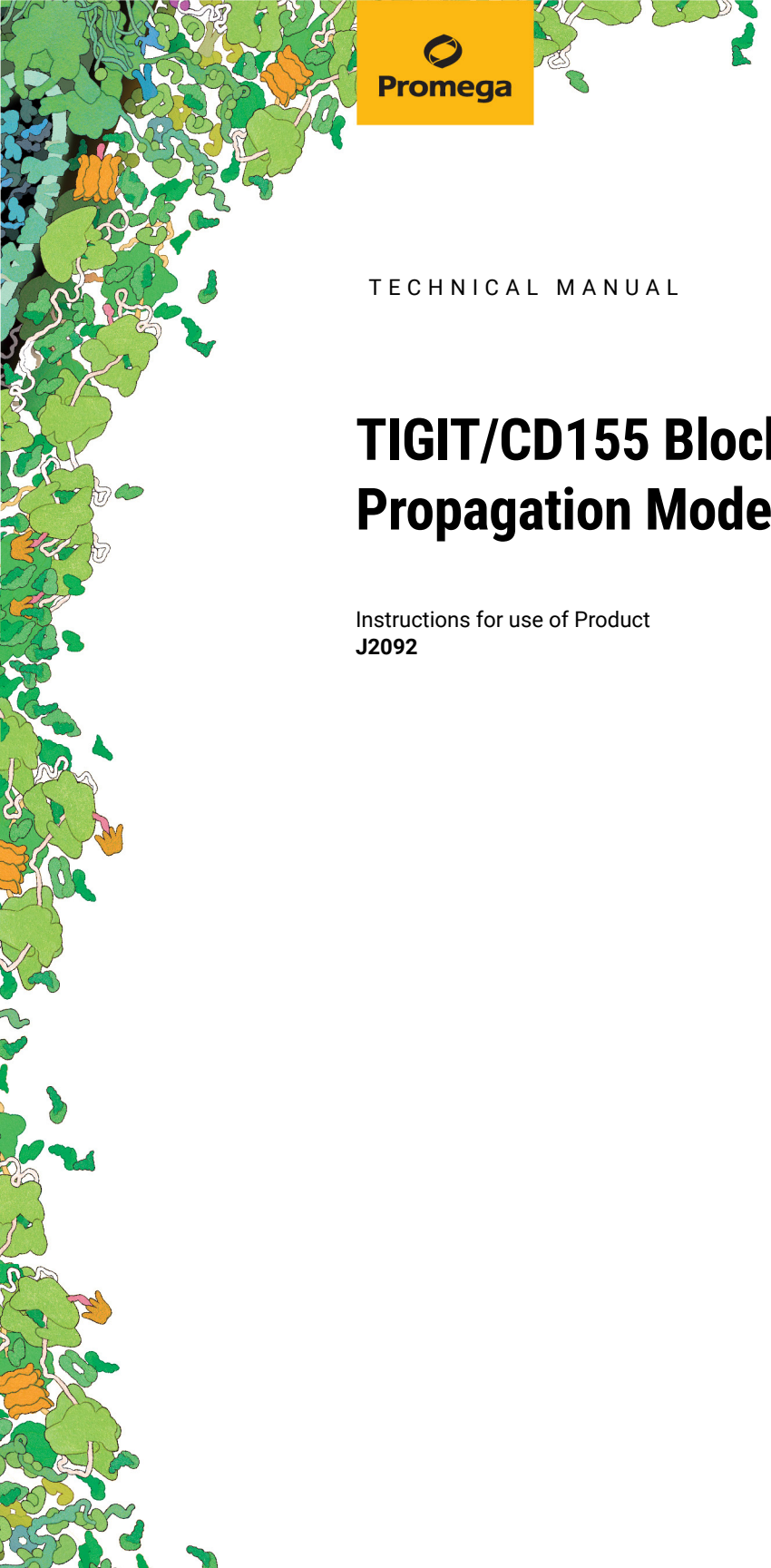


TECHNICAL MANUAL

TIGIT/CD155 Blockade Bioassay, Propagation Model

Instructions for use of Product
J2092

Revised 8/26
TM533

Promega

TIGIT/CD155 Blockade Bioassay, Propagation Model

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 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), and Lymphocyte Activation Gene-3 (LAG-3) have been identified. 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).

