APPLICATION NOTE

Endogenous detection of toll-like receptor-mediated IRF5 signaling using AlphaLISA *SureFire® Ultra.*

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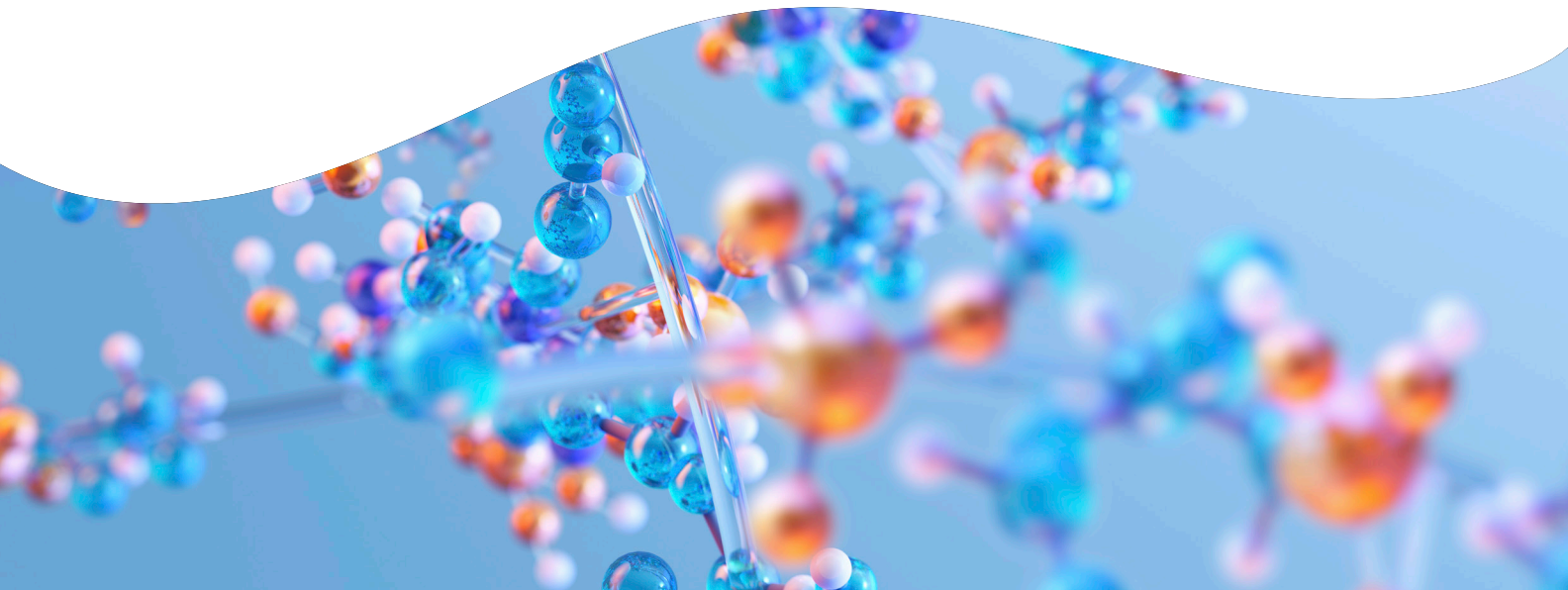
Overview

- **Revvity's AlphaLISA™ *SureFire® Ultra™* technology is a robust, no-wash assay platform ideal for studying protein interactions and post-translational modifications with unmatched sensitivity.**
- **This application note highlights the utility of AlphaLISA *SureFire® Ultra* for detecting total IRF5, phosphorylated IRF5 (Ser446), and aggregated (dimerized) levels in endogenous cells.**

1. Introduction

1.1. IRF5: A central regulator of innate immunity

Inflammation is a vital defense mechanism of the immune system, protecting the host against microbial infections and maintaining tissue homeostasis. However, when inflammation is exacerbated, it can drive the progression of various human diseases, including autoimmune and autoinflammatory disorders. At the center of this inflammatory response is **interferon regulatory factor 5 (IRF5)**, a pivotal transcription factor involved in antiviral defense mechanisms and inflammatory pathways that regulate type I interferon (IFN-α and IFN-β) production and pro-inflammatory cytokine responses, including interleukin-6 (IL-6), IL-12, IL-23 and tumor necrosis factor-alpha (TNF-α) [1, 2].



1.2. IRF5 activation, phosphorylation, and dimerization

IRF5 exists in an inactive monomeric form within the cytoplasm. Upon stimulation of toll-like receptor (TLRs) — including **TLR7**, **TLR8**, and **TLR9**—via MyD88-dependent signaling, upstream components of the IRF5 pathway, such as **IRAK4**, **TAK1**, **TRAF6**, **IKKα**, and **IKKβ** are activated. This activation leads to the phosphorylation of **p65 (NFκB)** at **serine 536 (Ser536)** and **IRF5** at **serine 446 (Ser446)**, both of which are crucial for initiating the inflammatory response **(Figure 1).**

This post-translational modification induces a conformational change in an autoinhibitory region, enabling IRF5 homodimerization and subsequent translocation to the nucleus. Once in the nucleus, IRF5 drives the transcription of type I IFNs and pro-inflammatory cytokines to amplify the immune response. Dysregulation of IRF5 activity can result in the uncontrolled production of interferon proteins, which are associated with pathogenic inflammation. Furthermore, elevated and sustained phosphorylation levels have been implicated in various types of cancer and autoimmune diseases, such as systemic lupus erythematosus (SLE) [3]. Consequently, IRF5 has emerged as a key therapeutic target for arresting the pathogenesis associated with unregulated IRF5 signaling.

