TECHNICAL MANUAL

LAG-3/MHCII Blockade Bioassay, Propagation Model

Instructions for Use of Product
JA1112

7/19
TM571

LAG-3/MHCII Blockade 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

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 they 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 is an effective strategy to enhance anti-tumor immune responses and promote immune-mediated tumor rejection (1, 2).

LAG-3, also known as CD223, is an immune checkpoint receptor expressed on activated $CD4^+$ and $CD8^+$ T cells, and natural killer (NK) cells (3). Expression of LAG-3 is higher on regulatory T cells compared to conventional $CD4^+$ T cells (4). The best characterized ligand for LAG-3 is major histocompatibility complex II (MHCII) (5), although alternate ligands, including fibrinogen-like protein 1 (FGL1), have been described (6, 7). Crosslinking of LAG-3 inhibits calcium signaling from the T-cell receptor (TCR) complex and reduces cytokine production and proliferation of activated T cells (8). While the molecular mechanisms of T-cell inhibition are not clear, a cytoplasmic KIEELE motif is required for inhibitory function in T cells (9).

There are no easy-to-use functional LAG-3 bioassays available to measure the in vitro potency of biologic drugs under development and in early clinical trials. Quantitative bioassays are needed in the development of biologic drugs targeting LAG-3. Current methods used to measure the activity of drugs targeting LAG-3/MHCII rely on primary human T cells and measurement of functional endpoints such as cell proliferation, cell surface marker expression and cytokine production. These assays typically require the use of *Staphylococcus aureus* enterotoxins (e.g., SEB) which exhibit significant batch-to-batch variability, and have limited availibility and usage restrictions due their classification as "select agents" by the United States Department of Health and Human Services. These assays are also laborious and highly variable due to their reliance on donor primary cells, complex assay protocols and unqualified assay reagents. As a result, these assays are difficult to establish in quality-controlled drug development settings. Preclinical cancer studies suggest that blocking LAG-3 re-activates the immune system to kill tumor cells. Several LAG-3 blocking antibodies are already in clinical trials for cancer indications.

The LAG-3/MHCII Blockade Bioassay, Propagation Model[a–c] (Cat.# JA1112), is a bioluminescent cell-based assay that overcomes the limitations of existing assays. It can be used to measure the potency and stability of antibodies and other biologics targeting LAG-3/MHCII (10, 11). The assay consists of a genetically engineered T-cell line paired with an MHCII-positive cell line:

- **LAG-3 Effector Cells:** Jurkat T cells expressing human LAG-3 and a luciferase reporter driven by T-cell activation pathway-dependent response elements
- **MHCII APC Cells:** MHCII-positive human cell line

The LAG-3 Effector Cells and MHCII APC Cells are provided in Cell Propagation Model (CPM) format, which includes cryopreserved cells that can be thawed, propagated and banked for long-term use.

In addition, TCR Activating Antigen is available separately. The TCR Activating Antigen is a proprietary peptide that is presented by MHCII on the MHCII APC Cells to specifically activate the TCR on the LAG-3 Effector Cells.

When the two cell types are co-cultured in the presence of TCR Activating Antigen (Ag), MHCII on the APC cells presents the Ag to the TCR on the LAG-3 Effector Cells to activate the Effector Cells. LAG-3 on the Effector Cells inhibits TCR-induced activation and promoter-mediated luminescence. Addition of an anti-LAG-3 blocking antibody releases LAG-3-mediated inhibition and results in increased promoter-mediated luminescence (Figure 1). The bioluminescent signal is quantified using the Bio-Glo™ Luciferase Assay System (Cat.# G7941), and a standard luminometer such as the GloMax® Discover System (see Section 7.C, Related Products).

In addition to the LAG-3/MHCII Blockade Bioassay, we offer Control Ab, Anti-LAG-3 (Cat.# K1150) blocking antibody for use as a positive control.

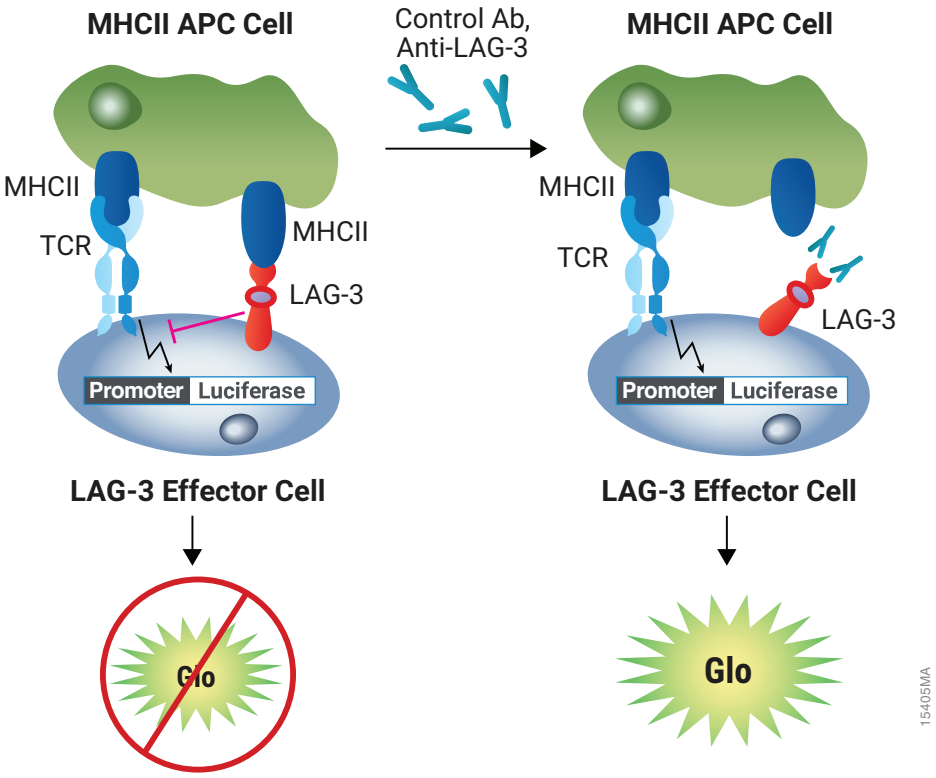

Figure 1. Representation of the LAG-3/MHCII Blockade Bioassay. The bioassay consists of two cell lines, LAG-3 Effector Cells and MHCII APC Cells. When co-cultured, LAG-3 inhibits TCR pathway-activated luminescence. The addition of anti-LAG-3 antibody blocks LAG-3 binding to MHCII, resulting in full TCR pathway activation, which can be detected in a dose-dependent manner by addition of Bio-Glo™ Reagent and quantitation with a luminometer.

