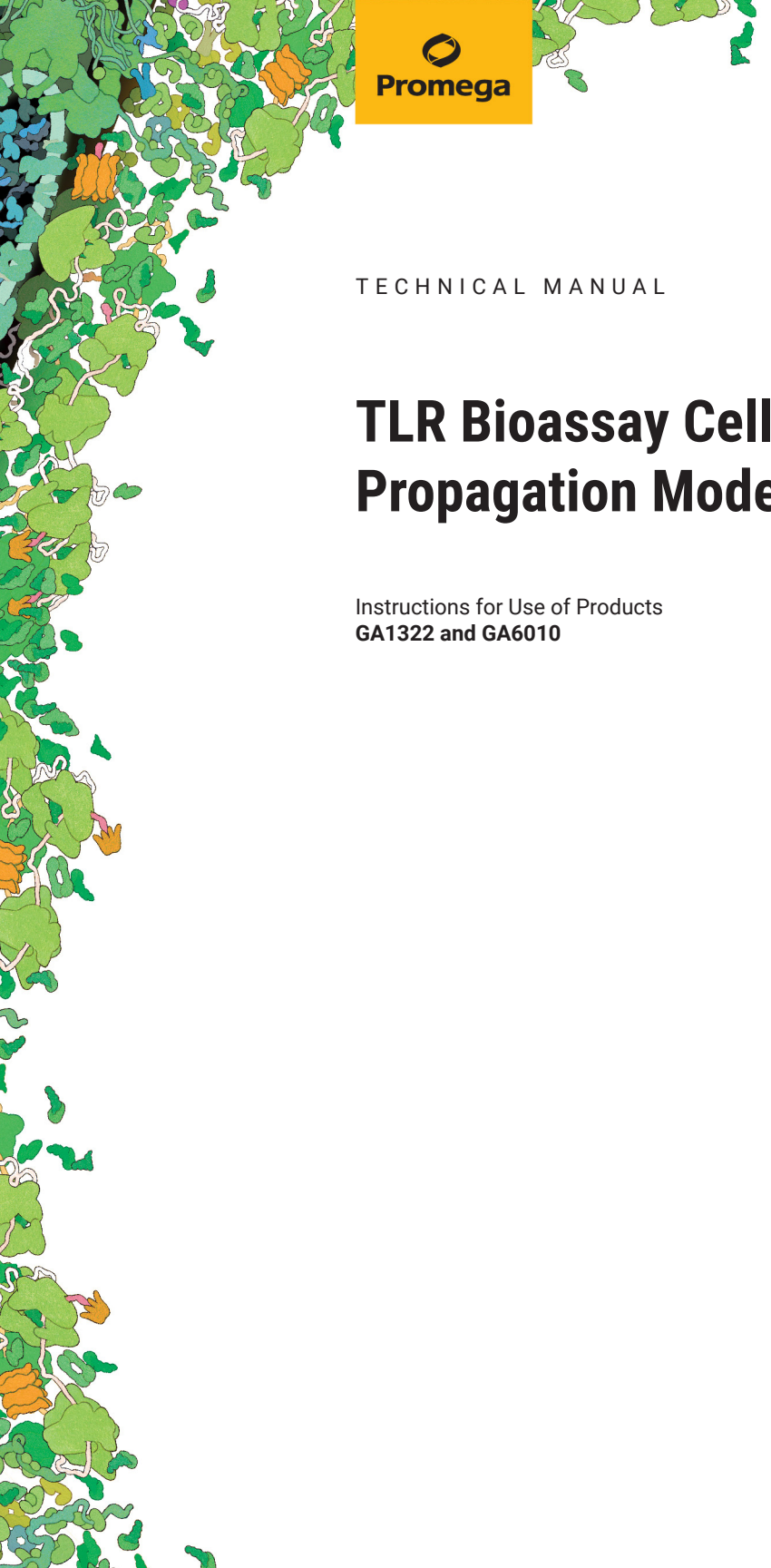

TECHNICAL MANUAL

TLR Bioassay Cells, Propagation Model

Instructions for Use of Products
GA1322 and GA6010

Revised 4/25
TM704

Promega

TLR Bioassay Cells, 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 responds to conserved damage- and pathogen-associated molecular patterns (DAMPs and PAMPs, respectively) via pattern-recognition receptors (PRRs). One such family of PRRs is the Toll-like receptor (TLR) family. In humans, there are 10 known TLRs that enable the immune system to sense and respond to damage and danger signals. TLRs are type I transmembrane proteins sharing similar molecular structure including 20–27 extracellular leucine-rich repeats for DAMP/PAMP recognition, transmembrane domains and intracellular Toll/Interleukin-1 (IL-1) receptor (TIR) domains that activate downstream signaling pathways (1).

TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the plasma membrane. However, the nucleic acid sensors TLR3, TLR7, TLR8 and TLR9, as well as TLR10, are localized in the endosome (2). Activating TLRs initiates and propagates transcriptional programs that result in TLR-dependent pro-inflammatory cytokine and type 1 interferon (IFN) production. TLR activation recruits up to five signaling adaptor proteins, resulting in two predominant signaling pathways. The MyD88-dependent pathway is activated by all TLRs, except TLR3, and leads to pro-inflammatory cytokine production via phosphorylation of IκB and subsequent nuclear localization of NF-κB (3). TLRs 7–9 also activate interferon response factor 7 (IRF7) via MyD88. In contrast, the TIR domain-containing adaptor protein-inducing IFNβ- (TRIF-) dependent pathway is activated by TLR3 and TLR4, and results in IRF3 activation and type 1 IFN production, as well as maturation of dendritic cells (3). Details on the ligand specificity of the ten human TLRs are described in Table 1.

Table 1. TLR Ligand Specificity (1).

