

TECHNICAL MANUAL

TLR Bioassay

Instructions for Use of Products
JA9011 and JA9015

TLR Bioassay

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1. Description	2
2. Product Components and Storage Conditions	6
3. Before You Begin	6
3.A. Materials to Be Supplied by the User	7
4. Assay Protocol	8
4.A. Preparing Assay Buffer, Bio-Glo-NL™ Reagent and Agonist Samples	9
4.B. Plate Layout Design	10
4.C. Preparing and Plating TLR Bioassay Cells	11
4.D. Preparing and Plating Agonist Serial Dilutions	11
4.E. Preparing and Adding Bio-Glo-NL™ Luciferase Assay Reagent	13
4.F. Data Analysis	13
5. Troubleshooting	14
6. References	15
7. Appendix	15
7.A. Representative Assay Results	15
8. Summary of Changes	18

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 I κ B and subsequent nuclear localization of NF- κ B (3). TLRs 7–9 also activate interferon response factor 7 (IRF7) via MyD88. In contrast, the TIR domain-containing adaptor protein-inducing IFN β - (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^(a-d) (Cat. # JA9011, JA9015) 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 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.

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.

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- and 384-well plate formats used for agonist screening in early drug discovery (Figure 4).

1. Description (continued)

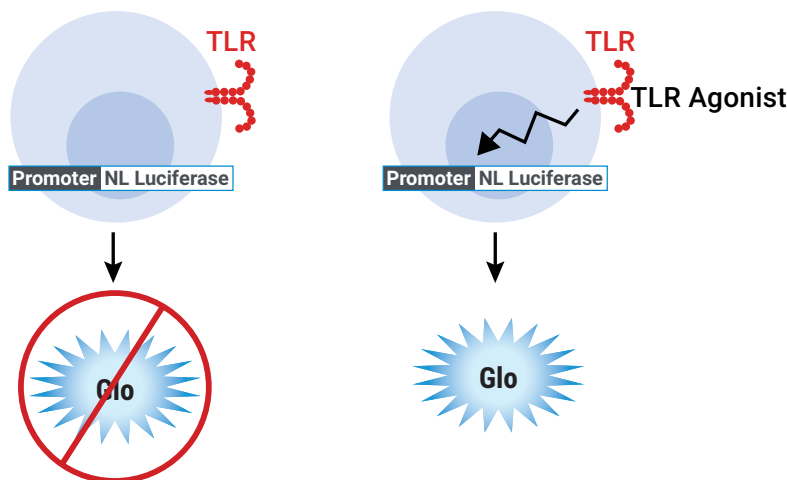


Figure 1. Representation of the TLR Bioassay. Treatment with a TLR agonist results in promoter-driven luminescence that can be detected and quantified using Bio-Glo-NL™ Reagent. The TLR Bioassay consists of a single genetically engineered cell line, TLR Bioassay Cells. In the absence of agonist, TLRs 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.

