

**A Novel Assay to Quantitatively Detect
Bacterial Endotoxin by Harnessing
PAMP-Triggered Immunity of *FRK1-LUC*
*Arabidopsis thaliana***



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Abstract:

Harvesting *Limulus polyphemus* (horseshoe crabs) to produce the *Limulus* amoebocyte lysate endotoxin assay for medical devices, pharmaceuticals, and drinking water is ravaging coastal ecosystems. This project develops a more sustainable and cost-effective quantitative endotoxin assay. The PAMP-Triggered Immunity response of *Arabidopsis thaliana* to pathogen-associated molecular patterns (PAMPs) was harnessed for quantitative determination of endotoxin presence based on induction of the *FRK1* gene. Transgenic *FRK1-LUC A. thaliana* were used to express luciferase (*LUC*) upon activation of *FRK1* by exposure to gram-negative bacteria. Luciferase enzyme control was first tested via plate reader, and luminescence produced by varying enzyme quantities was recorded. Next, *E. coli* ranging from 6×10^5 to 10^3 CFU/mL were infiltrated into the leaf apoplastic space of *FRK1-LUC* and wild-type plants. Luminescence of infiltrated leaf discs was measured after adding luciferin substrate to reconstitute functional luciferase. The results showed a direct relationship between bacteria concentration and luminescence. The *FRK1-LUC* luminescence versus endotoxin concentration results yielded a formula of $y = 1518e^{0.0196x}$ ($R^2 = 0.937$). Data suggest this assay achieves a sensitivity down to 18 endotoxin units/mL ($p < 0.001$, SEM = 1.76%). To determine specificity, the SeeSAR software was used to calculate binding affinities of endotoxin (LPS) and flg22 with several receptors. Results indicate high specificity in LORE-LPS binding, signifying the luminesce results were caused by LPS concentration. This method's sensitivity and specificity combined with its elimination of environmental impacts and low cost make it a promising new bacterial endotoxin assay for pharmaceutical and drinking water testing.

1. Introduction:

Endotoxins, or lipopolysaccharides (LPS), are a crucial component on the outer membrane of all Gram-negative bacteria, and consist of a Lipid A and an O-polysaccharide component [5, 6]. This molecule is severely toxic to humans and can cause effects such as septic shock. It is potentially lethal after entering the bloodstream or ingested. Endotoxins cause hypotension by eliciting the production of vasoactive amines, first directly affecting complement proteins, platelets, and neutrophils as well as indirectly through mast cell degranulation [1].

Currently, the FDA, European Pharmacopoeia, and other regulatory agencies require all parenteral drugs and medical devices to be tested for the presence of endotoxin following manufacturing. This ensures that humans are not exposed to pathogenic Gram-negative bacteria, including the common pathogen *E. coli*, through the bloodstream. Similarly, the Environmental Protection Agency (EPA) issues guidelines on the maximum limit of endotoxin in drinking water, with an acceptable concentration of 5.0 endotoxin units (EU)/kg body weight.

The most widely approved endotoxin test is the *Limulus* amoebocyte lysate (LAL) assay. Primarily, this is derived from the blood of *Limulus polyphemus*, the Atlantic horseshoe crab, as well as related species in other regions. Amoebocytes in its blood release coagulogen, and a clotting cascade is triggered when bacterial endotoxin is present [8, 11]. While this system confers high sensitivity, to 0.05 endotoxin units (EU) per mL [2], there are substantial drawbacks to the existing method.

For one, there is significant environmental damage caused by the harvesting of *L. polyphemus* for biomedical and water testing use. With minimal regulatory oversight over this industry, animal safety is not prioritized during the blood extraction process. Moreover, while harvesters claim to return all crabs back to their marine habitat, there is an estimated minimum 15% to 30% mortality rate [9]. The Atlantic States Marine Fisheries Commission has recorded declining horseshoe crab populations due to LAL assay production, and the species has been classified as vulnerable. Because *L. polyphemus* is a keystone species, other organisms of the Atlantic coast ecosystem such as the migratory *Calidris canutus* are also being threatened. This environmental damage is in turn causing economic devastation to coastal communities. Continued depletion of this limited resource ultimately threatens to destabilize the entire global marine ecosystem. Moreover, expenses of LAL are high, with *L. polyphemus* blood costing approximately \$60,000 per gallon.

One alternative is the Recombinant factor C (RfC) assay, which relies on the isolated coagulating protein from *L. polyphemus* blood. The drawbacks to this include similarly high cost, and lack of approval by regulatory authorities for almost all testing applications.

In order to solve the tremendous damage being caused by continued depletion of the limited *L. polyphemus* population, a novel endotoxin assay that overcomes the shortcomings of previous methods is needed. After analyzing organisms whose pathogen response pathways have been well-characterized, I selected *Arabidopsis thaliana*, a member of the Brassicaceae family.

These plants specifically respond to PAMPs (pathogen-associated molecular patterns), or MAMPs (microbe-associated molecular patterns), which includes LPS/endotoxin and the flg22 flagellin peptide, common components of Gram-negative bacteria. Activation of the pattern-triggered immunity (PTI) pathway of *A. thaliana* elicits an immune response based on the quantity of PAMPs/MAMPs binding to cell membrane pattern recognition receptors (PRRs) like LORE [12, 13], which is somewhat similar in physiology to the mammalian pathway [3] (fig. 1).