TIGIT, also known as WUCAM and Vstm3, is an immune checkpoint protein expressed on lymphocytes. Highest expression levels are observed on effector $CD4^+$ and $CD8^+$ T cells, regulatory T cells, and NK cells (3). TIGIT has several distinct mechanisms of action that inhibit lymphocyte activation. First, it is an inhibitory counterpart of the costimulatory 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; 3). Therefore, TIGIT will outcompete CD226 for CD155 binding and thus negate CD226 co-stimulation. Second, TIGIT inhibits CD226 homodimerization in cis, preventing CD226 signaling (4). 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 (5).

Current methods used to measure the activity of biologic drugs targeting 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 TIGIT/CD155 Blockade Bioassay, Propagation Model[a,b] (Cat.# J2092), 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 TIGIT (6,7). The assay consists of two genetically engineered cell lines:

- **TIGIT Effector Cells:** Jurkat T cells expressing human TIGIT with a luciferase reporter driven by a native promoter that can respond to both TCR activation and CD226 co-stimulation
- **CD155 aAPC/CHO-K1 Cells:** CHO-K1 cells engineered to express human CD155 with an engineered cell-surface protein designed to activate the T cell receptor (TCR) complex in an antigen-independent manner.

The TIGIT Effector Cells and 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 cocultured, TIGIT inhibits CD226 activation and promoter-mediated luminescence. Addition of an anti-TIGIT antibody blocks the interaction of TIGIT with CD155 and/or inhibits the ability of TIGIT to prevent CD226 homodimerization, resulting in promoter-mediated luminescence (Figure 1). The bioluminescent signal is quantified using the Bio-Glo™ Luciferase Assay System and a standard luminometer such as the GloMax® Discover System.

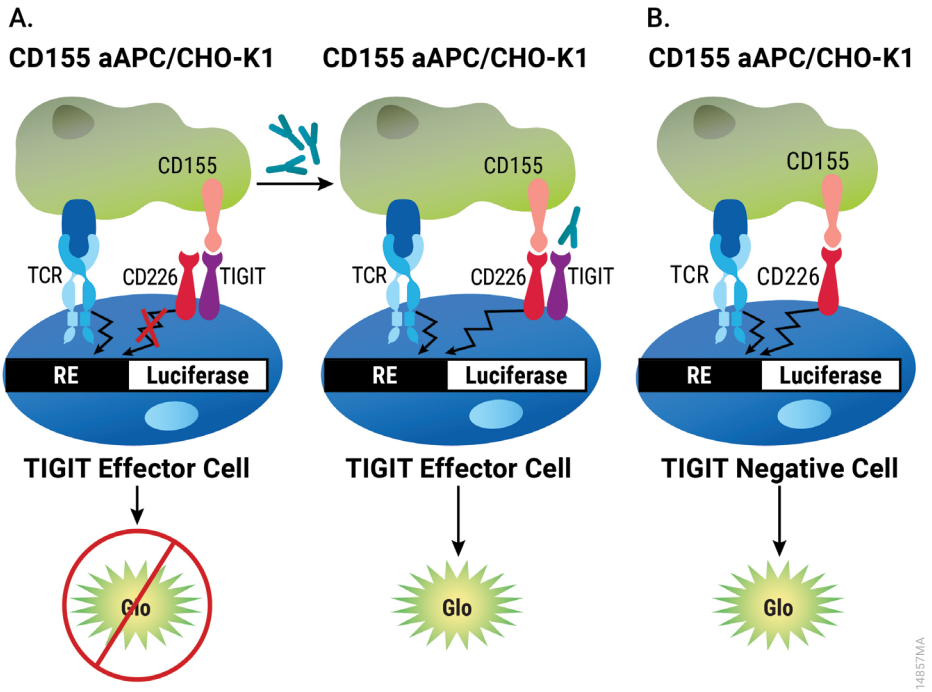


Figure 1. Representation of the TIGIT/CD155 Blockade Bioassay. The bioassay consists of two genetically engineered cell lines, TIGIT Effector Cells and CD155 aAPC/CHO-K1 Cells. **Panel A.** When co-cultured, TIGIT inhibits CD226 pathway-activated luminescence. The addition of anti-TIGIT antibody blocks the TIGIT/CD155 interaction, thereby re-establishing CD226 pathway-activated luminescence, which 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 non-TIGIT-expressing Effector Cells, TCR activation and CD226/CD155 induces luminescence by activation of the CD226 pathway.