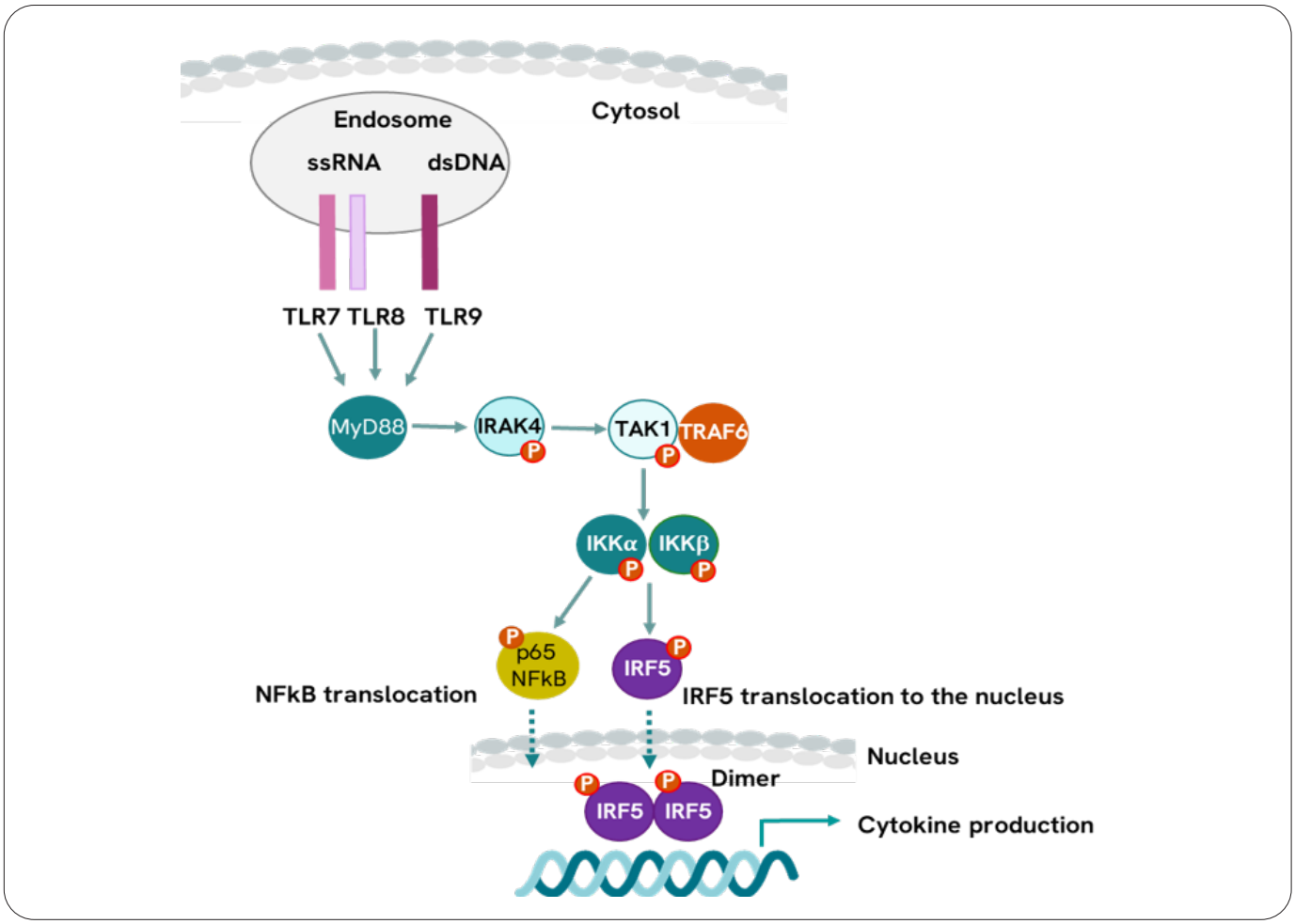


Figure 1: **Canonical IRF5 signaling cascade.** A schematic representation of the proposed mechanisms of IRF5 activation. Stimulation of TLR7, TLR8, and/or TLR9 on cell membranes via binding of pathogen-associated molecular patterns (PAMPs) triggers a signaling cascade through MyD88-dependent signaling. This activates IRAK4 and TAK1 via phosphorylation, leading to the subsequent activation of TRAF6, which, in turn, promotes the phosphorylation of IKKα and IKKβ. These kinases initiate downstream signaling events that contribute to the activation of IRF5 and NFκB. Post-translational modifications of IRF5 promote its homodimerization and translocation to the nucleus, ultimately resulting in the production of proinflammatory cytokines such as IL-6, IL-12, and TNF-α.

