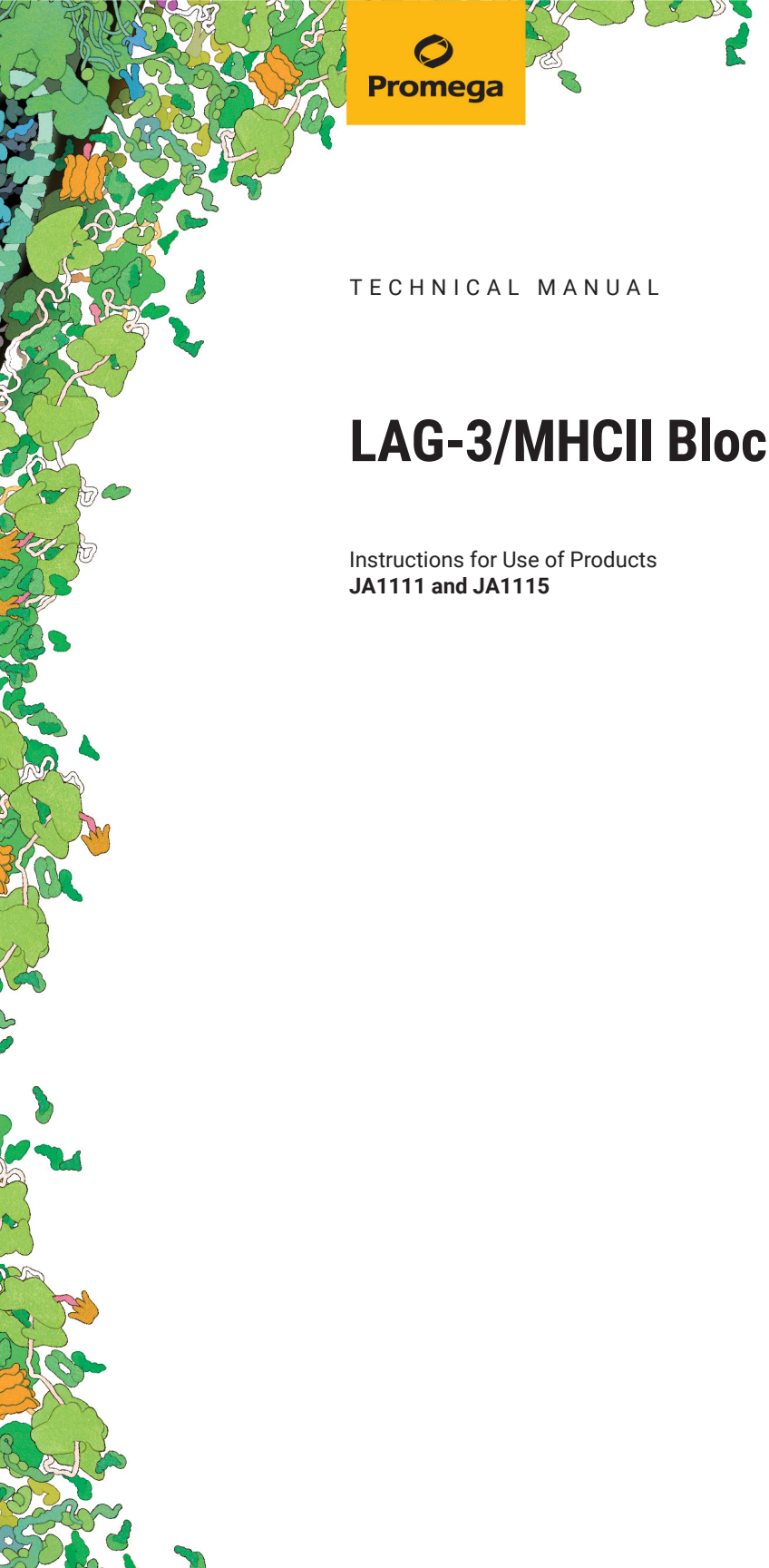

TECHNICAL MANUAL

LAG-3/MHCII Blockade Bioassay

Instructions for Use of Products
JA1111 and JA1115

Revised 4/25
TM577

LAG-3/MHCII Blockade Bioassay

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

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1. Description

The human immune system is regulated by a complex network of inhibitory and stimulatory receptors that facilitate the elimination of pathogens, while maintaining tolerance to self-antigens. Inhibitory immune checkpoint receptors have been shown to perform critical roles in the maintenance of immune homeostasis, but 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[a–d] (Cat.# JA1111, JA1115), 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 thaw-and-use format as cryopreserved cells that can be thawed, plated and used in an assay without the need for cell culture and propagation. In addition, TCR Activating Antigen is included in the assay kits. 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 cocultured 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.B, 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.