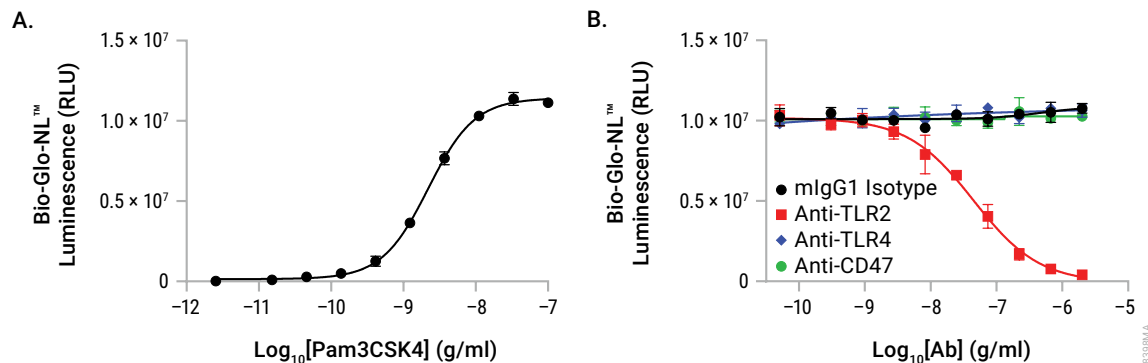


Figure 2. The TLR Bioassay reflects the MOA and specificity for TLR agonists. **Panel A.** TLR Bioassay Cells were treated with a serial titration of TLR1/2 agonist Pam3CSK4. **Panel B.** TLR Bioassay Cells were treated with 6ng/ml of Pam3CSK4 in the presence of serial titrations of blocking antibodies 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.

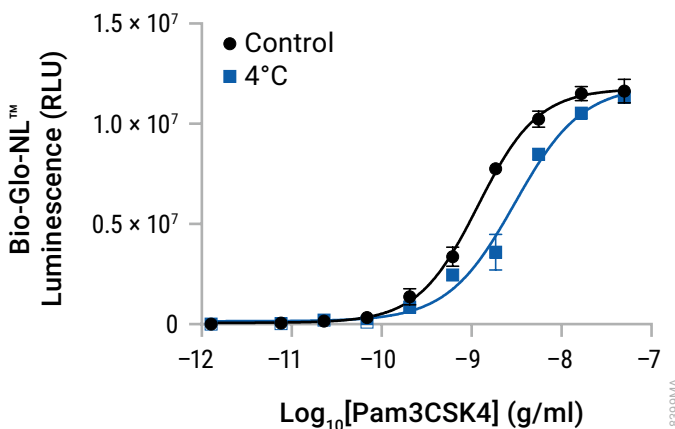


Figure 3. The TLR Bioassay is stability-indicating. Samples of the TLR1/2 agonist Pam3CSK4 were maintained at -80°C (control) or stored at 4°C for one year, then analyzed using the TLR Bioassay. Bio-Glo-NL™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were fitted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

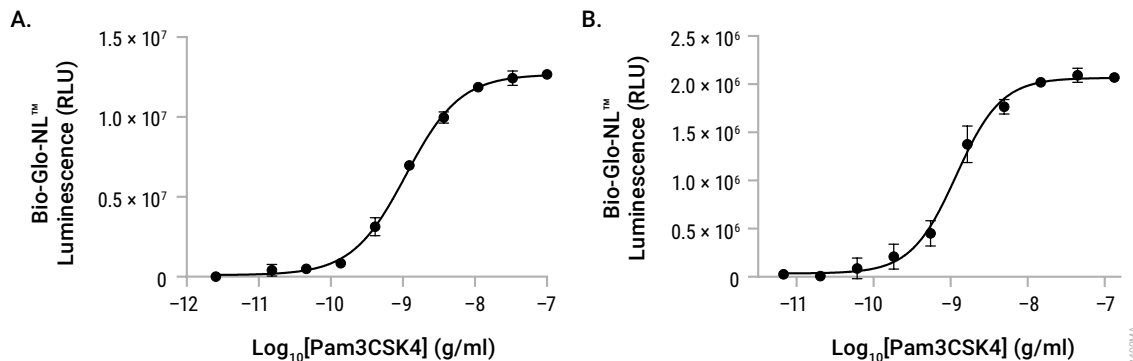


Figure 4. The TLR Bioassay is amenable to 384-well plate format. **Panel A.** The TLR Bioassay was performed in 96-well plates as described in this technical manual, with a titration of the TLR1/2 agonist Pam3CSK4. **Panel B.** The TLR Bioassay was performed in 384-well format as briefly described here. Thaw-and-use TLR Bioassay Cells were thawed and plated at 1.2×10^4 cells/20 μl /well 20 hours prior to assay, in a 384-well white assay plate (e.g., Corning® Cat.# 3570). On the day of the assay, 5 μl of a serial dilution of 5X concentrated TLR1/2 agonist Pam3CSK4 was added. After a 4-hour incubation at 37°C , 5% CO_2 , 25 μl 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	1 each	JA9011