1.3. Application of AlphaLISA SureFire® Ultra technology

This study showcases innovative/novel immunoassays developed for the intracellular detection of total IRF5, phospho-IRF5 (Ser446), and IRF5 dimer/aggregate levels in an endogenous cell system. Additionally, the phosphorylation status of key associated signaling molecules, including TAK1, IKKα, IKKβ, and NFκB, were assessed. The data presented demonstrates the capabilities of AlphaLISA *SureFire® Ultra* technology for precisely measuring the activation of various key components involved in the IRF5 signaling cascade, including:

- **Total IRF5**
- **Phospho-IRF5 (Ser466)**
- **Dimerized (aggregated) IRF5**
- **Phospho-NFκB p65 (Ser536)**
- **Phospho-IKKα (Ser176/180)**
- **Phospho-IKKβ (Ser177/181)**
- **Phospho-TAK1 (T184/187)**

2. Materials and methods

2.1. Cell lines and treatments

RPMI 8226 cells (ATCC, Cat. # CCL-155) were cultured at 37°C with 5% CO_2 in RPMI medium (Gibco, Cat. # 21870076) supplemented with 10% inactivated fetal bovine serum (Gibco, Cat. # 16000-044) and 1% penicillin-streptomycin (Gibco, Cat. # 15140122).

2.2. CpG time course experiment

RPMI 8226 cells were harvested and seeded at a density of 200,000 cells per well in a 96-well round bottom culture plate and were then incubated for approximately 10 minutes at 37°C with 5% CO_2. Following incubation, cells were treated with 1 µM CpG (ODN 2006, MedChem Express, Cat. # HY-150218) at the specified time points (0, 0.5, 2, 4, 6 hours). After treatment, cells were washed via centrifugation at 300 RCF for 5 minutes in Hanks Balanced Salt Solution (HBSS; Gibco, Cat. # 14175095) containing 0.1% bovine serum albumin (BSA, Jackson ImmunoDiagnostics, Cat. # 001-000-173) and were then lysed with 100 µL of 1X Lysis Buffer (Revvity, Cat. # ALSU-LB-100mL). For the detection step, 10 µL of cell lysate was used for the respective AlphaLISA *SureFire® Ultra* assays, equivalent to approximately 20,000 cells per datapoint.

2.3. Cell density evaluation

RPMI 8226 cells were harvested and seeded at densities ranging from 100,000 to 500,000 cells per well in a 96-well round bottom culture plate and were then incubated for approximately 10 minutes at 37°C with 5% CO_2. Following incubation, cells were treated with CpG (ODN 2006, MedChem Express, Cat. # HY-150218) at various concentrations for 6 hours. After treatment, cells were washed via centrifugation at 300 RCF for 5 minutes in Hanks Balanced Salt Solution (HBSS; Gibco, Cat. # 14175095) containing 0.1% bovine serum albumin (BSA, Jackson ImmunoDiagnostics, Cat. # 001-000-173), and then lysed with 100 µL of 1X Lysis Buffer (Revvity, Cat. # ALSU-LB-100mL). For the detection step, 10 µL of cell lysate was used for the respective AlphaLISA *SureFire® Ultra* assays, with the approximate number of cells per datapoint indicated.

2.4. Measurement of IRF5 phosphorylation and dimerization induction

- ***CpG treatment***
  - RPMI 8226 cells were harvested and seeded at 100,000 cells per well in a 96-well round bottom culture plate and were then incubated for approximately 10 minutes at 37 °C with 5% CO_2. Following incubation, cells were treated with CpG (ODN 2006, MedChem Express, Cat. #HY-150218) at the indicated concentrations for 3 hours. After treatment, cells were washed via centrifugation at 300 RCF for 5 minutes in Hanks Balanced Salt Solution (HBSS; Gibco, Cat. # 14175095) containing 0.1% bovine serum albumin (BSA, Jackson ImmunoDiagnostics, Cat. # 001-000-173), and then lysed with 50 µL of 1X Lysis Buffer (Revvity, Cat. # ALSU-LB-100mL). For the detection step, 10 µL of cell lysate was used for the respective AlphaLISA *SureFire® Ultra* assays, equivalent to approximately 20,000 cells per datapoint.

- ***R848 (TLR7/8 agonist) treatment***
  - RPMI 8226 cells were harvested and seeded at a density of 200,000 cells per well in a 96-well round bottom culture plate and incubated for approximately 10 minutes at 37 °C with 5% CO_2. Following incubation, cells were treated with 1 µM R848 (MedChem Express, Cat. # HY-13740) for up to 4 hours. After the time course was complete, cells were washed via centrifugation at 300 RCF for 5 minutes in Hanks Balanced Salt Solution (HBSS; Gibco, Cat. # 14175095) containing 0.1% bovine serum albumin (BSA, Jackson ImmunoDiagnostics, Cat. # 001-000-173), and then lysed with 50 µL of 1X Lysis Buffer (Revvity, Cat. # ALSU-LB-100mL). For the detection step, 10 µL of cell lysate was used to evaluate IRF5 Aggregate and Phospho (Ser446) expression using the respective AlphaLISA *SureFire® Ultra* assays. Equivalent to approximately 40,000 cells per datapoint.

