revvity

APPLICATION NOTE

Characterize STING agonists with HTRF human IFN-β.

This application note demonstrates how HTRF® human IFN-β assay enables a rapid and reliable identification and characterization of STING targeting compounds.

Introduction

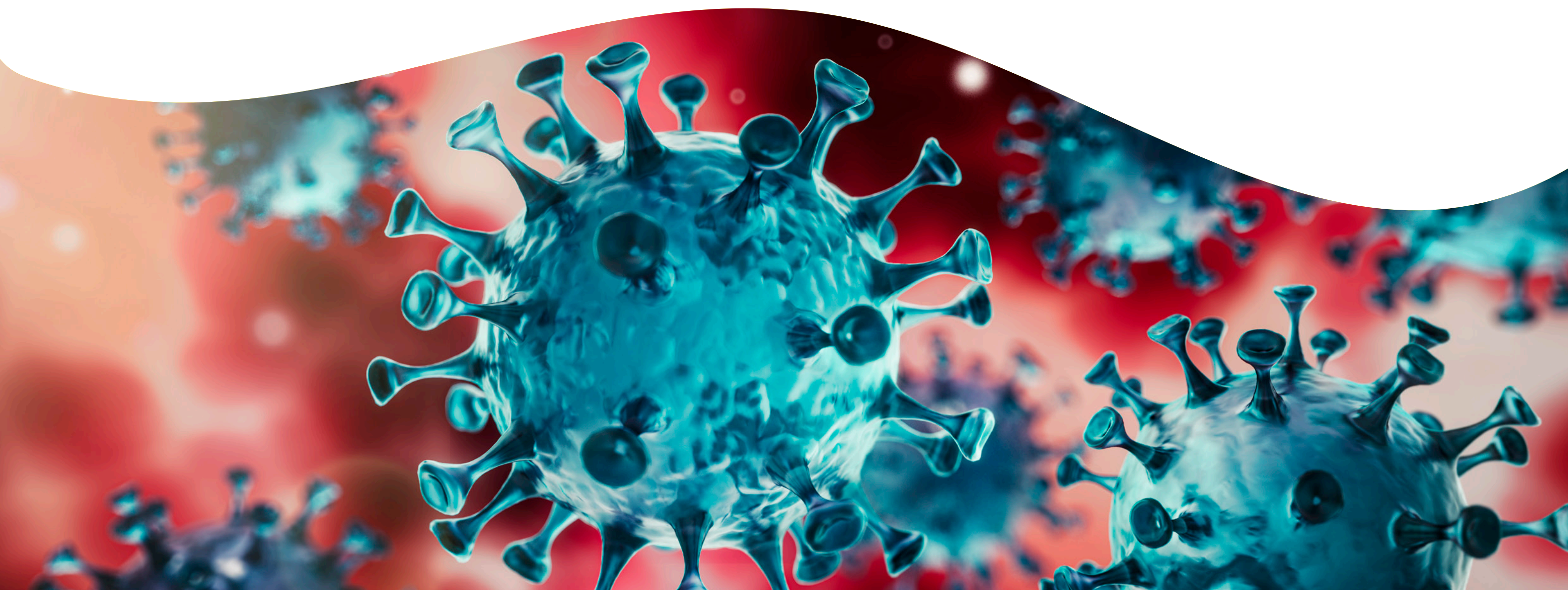
IFN-β along with IFN-a belongs to type I IFN and is rapidly produced in response to infection. Although the crucial role of IFN-β has been well established in innate defense to viral infection, this cytokine is also an important factor linking innate and adaptive immunity. IFN-β has been shown to induce dendritic cell maturation and the cross-priming of cytotoxic CD8+ T-cells.

The production of IFN-β is ultimately controlled by the Interferon Regulatory transcription Factors, IRF3, IRF7, and IRF5, downstream of Pattern Recognition Receptors including Toll-Like receptors, the RIG-I-Like receptors, or the DNA cytosolic sensor cGAS.

In cancer therapy, boosting innate immunity has gained wide recognition. Emerging strategies exploit the STING signaling pathway to enhance anticancer immune responses, as represented in the diagram below. As an example, a first clinical trial combining a STING protein agonist, ADUS100, and a PD-1 checkpoint inhibitor has recently been initiated (1, 2).

Monitoring IFN-β release can be performed by using a recombinant reporter cell line, as well as immunoassay methods such as ELISA.

For research purposes only. Not for use in diagnostic procedures.



Here we demonstrate that HTRF® human IFN-β provides a straightforward assay for accurately quantifying the secretion of human IFN-β from native cells, like THP1 or PBMCs. Using small amounts of samples, this HTRF human IFN-β assay enables a rapid and reliable identification and characterization of STING targeting compounds.