The LAG-3/MHCII Blockade Bioassay combines a simple, add-mix-read two-day workflow with LAG-3 Effector Cells and MHCII APC Cells provided in a frozen thaw-and-use format, and an optimized protocol, that together yield a quantitative bioassay that exhibits low variability and high accuracy. The thaw-and-use cells provided in the LAG-3/MHCII Blockade Bioassay kits are manufactured under stringent quality control to provide high assay reproducibility with the convenience of an assay reagent that eliminates the need for continuous cell propagation.

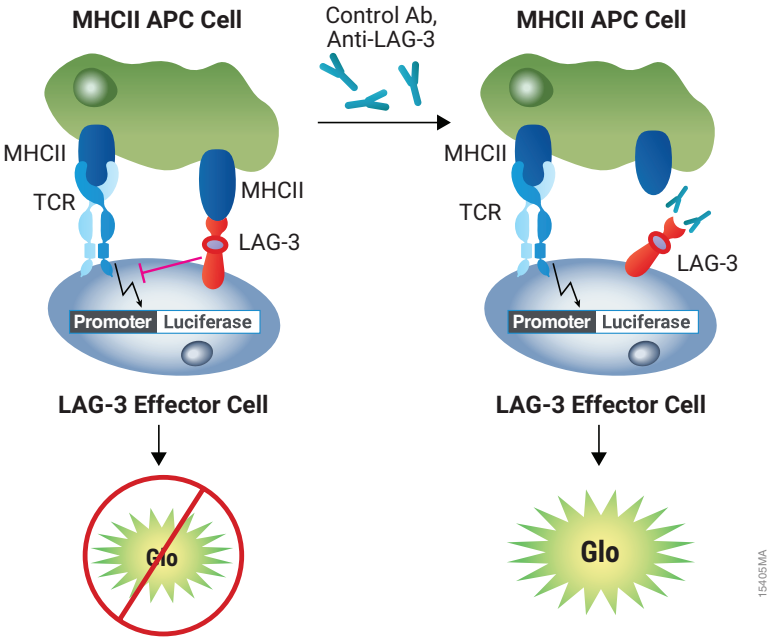

Figure 1. Representation of the LAG-3/MHCII Blockade Bioassay. The bioassay consists of two cell lines, LAG-3 Effector Cells and MHCII APC Cells. When co-cultured, LAG-3 inhibits TCR pathway-activated luminescence. The addition of anti-LAG-3 antibody blocks LAG-3 binding to MHCII, resulting in full TCR 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 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 to the MHCII APC Cells when plating (Figure 3). According to International Council for Harmonisation 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, with LAG-3 Effector Cells (Figure 7).

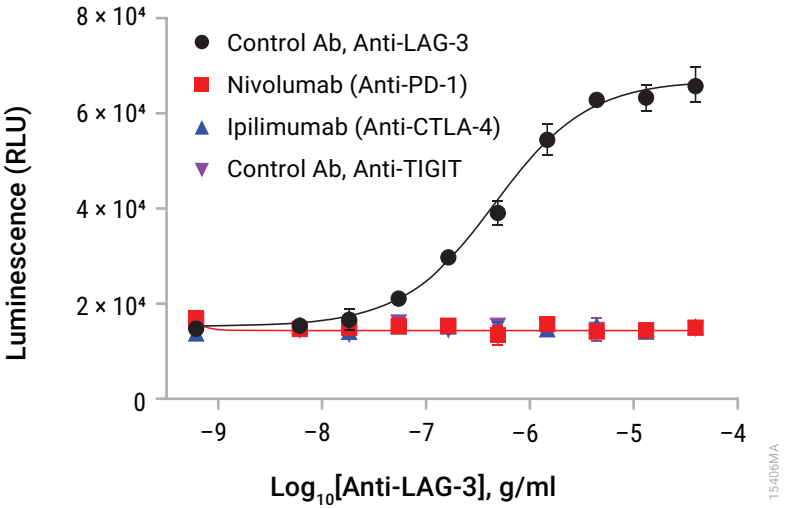

Figure 2. The LAG-3/MHCII Blockade Bioassay reflects the mechanism of action (MOA) and specificity of biologics designed to block the LAG-3/MHCII interaction. LAG-3 Effector Cells were incubated with TCR Activating Antigen and MHCII APC Cells in the presence of serial titrations of blocking antibodies 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 System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