2.5. AlphaLISA SureFire® Ultra assay principle

AlphaLISA technology is a fast, highly sensitive and homogeneous no-wash assay platform with a broad dynamic range that can be performed in a microplate format. These features make AlphaLISA technology well-suited for measuring both very low and high levels of analytes across various matrix types. AlphaLISA assays utilize two types of beads: Donor beads and Acceptor beads. In an AlphaLISA *SureFire® Ultra* assay, Donor beads are coated with streptavidin to capture a biotinylated detection antibody, while Acceptor beads are coated with a proprietary Capt*Sure*™ agent to immobilize the other detection antibody labeled with a Capt*Sure* tag. In the presence of a target protein, the antibodies bring the Donor and Acceptor beads into proximity. Upon excitation at 680 nm, a photosensitizer within the Donor bead converts ambient oxygen into an excited singlet state, which diffuses up to 200 nm and produces a chemiluminescent reaction in the Acceptor bead, emitting light at 615 nm. If an Acceptor bead is not in proximity to a Donor bead, little-to-no signal is produced over the background. A schematic of the AlphaLISA *SureFire® Ultra* assay is shown in **Figure 2.**

AlphaLISA *SureFire® Ultra* kits offer the option for either 1- or 2-plate assay protocols, where the cell culture and assay can be conducted in a single plate or in two separate plates, respectively. For this study, the 2-plate assay protocol was followed for all assays as outlined in the AlphaLISA *SureFire® Ultra* manual. Briefly, 10 µL of prepared cell lysate was transferred from the 96-cell to well culture plate to a white 384-well OptiPlate (Revvity, Cat. # 6007299), followed by the addition of 5 µL of Acceptor Mix from the respective AlphaLISA *Surefire® Ultra* assay kit. The plate was incubated for 1 hour at room temperature and then 5 µL of Donor Mix was added to each well. The plate was incubated for another hour at room temperature and protected from light. Plates were read on an EnVision™ 2105 Multimode Plate Reader using default AlphaLISA settings.

The following AlphaLISA *SureFire® Ultra* assay kits (Revvity) were used for each target as listed:

1. Human and Mouse IRF5 Total (Cat. # ALSU-TIRF5-A500)
2. Human and Mouse Phospho-IRF5 (Ser446) (Cat. # ALSU-PIRF5-A500)
3. Human IRF5 Aggregate (dimerized) (Cat. # ALSU-AIRF5-A500)
4. Human and Mouse Phospho-NFκB p65 (Ser536) (Cat. # ALSU-PNFKB-A500)
5. Human and Mouse Phospho-IKKα (Ser176/180) (Cat. # ALSU-PIKKA-A500)
6. Human and Mouse Phospho-IKKβ (Ser177/181) (Cat. # ALSU-PIKKB-A500)
7. Human and Mouse Phospho-TAK1 (Thr184/187) (Cat. # ALSU-PTAK-A400)

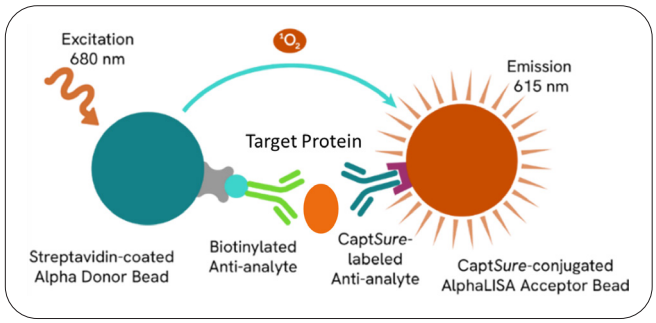


Figure 2: **AlphaLISA *SureFire® Ultra* (ALSU) assay schematic demonstrating capture of a target protein.** The target protein is detected in a sandwich assay using specific antibodies. One antibody is directed against a specific epitope on the analyte, while the other antibody is directed against another epitope on a distal part of the analyte. The resulting AlphaLISA signal in the immunoassay is directly proportional to the amount of target analyte present in the sample.

3. Results

3.1. Phospho-IRF5 (Ser466) activation peaks after 6 hours following CpG treatment in RPMI 8226 cells

The primary role of TLR9 is to recognize unmethylated cytosine-phosphate-guanine (CpG) motifs in bacterial and viral genomes and activate the signaling pathways that elicit a strong pro-inflammatory cytokine response [4-5]. The use of synthetic CpG-oligodeoxynucleotides (ODNs) has been crucial to studying TLR9-mediated activation and subsequent initiation of the innate and acquired immune responses [4].