2. Hypothesis:

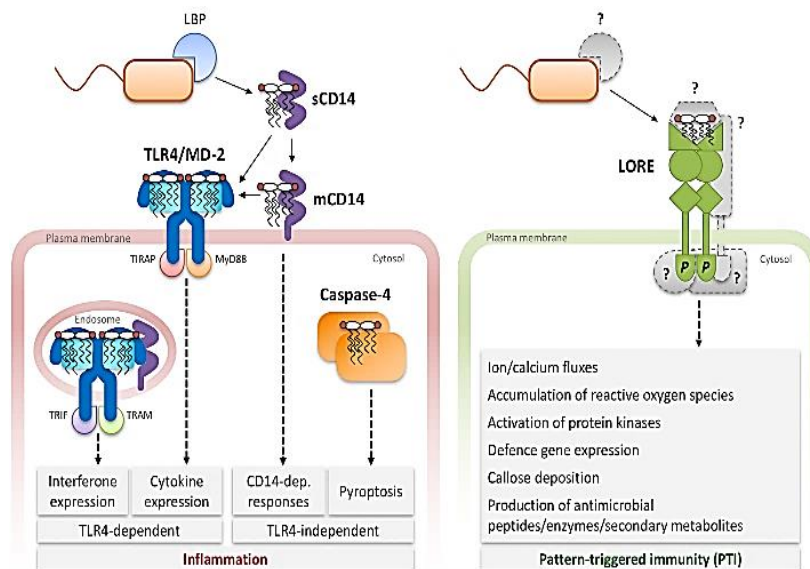


Fig 1: The *A. thaliana* pathogen defense pathway (right) compared with the mammalian system (left)

The primary gene this study focuses on is *FRK1* (flagellin receptor kinase), which is activated by LPS binding to the LORE receptor as part of the PTI pathway. I hypothesized that the level of induction of this gene could indicate the concentration of endotoxin present. The transgene I harnessed, *FRK1-LUC*, (fig. 2a) features the *FRK1* promoter attached to a luciferase gene.

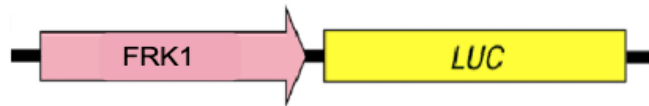


Fig 2a: The transgene design harnessed, featuring an *FRK1* promoter upstream of the *LUC* (luciferase) gene

For plants with this genetic modification, I predicted that greater luminescence would indicate higher endotoxin concentration since increased luciferase expression and resulting luminosity (fig. 2b) indicates *FRK1* induction. I also hypothesized that due to specificity of the binding between each membrane protein and its respective PAMP, the luminescence results would be significant.

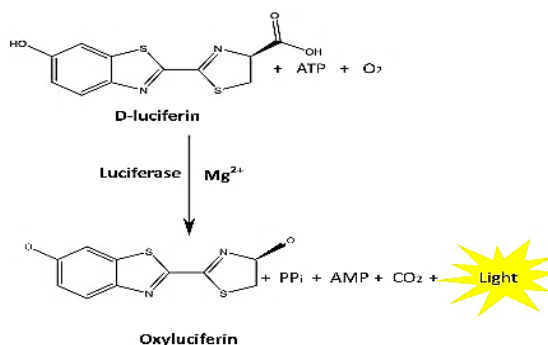


Fig 2b: The luciferin-luciferase reaction

3. Purpose:

The ultimate objective of this research is to measure induction of *FRK1* via luminescence by luciferase expression from the *FRK1-LUC* transgene as a means to determine the concentration of endotoxin in a sample, thus serving as a potentially viable replacement for LAL, RfC, and other existing assays.

4. Materials and Procedures:

Stage 1: Transgenic Plant Development and Growth

Transgenic *FRK1-LUC* and *WRKY46-LUC Arabidopsis thaliana* seeds were first produced by transforming each of these constructs into wild-type *A. thaliana* using an *Agrobacterium* vector. I selected *FRK1* because this is a key gene involved in the *Arabidopsis* response to PAMPs, particularly to LPS/endotoxin. I selected *WRKY46* because it is not induced by the PAMPs tested in this research and instead involved in the separate process of aluminum-induced malate secretion as well as in the slower effector-triggered immunity (ETI) pathogen response pathway [15, 16]. Comparing these two served to verify whether *FRK1* induction by endotoxin caused luminescence, not other potential inducers. The 3' end of each of the promoters and 5' end of *LUC* were cleaved

with the *MaeII* restriction endonuclease to create sticky ends, which were then ligated together.

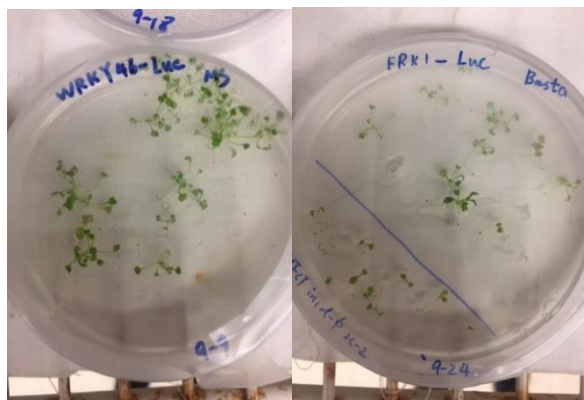


Fig. 3: *WRKY46-LUC* and *FRK1-LUC* *A. thaliana* seedlings growing on Murashige-Skoog (MS) medium

The transgenes were then inserted into wild-type Col-0 *A. thaliana* plants using *Agrobacterium tumefaciens*-mediated floral dipping transformation. Flg22 is a known inducer of *FRK1* and therefore selected to initially screen for transgenic plants. Flg22-inducible seedlings were then germinated on Murashige-Skoog (MS) medium and treated with 10 nM flg22 for a 12-hour period following 14 days growth. Finally, 20 mM luciferin substrate was added, and luminescence-positive samples were transferred to be grown on soil. I then obtained seeds of these plants and further optimized them for use in this assay by selecting the most sensitive plants in successive generations. I selected the most sensitive plants in each generation, and obtained seeds from them. Ultimately, I germinated 8th generation seeds in Petri dishes with MS medium (fig. 3). After 1 week of growth, I collected all surviving seedlings, and planted them into Pro mix BK25-V soil, with each in an individual pod (fig. 4a).

I set the growth chamber to 16 hours light and 8 hours dark per 24-hour period for 3 weeks, then switched it to 10 hours light and 14 hours dark. In addition, I grew wild-type (WT) Col-0 *A. thaliana* to use as another control. These were under identical growth conditions to the transgenic experimental plants (fig. 4b).