TLR	PAMPs	DAMPs
TLR1/TLR2	Triacylated lipoproteins (e.g., Pam3CSK4), Peptidoglycans	HMGB1, HSP60, HSP70, Gp76, Peptidoglycans
TLR2/TLR6	Diacylated lipoproteins (e.g., FSL-1), LTA, Zymosan	HMGB1, HSP60, HSP70, Gp76, Peptidoglycans
TLR3	dsRNA (e.g., Poly (I:C))	mRNA, tRNA
TLR4	Lipopolysaccharide (LPS)	HMGB1, Peptidoglycans HSP22, HSP60, HSP70, HSP72, Gp96
TLR5	Flagellin	
TLR7	ssRNA, Imidazoquinolines (e.g., R848), Guanosine analogs	ssRNA
TLR8	ssRNA, Imidazoquinolines (e.g., R848)	ssRNA
TLR9	CpG DNA, CpG ODNs	Chromatin IgG complex
TLR10	Profilin-like protein	

Modulating the TLR system, either by agonism or antagonism, has the potential to modulate immune and inflammatory responses. Blocking TLRs may inhibit inflammatory responses, while TLR agonists may promote immune responses against pathogens or cancer (4,5). Current methods for assessing the activity of TLR modulators typically rely on primary monocyte-derived macrophages and measuring functional endpoints, such as cytokine production. These assays are laborious and highly variable due to their reliance on donor cells, complex assay protocols and unqualified assay reagents. As a result, these assays are difficult to establish in a quality-controlled setting.

The TLR Bioassay Cells, Propagation Model[a–c] (Cat.# GA1322) is a bioluminescent reporter cell-based assay that overcomes the limitations of existing assays. The bioassay is easy-to-use, quantitative and demonstrates a functional readout of agents that activate TLRs. The TLR Bioassay can be used to measure the potency of agonists of TLRs 1, 2, 4, 5, 6, 7 and 8. It can also be used to measure the potency of inhibitors of TLRs. The assay consists of one genetically engineered cell line:

- **TLR Bioassay Cells**: Monocytic cells endogenously expressing TLRs 1, 2, 4, 5, 6, 7 and 8, with a stably integrated NanoLuc® (NL) luciferase reporter driven by TLR pathway-dependent response elements.

The TLR Bioassay Cells are provided in Cell Propagation Model (CPM) format, which includes cryopreserved cells that can be thawed, propagated and banked for long-term use. Cell banks for the TLR Bioassay Cells (Cat.# GA6010) are also available.

Treatment of the TLR Bioassay Cells with a TLR agonist results in TLR signaling and promoter-driven luminescence (Figure 1). The bioluminescent signal is quantified using the Bio-Glo-NL™ Luciferase Assay System and a standard luminometer such as the GloMax® Discover System (Section 8.C, Related Products).

The TLR Bioassay reflects the mechanism of action (MOA) of agents designed to activate TLRs. Specifically, TLR activation-mediated luminescence is increased after adding a TLR agonist, but not in the presence of specific TLR blocking antibodies (Figure 2). The TLR Bioassay can be used to measure the stability of TLR agonists (Figure 3). The bioassay can be performed in a two-day timeframe, and the workflow is simple, robust and compatible with both 96-well and 384-well plate formats used for agonist screening in early drug discovery (Figure 4).

1. Description (continued)

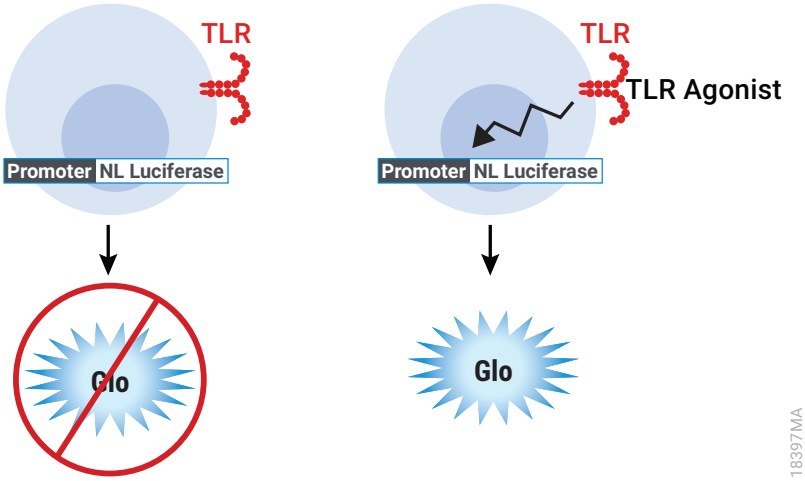

Figure 1. Representation of the TLR Bioassay. Treatment with a TLR agonist results in promoter-driven luminescence that can be detected and quantified using Bio-Glo-NL™ Reagent. The TLR Bioassay consists of a single genetically engineered cell line, TLR Bioassay Cells. In the absence of agonist, TLRs are not activated and the luminescence signal is low. Treatment with a TLR agonist results in promoter-driven luminescence that can be detected upon addition of Bio-Glo-NL™ Reagent and quantified with a luminometer.