In this study, AlphaLISA *SureFire® Ultra* technology was utilized to measure phosphorylation of IRF5 and other associated signaling molecules in an endogenous cell system upon activation with CpG-ODNs. RPMI 8226 cells were treated with a fixed dose of CpG for up to 6 hours.

Early activation of TAK1, NFκB p65, IKKα, and IKKβ was observed following treatment with the TLR9 agonist **(Figure 3)**. NFκB p65 (Ser536) phosphorylation levels peaked at 0.5 hours post-treatment, while phosphorylation of TAK1 (Thr184/187), IKKα (Ser176/180), and IKKβ (Ser177/181) peaked at 2 hours with a 4-5-fold increase in Alpha signal. The phosphorylation levels of these kinases decreased after 2 hours but persisted above basal levels for up to 6 hours.

Downstream phosphorylation of IRF5 (Ser446) was markedly increased following 2 hours of CpG treatment, peaking at 6 hours with a 12-fold increase in signal. These results, in agreement with the literature [6], demonstrated the role of TAK1/IKKα/IKKβ kinases in the induction of IRF5 phosphorylation and its involvement in late stages of the inflammatory response. Overall, these findings demonstrated a temporal and robust progression of the IRF5 signaling cascade induced by CpG treatment, which was accurately captured by AlphaLISA *SureFire® Ultra* technology.

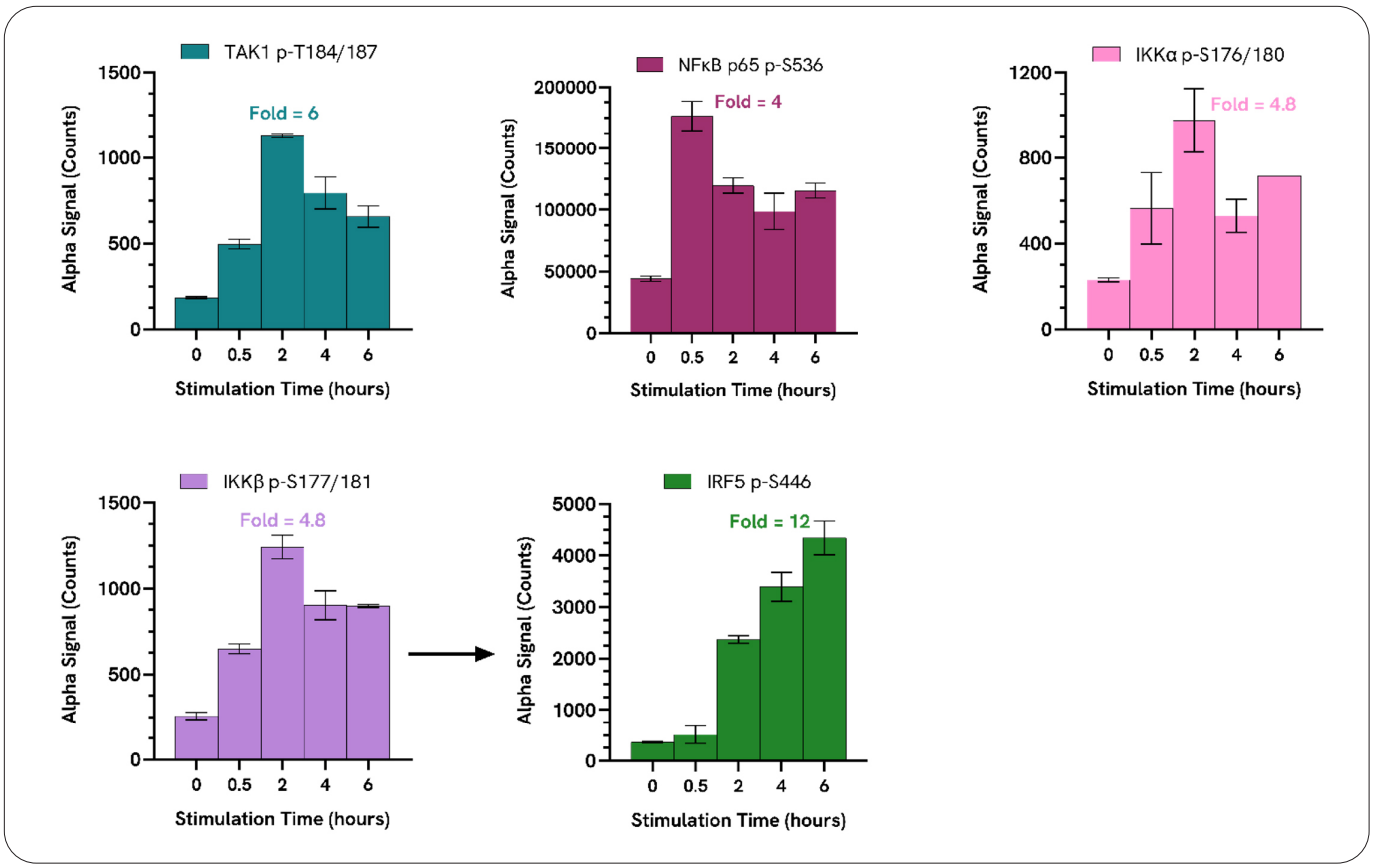


Figure 3: **TLR9 activator CpG induces MyD88-IRF5 signaling activation in RPMI 8226 cells.** Cells were treated with 1 µM CpG (ODN 2006) at the indicated timepoints. Alpha signal was measured for each target using the respective AlphaLISA *SureFire®* Ultra assay kit. Equivalent to approximately 20,000 cells per datapoint.