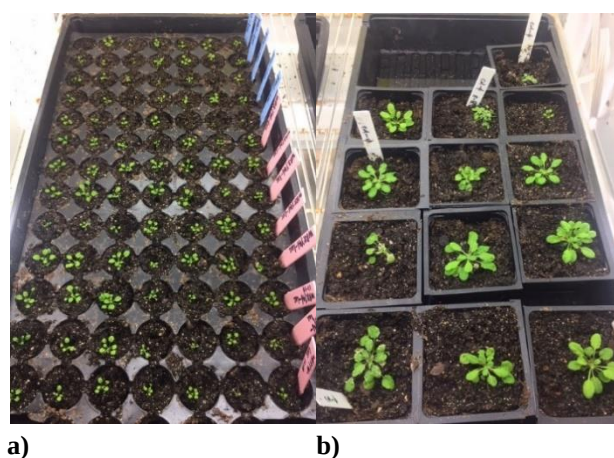


Fig. 4: WT (white), *FRK1-LUC* (blue), and *WRKY46-LUC* (pink) *A. thaliana* seedlings

Stage 2: Culturing and Dilution of DH-5 α *E. coli*

I generated the bacteria cultures and dilutions to produce a range of endotoxin concentrations. First, I made standard lysogeny broth (LB), composed of 25 g tryptone + agar dissolved in 1 L sterile ddH₂O which was then autoclaved. Then, I added 5 mL LB into each of 2 bacteria culture tubes. In one of the tubes, I inserted 5 μ L DH-5 α *Escherichia coli*.

I incubated the tubes in 37° C for 15 hours overnight. Following this, I ensured the control tube did not have any contamination or bacterial growth. Then, I performed dilutions of the *E. coli* culture with ddH₂O to obtain 18 samples with bacteria concentrations ranging from 6×10^5 to 10^3 CFU/mL. Using an absorbance reader, I measured the optical density (OD) values of each of these samples at $\lambda = 600\text{nm}$. Then, I plated 20 μ L of each sample on a Petri dish with solid LB (composed of 25 g tryptone + agar, 15 g agar, and 1 L sterile ddH₂O). There were 12 replicates per concentration, which I incubated under the same conditions of 37° C for 15 hours overnight. Finally, I calculated CFU/mL based on the quantity of bacteria colonies observed in each dish (fig. 5). I subsequently determined a relationship between OD₆₀₀ and CFU/mL. I took this measure of bacteria concentration in order to later verify the results given by my assay.

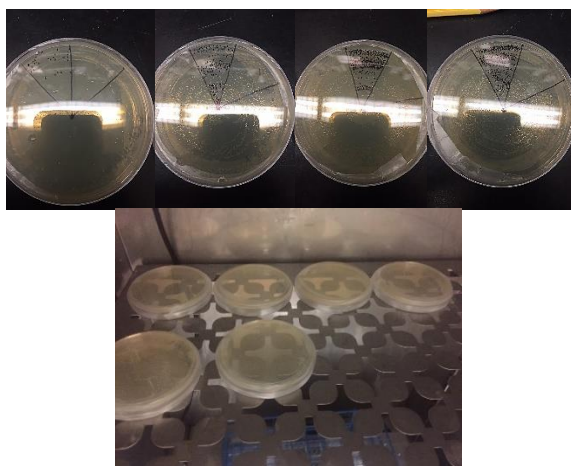


Fig. 5: Incubation of DH-5 α *E. coli* bacteria colonies

Stage 3: Control Experiment with Wild-Type *Arabidopsis Thaliana*

Next, I conducted the control experiment with the wild-type Col-0 *A. thaliana* plants. I performed bacteria infiltration after the plant leaves had matured, at 4 weeks of growth. I treated 3 plants with 10 nM flg22, another 3 with 30 nM flg22 and another 3 with 50 nM flg22 all for 12 hours overnight, and then obtained one leaf disc each from each of the plants. I placed each of these in individual wells of a microtiter plate, sprayed them with 0.2 mM luciferin, and kept them in the dark for 20 minutes. I also added 16.54 μ L dimethyl sulfoxide (DMSO) buffer + nutrients.

In addition to the flg22 control treatment, I infiltrated each concentration of *E. coli* produced in the prior stage into three individual plants. For this, I used 4-week-old *A. thaliana*. First, I drew up 1 mL of each concentration bacteria sample with a needleless syringe and injected this into the apoplastic space of a leaf to permit binding with cell membrane LORE receptors. There were three

replicates for each of the 18 dilutions, meaning 54 total infiltrates. I ensured plant and leaf sizes were identical or as similar as possible to eliminate any confounding variables. After 4 hours of exposure, I obtained leaf discs from each of the infiltrated leaves and placed these into individual wells of a microtiter plate. Identical to the flg22 treatment, I added 16.54 μL dimethyl sulfoxide (DMSO) buffer + nutrients to each well. I also then evenly sprayed each of these discs with 0.2 mM luciferin and left them to rest for 20 minutes to allow the reactants to interact. I recorded luminescence with a plate reader, taking measurements in 30 second intervals for a total of 30 minutes (fig. 6, fig. 7).



Fig. 6: Infiltration of bacteria samples into leaf apoplastic space of wild-type *A. thaliana*



Fig. 7: Microtiter plate with leaf discs from infiltrated plants

Stage 4: In Vitro Testing of Luciferase Enzyme

The subsequent stage involved testing the luminescence generated from different isolated luciferase enzyme concentrations in standardized quantities of buffer, adenosine triphosphate (ATP), and luciferin substrate. I did this to have an initial verification of the reliability of measuring luminescence from *LUC* gene expression. I first dissolved luciferin substrate in 100 μL Assay Reagent solvent. I used Reagent Buffer to dissolve the luciferase enzyme, with 200 μL 1 M Tris-HCl stabilizing the pH at 8.0. I added 50 μL assay buffer + luciferin substrate to each well, then 0 μL luciferase in one well as a control, 0.5 μL in 2 wells, 1 μL in 2 wells, 3 μL in 2 wells, and 5 μL in 2 wells. I immediately placed the microtiter plate into a plate reader to observe luminescence measurements. I set the instrument to kinetic readings, mixing before the first reading, and taking measurements from the bottom of the plate in 30 second intervals for a total of 30 minutes (fig. 8, fig. 9).