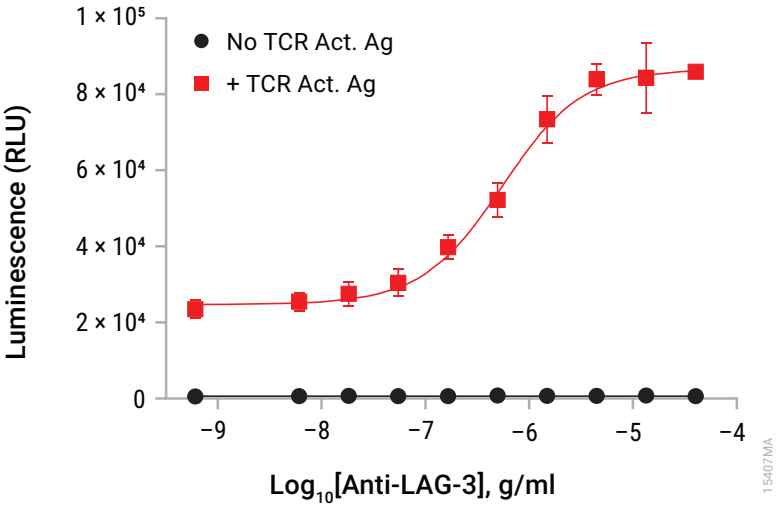

Figure 3. The LAG-3/MHCII Blockade Bioassay is dependent on TCR Activating Antigen. MHCII APC Cells were plated with and without TCR Activating Antigen. The next day, a titration of Control Ab, Anti-LAG-3 was plated followed by addition of LAG-3 Effector Cells. After a 5-hour induction, Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover 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 System. Data were analyzed and relative potencies calculated after parallelism determination using JMP® software. Data were generated using thaw-and-use cells.

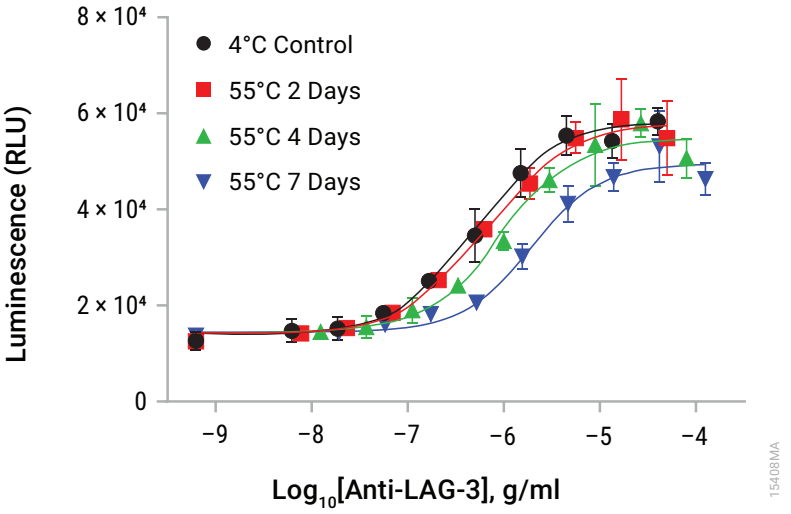

Figure 4. The LAG-3/MHCII Blockade Bioassay indicates antibody stability. Samples of Control Ab, Anti-LAG-3 were maintained at 4°C (control) or heat-denatured at 55°C for the indicated times, then analyzed using the LAG-3/MHCII Blockade Bioassay. Bio-Glo™ Reagent was added and luminescence quantified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