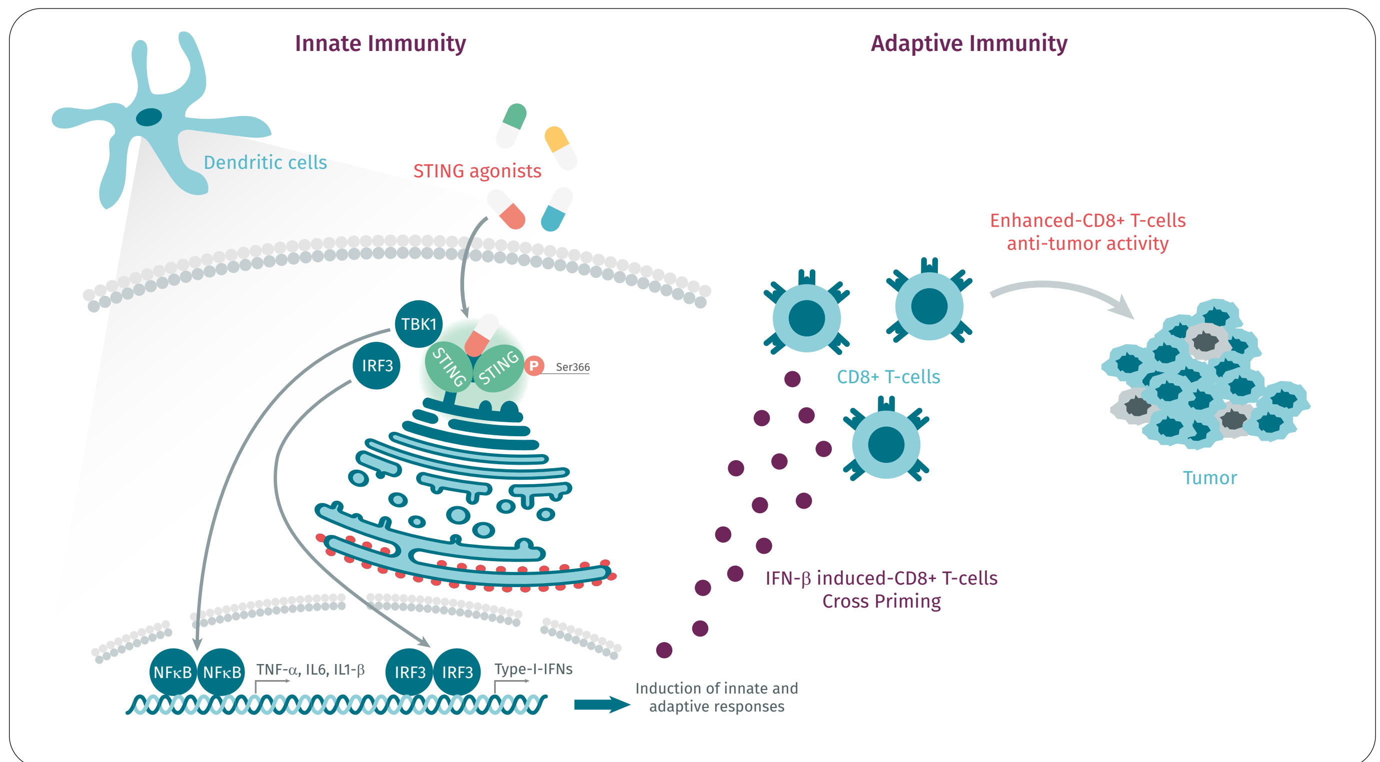


Figure 1: HTRF assay protocol description.

Principle of no-wash HTRF IFN-β assay

After cell treatment, 14µL of cell supernatants are transfered to a HTRF detection microplate, followed by 2µL of activation reagent and 4µL of pre-mixed HTRF antibodies. The HTRF signal is recorded after a 3 hour incubation at RT.