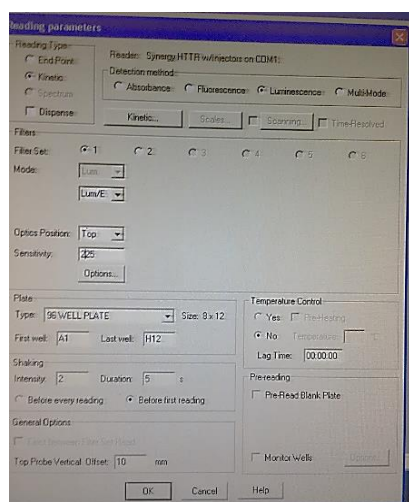


Fig. 8: Plate reader settings, with kinetic luminescence measurements every 30 seconds



Fig. 9: Insertion of plate with leaf discs into plate reader

Stage 5: Transgenic *Arabidopsis thaliana* Bacteria Infiltration

Finally, the last stage of experimentation entailed infiltrating flg22 peptide and bacteria into 4-week-old transgenic (*FRK1-LUC* and *WRKY46-LUC*) plants to observe any correlation between endotoxin concentration and luminescence based on luciferase expression. *FRK1* is specifically activated in the presence of the conserved flg22 domain from bacterial flagellin, so this infiltrate was a positive control. This ensured that the PTI pathway in the transgenic plants were functioning normally. Meanwhile, *WRKY46-LUC* was a negative control, because *WRKY46* is not induced in the PTI pathway, so I did not expect it to produce significant luminescence during the 30-minute period of recording.

I made a 1 mM stock solution of flg22 peptide by dissolving 0.00227 g of flg22 into 1 mL sterile ddH₂O, which I then aliquoted into 20 μ L proportions. By adding sterile ddH₂O, I produced final flg22 concentrations of 10 nM, 30 nM, and 50 nM.

In addition, I made a luciferin solution to serve as a standardized quantity of substrate for any amount of luciferase enzyme produced by each plant sample. I dissolved 0.001 g luciferin in 165.4 μ L sterile ddH₂O to produce a 20 mM luciferin stock and aliquoted this amongst 10 tubes. Immediately prior to testing, I added 1637.46 μ L sterile ddH₂O to each tube, to obtain a consistent final concentration of 0.2 mM luciferin.

Furthermore, I made a nutrient mixture to ensure the survival of leaf disc cells during the assay. I used a ratio of 4.43 g Murashige and Skoog Basal medium with vitamin dissolved in 1 L sterile ddH₂O, pH adjusted to 5.7 using 1 M NaOH.

The bacteria I used for infiltration were the DH-5 α *Escherichia coli* I previously cultured. I stored these at 4°C to ensure that samples remained at original concentrations without additional bacterial growth. Finally, the last step of the procedure was sample infiltration into the *FRK1-LUC* and *WRKY46-LUC* *A. thaliana*. Using a syringe, I infiltrated 1 mL of each of the 18 *E. coli* dilutions as well as the three flg22 control preparations into the leaf apoplastic space of individual plants of each genotype (fig. 10).



Fig. 10: Infiltration of bacteria test samples into transgenic *A. thaliana*

Per treatment, there were 3 replicates for the *FRK1-LUC* and 3 for *WRKY46-LUC*, and I conducted three of these entire experiment cycles to ensure consistency. Thus, I had 324 infiltration tests in total with the transgenic plants (162 in *FRK1-LUC* and 162 in *WRKY46-LUC*), and a further 162 tests with the wild-type plants.

I left all the plants to rest for 4 hours after infiltration due to the time needed to activate the PTI response. Following this rest period, I obtained equal-sized leaf discs from each of the infiltrated leaves and placed them into individual wells of a microtiter plate. To each of these, I added 100 μ L 0.2 mM luciferin to ensure sufficient substrate for any luciferase expressed, in order for luminescence to be produced in samples where FRK1 was sufficiently induced. I added neither ATP nor O₂ for this in vivo testing due to their presence already in the leaves. I left these in the dark for 20 minutes before measuring them for luminescence using identical plate reader settings as previous stages.

5. Results and Data Analysis:

The first main component of this research was the bacteria infiltration with wild-type *A. thaliana* as the negative control, with no expected luciferase expression. Luminescence readings showed, after accounting for noise and instrumental fluctuations with boxcar averaging, approximately 0 luminescence (fig. 11).

Wild-Type Col-0 *Arabidopsis thaliana* lack statistically significant PAMP-dependent luminescence

Wild-Type *A. Thaliana* PAMP Response

Negative Control

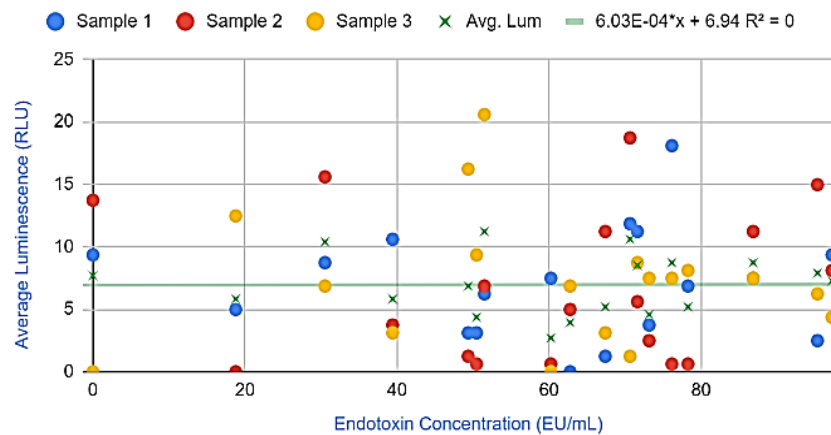


Fig. 11: Average luminescence values of WT Col-0 *A. thaliana*, indicating no significant background luminescence

An average plate reader measurement corroborates this (fig. 12).