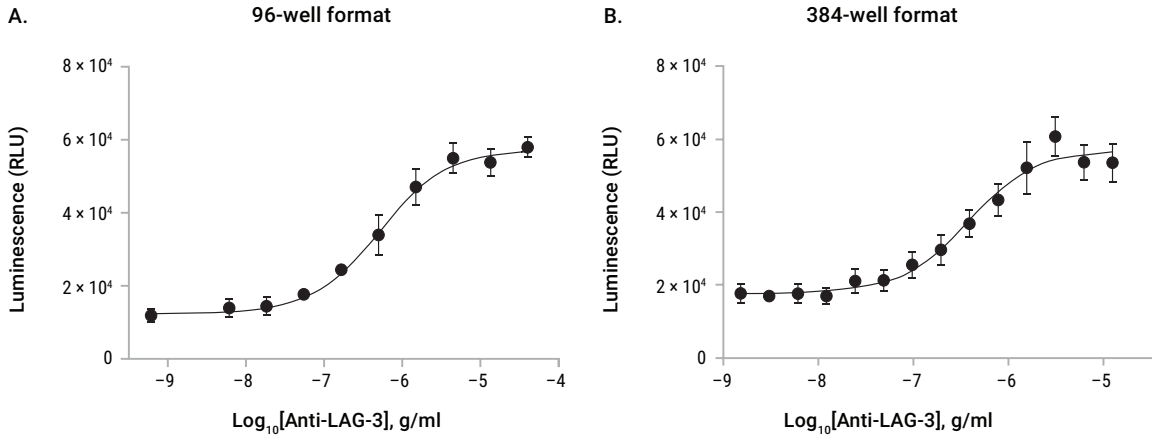

Figure 5. The LAG-3/MHCII Blockade Bioassay is amenable to 384-well plate format. Panel A. The LAG-3/MHCII Blockade Bioassay was performed in 96-well plates as described in this technical manual with a titration of Control Ab, Anti-LAG-3. **Panel B.** The LAG-3/MHCII Blockade Bioassay was performed in 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 induction, 25µl 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 500ng/ml and 380ng/ml, and fold inductions were 4.8 and 3.2 for 96-well and 384-well formats, respectively. Data were generated using thaw-and-use cells.

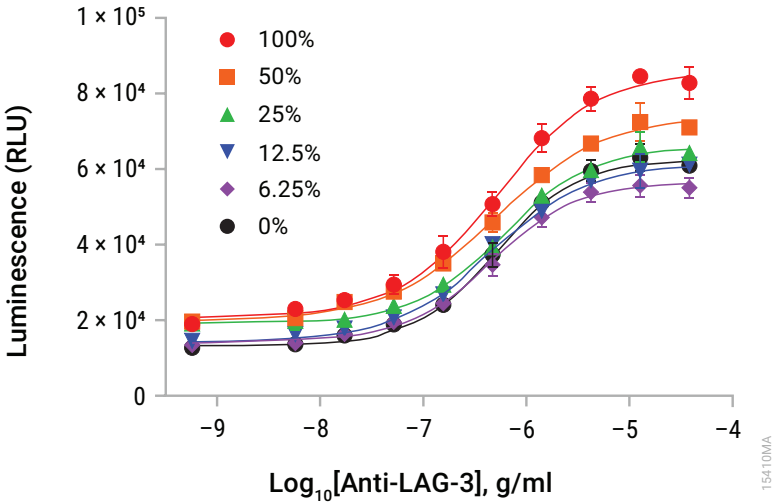

Figure 6. The LAG-3/MHCII Blockade Bioassay tolerates human serum. Control Ab, Anti-LAG-3 was analyzed in the absence or presence of increasing concentrations of pooled normal human serum (0–100% in the antibody sample). Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover 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)