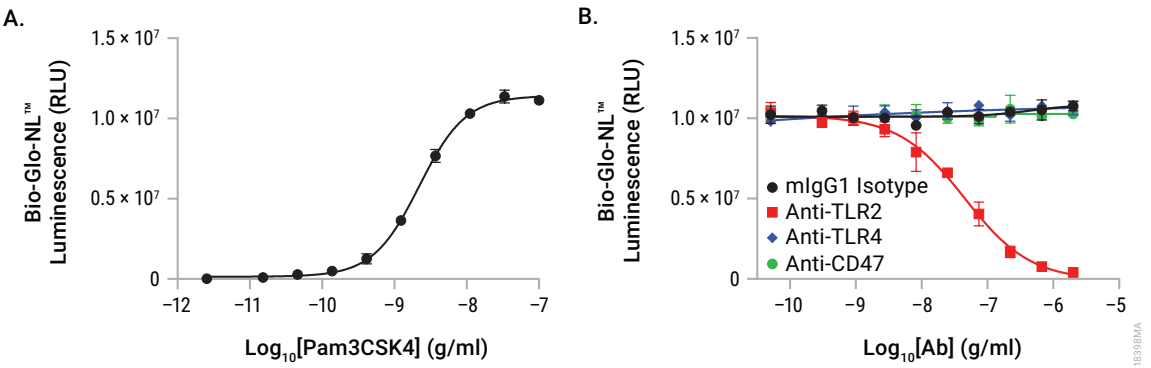

Figure 2. The TLR Bioassay reflects the MOA and specificity for TLR agonists. Panel A. TLR Bioassay Cells were treated with a serial titration of TLR1/2 agonist Pam3CSK4. **Panel B.** TLR Bioassay Cells were treated with 6ng/ml of Pam3CSK4 in the presence of serial titrations of blocking antibodies as indicated. After a 4-hour induction, Bio-Glo-NL™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were fitted to four-parameter logistic curves using GraphPad Prism® software. Data were generated using thaw-and-use cells.

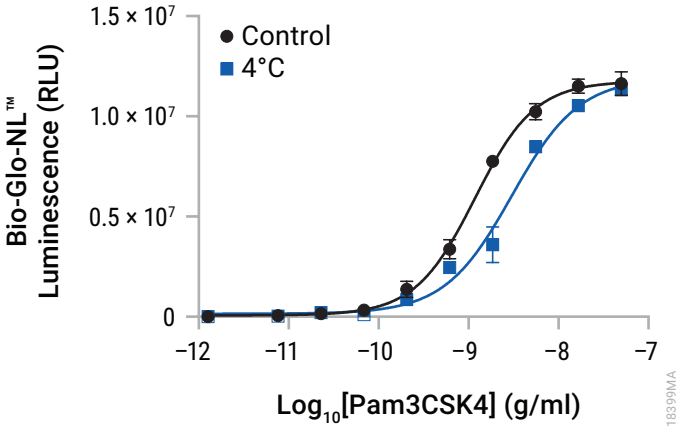

Figure 3. The TLR Bioassay is stability-indicating. Samples of the TLR1/2 agonist Pam3CSK4 were maintained at −80°C (control) or stored at 4°C for one year, then analyzed using the TLR Bioassay. Bio-Glo-NL™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were fitted to four-parameter logistic curves using GraphPad Prism® software. Data were generated using thaw-and-use cells.

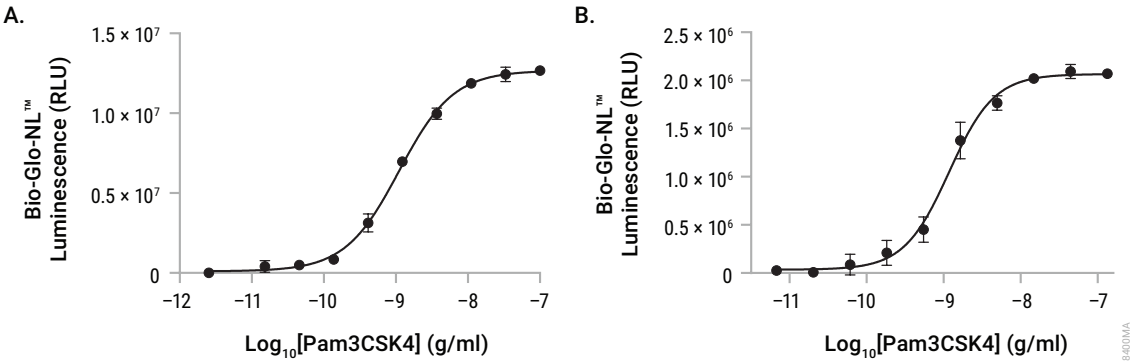

Figure 4. The TLR Bioassay is amenable to 384-well plate format. Panel A. The TLR Bioassay was performed in 96-well plates as described in the *TLR Bioassay Technical Manual*, #TM705, with a titration of the TLR1/2 agonist Pam3CSK4. **Panel B.** The TLR Bioassay was performed in 384-well format as briefly described here. Thaw-and-use TLR Bioassay Cells were thawed and plated at 1.2 × 10^4 cells/20µl/well 20 hours prior to assay, in a 384-well white assay plate (e.g., Corning® Cat.# 3570). On the day of the assay, 5µl of a serial dilution of 5X concentrated TLR1/2 agonist Pam3CSK4 was added. After a 4-hour incubation at 37°C, 5% CO_2, 25µl of Bio-Glo-NL™ Reagent was added per well and luminescence quantified using the GloMax® Discover System. Data were fitted to four-parameter logistic curves using GraphPad Prism® software. The EC_{50} values were 1.1 and 1.2ng/ml for the 96- and 384-well formats, respectively, and the fold induction was 1,160 and 1,600 for 96- and 384-well formats, respectively. Data were generated using thaw-and-use cells.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
TLR Bioassay Cells, Propagation Model	**1 each**	**GA1322**

Not for Medical Diagnostic Use. Includes:

- 2 vials TLR Bioassay Cells (CPM), 1.32 × 10^7 cells/ml (1.0ml per vial)

PRODUCT	SIZE	CAT.#
TLR Bioassay Cells, Cell Bank	**1 each**	**GA6010**

Not for Medical Diagnostic Use. Includes:

- 50 vials TLR Bioassay Cells (CPM), 1.32 × 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 remaining vial(s) 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, 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 web site, such as the Certificate of Analysis.

Note: The TLR Bioassay Cells, Propagation Model, uses the Bio-Glo-NL™ Luciferase Assay System (Cat.# J3081, J3082, J3083) for detection. **Do not** use the Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941).