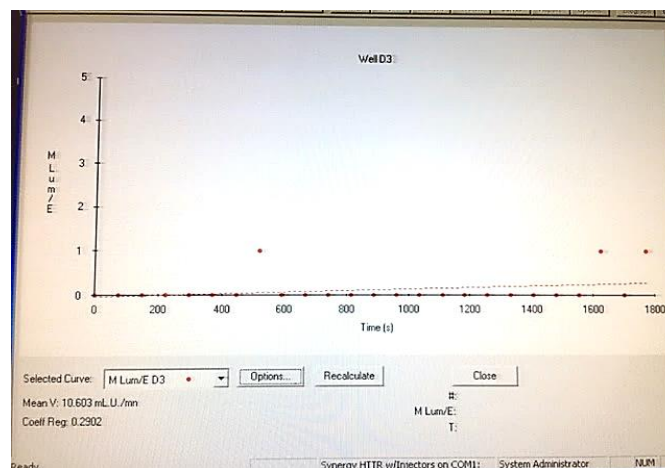


Fig. 12: Luminescence measurements from WT *A. thaliana* after bacteria infiltration

Next, the results from the in vitro experiment with varying luciferase enzyme concentrations allow determination of whether there is a reliable relationship between luciferase concentration and significant luminescence. After averaging 10 replicates for each variation in enzyme concentration,

there is a definite observed relationship between increasing luciferase concentration and luminosity, with and without ATP (fig. 13). The $R^2 = 0.684$ and 0.736 with and without $25 \mu\text{L}$ ATP, respectively, indicates direct correlation between higher luciferase concentration and luminescence. This confirmed the validity of one aspect of the hypothesis that increased luciferase expression with a constant level of luciferin substrate would yield a higher luminosity.

Luciferin-Luciferase reaction has [Luciferase] - dependent rate

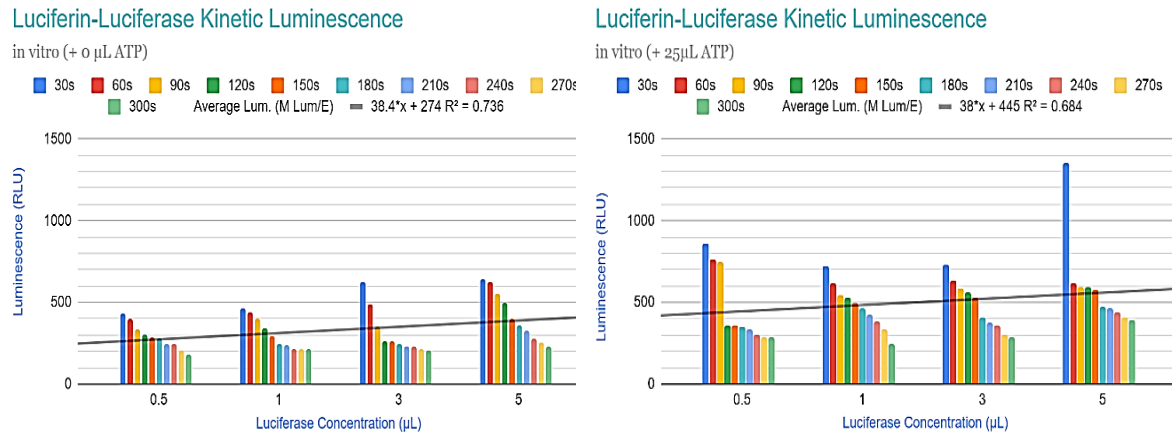


Fig. 13: Direct correlation between luciferase concentration and luminescence in luciferin-luciferase reaction

There is also an evident direct relationship between luminescence and endotoxin or flg22 peptide concentration (fig. 14, fig. 15). Based on the equation I developed by regression analysis, displayed

FRK1-LUC transgenic *Arabidopsis thaliana* displays expected results with positive control flg22 (known FRK1 inducer)

<i>FRK1-LUC</i>		<i>WRKY46-LUC</i>	
[flg22]	Luminescence (RLU)	[flg22]	Luminescence (RLU)
50nM	17621	50nM	2228.1
30nM	12463.66667	30nM	2191.55
10nM	11997.33333	10nM	2115

Fig. 14: Luminescence resulting from infiltration of positive control flg22 peptide into *FRK1-LUC* and *WRKY46-LUC* plants

on fig. 15, the steps outlined in the Materials and Procedures section can be used to infiltrate a sample into *FRK1-LUC A. thaliana*, and determine endotoxin concentration (EU) based on observed luminescence.

FRK1-LUC transgenic *Arabidopsis thaliana* displays endotoxin concentration-dependent luminescence