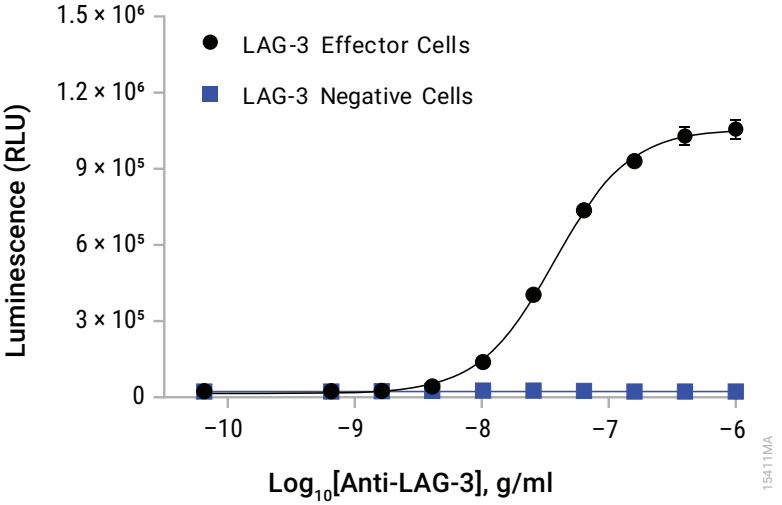

Figure 7. The LAG-3/MHCII Blockade Bioassay can be used to measure ADCC activity. A 1:1 ratio of LAG-3 Effector Cells (used as target cells in this application) or LAG-3-negative Jurkat cells (TIGIT Negative Cells, Cat.# J1921) and mADCC Effector Cells (mouse FcγRIII) available separately, were incubated 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 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	**1 each**	**JA1111**

Not for Medical Diagnostic Use. Includes:

- 1 vial LAG-3 Effector Cells (0.5ml)
- 1 vial MHCII APC Cells (0.5ml)
- 1 vial TCR Activating Antigen Stock Solution (60µl)
- 36ml RPMI 1640 Medium
- 60ml DMEM Medium
- 4ml Fetal Bovine Serum
- 1 vial Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 10ml Bio-Glo™ Luciferase Assay Buffer

PRODUCT	SIZE	CAT.#
LAG-3/MHCII Blockade Bioassay	**5 each**	**JA1115**

Not for Medical Diagnostic Use. Includes:

- 5 vials LAG-3 Effector Cells (0.5ml)
- 5 vials MHCII APC Cells (0.5ml)
- 5 vials TCR Activating Antigen Stock Solution (60µl)
- 5 × 36ml RPMI 1640 Medium
- 5 × 60ml DMEM Medium
- 5 × 4ml Fetal Bovine Serum
- 5 vials Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 5 × 10ml Bio-Glo™ Luciferase Assay Buffer

Note: The LAG-3/MHCII Blockade Bioassay components are shipped separately due to differing temperature requirements. The LAG-3 Effector Cells and MHCII APC Cells are shipped on dry ice. The TCR Activating Antigen, Bio-Glo™ Luciferase Assay System and Fetal Bovine Serum are shipped on dry ice, separately from the cells. The RPMI 1640 Medium and DMEM Medium are shipped at ambient temperature.

Storage Conditions:

- **Upon arrival, immediately transfer the cell vials to below –140°C (freezer or liquid nitrogen vapor phase) for long-term storage. Do not store cell vials submerged in liquid nitrogen. Do not store cell vials at –80°C as this will negatively impact cell viability and cell performance.**
- **Store TCR Activating Antigen, Bio-Glo™ Luciferase Assay Substrate, Bio-Glo™ Luciferase Assay Buffer, Bio-Glo™ Luciferase Assay Buffer and Fetal Bovine Serum at –30°C to –10°C. Avoid multiple freeze-thaw cycles of the antigen and serum.**
- **For optimal performance, use reconstituted Bio-Glo™ Reagent on the day of preparation. However, once reconstituted, Bio-Glo™ Reagent can be stored at –30°C to –10°C for up to 6 weeks.**
- **Store RPMI 1640 Medium and DMEM Medium at +2°C to +10°C protected from fluorescent light.**

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3. Before You Begin

The LAG-3/MHCII Blockade Bioassay 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.

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

The LAG-3 Effector Cells and MHCII APC Cells are provided in frozen, thaw-and-use format and are ready to be used without any additional cell culture or propagation. When thawed and diluted as instructed, the cells will be at the appropriate concentration for the assay. The cells are sensitive, and care should be taken to follow cell thawing and plating procedures as described.