The TIGIT/CD155 Blockade Bioassay, Propagation Model, reflects the mechanism of action (MOA) of biologics designed to block the TIGIT/CD155 interaction. Specifically, CD226-mediated luminescence is detected following the addition of anti-TIGIT blocking antibodies but not following addition of anti-PD-1, anti-PD-L1 or anti-CTLA-4 blocking antibodies (Figure 2). The bioassay is prequalified according to ICH guidelines and shows the precision, accuracy and linearity required for routine use in potency and stability studies (Table 1 and Figure 3). The assay 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 4). In addition, the bioassay can be used with up to 100% human serum (in antibody samples) with minimal impact on fold induction (Figure 5), indicating potential for further development into a neutralizing antibody bioassay.

It is increasingly common during drug development to analyze potential therapeutic antibodies for Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC activity). Another application of the TIGIT/CD155 Blockade Bioassay is the ability to measure ADCC activity of anti-TIGIT-blocking antibodies by combining ADCC Reporter Bioassay Jurkat Effector cells, available separately (Cat.# G7010), with TIGIT Effector Cells (Figure 6).

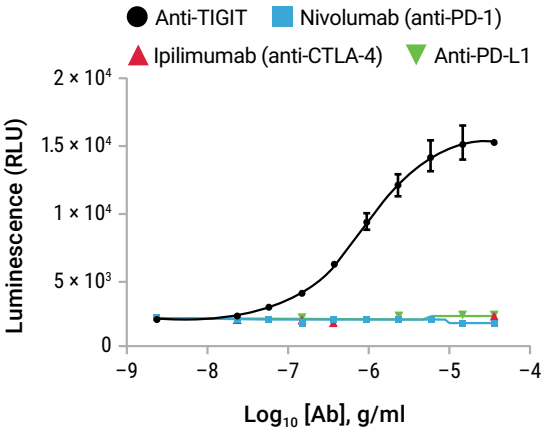


Figure 2. The TIGIT/CD155 Blockade Bioassay reflects the mechanism of action (MOA) and specificity of biologics designed to block the TIGIT/CD155 interaction. TIGIT Effector Cells were incubated with CD155 aAPC/CHO-K1 Cells in the presence of a serial titration of Control Ab, Anti-TIGIT (Cat.# J2051), anti-PD-1 (nivolumab), anti-CTLA-4 (ipilimumab) or anti-PD-L1-blocking antibodies 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 GraphPad Prism® software. Data were generated using thaw-and-use cells.

Table 1. The TIGIT/CD155 Blockade Bioassay Shows Precision, Accuracy and Linearity.

Parameter	**Results**	
Accuracy	% Expected Relative Potency	% Recovery
	50	101.8
	70	102.8
	140	101.0
	200	98.5
Repeatability (% CV)	100% (Reference)	11.0
Intermediate Precision (% CV)		9.7
Linearity (r^2)		0.999
Linearity (y = mx + b)		y = 0.974x + 3.33

A 50–200% theoretical potency series of 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.