1. Description (continued)

The LAG-3/MHCII Blockade Bioassay, Propagation Model reflects the mechanism of action (MOA) of biologics designed to block LAG-3/MHCII interactions. Specifically, TCR activation-mediated luminescence is enhanced following the addition of anti-LAG-3 blocking antibody but not following addition of anti-PD-1 (nivolumab), anti-CTLA-4 (ipilimumab) or anti-TIGIT blocking antibodies (Figure 2). The bioassay is reliant on antigen-dependent activation of the LAG-3 Effector Cells. Therefore, it is **critical** to add the TCR Activating Antigen (Cat.# K1201) to the MHCII APC Cells when plating (Figure 3). According to International Council for Homogenization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines, this bioassay is prequalified and shows the precision, accuracy and linearity required for routine use in potency and stability studies (Table 1 and Figure 4). The workflow is simple, robust and compatible with both 96-well and 384-well plate formats used for antibody screening in early drug discovery (Figure 5), and can be performed in a two-day timeframe. 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 6), 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 LAG-3/MHCII Blockade Bioassay is the ability to measure ADCC activity of anti-LAG-3 antibodies by combining ADCC Reporter Bioassay Effector Cells, available separately (Cat.# G7102), with LAG-3 Effector Cells (Figure 7).

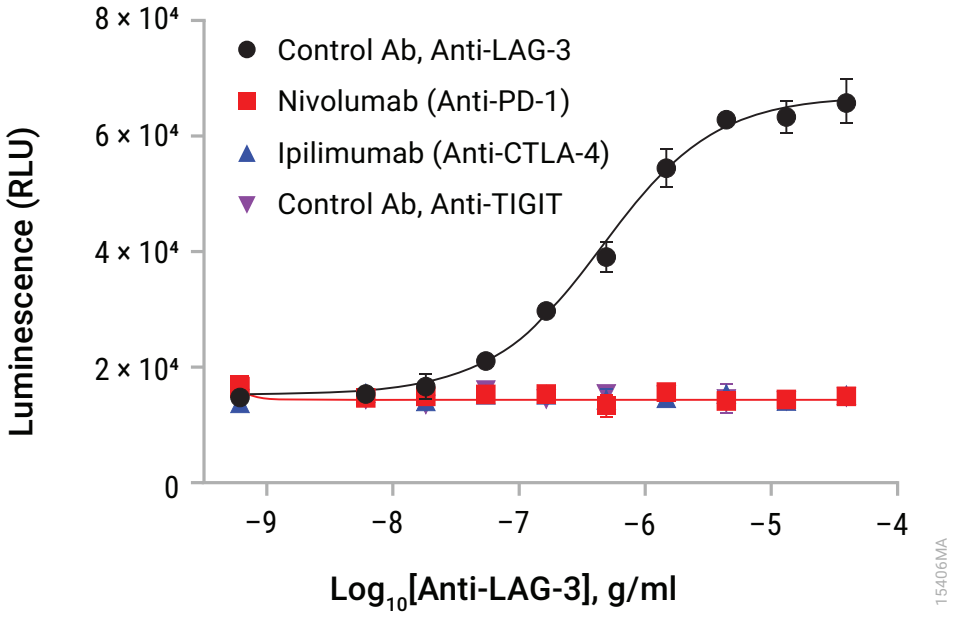

Figure 2. The LAG-3/MHCII Blockade Bioassay reflects the mechanism of action (MOA) and specificity of biologics designed to block the LAG-3/MHCII interaction. LAG-3 Effector Cells were incubated with TCR Activating Antigen and MHCII APC Cells in the presence of serial titrations of blocking antibodies Control Ab, Anti-LAG-3, anti-PD-1, anti-CTLA-4 or anti-TIGIT, as indicated. After a 5-hour induction, Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover Detection System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

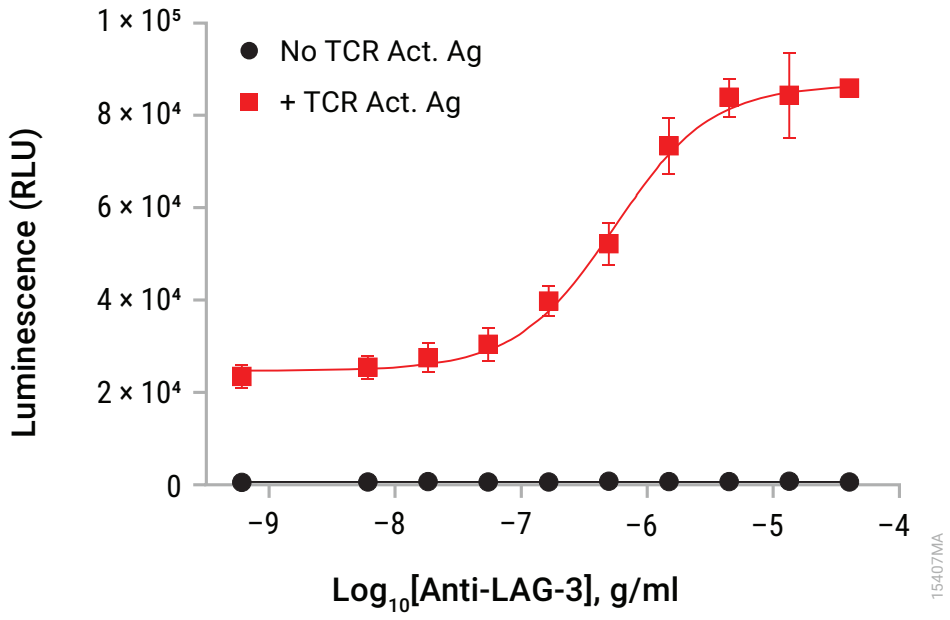

Figure 3. The LAG-3/MHCII Blockade Bioassay is dependent on TCR Activating Antigen. MHCII APC Cells were plated with and without TCR Activating Antigen. The next day, a titration of Control Ab, Anti-LAG-3 was plated followed by addition of LAG-3 Effector Cells. After a 5-hour induction, Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover Detection 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)

Table 1. The LAG-3/MHCII Blockade Bioassay Shows Precision, Accuracy and Linearity.