3.2. R848-mediated activation of TLR7/8 induces IRF5 phosphorylation in RPMI 8226 cells

Following the demonstration of CpG-mediated TLR9 activation and its role in inducing IRF5 phosphorylation in RPMI 8226 cells, we investigated whether R848—a TLR7/8 agonist—would similarly activate IRF5. Upon treatment with R848, phosphorylation of IKKα (Ser176/180) and IKKβ (Ser177/181) occurred within 1 hour of stimulation, with maximal signals observed in both at 3 hours post-treatment **(Figure 4)**. Phosphorylation of IRF5 (Ser446) peaked at 6 hours, reiterating the activation previously observed following CpG treatment.

Overall, the phosphorylation levels of IKKα, IKKβ, and IRF5 detected in RPMI 8226 cells stimulated with R848 were more modest compared to the levels induced by CpG **(Figure 3)**. However, the exceptional sensitivity of AlphaLISA *SureFire® Ultra* platform allowed for a comprehensive evaluation of the IRF5 pathway in response to a TLR7/8 agonist.

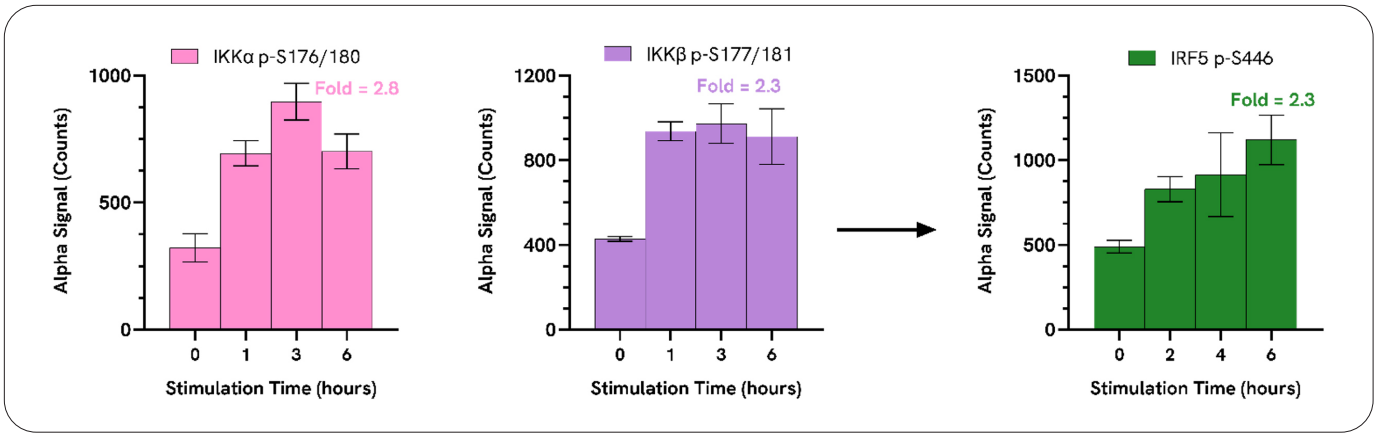


Figure 4: **R848-mediated activation of TLR7/8 induces IRF5 phosphorylation in RPMI 8226 cells.** RPMI 8226 cells were treated with 1 µM R848 at the indicated time points. Alpha signal was measured for each target using the respective AlphaLISA *SureFire® Ultra* assay kit. Equivalent to approximately 20,000 cells per datapoint.

3.3. Optimization of cell density to maximize the IRF5 phosphorylation signal window using AlphaLISA SureFire® Ultra

Following the time course evaluation, where peak activation of IRF5 was detected 6 hours post-CpG and R848 treatment, we used this timepoint to further examine the impact of cell density on IRF5 phosphorylation levels using AlphaLISA *SureFire® Ultra* to optimize assay conditions for achieving the best signal window. This approach allowed for an in-depth evaluation of the assay's sensitivity and performance across varying cell densities.

To assess the impact of seeding density on IRF5 phosphorylation in response to CpG treatment, cells were seeded at densities ranging from 100,000 to 500,000 cells per well (corresponding to 10,000 to 50,000 cells per datapoint) and treated with CpG at varying concentrations for 6 hours. IRF5 activation was then quantified by measuring fold changes in phospho-IRF5 levels across the different cell densities.

Phosphorylation of IRF5 increased in a dose-dependent manner, while IRF5 Total levels remained unchanged **(Figure 5)**. At the lowest cell density (10,000 cells per datapoint), CpG treatment resulted in a 10-fold increase in phospho-IRF5 (Ser446) levels **(Figure 5)**. Increasing the seeding density resulted in greater fold changes in phospho-IRF5 (Ser446), with the highest density (50,000 cells per datapoint) producing a 37-fold increase in signal.