The 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.B, 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

- user-defined anti-LAG-3/MHCII blocking antibodies or other biologics samples
- sterile clear V-bottom 96-well plate with lid (e.g., Costar® Cat.# 3896) for preparing antibody dilutions
- 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
- 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)

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

1. **Cell Recovery Medium:** On the day before the assay, prepare 14.5ml of Cell Recovery Medium (90% DMEM/10% FBS) in a 50ml conical tube. Thaw the Fetal Bovine Serum (FBS) overnight at 4°C or in a 37°C water bath on the day of use. Add 1.5ml of FBS to 13ml of DMEM Medium to yield 90% DMEM/10% FBS. Mix well and warm to 37°C prior to use. Warm the remaining DMEM Medium to 37°C. For reference, 14.5ml of Cell Recovery Medium is sufficient to thaw 1 vial of MHCII APC Cells. If multiple vials of MHCII APC Cells will be used, then scale the amount of Cell Recovery Medium appropriately. Store the remaining FBS at 4°C for use in preparing the assay buffer on the day of the assay.
2. **TCR Activating Antigen:** Once the Cell Recovery Medium is prepared, add 45µl of TCR Antigen Stock Solution into the 14.5ml Cell Recovery Medium. Mix well by gentle tube inversion.
3. **Assay Buffer:** On the day of the assay, prepare 20ml of assay buffer in a 50ml conical tube. Add 2ml of FBS to 18ml of RPMI 1640 Medium to yield 90% RPMI 1640/10% FBS. Mix well and warm to 37°C prior to use. Warm the remaining RMPI 1640 Medium to 37°C on the day of the assay.

   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.

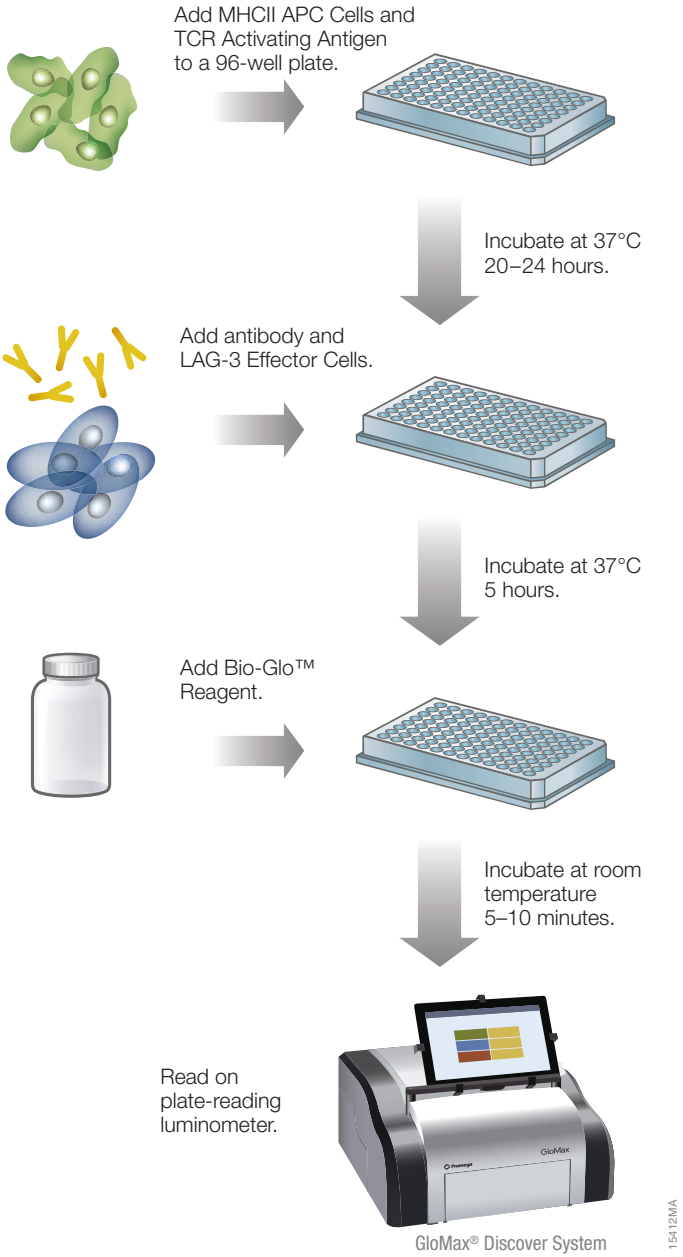

Figure 8. Schematic protocol for the LAG-3/MHCII Blockade Bioassay.

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 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	RPMI Medium (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	RPMI Medium (B)

Figure 9. Example plate layout showing non-clustered sample locations of test and reference antibody dilution series and wells containing RPMI medium (denoted by "B") alone.

4.C. Preparing and Plating MHCII APC Cells

The thaw-and-use MHCII APC Cells included in this kit are sensitive, and care should be taken to follow the cell thawing and plating procedures **exactly** as described. **Do not** overmix or overwarm the cell reagents. No additional cell culture or manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at one time.