Not for Medical Diagnostic Use. Includes:

- 1 vial TLR Bioassay Cells (0.5ml per vial)
- 36ml RPMI 1640 Medium
- 4ml Fetal Bovine Serum
- 1 vial Bio-Glo-NL™ Luciferase Assay Substrate
- 10ml Bio-Glo-NL™ Luciferase Assay Buffer

PRODUCT	SIZE	CAT.#
TLR Bioassay, 5X	1 each	JA9015

Not for Medical Diagnostic Use. Includes:

- 5 vials TLR Bioassay Cells (0.5ml per vial)
- 5 × 36ml RPMI 1640 Medium
- 5 × 4ml Fetal Bovine Serum
- 5 vials Bio-Glo-NL™ Luciferase Assay Substrate
- 5 × 10ml Bio-Glo-NL™ Luciferase Assay Buffer

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.

Store Bio-Glo-NL™ Luciferase Assay Substrate, Bio-Glo-NL™ Luciferase Assay Buffer and Fetal Bovine Serum (FBS) at –30°C to –10°C. Avoid multiple freeze-thaw cycles of the serum. The Bio-Glo-NL™ Luciferase Assay Substrate remains liquid and does not freeze.

Store RPMI 1640 Medium at +2°C to +10°C, protected from fluorescent light.

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 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 is intended for use with user-provided TLR agonists or TLR blocking antibodies in the presence of TLR agonists. The recommended induction time and assay buffer components described in Section 4 were established using the TLR1/2 agonist Pam3CSK4 and TLR4 agonist lipopolysaccharide (LPS). Representative data generated using several TLR agonists are shown in Section 7.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.

The TLR Bioassay produces a bioluminescent signal and requires a sensitive luminescence plate reader. Bioassay development and performance data included in this Technical Manual were generated using the GloMax® Discover System. An integration time of 0.5 second/well was used for all readings. 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 adjustments 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 TLR agonists or TLR agonist/antagonist antibodies
- **optional:** TLR1/2 agonist Pam3CSK4 (e.g., InvivoGen Cat.# tlrl-pms)

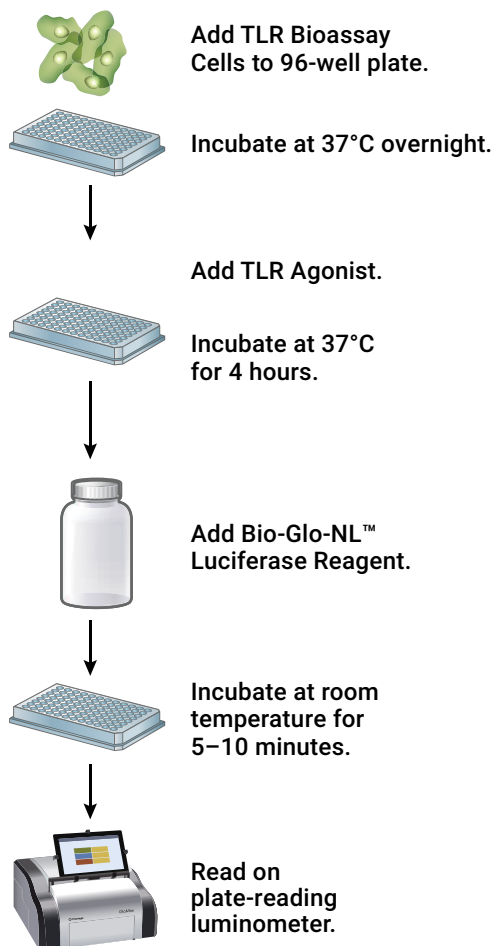
Supplies and Equipment

- 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 (e.g., 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% CO₂ incubator
- 37°C water bath
- sensitive plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System or equivalent system)

4. Assay Protocol

This procedure illustrates use of the TLR Bioassay to test two TLR agonist samples against a reference sample in a single assay run using the TLR Bioassay thaw-and-use 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.



GloMax® Discover System

18401MA

Figure 5. Schematic protocol for the TLR Bioassay.