Parameter	Results	
Accuracy	% Expected Relative Potency	% Recovery
	50	94.4
	70	94.8
	100	100.3
	140	103.6
	200	99.8
Repeatability (% CV)	100% (Reference)	7.5
Intermediate Precision (% CV)		9.7
Linearity (r^2)		0.998
Linearity (y = mx + b)		y = 1.03x – 3.65

A 50–200% theoretical potency series of Control Ab, Anti-LAG-3 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.

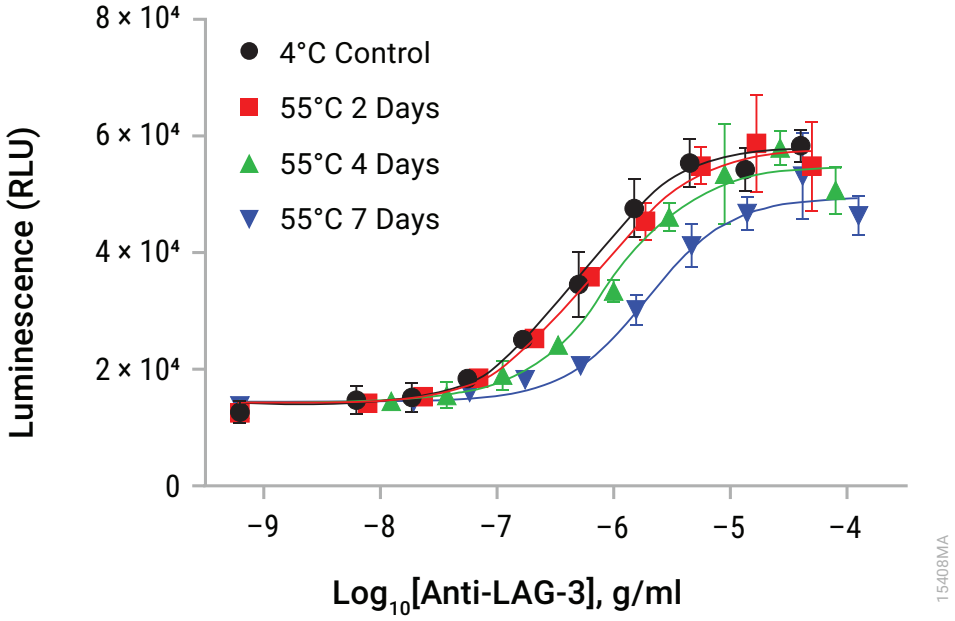

Figure 4. The LAG-3/MHCII Blockade Bioassay indicates antibody stability. Samples of Control Ab, Anti-LAG-3 were maintained at 4°C (control) or heat-denatured at 55°C for the indicated times, then analyzed using the LAG-3/MHCII Blockade Bioassay. Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover Detection 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)