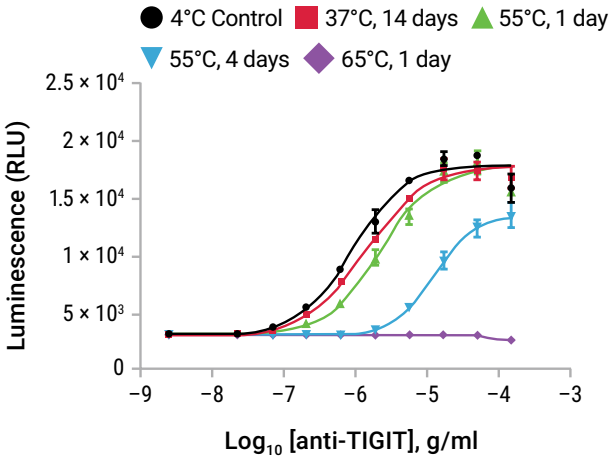


Figure 3. The TIGIT/CD155 Blockade Bioassay is stability-indicating. Samples of Control Ab, Anti-TIGIT, were maintained at 4°C (control) or heat-treated at the indicated temperatures and times, and then analyzed using the TIGIT/CD155 Blockade Bioassay. Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

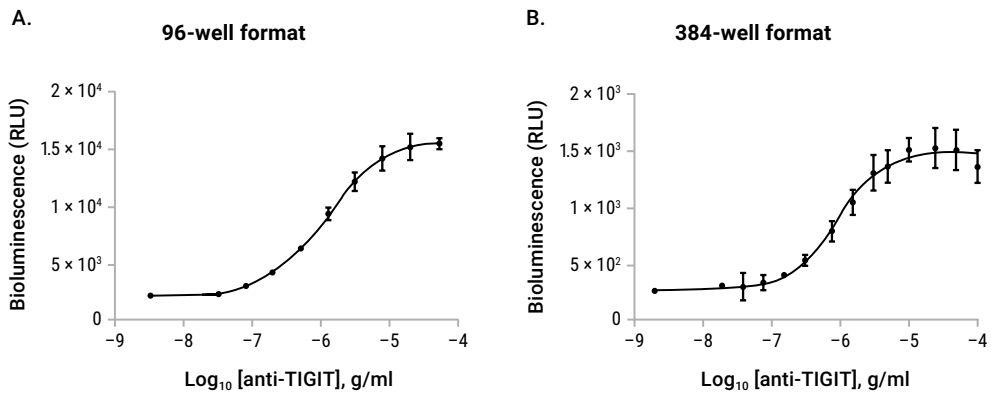


Figure 4. The assay is amenable to 384-well plate format and compatible with laboratory automation. Panel A. The TIGIT/CD155 Blockade Bioassay was performed in 96-well plates as described in this technical manual using Control Ab, Anti-TIGIT. **Panel B.** The TIGIT/CD155 Blockade Bioassay was performed in 384-well format using a Multidrop™ Combi nL (ThermoFisher) dispenser. TIGIT Effector Cells were plated at 6 × 10^4 cells/10µl/well. Next Control Ab, Anti-TIGIT, was serially diluted and added to the plate at 5µl/well. Finally, CD155 aAPC/CHO-K1 cells were added at 1.5 × 10^4 cells/5µl/well. After a 6-hour 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 1.1µg/ml and 0.9µg/ml, and the fold inductions were 8.0 and 6.1 for 96- and 384-well format, respectively. Data were generated using thaw-and-use cells.

1. Description (continued)

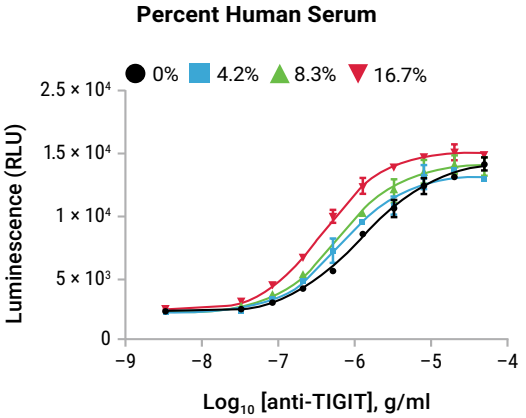


Figure 5. The TIGIT/CD155 Blockade Bioassay is tolerant to human serum. Control Ab, Anti-TIGIT, was analyzed in the absence or presence of increasing concentrations of pooled normal human serum (0–100% in the antibody sample), resulting in final assay concentration of human serum (0–16.7%). Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells. The TIGIT blockade assay is tolerant to serum with this human serum pool. A different human serum pool showed similar effects on the assay (data not shown).