4.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 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.

4.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 Agonist	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference
C	B	No Agonist	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test
D	B	No Agonist	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference
E	B	No Agonist	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test
F	B	No Agonist	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference
G	B	No Agonist	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”).

4.C. Preparing and Plating TLR Bioassay Cells

Thaw-and-use TLR Bioassay Cells 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.

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

Perform the following steps in a sterile cell culture hood.

1. Add 9.5ml of prewarmed (37°C) assay buffer to a 15ml conical tube.
2. Remove one vial of TLR Bioassay 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 the vial.
3. Gently mix the cell suspension by pipetting, then transfer 0.5ml cells to the 15ml conical tube containing 9.5ml 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 60µl of the cell suspension to each of the inner 60 wells of the assay plates.
5. Add 80µl of RPMI 1640 Medium (prewarmed to 37°C) to each of the outside wells of the assay plates.
6. Cover the assay plates with lids and incubate in a 37°C, 5% CO₂ incubator for 18–22 hours.

4.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 4.A in a 37°C water bath.
2. Prepare initial dilution of samples according to the instructions in Section 4.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.

4.D. Preparing and Plating Agonist Serial Dilutions (continued)

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% CO₂ 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 Agonist	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference
B		No Agonist	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference
C													
D													
E		No Agonist	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test
F													
G		No Agonist	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test
H													

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

4.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 EC₅₀ value and maximum fold induction.

4.F. Data Analysis

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

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

3. Graph data as RLU versus Log₁₀ [agonist] and fold induction versus Log₁₀[agonist]. Fit curves and determine the EC₅₀ value of agonist response using appropriate curve fitting software (such as GraphPad Prism® software).

5. Troubleshooting

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

Symptoms	Causes and Comments
Low luminescence measurements (RLU readout)	Choose an instrument designed for plate-reading luminescence detection. Instruments designed primarily for fluorescence detection are not recommended. Luminometers measure and report luminescence as relative values, and actual RLU numbers will vary between instruments. Some 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.
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 EC₅₀ value obtained with the TLR Bioassay Cells may vary from the EC₅₀ 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	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.

6. References

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4. Xun, Y., et al. (2021) Toll-like receptors and toll-like receptor-targeted immunotherapy against glioma. *J. Hematol. Oncol.* **14**, 176.
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7. Appendix

7.A. Representative Assay Results

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

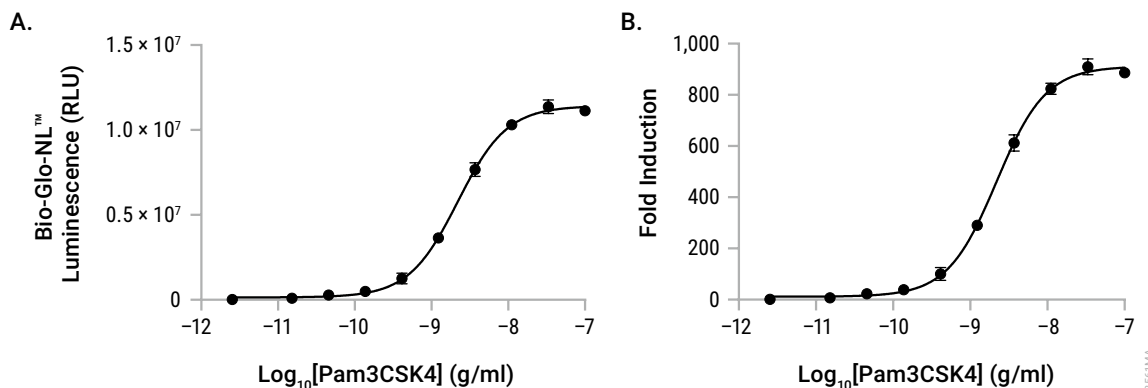


Figure 8. The TLR Bioassay measures the activity of TLR1/2 agonist Pam3CSK4. TLR Bioassay Cells were added to a 96-well assay plate 20 hours prior to the assay. On the day of assay, a titration of Pam3CSK4 was added. After a 4-hour induction at 37°C, Bio-Glo-NL™ Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. **Panel A.** The EC₅₀ was 2.2ng/ml. **Panel B.** The fold induction was 910.