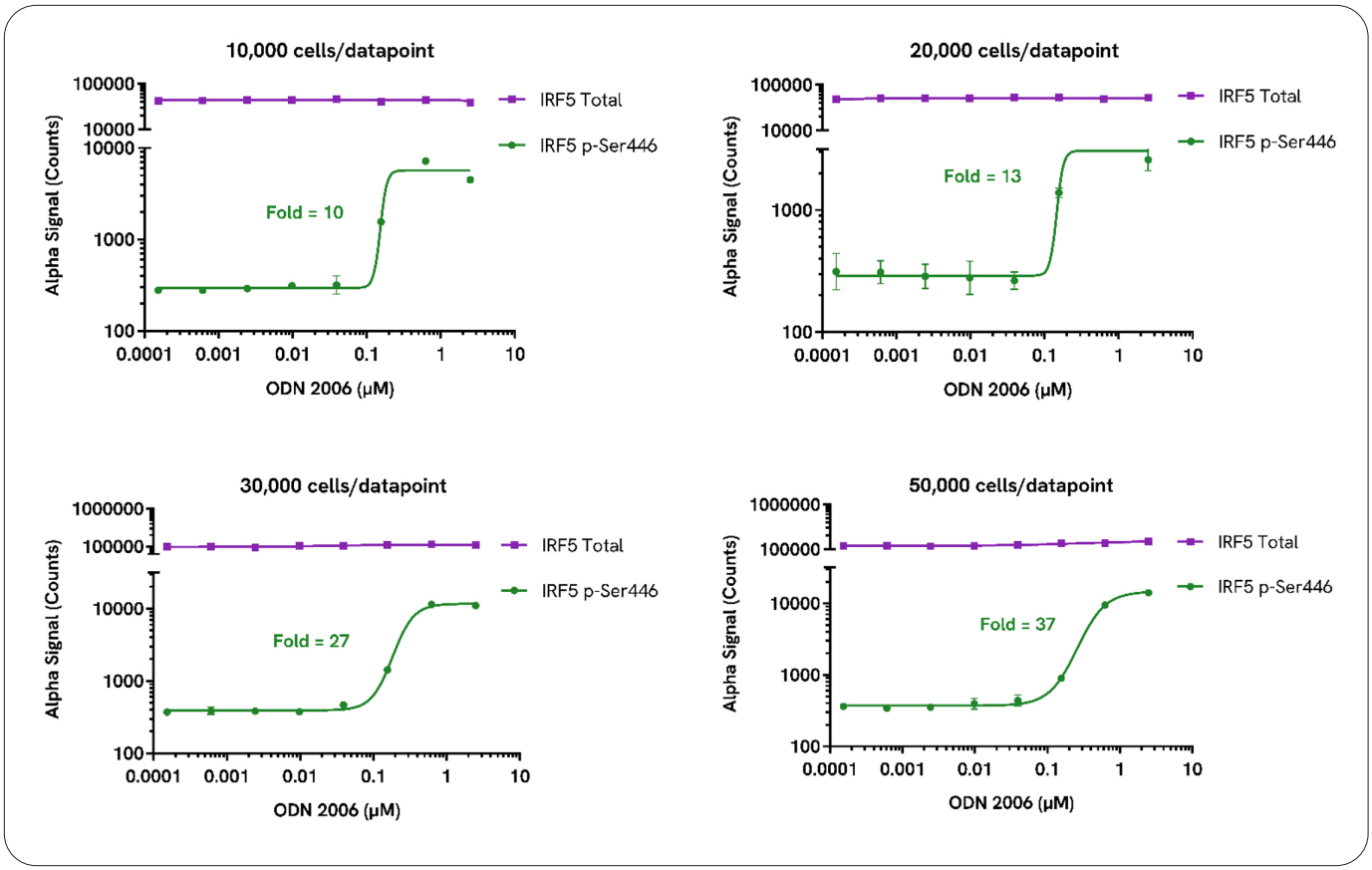


Figure 5: **Optimization of cellular conditions for IRF5 phosphorylation.** RPMI 8226 cells were treated with CpG (ODN 2006) at the indicated concentrations for 6 hours. Various cell densities were evaluated and measured to evaluate Phospho (Ser446) and Total IRF5 levels using the respective AlphaLISA *SureFire® Ultra* assay kits.

3.4. TLR activation induces IRF5 phosphorylation followed by dimerization

Following the optimization of cell density for maximizing the IRF5 phosphorylation signal window, we expanded our investigation to determine whether phosphorylation leads to IRF5 dimerization (aggregation) and whether AlphaLISA *SureFire® Ultra* could accurately reflect these events.

Both IRF5 phosphorylation and dimerization were induced in a dose-dependent manner in RPMI 8226 cells treated with CpG **(Figure 6A)**. A 25-fold increase in IRF5 phosphorylation was observed, along with a 20-fold induction in dimer formation. As expected, Total IRF5 levels remained unchanged.