The TLR Bioassay Cells, Propagation Model, is intended for use with user-provided TLR agonists or TLR blocking antibodies in the presence of TLR agonists. The recommended cell plating densities, induction time and assay buffer components described in Section 5 were established using the TLR1/2 agonist Pam3CSK4 and TLR4 agonist lipopolysaccharide (LPS). Representative data generated using several TLR agonists are shown in Section 8.A., Representative Assay Results. You may need to adjust the parameters provided here and optimize assay conditions for your own TLR agonist, antibody or biologic samples.

Cell thawing, propagation and banking should be performed exactly as described in Section 4. 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 TLR Bioassay Cells produce a bioluminescent signal and require a sensitive luminescence plate reader. Bioassay development and performance data included in this Technical Manual were generated using the GloMax® Discover System (see Section 8.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 (RLU) readings will vary with the sensitivity and settings of each instrument. If using a reader with adjustable gain, we recommend a high-gain setting. The use of different instruments and gain adjustment will affect the magnitude of the raw data but should not affect the measured relative potency of test samples.

3.A. Materials to Be Supplied by the User

(Composition of Buffers and Solutions is provided in Section 8.B.)

Reagents

- user-defined TLR agonists or TLR agonist/antagonist antibodies
- RPMI 1640 medium with L-glutamine and HEPES (e.g., Corning® Cat.# 10-041-CV or GIBCO® Cat.# 22400105)
- fetal bovine serum (e.g., VWR Cat.# 89510-194, GIBCO® Cat.# 35-015-CV or HyClone Cat.# SH30071.03)
- hygromycin B (e.g., GIBCO® Cat.# 10687010)
- DMSO (e.g., Sigma Cat.# D2650)
- Bio-Glo-NL™ Luciferase Assay System (Cat.# J3081, J3082, J3083)
- Trypan blue solution (e.g., Sigma Cat.# T8154)
- **optional:** TLR1/2 agonist Pam3CSK4 (e.g., InvivoGen Cat.# tlrl-pms)

Supplies and Equipment

- solid-white, flat-bottom 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) for low volume antibody preparation (100–300µl per dilution), or sterile dilution reservoirs with lid (Dilux®, Cat.# D-1002) for high volume antibody preparation (300µl–5ml per dilution)
- 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®/Costar® Cat.# 4870)
- 37°C, 5% CO_2 incubator
- 37°C water bath
- sensitive plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System or equivalent system)

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4. Preparing TLR Bioassay Cells

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

4.A. Cell Thawing and Initial Cell Culture

1. Prepare 20ml of initial cell culture medium by adding 2ml of FBS to 18ml 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 15ml conical tube.
3. Remove one vial of TLR Bioassay Cells from storage at –140°C and thaw in a 37°C water bath with gentle agitation (do not invert cell vial) until just thawed (typically 2–3 minutes).
4. Transfer all of the cells (approximately 1ml) to the 15ml conical tube containing 9ml of prewarmed initial cell culture medium.
5. Centrifuge at 150 × *g* for 10 minutes.
6. Carefully aspirate the medium and resuspend the cell pellet in 7ml of prewarmed initial cell culture medium.
7. Transfer the cell suspension to a T25 tissue culture flask and place the flask horizontally in a humidified 37°C, 5% CO_2 incubator.
8. Incubate for approximately 24 hours before passaging the cells.
9. Passage the cells at a seeding density of 5×10^5 viable cells/ml using cell growth medium containing hygromycin B.

Note: When passaging cells for the first time after thawing, it is critical to use a minimum seeding density of 5×10^5 viable cells/ml. Lower densities may reduce cell viability and growth.

10. Incubate for approximately 48 hours before passaging the cells according to the schedule outlined in Section 4.B.

4.B. Cell Maintenance and Propagation

For cell maintenance and propagation, use cell growth medium containing hygromycin B 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 48 hours. Passage number should be recorded for each passage. In our experience, cells maintain their functionality for up to 25 passages, or 29 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 two days (e.g., Monday-Wednesday or Wednesday-Friday) or 3×10^5 cells/ml if passaging every three days (e.g., Friday-Monday). Always maintain the flasks in a horizontal position in the incubator. Do not allow the cells to grow to a density greater than 1×10^6 cells/ml.
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 humidified, 37°C, 5% CO_2 incubator.

4.C. Cell Freezing and Banking

1. On the day of cell freezing, prepare fresh cell freezing medium and keep on ice.
2. Gently mix the cells with a pipette to create a homogenous cell suspension.
3. Remove a sample for cell counting by Trypan blue staining. Calculate the volume of cell freezing medium needed based on desired cell freezing densities of 1×10^7–2×10^7 cells/ml.
4. Transfer the cell suspension to 50ml sterile conical tubes or larger sized centrifuge tubes, and centrifuge at 150 × *g*, 4°C 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 1×10^7– 2×10^7 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 –140°C or below (freezer or liquid nitrogen vapor phase) for long-term storage.

5. Assay Protocol

This procedure illustrates the use of TLR Bioassay Cells to test two TLR agonist samples against a reference sample in a single assay run using the TLR Bioassay Cells, Propagation Model format (Figure 5). Each test and reference agonist is run in triplicate, in a ten-point dilution series, in a single 96-well assay plate using the inner 60 wells (Figure 6). Other experimental and plate layouts are possible but may require further optimization.

Note: When preparing test and reference TLR agonists, choose an appropriate starting concentration and dilution scheme to achieve a complete dose-response curve with proper upper and lower asymptotes and sufficient points on the slope. For reference, we use 100ng/ml as a starting concentration (1X) and a threefold serial dilution when testing Pam3CSK4.