7.A. Representative Assay Results (continued)

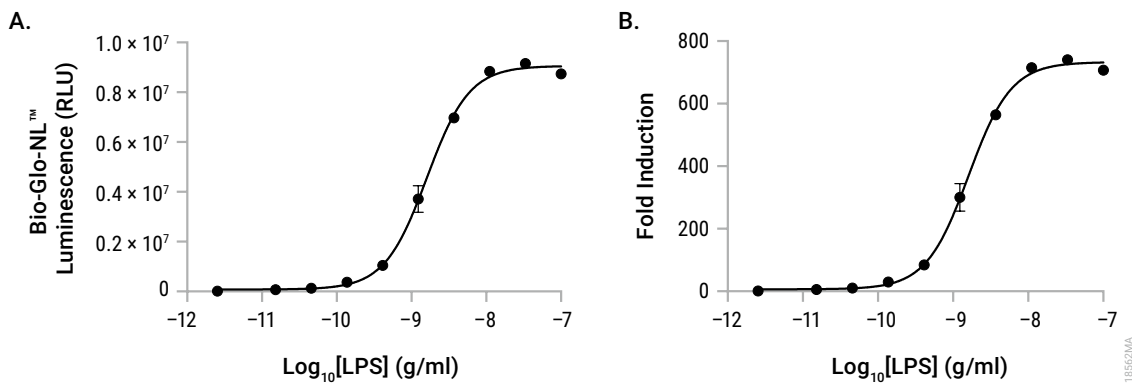


Figure 9. The TLR Bioassay measures the activity of TLR4 agonist LPS. TLR Bioassay Cells were added to a 96-well assay plate 20 hours prior to the assay. On the day of assay, a titration of LPS (InvivoGen Cat.# tlr1-eklp) was added. For reference, we used 100ng/ml as a starting concentration (1X) and 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 EC₅₀ was 1.6ng/ml. **Panel B.** The fold induction was 730.

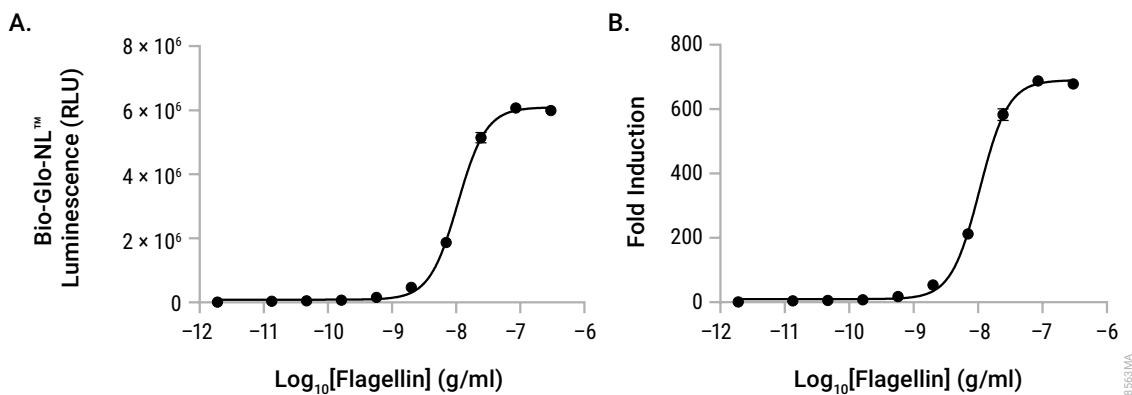


Figure 10. The TLR Bioassay measures the activity of TLR5 agonist flagellin. TLR Bioassay Cells were added to a 96-well assay plate 20 hours prior to the assay. On the day of assay, a titration of flagellin (InvivoGen Cat.# tlr1-stfla) was added. For reference, we used 300ng/ml as a starting concentration (1X) and a 3.5-fold 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 EC₅₀ was 11ng/ml. **Panel B.** The fold induction was 690.