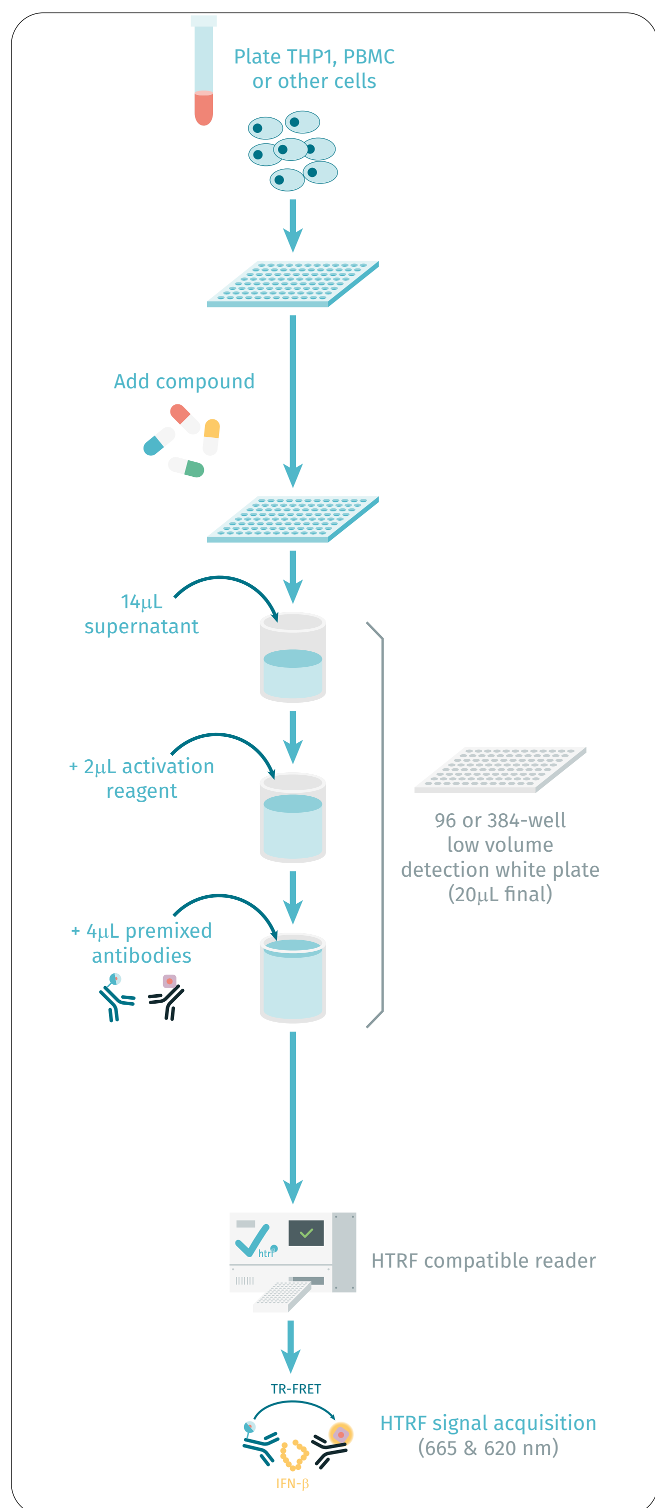


Figure 2: Principle of no-wash HTRF IFN-β assay.

HTRF IFN-β is a reliable readout of the sting-induced IRF dependent pathway

HTRF quantification of IFN-β, IL1-β, and TNF-α in THP1 cells

THP1 WT-cells were seeded at 1,000,000 cells per well in 275µL RPMI supplemented with 10% FCS.

After incubation at 37°C, 5% CO_2, the cells were stimulated or not with 30µL of the STING agonist 2’3’ cGAMP used at 400µM final. Stimulation was performed for 24 hours, then either 14µL or 16µL of cell supernatants were transfered to a detection plate prior to the quantification of IFN-β, IL6, and TNF-α, according the corresponding protocols.

Figure 3: HTRF Quantification of IFN-β, IL1-b, and TNF-α in THP1 cells.

As shown in the 3 graphs above, IFN-β and TNF-α are efficiently secreted after 2′-3′ cGAMP stimulation, whereas the production of IL1-β remains very low, similar to the basal level.

2′-3′ cGAMP- induced STING activation triggers both TNF-α secretion through NF-κB dependent gene transcription, and IFN-β production through IRF dependent gene transcription. Thus HTRF IFN-β and TNF-α represent complementary assays for monitoring the STING pathway.

HTRF IFN-β assay is well correlated with the gene reporter approach

Correlation between HTRF and gene reporter assays

Activation of the STING pathway was induced either by 2′-3′ cGAMP or G3-YSD, a Y-form DNA cGAS ligand.

These two compounds were applied on a THP1 reporter cell line expressing two inducible reporter genes:

An SEAP reporter gene controlled by NF-κB transcriptional response element, thereby enabling NF-κB pathway study through monitoring the activity of SEAP.

A luciferase reporter gene, under the control interferon-stimulated response elements which enable the study of the IRF pathway by monitoring the activity of luciferase.