5. Assay Protocol (continued)

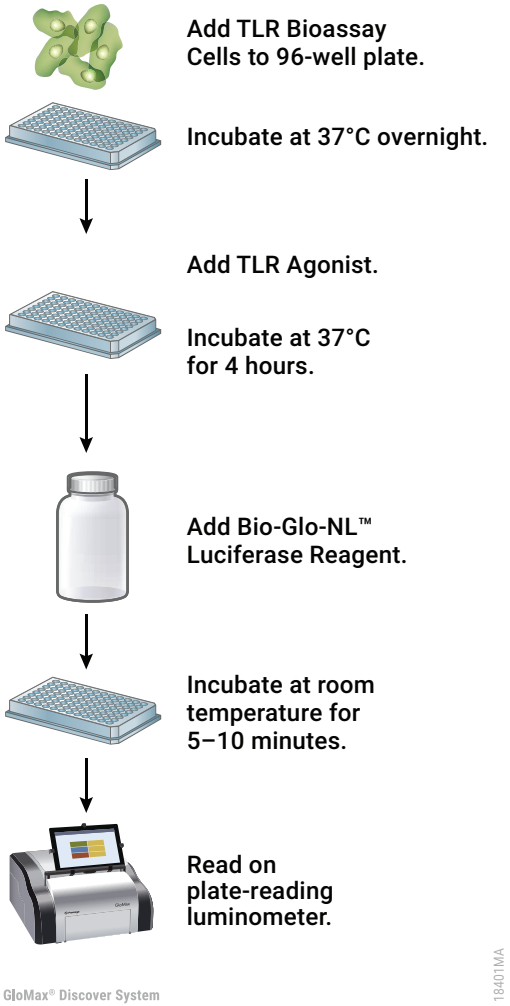

Figure 5. Schematic protocol for the TLR Bioassay Cells, Propagation Model.

5.A. Preparing Assay Buffer, Bio-Glo-NL™ Reagent and Agonist Samples

Assay Buffer: On the day of cell plating, prepare an appropriate amount of assay buffer (90% RPMI 1640/10% FBS). Mix well and warm to 37°C before use. For reference, 40ml of assay buffer is typically sufficient for 120 wells in a 96-well assay format using the inner 60 wells. Store unused assay buffer overnight at 4°C for use on the day of the assay.

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

Bio-Glo-NL™ Luciferase Assay Reagent: For reference, 10ml of Bio-Glo-NL™ Reagent is sufficient to assay 120 wells in a 96-well assay format. Bio-Glo-NL™ Luciferase Assay Substrate should always be stored at –30°C to –10°C. Thaw the Bio-Glo-NL™ Luciferase Assay Buffer at room temperature (do not exceed 25°C) during the 4-hour assay induction period. We recommend preparing the reconstituted Bio-Glo-NL™ Reagent immediately before use. For instructions on use of the Bio-Glo-NL™ Luciferase Assay System, please refer to the *Bio-Glo-NL™ Luciferase Assay System Quick Protocol,* #FB227.

Note: The TLR Bioassay Cells, Propagation Model, are compatible only with the Bio-Glo-NL™ Luciferase Assay Reagent. **Do not** use the Bio-Glo™ Luciferase Assay Reagent with the TLR Bioassay.

Test and Reference Samples: Using assay buffer as the diluent, prepare stock starting dilutions (dilu1, 4X final concentration) of two test samples (150µl each) and one reference sample (300µl) in 1.5ml tubes. Store the tubes containing starting dilutions appropriately before making serial dilutions.

Note: If you are using TLR1/2 agonist Pam3CSK4 as a reference in your assay, prepare a 300µl starting dilution of 400ng/ml (dilu1, 4X final concentration) in assay buffer.

5.B. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 6 as a guide. The protocol describes serial replicate dilutions (n = 3) of test and reference agonists 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
C	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test
D	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference
E	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test
F	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference
G	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test
H	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)

Figure 6. Example plate layout showing nonclustered sample locations of reference and test agonist dilution series and wells containing assay buffer only (denoted by "B").

5.C. Preparing and Plating TLR Bioassay Cells

While maintaining the TLR Bioassay Cells, 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, and when cell viability is ≥90%.

1. Passage the cells two days before performing the assay as described in Section 4.B.
2. Count the TLR Bioassay Cells by Trypan blue staining and calculate the cell density and viability.
3. Transfer an appropriate amount of TLR Bioassay Cells from the culture vessel to a 50ml conical tube or larger-sized centrifuge tube.
4. Pellet the cells at 150 × *g* for 10 minutes at ambient temperature, and resuspend the pellet in assay buffer at 70% of the full volume needed to generate the targeted final cell density of 6×10^5 cells/ml.

5. Count the cells again and adjust the volume of assay buffer to achieve a final cell density of 6×10^5 cells/ml. You will need at least 9.5ml of TLR Bioassay Cells to fill 120 assay wells, or the inner 60 wells of two assay plates.
6. Mix the TLR Bioassay Cells by inverting the tube and transfer the cell suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 60µl of the cell suspension to each of the inner 60 wells of the assay plates.
7. Add 80µl of assay buffer to each of the outside wells of the assay plates.
8. Cover the assay plates with lids and incubate in a 37°C, 5% CO_2 incubator for 18–22 hours.

5.D. Preparing and Plating Agonist Serial Dilutions

The instructions described here are for preparation of a threefold serial dilution of a single TLR agonist for analysis in triplicate (100µl of each dilution provides a sufficient volume for analysis in triplicate). Alternatively, you can prepare three independent stocks of serial dilutions to generate triplicate samples. To prepare threefold serial dilutions, you will need 300µl of reference agonist at 4X the highest concentration in your dose-response curve. You will need 150µl of each test agonist at 4X the highest concentration in each of the test dose-response curves. For other dilution schemes, adjust the volumes accordingly.

Note: If you are using Pam3CSK4, as a control in the assay, follow the instructions below to prepare threefold serial dilutions.

1. On the day of the assay, warm assay buffer prepared in Section 5.A in a 37°C water bath.
2. Prepare initial dilution of samples according to the instructions in Section 5.A.
3. Add 150µl of reference starting dilution (dilu1, 4X final concentration) to wells A11 and B11 of a sterile clear v-bottom 96-well plate (Figure 7).
4. Add 150µl of each test starting dilution (dilu1, 4X final concentration) to wells E11 and G11, respectively (Figure 7).
5. Add 100µl of assay buffer to other wells in these four rows, from well 10 to well 2.
6. Transfer 50µl of the agonist starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
7. Repeat equivalent threefold serial dilutions across the dilution reservoirs from right to left through well 3. Do not dilute into well 2.