Notes:

- Perform the following steps using aseptic technique in a sterile cell culture hood.
- Follow institutional guidelines for handling, including use of personal protective equipment (PPE), and waste disposal for biohazardous material.

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

4.C. Preparing and Plating MHCII APC Cells (continued)

1. On the day before performing the assay, add 1.5ml of FBS to 13ml of DMEM Medium in a 50ml conical tube to make 14.5ml of Cell Recovery Medium (90% DMEM/10% FBS). Warm in a 37°C water bath. Warm the remaining DMEM Medium to 37°C in a water bath.
2. Remove one vial of TCR Activating Antigen Stock Solution (Ag) from storage at –20°C and thaw on the bench top. Once thawed, mix by gently vortexing.
3. Add 45µl of Ag to the tube containing 14.5ml of Cell Recovery Medium. Mix by inverting the tube.
4. Remove one vial of MHCII APC Cells from storage at –140°C and transfer to the bench on dry ice. Warm the cells in a 37°C water bath until just thawed (about 2 minutes). While thawing, gently agitate and visually inspect.
5. Gently mix the cell suspension by pipetting, then transfer the cells (0.5ml) to the 50ml conical tube containing 14.5ml of prewarmed (37°C) Cell Recovery Medium/Ag. Mix well by gently inverting.
6. Transfer the cell 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 two 96-well white, flat-bottom, tissue culture-treated assay plates.
7. Add 100µl of prewarmed (37°C) DMEM Medium to each of the outside wells of the assay plates.
8. Cover the assay plates with a lid and incubate the cells overnight (20–24 hours) in a humidified 37°C, 5% CO_2 incubator.

4.D. Preparing Antibody Serial Dilutions

The instructions described here are for preparing of a single stock of three-fold 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 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.

7. 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 plates on paper towels for 5–10 seconds to drain any remaining medium. Alternatively, carefully use a manual multichannel pipette to remove 95µl of medium from each of the wells. **Do not** use a vacuum aspirator to remove the media.
8. 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.
9. Add 80µl of RPMI 1640 Medium to each of the outside wells of the assay plates.
10. Cover the assay plates with a lid and keep at ambient temperature (22–25°C) while preparing the 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.

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4.E. Preparing LAG-3 Effector Cells

Note: The thaw-and-use LAG-3 Effector Cells included in this kit are sensitive, and care should be taken to follow the cell thawing and plating procedures **exactly** as described. **Do not** overmix or overwarm the cell reagents. No additional cell culture or manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at any one time.

1. Add 7ml of prewarmed (37°C) assay buffer to a 15ml conical tube.
2. Remove one vial of LAG-3 Effector Cells from storage at –140°C and transfer to the bench on dry ice. Thaw the cells in a 37°C water bath until just thawed (about 2 minutes). While thawing, gently agitate and visually inspect.
3. Gently mix the cell suspension by pipetting, then transfer the cells (0.5ml) to the 15ml conical tube containing 7ml of assay buffer. Mix well by gently inverting the tube.
4. Transfer the cell suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 40µl of the cell suspension to inner 60 wells of the assay plates.
5. Cover the assay plates with a lid and incubate for 5 hours in a humidified 37°C, 5% CO_2 incubator.

4.F. Adding Bio-Glo™ Reagent

Note: Bio-Glo™ Reagent should be at ambient temperature (22–25°C) when added to assay plates.

1. 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.G. 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**. Email: **techserv@promega.com**

Symptoms	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. 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 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 luminescence measurements (RLU readout)	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). 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.

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6. References

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7. Appendix

7.A. Representative Assay Results

The following data were generated using the LAG-3/MHCII Blockade Bioassay, using Control Ab, Anti-LAG-3 (Figure 11).