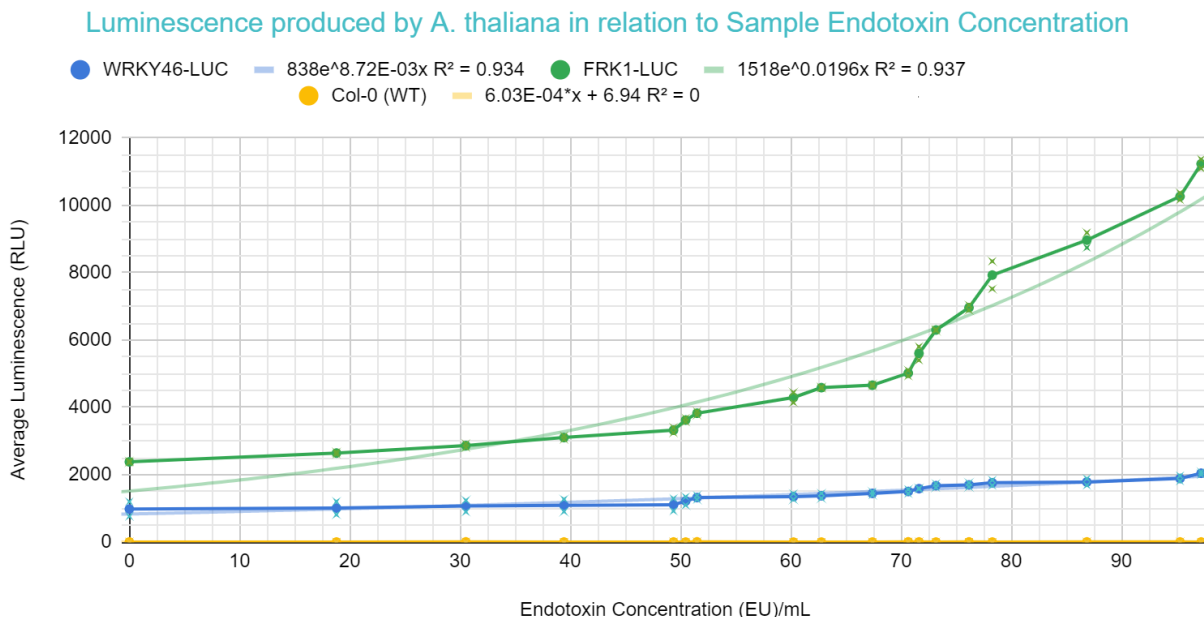


Fig. 15: Luminescence data of *FRK1-LUC*, *WRKY46-LUC*, and WT (negative control) *Arabidopsis thaliana* after infiltration with different concentrations of DH-5α *Escherichia coli*

The *FRK1-LUC* genotype demonstrates a clear positive relationship between endotoxin concentration and average luminescence (exponential $R^2 = 0.937$). Low standard error (average 1.76%) as compared with the bacteria concentration values measured via optical density indicates fairly high reliability. *WRKY46-LUC* displays a lower relationship between endotoxin concentration and luminescence, although some is observed (exponential $R^2 = 0.934$, average standard error = 8.27%), as expected since *WRKY46* is not directly involved in the response to PAMPs such as endotoxins. Wild-type plants in contrast demonstrate no correlation ($R^2 = 0$). The p-value I calculated by paired t-test is $7.967 \cdot 10^{-7}$ ($p < 0.001$), allowing rejection of the null hypotheses that both the *FRK1-LUC* plants and negative control plants display similar luminosity at each endotoxin concentration. Thus, there is a significant relationship, and greater endotoxin concentration results in higher luminescence. Using the general conversion of 20 ng endotoxin = 200 endotoxin units (EU) = 10^6 CFU *E. coli*, this assay has a sensitivity of at least 18 EU/mL.

6. Discussion and Applications:

Harnessing the PAMP-Triggered Immunity Pathway of *A. thaliana*

In recent years, the pathogen-sensing immune pathway of *Arabidopsis thaliana* has been well-characterized. It is known that *FRK1* is one of the main genes expressed when PAMP receptors are activated.

Past studies have also demonstrated the effects of PAMPs/MAMPs on *A. thaliana* at the genetic level. Specific pathways are activated only in the presence of certain triggers, and hence are specialized for defense against pathogens [4]. Therefore, activation of these pathways indicates presence of inducers such as LPS [14] (fig. 16).

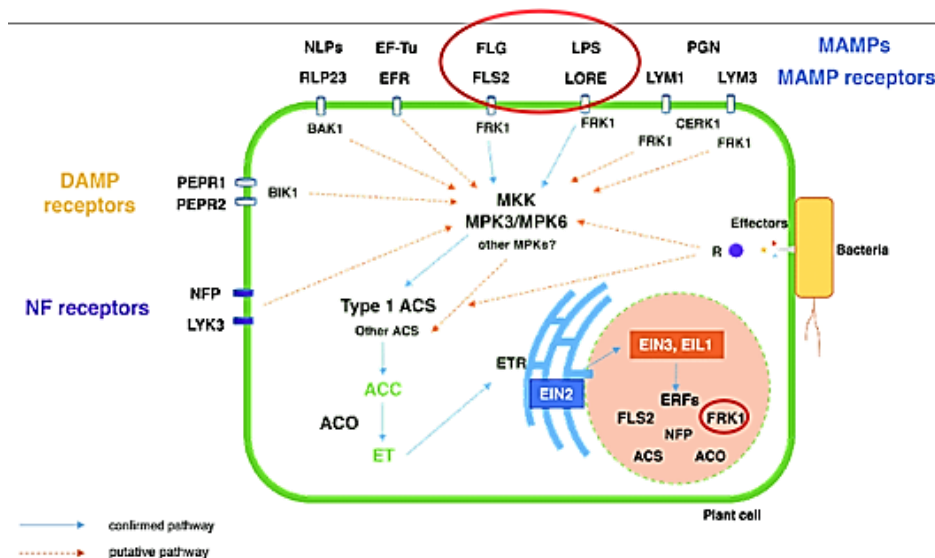


Fig. 16: The PTI signal transduction pathway in response to flg22 peptide and LPS

This project effectively harnesses the PAMP-triggered immunity pathway of *Arabidopsis thaliana* through genetic modification to determine the presence of endotoxin, or LPS, based on *FRK1* expression. LPS binds with the LORE (lipooligosaccharide-specific reduced elicitation) protein on the cell membrane. Since LPS is a conserved structure amongst all Gram-negative bacteria, *A. thaliana* uses recognition of this molecule to determine the presence of a bacterial pathogen. Binding initiates a mitogen-activated protein kinase (MPK) pathway that then leads to activation of ACC synthase. Finally, this pathway results in induction of *FRK1* in the nucleus.

Potential Interference by Other Molecules

Although flg22 also results in *FRK1* induction, the concentration of flagellin is directly correlated with LPS concentration since both molecules are conserved amongst Gram-negative bacteria. Thus, any interference from this would not have altered the relative luminescence measurements, and *FRK1* induction would indicate bacterial presence in either scenario. In addition, the relatively consistent luminescence across all endotoxin concentrations produced by the *WRKY46-LUC* plants indicates that other molecules from the bacteria did not cause differences in luminescence in the *FRK1-LUC* plants, since *WRKY46* is induced by bacterial effector proteins such as *avrRpm1* and *avrRpt2*. I can thus rule out any major difference in relative luminescence caused by injection of effector proteins by bacteria in the *FRK1-LUC* plants.