   Note: Well 2 of each dilution reservoir contains 100µl of assay buffer without agonist as a negative control.
8. Take the 96-well assay plates containing TLR Bioassay Cells out of the incubator.
9. Using a multichannel pipette, add 20µl of the appropriate agonist dilution (Figure 7) to the assay plates according to the plate layout in Figure 6. Swirl plate gently to ensure mixing of the cells and agonist.
10. Cover the assay plates with lids and incubate in a 37°C, 5% CO_2 incubator for 4 hours.

    Note: The 4-hour incubation was optimized using the TLR1/2 and TLR4 agonists Pam3CSK4 and LPS. We recommend optimizing assay time (3–24 hours) with your TLR agonist, antibody or other biologic samples.

Recommended Plate Layout for Agonist Dilutions Prepared from a Single Agonist 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
B		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference
C													
D													
E		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test
F													
G		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test
H													

Figure 7. Example dilution plate layout showing TLR agonist serial dilutions.

5.E. Preparing and Adding Bio-Glo-NL™ Luciferase Assay Reagent

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

Note: The TLR Bioassay Cells are compatible only with Bio-Glo-NL™ Luciferase Assay Reagent. **Do not** use Bio-Glo™ Luciferase Assay Reagent with the TLR Bioassay.

1. Remove the Bio-Glo-NL™ Luciferase Assay Substrate from –30°C to –10°C storage. Briefly centrifuge the tubes if the substrate has collected in the cap or on the sides of the tubes.
2. Prepare the desired amount of reconstituted Bio-Glo-NL™ Reagent by combining one volume of substrate with 50 volumes of buffer. For example, if the experiment requires 10ml of reagent, add 200µl of substrate to 10ml of buffer. Ten milliliters (10ml) of Bio-Glo-NL™ Reagent is sufficient for 120 wells (two assay plates, using the inner 60 wells of each plate).
3. Remove assay plates from the incubator after the incubation period and equilibrate to room temperature for 15 minutes.
4. Using a manual multichannel pipette, add 80µl of Bio-Glo-NL™ Reagent to the inner 60 wells of the assay plates, taking care not to create bubbles.
5. Add 80µl of Bio-Glo-NL™ Reagent to wells B1 and F1 of each assay plate to measure background signal.

6. Wait 5–10 minutes, then measure the luminescence in a GloMax® Discover System or a plate reader with glow-type luminescence reading capabilities. The luminescence intensity will decay gradually, with a signal half-life of approximately 120 minutes at room temperature.

   Note: Varying the Bio-Glo-NL™ Reagent incubation time will affect the raw relative light unit (RLU) values but should not significantly change the EC_{50} value and maximum fold induction.

5.F. Data Analysis

1. Determine the plate background by calculating the average RLU from wells B1 and F1.
2. Calculate fold induction:

$$\text{Fold Induction} = \frac{\text{RLU (induced} - \text{background)}}{\text{RLU (no agonist control} - \text{background)}}$$

3. Graph data as RLU versus Log_{10} [agonist] and fold induction versus Log_{10}[agonist]. Fit curves and determine the EC_{50} value of agonist response using appropriate curve fitting software (such as GraphPad Prism® software).

6. 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)	Ensure that you are using Bio-Glo-NL™ Reagent, which is designed for NanoLuc® Luciferase reporter bioassays. TLR Bioassay Cells are not compatible with Bio-Glo™ Reagent, which is designed for firefly luciferase reporter bioassays.
	Choose an instrument designed for plate-reading luminescence detection. Instruments designed primarily for fluorescence detection are not recommended. Luminometers measure and report luminescence as relative values, and actual RLU numbers will vary between instruments.
	Some models of luminometers with low sensitivity should be avoided. If using a reader with an adjustable gain, we recommend a high-gain setting.
	Insufficient cells per well can lead to low RLU. Handle and plate cells according to the instructions to ensure a sufficient number of viable cells per well.
	Low activity of Bio-Glo-NL™ Reagent leads to low RLU. Store and handle Bio-Glo-NL™ Reagent according to the instructions. For best results, prepare immediately before use.

6. Troubleshooting (continued)

Symptoms	Causes and Comments
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 with the TLR Bioassay Cells may vary from the EC_{50} value obtained using other methods such as primary cell assays.
	The assay is sensitive to the concentration of FBS in assay buffer. Optimize the FBS concentration from 0.5–10% in assay buffer if assay performance is not ideal.
	Optimize the assay incubation time within a range of 3–24 hours.
	If untreated control RLU is less than 100-fold above plate reader background RLU, subtract plate background RLU from all samples before calculating fold induction.
Variability in assay performance	Variations in cell growth conditions including cell plating, harvest density, cell viability and cell doubling time can cause low assay performance and high assay variation. Avoid one-day cell passages whenever possible. Use high-quality cell culture reagents (especially serum) and plasticware for maintaining cells in culture. Ensure consistent cell growth by handling the cells exactly according to the instructions.
	Inappropriate cell handling during cell harvest, including long centrifuge times and high centrifuge speeds can cause low assay performance and high assay variation. Centrifuge the cells exactly according to the instructions.
	Inappropriate cell freezing/DMSO exposure can cause low assay performance and high assay variation. Freeze the cells exactly according to the instructions.
	Inappropriate cell counting methods can lead to variation in cell numbers in culture and assays and cause high assay variation. Ensure consistent and accurate cell counting methods.