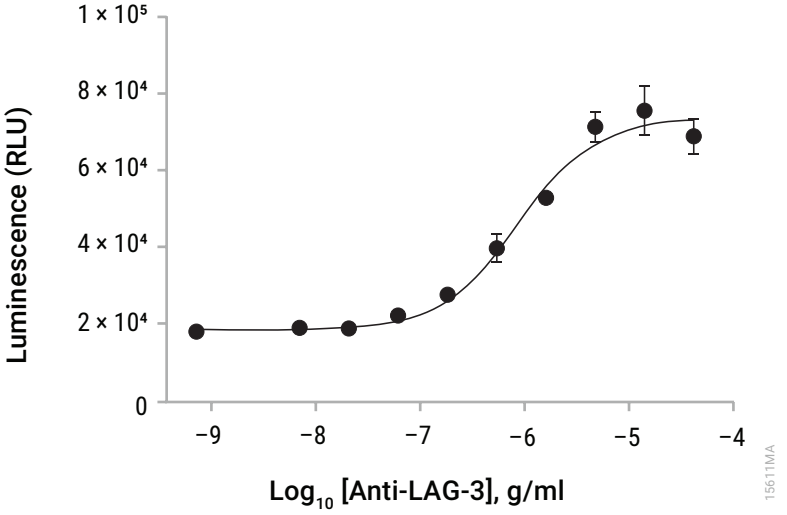

Figure 11. The LAG-3/MHCII Blockade Bioassay measures the activity of Control Ab, Anti-LAG-3. MHCII APC Cells and TCR Activating Antigen were plated overnight. The following day, a titration of Control Ab, Anti-LAG-3 (Cat.# K1150) was added followed by LAG-3 Effector Cells. After 5 hours, Bio-Glo™ Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using using GraphPad Prism® software. The EC_{50} value was 790ng/ml and the fold induction was 4.3.

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7.B. Related Products

Fc Effector Bioassays

Product	Size	Cat.#
ADCC Reporter Bioassay, Complete Kit (Raji)*	1 each	G7015
ADCC Reporter Bioassay, Core Kit*	1 each	G7010
ADCC Reporter Bioassay, F Variant, Core Kit**	1 each	G9790
ADCC Reporter Bioassay, Target Kit (Raji)*	1 each	G7016
FcγRIIa-H ADCP Reporter Bioassay, Complete Kit**	1 each	G9901
FcγRIIa-H ADCP Reporter Bioassay, Core Kit**	1 each	G9991
Mouse FcγRIV ADCC Bioassay, Complete Kit	1 each	M1201
Mouse FcγRIV ADCC Bioassay, Core Kit	1 each	M1211
Membrane TNFα Target Cells**	1 each	J3331
Membrane RANKL Target Cells**	1 each	J3381

*For Research Use Only. Not for use in diagnostic procedures.
**Not for Medical Diagnostic Use.
Additional kit formats are available.

Fc Effector Immunoassay

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

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

Immune Checkpoint Bioassays

Product	Size	Cat.#
4-1BB Bioassay	1 each	JA2351
CD28 Bioassay	1 each	JA6701
CD28 Blockade Bioassay	1 each	JA6101
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
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

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
T Cell Activation Bioassay (TCRαβ-KO, CD4+)	1 each	GA1172
T Cell Activation Bioassay (TCRαβ-KO, CD8+)	1 each	GA1162
T Cell Activation Bioassay (TCRαβ-KO, CD4+, CD8+)	1 each	GA1182

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7.B. Related Products (continued)

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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Control Antibodies and Proteins

Product	Size	Cat.#
Control Ab, Anti-4-1BB	50µg	K1161
Control Ab, Anti-CD20	5µg	GA1130
Control Ab, Anti-CD40	50µg	K1181
Control Ab, Anti-CTLA-4	100µg	JA1020
Control Ab, Anti-LAG-3	100µg	K1150
Control Ab, Anti-OX40	50µg	K1191
Control Ab, Anti-PD-1	100µg	J1201
Control Ab, Anti-SIRPα	50µg	K1251
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™ Luciferase Assay System	10ml	G7941
Bio-Glo-NL™ Luciferase Assay System	10ml	J3081

Not for Medical Diagnostic Use. Additional sizes are available.

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, Cytokine, Macrophage, Primary Cell and Target Cell Killing Bioassays are available. To view and order Promega Bioassay products visit: **www.promega.com/products/reporter-bioassays/** or email: **EarlyAccess@promega.com**. For information on custom bioassay development and services visit the Promega Tailored R&D Solutions website: **www.promega.com/custom-solutions/tailored-solutions/**

8. Summary of Changes

The following changes were made to the 4/25 revision of this document:

1. Removed an expired patent statement.
2. Text about the product label was revised and moved to Section 3.

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