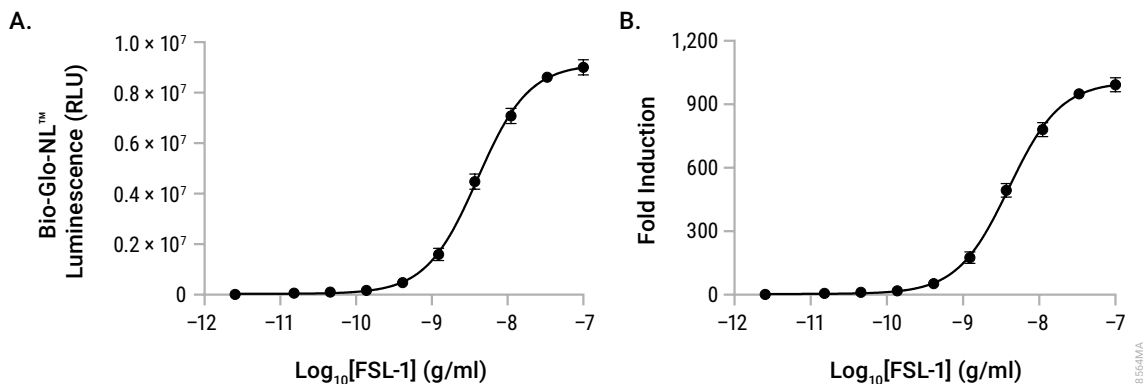


Figure 11. The TLR Bioassay measures the activity of TLR6/2 agonist FSL-1. TLR Bioassay Cells were added to a 96-well assay plate 20 hours prior to the assay. On the day of assay, a titration of FSL-1 (InvivoGen Cat.# tlr-fsl) was added. For reference, we used 100ng/ml as a starting concentration (1X) 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 4.0ng/ml. **Panel B.** The fold induction was 1,010.

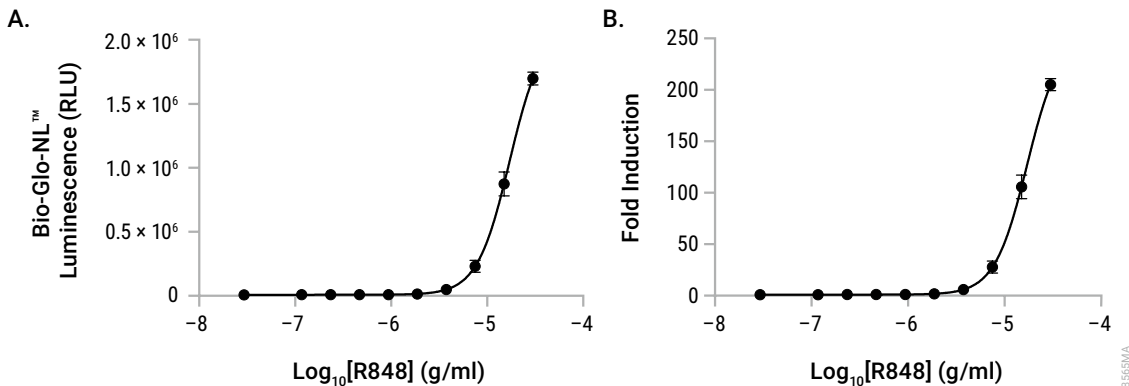


Figure 12. The TLR Bioassay measures the activity of TLR7/8 agonist R848. TLR Bioassay Cells were added to a 96-well assay plate 20 hours prior to the assay. On the day of assay, a titration of R848 (InvivoGen Cat.# tlr-r848) was added. For reference, we used 30µg/ml as a starting concentration (1X) 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. Summary of Changes

The following changes were made to the 7/26 revision of this document:

1. Removed Section 7.B, Related Products.
2. Made minor text edits.

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