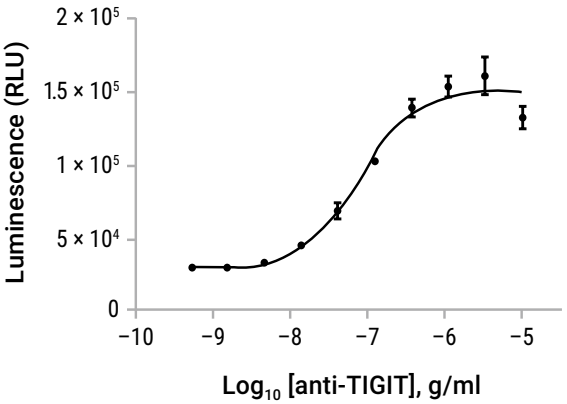


Figure 6. The TIGIT/CD155 Blockade Bioassay can be used to measure ADCC activity. A 1:1 ratio of TIGIT Effector Cells (used as Target cells in this application) and ADCC Effector Jurkat cells, available separately (Cat.# G7010), were incubated for 6 hours in the presence of a research-grade anti-TIGIT antibody (human IgG1 isotype). 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. Data were generated using thaw-and-use cells.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
TIGIT/CD155 Blockade Bioassay, Propagation Model	**1 each**	**J2092**

Not for Medical Diagnostic Use. Includes:

- 2 vials TIGIT Effector Cells (CPM), 2.3×10^7 cells/ml (1.0ml per vial)
- 2 vials CD155 aAPC/CHO-K1 cells (CPM), 1.2×10^7 cells/ml (1.0ml per vial)

Note: Thaw and propagate one vial to create frozen 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 decrease 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.

Note the catalog number and lot number from the cell vial box label. This information can be used to download documents for the specified product from the website such as Certificate of Analysis.

The TIGIT/CD155 Blockade Bioassay, Propagation Model, is intended to be used with user-provided antibodies or other biologics designed to block the interaction of TIGIT with its ligand, CD155. Control Ab, Anti-TIGIT and TIGIT Negative Cells (Cat.# J1921) are available separately for use in assay optimization and routine quality control. We strongly recommend including Control Ab, Anti-TIGIT, as a positive control in the first few assays to gain familiarity with the assay. Data generated using these reagents are shown in Figures 2–5 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 were established using Control Ab, Anti-TIGIT. You may need to adjust the parameters provided here and optimize assay conditions for your own antibody or biologic.

The TIGIT/CD155 Blockade Bioassay produces a bioluminescent signal and requires a sensitive luminometer or luminescence plate reader for the detection of luminescence. Bioassay development and performance data included in this Technical Manual were generated using the GloMax® Discover System. An integration time of 0.5 second/well was used for all readings.

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3.A. Materials to Be Supplied by the User

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

Reagents

- user-defined anti-TIGIT blocking antibodies 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 (e.g., HyClone Cat.# SH300070.03 or GIBCO Cat.# 16000044)
- hygromycin B (e.g., GIBCO® Cat.# 10687010)
- 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. 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 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 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 conical tube containing 9ml of prewarmed initial cell culture medium.
5. Centrifuge at 90 × *g* for 10 minutes.
6. Carefully aspirate the medium, and resuspend the cell pellet in 25ml of prewarmed initial cell culture medium.
7. Transfer the cell suspension to a T75 tissue culture flask, and place the flask horizontally in a 37°C, 5% CO_2 incubator.
8. Incubate for approximately 48 hours before passaging the cells.

Cell Maintenance and Propagation

Note: For cell maintenance and propagation starting from the second cell passage, use the cell growth medium containing antibiotics and monitor cell viability and doubling rate during propagation. The cell growth rate will stabilize by 7–10 days post-thaw, at which time cell viability is typically >90%, and the average cell doubling rate is 36–40 hours. Passage number should be recorded for each passage. In our experience, cells maintain their functionality for up to 25 passages, or 37 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 6×10^5 cells/ml if passaging every 2 days (e.g., Monday-Wednesday or Wednesday-Friday) or 4×10^5 cells/ml if passaging every 3 days (e.g., Friday-Monday). Always maintain the flasks in a horizontal position in the incubator.
11. Maintain the cell culture by adding fresh cell growth medium to the cell suspension in the original flask or by transferring the cells to a new flask while maintaining a consistent ratio of culture volume to flask surface area (e.g., 25ml volume per T75 flask or 50ml volume per T150 flask).
12. Place the flasks horizontally in a 37°C, 5% CO_2 incubator.