Similarly, R848-induced TLR7/8 activation increased both phosphorylated and dimerized IRF5 after 1 hour of treatment, peaking at 4 hours with a 6-fold increase in Alpha signal. Phospho (Ser446) and dimerized IRF5 levels decreased after this peak but remained above basal levels for up to 7 hours **(Figure 6B)**.

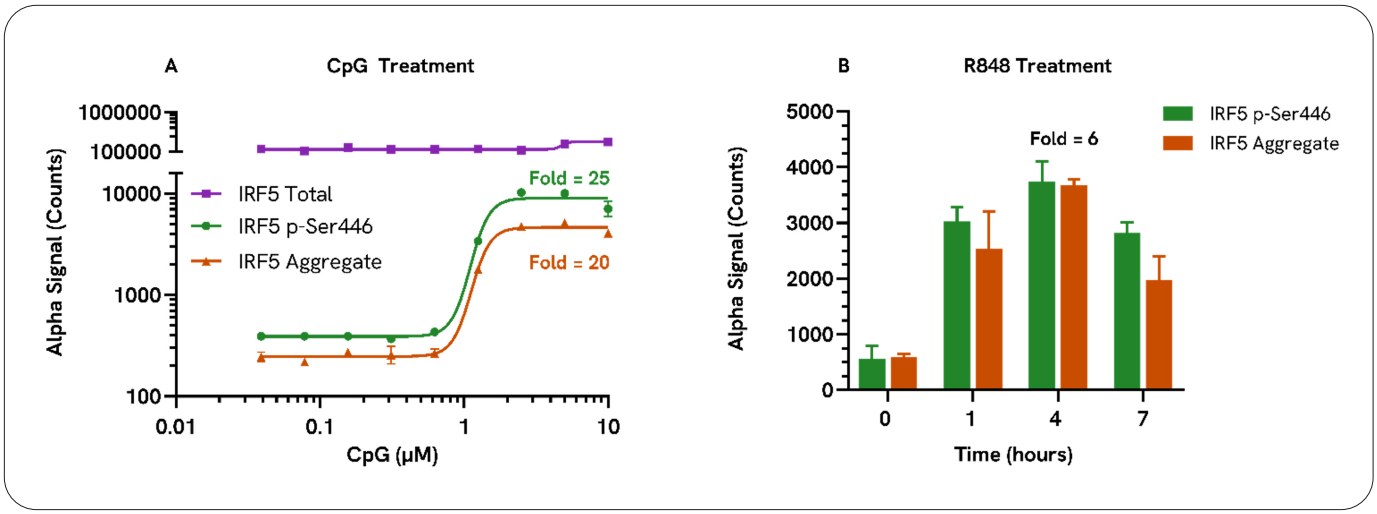


Figure 6: **TLR-mediated stimulation induces IRF5 phosphorylation and subsequent dimerization.**

A. TLR9 activator CpG induced IRF5 phosphorylation and dimerization. RPMI 8226 cells were treated with CpG (ODN 2006) at the indicated concentrations for 3 hours. Alpha Signal was measured for each IRF5 target using the respective AlphaLISA *SureFire® Ultra* assay kits. Equivalent to approximately 20,000 cells per datapoint.

B. **TLR7/8 agonist R848 induced IRF5 phosphorylation and dimerization.** RPMI 8226 cells were treated with 1 µM R848 at the indicated timepoints. Alpha Signal was measured for Phospho (Ser446) and Dimerized (Aggregate) IRF5 using the respective AlphaLISA *SureFire® Ultra* assay kits. Equivalent to approximately 40,000 cells per datapoint.

4. Conclusion

This study demonstrates the utility of AlphaLISA *SureFire® Ultra* technology for the precise and sensitive detection of key signaling molecules, including total, phosphorylated, and dimerized (aggregated) IRF5 in immune cells. Additionally, the assay platform enabled the detection of critical signaling proteins involved in the IRF5 pathway, such as phosphorylated NFκB p65 (Ser536), IKKα (Ser176/180), IKKβ (Ser177/181), and TAK1 (T184/187). The versatility in detecting multiple targets with high precision allows for a complete and comprehensive analysis of immune signaling dynamics that is applicable across multiple research areas.

The data highlight AlphaLISA *SureFire® Ultra*'s ability to detect endogenous IRF5 phosphorylation using minimal cell numbers, making it ideal for studies with limited sample availability. Optimizing cell density was crucial for maximizing the assay's performance, enabling the detection of both phosphorylated and dimerized forms of IRF5 with a broad signal window. This emphasizes the significance of optimizing this parameter during assay development to ensure precise and accurate detection of signaling events with a window that captures both early phosphorylation and subsequent events, such as dimerization.

Overall, these findings underscore the potential of AlphaLISA *SureFire® Ultra* assays to accelerate research in IRF5 signaling and related therapeutic areas, providing researchers with a powerful platform for exploring complex signaling pathways. The ability to profile both early and late signaling events with high sensitivity and temporal resolution positions this technology as an invaluable tool in immunological research, particularly in studying immune response regulation and therapeutic development.

5. References

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