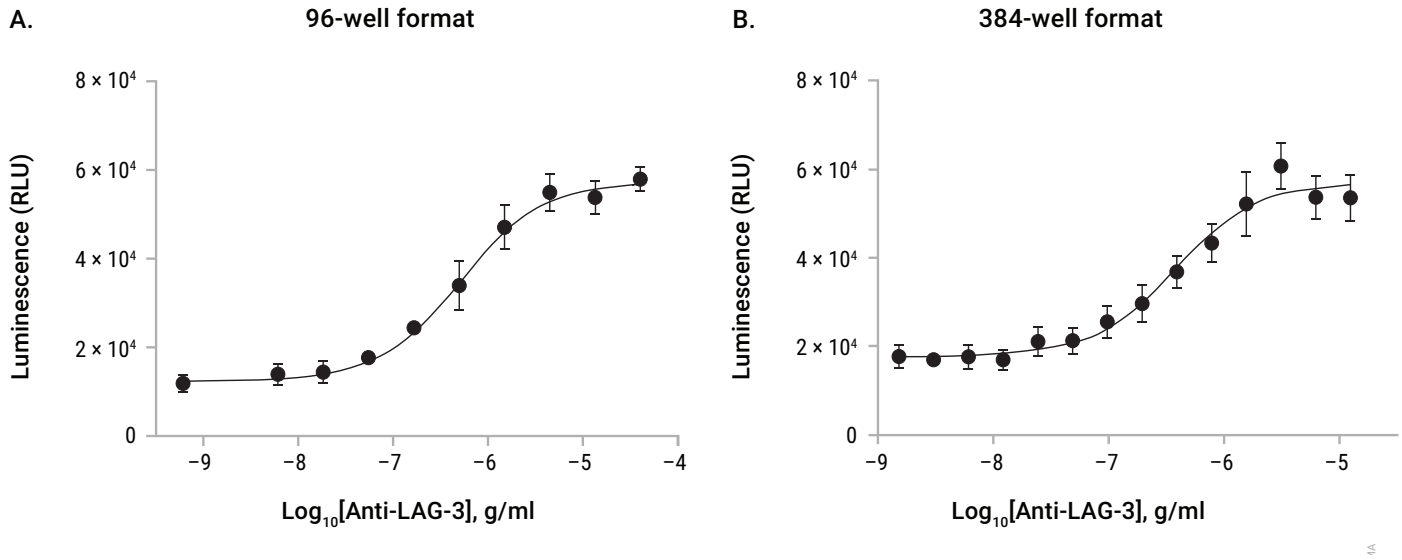

Figure 5. The LAG-3/MHCII Blockade Bioassay is amenable to 384-well plate format. Panel A: The LAG-3/MHCII Blockade Bioassay was performed in 96-well plates as described in this technical manual with a titration of Control Ab, Anti-LAG-3. **Panel B:** The LAG-3/MHCII Blockade Bioassay was performed in 384-well format as briefly described here. TCR Activating Antigen at 1X and MHCII aAPC Cells at 3×10^3 cells/15µl/well were plated in RPMI + 10% FBS in a 384-well white assay plate and incubated overnight at 37°C, 5% CO_2. Concentrated (5X) Control Ab, Anti-LAG-3 (Cat.# K1150) was serially diluted and added to the plate at 5µl/well. LAG-3 Effector Cells were then added at 2×10^4 cells/5µl/well. After a 6-hour incubation, 25µl Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover Detection System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. The EC_{50} values were 500ng/ml and 380ng/ml, and the fold inductions were 4.8 and 3.2 for 96-well and 384-well formats, respectively. Data were generated using thaw-and-use cells.

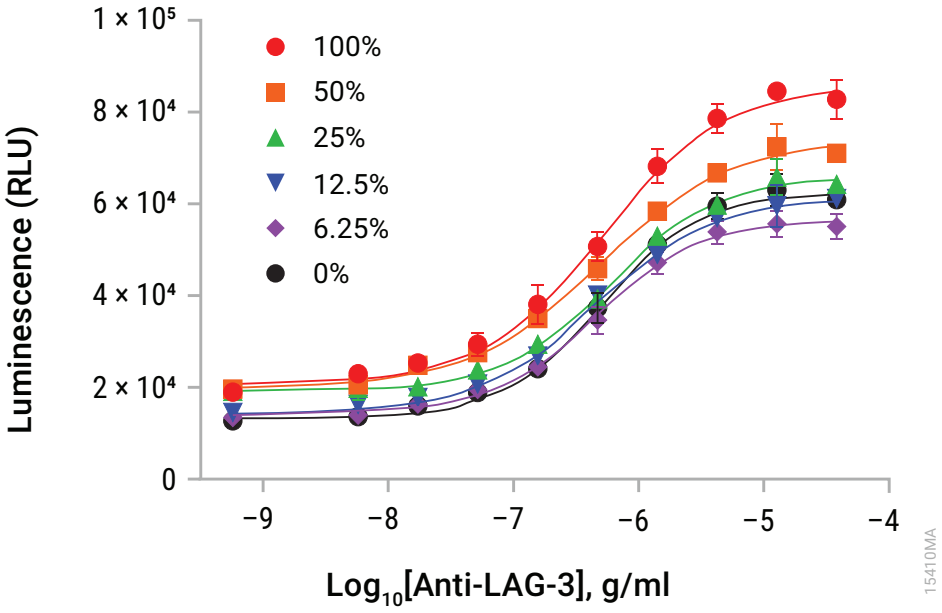

Figure 6. The LAG-3/MHCII Blockade Bioassay tolerates human serum. Control Ab, Anti-LAG-3 was analyzed in the absence or presence of increasing concentrations of pooled normal human serum (0–100% in the antibody sample). Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover Detection System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. The LAG-3 Blockade 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.

1. Description (continued)

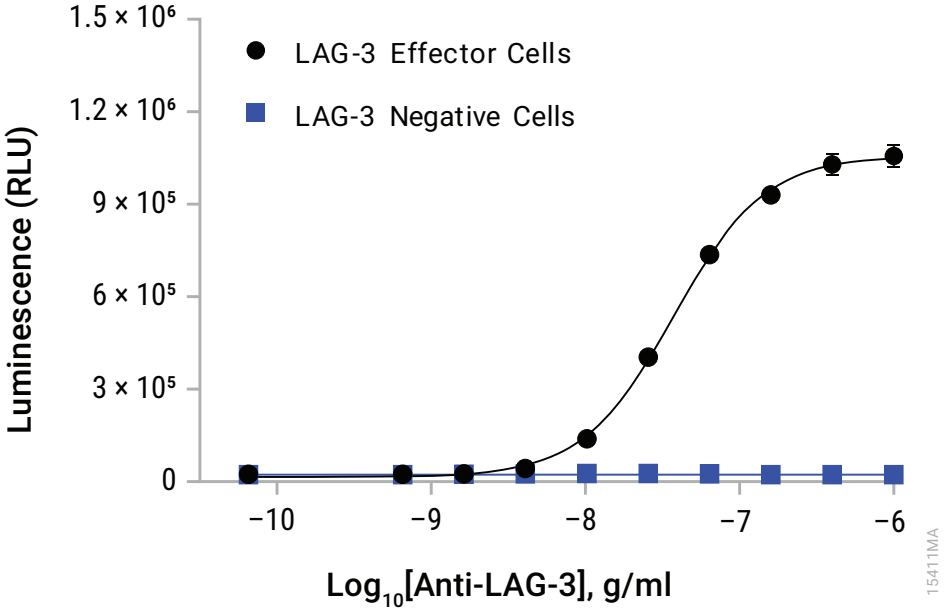

Figure 7. The LAG-3/MHCII Blockade Bioassay can be used to measure ADCC activity. A 1:1 ratio of LAG-3 Effector Cells (used as target cells in this application) or LAG-3-negative Jurkat cells (Cat.# J1921) and mADCC Effector Cells (mouse FcγRIII) available separately, were incubated for 6 hours in the presence of a titration of Control Ab, Anti-LAG-3 (Cat.# K1150). Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover Detection 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.#
LAG-3/MHCII Blockade Bioassay, Propagation Model	**1 each**	**JA1112**

Not for Medical Diagnostic Use.

Includes:

- 2 vials LAG-3 Effector Cells (CPM), 3.3×10^7 cells/ml (1.0ml per vial)
- 2 vials MHCII APC Cells (CPM), 6×10^6 cells/ml (1.0ml per vial)
- 500µl TCR Activating Antigen Stock Solution

Note: Thaw and propagate one vial per cell line 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 as this will negatively impact cell viability and cell performance.