Other possible confounding variables could be the triggering of the DAMP receptors (PEPR1 and PEPR2) or the NF receptors (NFP and LYK3). The DAMP receptors likely were activated since I obtained leaf discs from each of the infiltrated leaves, which would have caused extracellular protein fragments, nucleotides, and amino acids to bind with these receptors. However, since my methods ensured the level of DAMP receptor activation remained consistent across assay testing

at all endotoxin concentrations because I took leaf discs from same-sized leaves using the same method in all trials, the relative luminescence levels measured at each endotoxin concentration are still significant. I thus considered any possible DAMP receptor activation-induced *FRK1* to be a consistent level of noise across all my samples. On the other hand, the NF receptors likely were not activated at all since they bind with nodulation factors produced by symbiotic nitrogen-fixing bacteria. The DH-5 α *E. coli* I used for testing are not nitrogen-fixing and do not produce these nodulation factors. This indicates that my results were in fact significant and caused by differences in the concentration of PAMPs/MAMPs (specifically endotoxin).

To test for interference between different PAMPs, I used the computational protein modeling and molecular docking software to compare the binding affinities of the flg22 peptide from flagellin and the Lipid A component of LPS with the FLS2 protein and LORE protein.

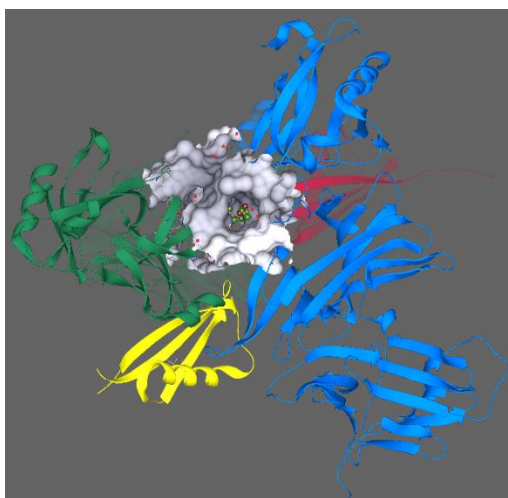


Fig. 17a: A molecular model depicting the binding of LPS (endotoxin) with a structural analog of the LORE protein

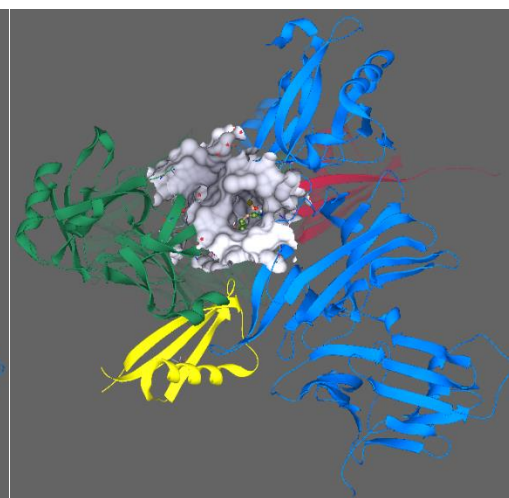


Fig. 17b: A molecular model depicting the binding of the flg22 peptide from flagellin with a structural analog of the LORE protein

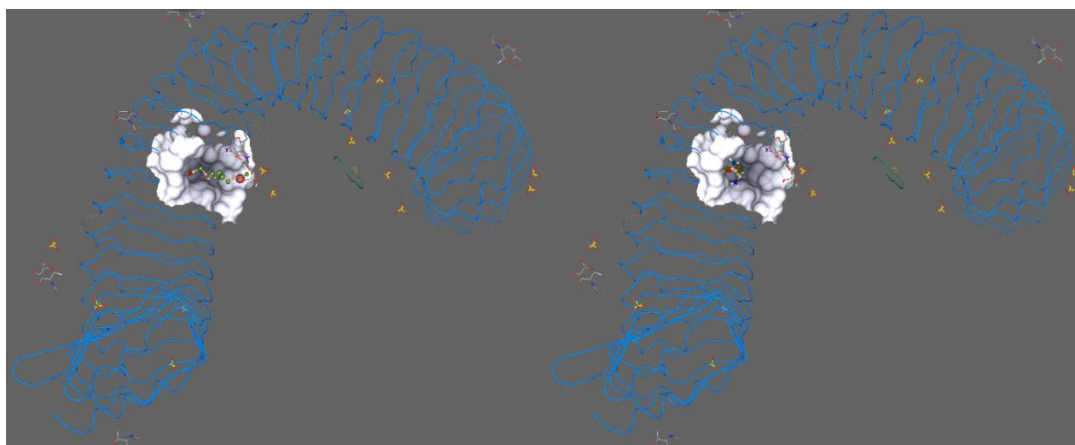


Fig. 18a: A molecular model depicting the binding of LPS (endotoxin) with the FLS2 protein

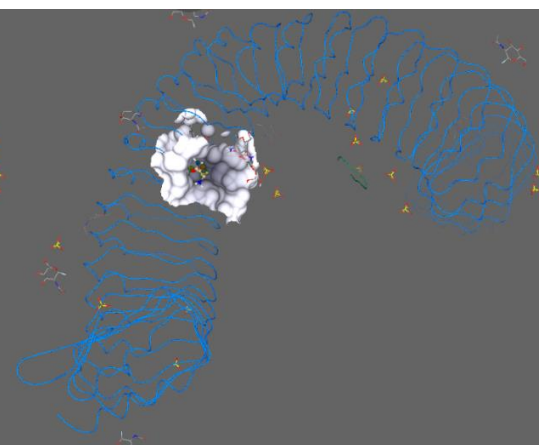


Fig. 18b: A molecular model depicting the binding of the flg22 peptide from flagellin with the FLS2 protein

Avg. Binding Affinities (K_D)	LORE Protein	FLS2 Protein
LPS (Endotoxin)	150,183.2136	101,456,896.5
Flg22 (Flagellin)	5,308,080.156	68,950,796.6

Fig. 19: Binding affinity calculations of LPS and flg22 with the LORE and FLS2 *A. thaliana* cell membrane proteins

Observing the LORE protein analog in SeeSAR, there are notably fewer molecular clashes (atoms highlighted in red) with the LPS (endotoxin) docking in comparison to the flg22 (flagellin) docking. Structurally, the LPS also fits better in the binding site than flg22. The calculated binding affinities also support LPS having a stronger binding affinity with LORE (fig. 19). The SeeSAR molecular model therefore indicates the LORE membrane protein has a higher binding affinity with LPS (fig. 17a) than with flg22 (fig. 17b).