Cell Freezing and Banking

13. On the day of cell freezing, make new cell freezing medium and keep on ice.
14. Gently mix the cells with a pipette to create a homogenous cell suspension.
15. Remove a sample for cell counting by Trypan blue staining. Calculate the volume of cell freezing medium needed based on desired cell freezing densities of 5×10^6–5×10^7 cells/ml.
16. Transfer the cell suspension to 50ml sterile conical tubes or larger sized centrifuge tubes, and centrifuge at 130 × *g,* 4°C, 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 suspensions into a single tube and dispense into cryovials.
19. Freeze the cells using a controlled-rate freezer (preferred), or a Mr. Frosty® or a Styrofoam® rack in a –80°C freezer overnight. Transfer the vials to at or below –140°C for long-term storage.

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3.C. Preparing CD155 aAPC/CHO-K1 Cells

Cell Thawing

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 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 the cells (approximately 1ml) to the 50ml conical 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 flask, and place the flask horizontally in a 37°C, 5% CO_2 incubator.
8. Incubate the cells for approximately 24 hours before passaging.

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 below. Do not allow cells to become 100% confluent prior to 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 a new flask.
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 TIGIT/CD155 Blockade Bioassay to test two antibody samples against a reference sample in a single assay run. Each test and reference antibody is run in triplicate, in a 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 100µg/ml as a starting concentration (1X) and 2.5-fold dilution when testing Control Ab, Anti-TIGIT.

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

1. **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 4 ml 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 before 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 Control Ab, Anti-TIGIT, we tested. If you experience assay performance issues when using this assay buffer, we recommend testing different serum concentrations in the range of 0.5–10%.

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

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 assay. Equilibrate the Bio-Glo™ Luciferase Assay Buffer to ambient temperature, protected from light. Transfer all of the Bio-Glo™ Luciferase Assay Buffer into the amber bottle containing the Bio-Glo™ Luciferase Assay Substrate and mix by inversion until the Substrate is thoroughly dissolved. Equilibrate and store the reconstituted Bio-Glo™ Reagent at ambient temperature (22–25°C) protected from light before adding to assay plates.

   If you are using a large (100ml) size of Bio-Glo™ Luciferase Assay System, dispense the reconstituted Bio-Glo™ Reagent into 10ml aliquots and store at –20°C for up to 6 weeks. Avoid repeated freeze-thaw cycles. On the day of the assay, thaw an appropriate amount of reconstituted Bio-Glo™ Reagent in a room temperature water bath for at least 1–2 hours before use. Approximate stability of Bio-Glo™ reagent after reconstitution is an 18% loss of luminescence after 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 (250µl each) and one reference antibody (500µl) in 1.5ml tubes. Store the tubes containing antibody starting dilutions appropriately before making antibody serial dilutions.

   Notes:

   If you are using Control Ab, Anti-TIGIT, as a reference antibody in your assay, prepare 500µl of 200µg/ml starting dilution (dilu1, 2X final concentration) by adding 100µl of Control Ab, Anti-TIGIT stock (1mg/ml) to 400µl 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 prior to harvesting and plating cells.