Note: Prior to storage, remove the product label from the box containing the cell vials 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 Promega web site, such as the Certificate of Analysis.

3. Before You Begin

The LAG-3/MHCII Blockade Bioassay, Propagation Model is intended for use with user-provided antibodies or other biologics designed to block the interaction of LAG-3/MHCII. Control Ab, Anti-LAG-3 (Cat.# K1150) is available separately for use in assay optimization and routine quality control. We strongly recommend including Control Ab, Anti-LAG-3 as a positive control in the first few assays to gain familiarity with the assay. Data generated using these reagents is shown in Section 7.A, Representative Assay Results.

Please read through the entire protocol to become familiar with the components and the assay procedure before beginning. 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-LAG-3. You may need to adjust the parameters provided here and optimize assay conditions for your own antibody or other biologic samples.

The LAG-3/MHCII Blockade Bioassay produces a bioluminescent signal and requires a luminometer or sensitive luminescence plate reader. 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 second/well was used for all readings. The bioassay is compatible with most other plate-reading luminometers, though relative luminescence unit readings will vary with the sensitivity and settings of each instrument. If you are using a reader with adjustable gain, we recommend a high-gain setting. The use of different instruments, gain adjustment and shorter or longer assay times will affect the magnitude of the raw data, but should not affect the measured relative potency of test samples.

3.A. Materials to Be Supplied by the User

Reagents

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

- user-defined anti-LAG-3/MHCII blocking antibodies or other biologics samples
- RPMI 1640 Medium with L-glutamine and HEPES (e.g., Corning Cat.# 10-041-CV or Gibco Cat.# 22400)
- DMEM Medium with high glucose, sodium pyruvate and L-glutamine (e.g., Gibco Cat.# 11995)
- fetal bovine serum (e.g., VWR Cat.# 89510-194, HyClone Cat.# SH30070.03 or Gibco Cat.# 35-015-CV)
- hygromycin B (e.g., Gibco Cat.# 10687-010)
- Blasticidin S HCl (e.g., ThermoFisher Cat.# A1113903)
- 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)
- TCR Activating Antigen Stock Solution (Cat.# K1201)
- Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941)

Supplies and Equipment

- white, flat-bottom, tissue-culture-treated 96-well assay plates (e.g., Corning Cat.# 3917) or 384-well assay plates (e.g., Corning Cat.# 3570) for plating and reading luminescence
- 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 system)

3.B. Preparing LAG-3 Effector Cells

Cell Thawing and Initial Cell Culture

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

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 LAG-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 place the flask horizontally in a 37°C, 5% CO_2 humidified incubator.
8. Incubate for approximately 24 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 after thawing, at which time cell viability is typically >90%, and the average cell doubling rate is 28 hours. Passage number should be recorded for each passage. In our experience, cells maintain their functionality for up to 25 passages or 50 cell doublings if passaging is performed on a Monday-Wednesday-Friday schedule.

1. On the day of cell passage, measure cell viability and density by Trypan blue staining.
2. Seed the cells at a density of 5×10^5 cells/ml if passaging every 2 days (e.g., Monday-Wednesday or Wednesday-Friday) or 3×10^5 cells/ml if passaging every 3 days (e.g., Friday-Monday).
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 horizontally in a 37°C, 5% CO_2 humidified incubator.

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 homogeneous 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 5×10^6–3×10^7 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, taking care not to disturb the cell pellet.
6. Carefully resuspend the cell pellet in ice-cold cell freezing medium to a final cell density of 5×10^6–3×10^7 cells/ml. Combine the cell suspensions into 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 long-term storage at or below –140°C.

3.C. Preparing MHCII APC Cells

Cell Thawing

1. Warm 50ml of cell culture medium to 37°C.
2. Transfer 9ml of prewarmed initial cell culture medium to a 50ml conical tube.
3. Remove one vial of MHCII APC 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 180 × *g* for 8 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 humidified 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, monitor cell viability and doubling rate during propagation. It is recommended that cells are not grown past 25 passages after vials are thawed. Maintain the cell density in the range of 5.0×10^3–2.5×10^5 cells/cm^2 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 18 hours when seeded at the densities listed in the table below. Passage number should be recorded for each passage. We recommend Accutase® for routine cell culturing and cell preparation for the assay.

Cell Passage Schedule	Cell Seeding Density
2 days	4×10^4 cells/cm^2
3 days	1.3×10^4 cells/cm^2
4 days	5.3×10^3 cells/cm^2

1. On the day of cell passage, carefully aspirate the cell culture medium and wash the cells with DPBS.
2. Add 2ml of Accutase® to each T75 flask (scale up for larger culture flasks) and place in a 37°C, 5% CO_2 humidified incubator for 5–7 minutes or until the cells round up and detach from the bottom of the flask.
3. Add 8ml of cell culture medium to each T75 flask. Transfer the cell suspension to a sterile 50ml (or larger) conical tube.
4. 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).
5. Add an appropriate amount of cell growth medium to achieve the desired cell seeding density per area.

6. Transfer the appropriate volume of cell suspension to new flasks.
7. Place the flasks in a 37°C, 5% CO_2 humidified incubator. Incubate the cells for 48–96 hours before passaging them.

Cell Freezing and Banking

1. On the day of cell freezing, make new cell-freezing medium and keep on ice.
2. Carefully aspirate the cell culture medium, and wash the cells with DPBS.
3. Add 2ml of Accutase® to each T75 flask, and place in a 37°C, 5% CO_2 humidified incubator for 5–7 minutes or until the cells round up and detach from the bottom of the flask.
4. Add 8ml of cell culture medium to each T75 flask. Transfer the cell suspension to a 50ml (or larger) conical centrifuge tube.
5. Count the cells by Trypan blue staining.
6. Centrifuge at 180 × *g* for 10 minutes.
7. Gently resuspend the cell pellet in ice-cold freezing medium to a final cell density of 3×10^6–3×10^7 viable cells/ml. Combine the cell suspensions in a single tube and dispense into cryovials.
8. 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.