Additionally, observing the FLS2 protein in SeeSAR, the molecular model of LPS and flg22 indicates a higher molecular favorability of flg22 binding than LPS binding. The models also indicate a greater structural favorability between flg22 and FLS2 than between LPS and FLS2. The calculated binding affinities also support flg22 having a stronger affinity with FLS2 (fig. 19). The SeeSAR molecular model therefore indicates that the FLS2 membrane protein has a higher binding affinity with flg22 (fig. 18b) than with LPS (fig. 18a).

These results lend support to there being relatively low interference between different PAMPs affecting the luminescence results, because when testing for LPS binding, there can be fairly high confidence that the LPS molecules themselves are binding to the LORE protein and not any flg22 peptides present in the bacteria sample.

Transgene Design and Application

For visualization of *FRK1* expression, I harnessed a transgene design attaching the *FRK1* promoter to the *LUC* gene. This permitted expression of luciferase, which generates luminescence after addition of the luciferin substrate, to indicate the level of *FRK1* induction. Greater luminescence therefore indicates greater *FRK1* induction, and this *FRK1* induction is directly related to LPS concentration. Hence, this assay design uses luminescence to quantify the concentration of endotoxin-containing bacteria in a sample. While this construct has been used in the past, investigators have only harnessed it to study the *A. thaliana* PAMP-triggered immunity response to the flg22 peptide, not for any direct external application. This is also why induction by this conserved flg22 molecule was used as a positive control in this study. This project goes further than existing studies by optimizing *FRK1-LUC A. thaliana* as an organism-level biosensor to quantitatively measure endotoxin concentration.

Data Variations

During testing there were some false positives (α /Type 1 error) and false negatives (β /Type 2 error). In the 0 EU/mL trials on *FRK1-LUC*, an average luminescence of 2384.9 RLU was produced. This indicates there was some background gene induction of *FRK1-LUC*. However, if this is considered to be a consistent background gene induction across all concentrations, then the RLU values at the other concentrations are still significant. For some concentrations, there was a

higher standard error, of which I will have to further investigate the cause. The overall reliability with low standard error for each concentration and statistically significant p-value indicates there is promise for applicability of this assay.

Real-World Assay Implementation

For future use of this assay for endotoxin testing, the tester can obtain 1 mL of their test sample, either liquid or eluted from a solid, and use a needleless syringe to pressure infiltrate this into the *FRK1-LUC A. thaliana* leaf apoplastic space. Following the waiting period for gene induction and addition of luciferin substrate, they can measure luminescence. Using the formula that I calculated by regression analysis ($y = 1518e^{0.0196x}$), the observed luminescence value can be substituted for the y-value, and then the x-value, endotoxin concentration, can be calculated. As shown in the model, 95% (up to two standard deviations) of results will be accurate to within 1.76% of the actual endotoxin concentration.

The data also indicates high versatility in using this novel method. Since all Gram-negative bacteria are characterized by endotoxin, or LPS, on their outer membrane, the assay can function to detect any species of this group of pathogens. Rather than detecting bacterial presence via metabolites specific to certain species or genera, this assay responds to the common endotoxin PAMP itself. Thus, it can likely detect various species of pathogenic gram-negative bacteria.

With continued refinement, this new endotoxin assay method has the potential for widespread applicability. It can replace LAL and RfC for contamination testing of medications and medical devices, and safety verification of potentially unsafe drinking water that may contain bacterial pathogens.

7. Conclusions:

1. *FRK1-LUC Arabidopsis thaliana* displays LPS/endotoxin concentration-dependent luminescence due to luciferase gene induction with the *FRK1* promoter in the PTI pathway.
2. Assay sensitivity increases from selecting more reactive plants in each generation, indicating that future development holds further potential for this method to detect lower endotoxin concentrations.
3. Results indicate potential for widespread applicability of this assay harnessing *FRK1-LUC A. thaliana* for endotoxin testing.

The novel assay developed by my study harnessing *FRK1-LUC Arabidopsis thaliana* permits specific and sensitive detection of endotoxin, found on all gram-negative bacteria including common infection-causing pathogens such as many strains of *E. coli*, *Cholera*, *Salmonella*, *Shigella*, and *Pseudomonas aeruginosa*. Regions with a contaminated water supply can use this assay for determining the safety of drinking water, pharmaceutical testers can harness it for assessment of endotoxin contamination, and environmental researchers can use this method as an easy metric by which to assess bodies of water for bacterial presence: this assay has global applications.

8. Future Work:

While this novel endotoxin assay I have developed is relatively effective, I aim to further increase its performance in the future. One objective is to increase sensitivity by testing at lower endotoxin concentrations and engineering *A. thaliana* to overexpress LORE receptors. I also will determine how to decrease false negatives at these low concentrations. I will confirm specificity by testing the system with additional types of PAMPs/MAMPs, as well as with other species of Gram-negative bacteria that are known to be human pathogens. I can potentially downregulate expression of non-LORE PAMP/MAMP, DAMP, and NF receptors in order to prevent potential interference from other bacterial molecules. I can also use molecular methods to confirm that there is no interference from DAMP or NF activation of FRK1. Finally, the ultimate goal is to test applicability with pharmaceutical and water samples to demonstrate ease of use and effectiveness, and implement this assay into test kits to be used for quantitative assessment of endotoxin or bacteria contamination in various settings.

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