7. References

1. De Nardo, D. (2015) Toll-like receptors: Activation, signalling and transcriptional modulation. *Cytokine* **74**, 181–9.
2. Nishiya, T. and DeFranco, A.L. (2004) Ligand-regulated chimeric receptor approach reveals distinctive subcellular localization and signaling properties of the Toll-like receptors. *J. Biol. Chem.* **279**, 19008–17.
3. Kawai, T. and Akira, S. (2007) TLR signaling. *Semin. Immunol.* **19**, 24–32.
4. Xun, Y., *et al.* (2021) Toll-like receptors and toll-like receptor-targeted immunotherapy against glioma. *J. Hematol. Oncol.* **14**, 176.
5. Leitner, G.R., *et al.* (2019) Targeting toll-like receptor 4 to modulate neuroinflammation in central nervous system disorders. *Expert Opin. Ther. Targets* **23**, 865–82.

8. Appendix

8.A. Representative Assay Results

The following data were generated using the TLR Bioassay Cells, Propagation Model, with various TLR agonists, as shown (Figures 8–12).

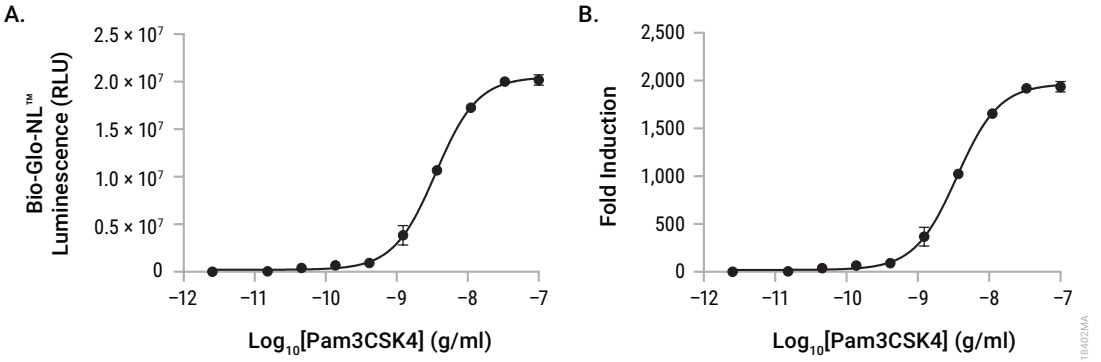

Figure 8. The TLR Bioassay measures the activity of TLR1/2 agonist Pam3CSK4. TLR Bioassay Cells were added to a 96-well assay plate 20 hours prior to the assay. On the day of assay, a titration of Pam3CSK4 was added. After a 4-hour induction at 37°C, Bio-Glo-NL™ Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. **Panel A**. The EC_{50} was 3.5ng/ml. **Panel B.** The fold induction was 1,970.

8.A. Representative Assay Results (continued)

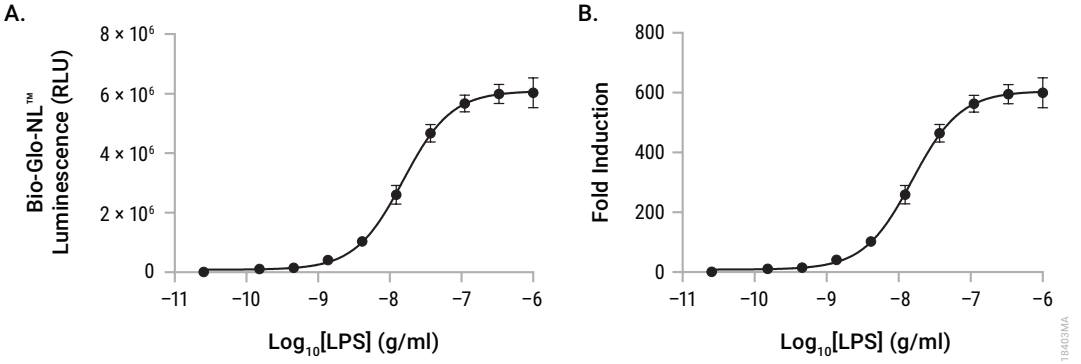

Figure 9. The TLR Bioassay measures the activity of TLR4 agonist LPS. TLR Bioassay Cells were added to a 96-well assay plate 20 hours prior to the assay. On the day of assay, a titration of LPS (InvivoGen Cat.# tlrl-eklps) was added. For reference, we used 1µg/ml as a starting concentration (1X) and a threefold serial dilution of LPS. After a 4-hour induction at 37°C, Bio-Glo-NL™ Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. **Panel A.** The EC_{50} was 16ng/ml. **Panel B.** The fold induction was 600.

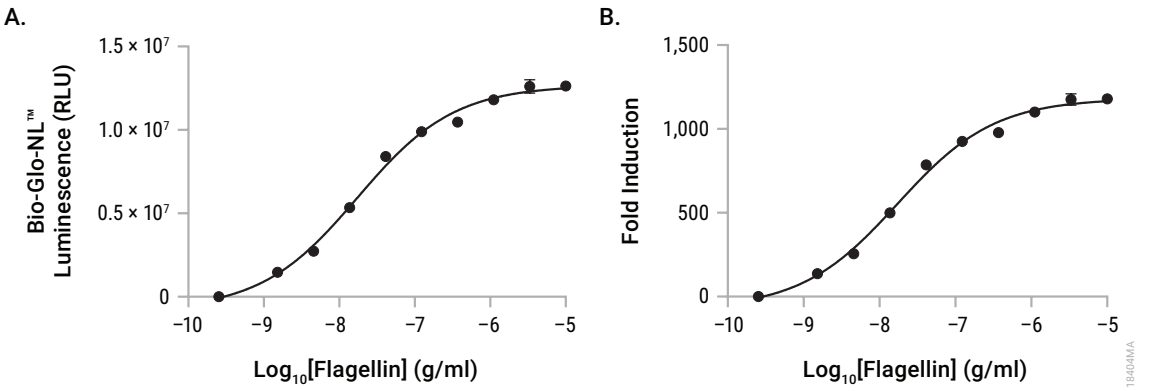

Figure 10. The TLR Bioassay measures the activity of TLR5 agonist flagellin. TLR Bioassay Cells were added to a 96-well assay plate 20 hours prior to the assay. On the day of assay, a titration of flagellin (InvivoGen Cat.# tlrl-stfla) was added. For reference, we used 10µg/ml as a starting concentration (1X) and a threefold serial dilution when testing flagellin. After a 4-hour induction at 37°C, Bio-Glo-NL™ Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. **Panel A.** The EC_{50} was 17ng/ml. **Panel B.** The fold induction was 1,180.