3.D. Preparing TCR Activating Antigen

TCR Activating Antigen Stock Solution (Cat.# K1201; must be purchased separately) is supplied as a 330X concentrate in PBS + 0.05% BSA. We recommend preparing single-use aliquots and storing at –20°C. **Do not** dilute TCR Activating Antigen Stock Solution for long-term storage. Avoid freeze/thawing of the Antigen Stock Solution. Undiluted TCR Activating Antigen Stock Solution can be stored at –20°C for up to 1 year with no change in performance.

4. Assay Protocol

This assay protocol illustrates the use of the LAG-3/MHCII 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 40µg/ml of Control Ab, Anti-LAG-3 as a starting concentration (1X) and threefold serial dilution.

4. Assay Protocol (continued)

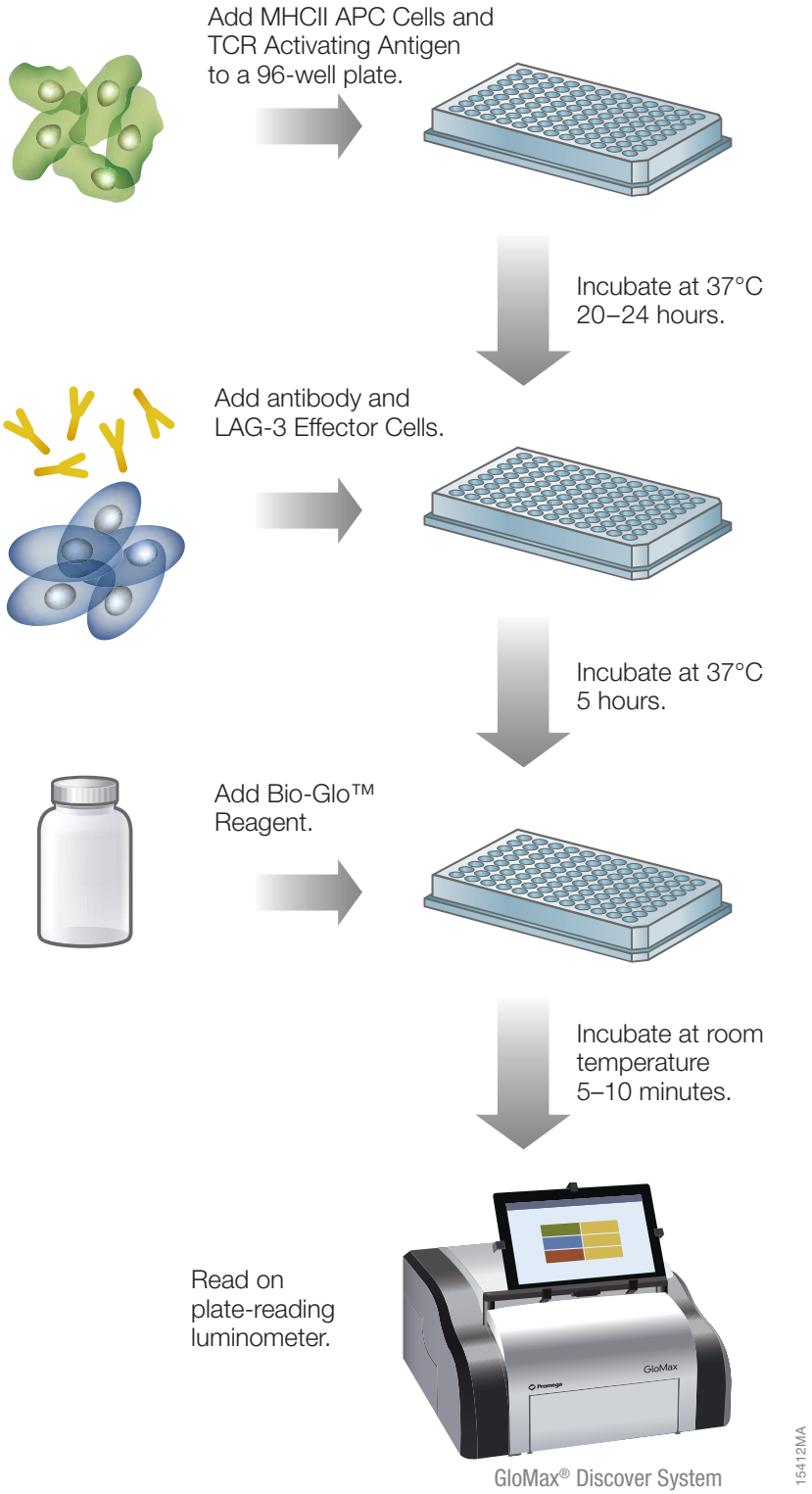

Figure 8. Schematic protocol for the LAG-3/MHCII Blockade Bioassay.

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

1. **Cell Plating Medium:** On the day before the assay, prepare an appropriate amount of fresh Cell Plating Medium (90% DMEM/10% FBS). Mix well and warm to 37°C prior to use. For reference, 25ml of Cell Plating Medium is typically sufficient for 120 wells in a 96-well assay format using the inner 60 wells.

2. **TCR Activating Antigen:** Once the MHCII APC Cells are prepared at 2×10^5 cells/ml in Cell Plating Medium, add the TCR Activating Antigen Stock Solution (Cat.# K1201) by diluting 330-fold into Cell Plating Medium containing MHCII APC Cells (e.g., for two assay plates in inner 60-well format, add 45µl TCR Antigen Stock Solution into 15ml MHCII APC Cells/Cell Plating Medium). Mix well by gentle inversion.

3. **Assay Buffer:** On the day of the assay, prepare an appropriate amount of assay buffer (90% RPMI 1640/10% FBS). 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-LAG-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%.

4. **Test and Reference Samples:** Using assay buffer as the diluent, prepare starting dilutions (dilu1, 2X final concentration) of two test antibodies (240µl each) and one reference antibody sample (480µl) in 1.5ml tubes. Store the tubes containing antibody starting dilutions appropriately before making antibody serial dilutions.