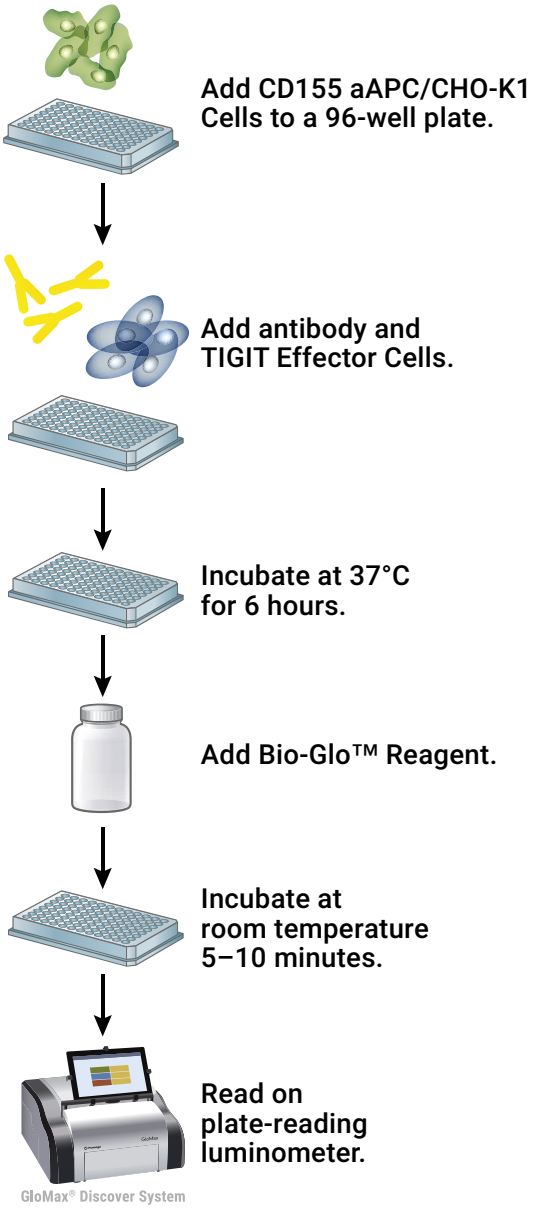


Figure 7. TIGIT/CD155 Blockade Bioassay schematic protocol.

4.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 two ten-point dose-response curves for each plate.

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

Figure 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.

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

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

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

1. We recommend passaging the 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 CD155 aAPC/CHO-K1 Cell Recovery Medium (Ham's F-12/ 10% FBS) for the CD155 aAPC/CHO-K1 Cells.
3. Aspirate the cell culture medium from the CD155 aAPC/CHO-K1 Cells and wash with DPBS.

4. Add 2ml of Accutase® solution to each T75 flask, and place the flask 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 CD155 aAPC/CHO-K1 Plating Medium to the flask. Transfer the cell suspension to a 50ml (or larger) conical centrifuge tube.
6. Gently mix and count the 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 preparation of a single stock of 2.5-fold serial dilutions of a single antibody for analysis in triplicate (250µl of each antibody dilution provides a sufficient volume for analysis in triplicate). Alternatively, you can prepare three independent stocks of serial dilutions to generate triplicate samples. To prepare 2.5-fold serial dilutions, you will need 500µl of reference antibody at 2X the highest antibody concentration in your dose-response curve. You will need 250µl of each test antibody at 2X the highest antibody concentration in each of the test antibody dose-response curves. For other dilution schemes, adjust the volumes accordingly.

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

1. On the day of assay, prepare an appropriate amount of assay buffer as described in Section 4.A.
2. To a sterile, clear V-bottom 96-well plate, add 250µl of reference antibody starting dilution (dilu1, 2X final concentration) to wells A11 and B11 (see Figure 9).
3. Add 250µl of test antibodies 1 and 2 starting dilution (dilu1, 2X final concentration) to wells E11 and G11, respectively (see Figure 9).
4. Add 150µl of assay buffer to other wells in these four rows, from column 10 to column 2.
5. Transfer 100µl of the antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
6. Repeat equivalent 2.5-fold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.
7. Cover the assay plate with a lid and keep at ambient temperature (22–25°C) while preparing the TIGIT Effector Cells.

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

4.D. Preparing Antibody Serial Dilutions (continued)

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 9. Example plate layout showing antibody serial dilutions.

4.E. Preparing TIGIT Effector Cells

While maintaining the 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 TIGIT Effector Cells by Trypan blue staining, and calculate the cell density and viability.
3. Transfer an appropriate amount of 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 3.75 × 10^6 cells/ml.
5. Count the cells again, and adjust the volume of assay buffer to achieve a final cell density of 3.75 × 10^6 cells/ml. You will need at least 6ml of TIGIT Effector Cells to fill 120 assay wells, or the inner 60 wells of two assay plates.