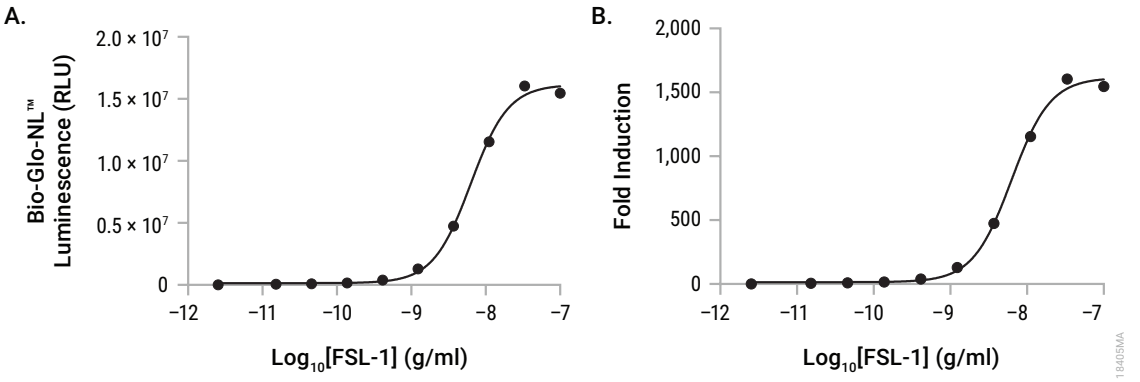

Figure 11. The TLR Bioassay measures the activity of TLR6/2 agonist FSL-1. TLR Bioassay Cells were added to a 96-well assay plate 20 hours prior to the assay. On the day of assay, a titration of FSL-1 (InvivoGen Cat.# tlrl-fsl) was added. For reference, we used 100ng/ml as a starting concentration (1X) and a threefold serial dilution for FSL-1. After a 4-hour induction at 37°C, Bio-Glo-NL™ Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. **Panel A.** The EC_{50} was 6.3ng/ml. **Panel B.** The fold induction was 1,620.

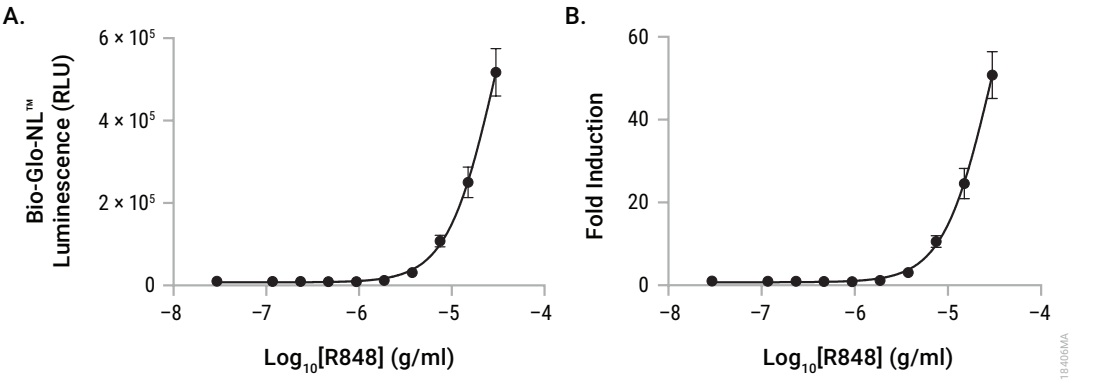

Figure 12. The TLR Bioassay measures the activity of TLR7/8 agonist R848. TLR Bioassay Cells were added to a 96-well assay plate 20 hours prior to the assay. On the day of assay, a titration of R848 (InvivoGen Cat.# tlrl-r848) was added. For reference, we used 30µg/ml as a starting concentration (1X) and a twofold serial dilution for R848. After a 4-hour induction at 37°C, Bio-Glo-NL™ Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software.

8.B. Composition of Buffers and Solutions

initial cell culture medium for TLR Bioassay Cells

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

cell growth medium for TLR Bioassay Cells

90%	RPMI 1640 with L-glutamine and HEPES
10%	FBS
200µg/ml	hygromycin B

cell freezing medium for TLR Bioassay Cells

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

assay buffer

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

8.C. 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 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
LAG-3/MHCII Blockade Bioassay	1 each	JA1111
OX40 Bioassay	1 each	JA2191
PD-1/PD-L1 Blockade Bioassay	1 each	J1250
PD-1+TIGIT Combination Bioassay	1 each	J2211
PD-L1 Negative Cells	1 each	J1191
TIGIT/CD155 Blockade Bioassay	1 each	J2201

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Macrophage-Directed Bioassays

Product	Size	Cat.#
SIRPα/CD47 Blockade Bioassay	1 each	JA6011
SIRPα/CD47 Blockade Bioassay, Fc-Dependent	1 each	JA4801
TLR Bioassay	1 each	JA9011

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8.C. Related Products (continued)

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

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

Cytokine and Growth Factor Bioassays

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

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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
	100ml	G7940
Bio-Glo-NL™ Luciferase Assay System	10ml	J3081
	100ml	J3082
	1,000ml	J3083

Not for Medical Diagnostic Use.

Luminometers

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

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

Note: Additional Fc Effector, Immune Checkpoint, T Cell Activation, 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/**

9. Summary of Changes

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

1. Lumit trademark was updated to registered.
2. In Section 3, text about the product label was revised.
3. Expired patent statement was removed.
4. A third party trademark was updated to registered.

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