   Note: If you are using Control Ab, Anti-LAG-3 as a reference antibody in your assay, prepare 480µl of 80µg/ml starting dilution (dilu1, 2X final concentration) by adding 38µl of Control Ab, Anti-LAG-3 stock (1mg/ml) to 442µl of assay buffer.

5. **Bio-Glo™ Luciferase Assay System:** 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. When stored appropriately, Bio-Glo™ Reagent will lose 18% activity after 24 hours at ambient temperature. For reference, 10ml of Bio-Glo™ Reagent is sufficient to assay 120 wells in a 96-well assay format.

4.B. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 9 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 9. 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 MHCII APC Cells

While maintaining the MHCII APC Cells, follow the recommended cell seeding density because 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.

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

TCR Activating Antigen must be added to the MHCII APC Cells prior to plating cells in the assay plate.

1. We recommend passaging the MHCII APC Cells 2 days before plating the assay as described in Section 3.C. to ensure optimal and consistent assay performance.
2. On the day before performing the assay, prepare fresh Cell Plating Medium (90% DMEM/10% FBS) for the MHCII APC Cells.
3. Carefully aspirate the cell culture medium from the MHCII APC Cells and wash with DPBS.

4. Add 2ml of Accutase® to each T75 flask, and place in a 37°C, 5% CO_2 humidified incubator for 5–7 minutes or until the cells round up and detach from the bottom of the flask.
5. Add 8ml of Cell Plating Medium to the flask. Transfer the cell suspension to a 50ml (or larger) conical centrifuge tube.
6. Centrifuge at 180 × *g* for 8 minutes.
7. Carefully aspirate the media and gently resuspend the cell pellet in 10ml cell plating medium per T75 flask.
8. Gently mix and count the MHCII APC Cells by Trypan blue staining.
9. Dilute the MHCII APC Cells in Cell Plating Medium to achieve a concentration of 2×10^5 viable cells/ml.
10. Remove an aliquot of TCR Activating Antigen Stock Solution (330X concentrated) from storage and thaw on the bench top. Once thawed, mix the solution by gently vortexing.
11. **Add TCR Activating Antigen 330-fold into the MHCII APC Cell suspension to achieve a final concentration of 1X. Mix well by gently inverting the tube.**
12. 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 2×10^4 cells/well.
13. Add 100µl of Cell Plating Medium to each of the outside wells of the assay plates.
14. Cover the assay plates with a lid and incubate the cells overnight (20–24 hours) in a 37°C, 5% CO_2 humidified incubator.

4.D. Preparing Antibody Serial Dilutions

The instructions described here are for preparing a single stock of threefold serial dilutions of a single antibody for analysis in triplicate (160µl 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 threefold serial dilutions, you will need 480µl of reference antibody at 2X the highest antibody concentration in your dose-response curve. You will need 240µl of each test antibody at 2X the highest antibody concentration in each of the test antibody dose-response curves. For other dilution schemes, adjust the volumes accordingly.

Note: If you are using Control Ab, Anti-LAG-3 (Cat.# K1150), as a control in the assay, follow the instructions below to prepare threefold serial dilutions.

1. On the day of the assay, prepare an appropriate amount of assay buffer as described in Section 4.A.
2. To a sterile, clear V-bottom 96-well plate, add 240µl of reference antibody starting dilution (dilu1, 2X final concentration) to wells A11 and B11 (see Figure 10).
3. Add 240µl of test antibodies 1 and 2 starting dilution (dilu1, 2X final concentration) to wells E11 and G11, respectively (see Figure 10).
4. Add 160µl of assay buffer to other wells in these four rows, from column 10 to column 2.
5. Transfer 80µl of the antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
6. Repeat equivalent threefold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.

   Note: Wells A2, B2, E2 and G2 contain 160µl of assay buffer without antibody as a negative control.
7. Cover the antibody dilution plate with a lid and keep at ambient temperature (22–25°C) while preparing the LAG-3 Effector Cells.

Recommended Plate Layout for Antibody Dilutions Prepared from a Single Antibody Stock													
	1	2	3	4	5	6	7	8	9	10	11	12	
A		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference Ab
B		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference Ab
C													
D													
E		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test Ab 1
F													
G		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test Ab 2
H													

Figure 10. Example plate layout showing antibody serial dilutions.

4.E. Preparing LAG-3 Effector Cells

While maintaining the LAG-3 Effector Cells, follow the recommended cell seeding density because 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 2 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 LAG-3 Effector Cells by Trypan blue staining, and calculate the cell density and viability.
3. Transfer an appropriate amount of LAG-3 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 6.5ml of LAG-3 Effector Cells to fill 120 assay wells, or the inner 60 wells of two assay plates.

4.F. Adding Antibody Samples and LAG-3 Effector Cells to Assay Plates

1. Remove the 96-well assay plates containing MHCII APC Cells from the incubator. Invert the assay plates above a sink to remove the medium. Then, place the inverted plate on paper towels for 5–10 seconds to drain any remaining medium from each of the wells. **Do not** use a vacuum aspirator to remove the media.
2. Using an electronic multichannel pipette, add 40µl of the appropriate antibody dilution (see Figure 10) to the pre-plated MHCII APC Cells according to the plate layout in Figure 9.
3. Transfer the LAG-3 Effector Cells prepared in Section 4.E. to a sterile reagent reservoir. Using a multichannel pipette, dispense 40µl (1×10^5 cells) of LAG-3 Effector Cells into the wells containing MHCII APC Cells and antibody.
4. Add 80µl of assay buffer to each of the outside wells of the assay plates.
5. Cover the assay plates with a lid and place in a 37°C, 5% CO_2 humidified incubator for 5 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. Following the 5-hour incubation time, 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 5–10 minutes.