4.F. Adding Antibody Samples and TIGIT Effector Cells to Assay Plates

1. Remove the 96-well assay plates containing 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 CD155 aAPC/CHO-K1 Cells (from Step 1) according to the plate layout in Figure 8.
3. Add 80µl of assay buffer to the outside wells of the 96-well assay plates.
4. Transfer the TIGIT Effector Cells prepared in Section 4.E to a sterile reagent reservoir. Using a multichannel pipette, dispense 40µl (1.5×10^5 cells) of TIGIT Effector Cells into the wells containing 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 the 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	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 older models of luminometers with low sensitivity should be avoided. We recommend using a high gain setting if you are using a luminometer with an adjustable gain.
	Insufficient cells per well can lead to low RLU. Handle and plate the cells according to the instructions to ensure a sufficient number of viable cells per well.
	Low activity of Bio-Glo™ Reagent leads to low RLU. Store and handle the Bio-Glo™ Reagent according to the instructions.
Variability in assay performance	Assay performance can be affected by variations in cell growth conditions including plating and harvest density, centrifuge times and speeds, and freezing and/or DMSO exposure times during cell banking.
	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 one-day cell passages whenever possible, especially with the TIGIT Effector Cells. Ensure you are using high-quality cell culture reagents (especially serum) and plasticware for maintaining cells in culture. Ensure accurate and consistent cell counting methods.
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 TIGIT/CD155 Blockade Bioassay may vary from the EC_{50} 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 reader background RLU from all samples prior to calculating fold induction.

6. References

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7. Appendix

7.A. Representative Assay Results

The following data were generated with the TIGIT/CD155 Blockade Bioassay, Propagation Model, using Control Ab, Anti-TIGIT, (Figure 10).

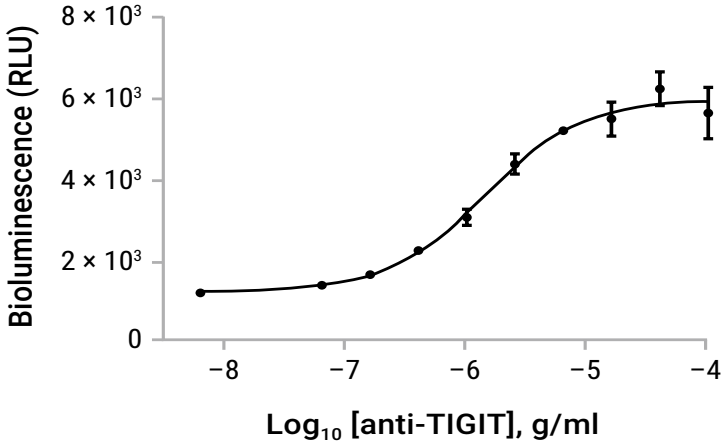


Figure 10. The TIGIT/CD155 Blockade Bioassay measures the activity of Control Ab, Anti-TIGIT. CD155 aAPC/CHO-K1 Cells were plated overnight. The following day, a titration of Control Ab, Anti-TIGIT, was added followed by TIGIT Effector Cells. After 6 hours, Bio-Glo™ Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. The EC_{50} value was 1.4µg/ml, and the fold induction was 5.1.

7.B. Composition of Buffers and Solutions

initial cell culture medium for TIGIT Effector Cells

90%	RPMI 1640 with L-glutamine and HEPES
10%	FBS

cell growth medium for TIGIT Effector Cells

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

cell freezing medium for TIGIT Effector Cells

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

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

90%	Ham's F-12
10%	FBS

cell growth medium for CD155 aAPC/CHO-K1 Cells

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

cell freezing medium for CD155 aAPC/CHO-K1 Cells

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

cell recovery medium for CD155 aAPC/CHO-K1

90%	Ham's F-12
10%	FBS

assay buffer

90%	RPMI 1640 with L-glutamine
10%	FBS

8. Summary of Changes

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

1. Removed Section 7.C, Related Products.
2. Made minor text edits.
3. Removed old patent statements.

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