Note: Varying the incubation time will affect the raw luminescence 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. Measure plate background by calculating the average relative light units (RLU) from wells B1, C1 and D1.
2. Calculate fold induction = RLU (induced–background)/RLU (no antibody control–background).
3. Plot 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**. E-mail: **techserv@promega.com**

Symptoms	Possible Causes and Comments
Low luminescence measurements (RLU readout)	Add the optimal concentration of the TCR Activating Antigen Stock Solution when plating the MHCII APC Cells. Failure to add the TCR Activating Antigen or addition of a lower concentration of Antigen than recommended will result in very low RLU numbers and no assay activity.
	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 Relative Light Units numbers will vary between instruments.
	Insufficient cells per well can lead to low luminescence signal. Handle and plate cells according to the instructions to ensure a sufficient number of viable cells per well.
	Low cell viability can lead to low luminescence readout and variability in assay performance.
	Low activity of Bio-Glo™ Reagent leads to low luminescence signal. Store and handle the Bio-Glo™ Reagent according to the instructions.
Weak assay response (low fold induction)	Optimize the concentration range of your test sample(s) to achieve a full dose response with complete upper and lower asymptotes. The EC_{50} value obtained in the LAG-3/MHCII Blockade Bioassay may vary from the EC_{50} obtained using other methods such as primary cell-based assays.
	For users with sensitive luminometers, lowering the amount of TCR Activating Antigen added can increase the assay window (fold induction). Users should titrate the amount of Antigen added to the assay to ensure the RLU readout is within the detection capability and linear range of the luminometer.
	The LAG-3/MHCII Blockade Bioassay may be sensitive to FBS. We recommend screening several sources of FBS to ensure a consistent supply of high-quality FBS for culturing the cells and performing the bioassay.

5. Troubleshooting (continued)

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 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 LAG-3 Effector Cells. 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.

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7. Appendix

7.A. Representative Assay Results

The following data were generated using the LAG-3/MHCII Blockade Bioassay, Propagation Model (Cat.# JA1112), using Control Ab, Anti-LAG-3 (Cat.# K1150) (Figure 11).

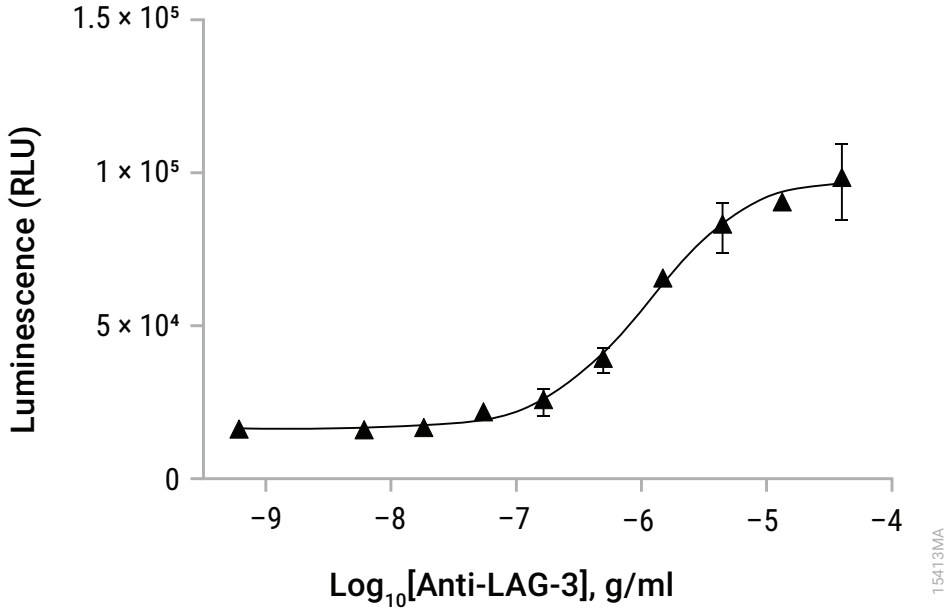

Figure 11. The LAG-3/MHCII Blockade Bioassay measures the activity of Control Ab, Anti-LAG-3. MHCII APC Cells and TCR Activating Antigen were plated overnight. The following day, a titration of Control Ab, Anti-LAG-3 (Cat.# K1150) was added followed by LAG-3 Effector Cells. After 5 hours, Bio-Glo™ Reagent 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 1.1µg/ml and the fold induction was 6.0.

7.B. Composition of Buffers and Solutions

Initial Cell Culture Medium for LAG-3 Effector Cells

90% RPMI 1640 with L-glutamine
10% FBS

Cell Growth Medium for LAG-3 Effector Cells

90% RPMI 1640 with L-glutamine
10% FBS
400µg/ml hygromycin B
10µg/ml blasticin S HCl

Cell Freezing Medium for LAG-3 Effector Cells

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

Cell Growth Medium for MHCII APC Cells

90% DMEM
10% FBS

Cell Freezing Medium for MHCII APC Cells

85% DMEM
10% FBS
5% DMSO

Cell Plating Medium for MHCII APC Cells

90% DMEM
10% FBS

Assay Buffer

90% RPMI 1640 with L-glutamine
10% FBS

7.C. Related Products

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 (NFAT), Propagation Model	1 each	J1601
T Cell Activation Bioassay (IL-2)	1 each	J1651
T Cell Activation Bioassay (IL-2) 5X	1 each	J1655
T Cell Activation Bioassay (IL-2), Propagation Model	1 each	J1631

Not for Medical Diagnostic Use.

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
Control Ab, Anti-PD-1	1 each	J1201
CTLA-4 Blockade Bioassay	1 each	JA3001
CTLA-4 Blockade Bioassay 5X	1 each	JA3005
Control Antibody, Anti-CTLA-4	100 µg	JA1020
TIGIT Negative Cells	1 each	J1921
PD-1+TIGIT Combination Bioassay	1 each	J2211
PD-1+TIGIT Combination Bioassay, 5X	1 each	J2215
Control Ab, Anti-TIGIT	100µg	J2051

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

Promega

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 are available.

Detection Reagent

Product	Size	Cat.#
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

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Note: Additional Immunotherapy and Fc Effector bioassays are available from: **https://www.promega.com/applications/biologics-drug-discovery/functional-bioassays/** or e-mail: **CAS@promega.com.**

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