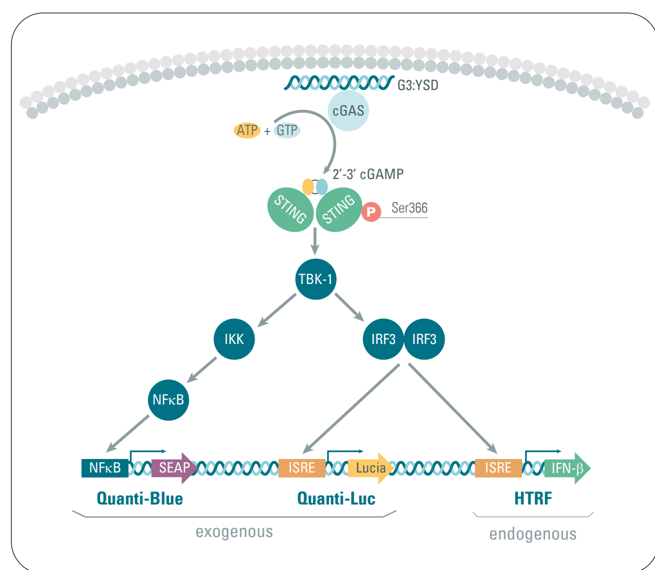


Figure 4: Assay principle description.

2′-3′ cGAMP dose response experiment reveals similar pharmacological behaviour between gene reporter and direct quantification of IFN-β with HTRF

Briefly, THP1 reporter cells were seeded at 100,000 cells per well in 180µL RPMI supplemented with 10% FCS.

After incubation at 37°C, 5% CO2, the cells were stimulated with 20µL of increasing concentrations of 2′3′ cGAMP for 24h.

Then cell supernatants were collected and IRF and NF-κB pathways were assessed using Quanti-Luc or Quanti-Blue reagents respectively (according to manufacturer's protocol), whereas IFN-β secretion was quantified in parallel with HTRF reagents.

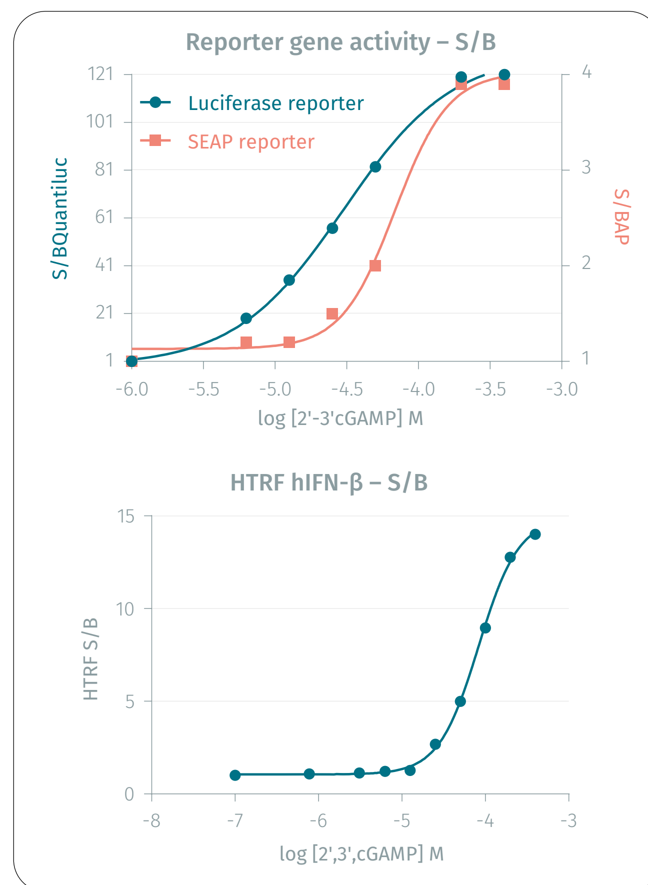


Figure 5: IRF and NF-κB pathways assessment on cells supernatants using Quanti-Luc (blue) or Quanti-Blue (orange) reagents respectively. HTRF IFN-β secretion quantification (second graph).

	Luciferase reporter	SEAP reporter	HTRF IFN-β
EC_{50} (μM)	30	70	83

As shown on the 2′-3′ cGAMP dose-response curves, a strong induction of IRF dependent gene transcription is revealed by the luciferase activity (blue curve) whereas the NF-κB dependent gene transcription is moderately promoted, as seen from the SEAP activity (red curve).
In the same experimental conditions, HTRF assay enables the detection of a significant induction of IFN-β secretion.
In addition, the determined 2′3′cGAMP EC_{50} are comparable across the 3 approaches.

Next we applied the same methodology to compare the efficacy of G3-YSD to 2′-3′ cGAMP.

Once again, gene reporter assays and HTRF IFN-β displayed similar G3-YSD efficacy, ranging from 10 to 30% compared to 2′-3′ cGAMP

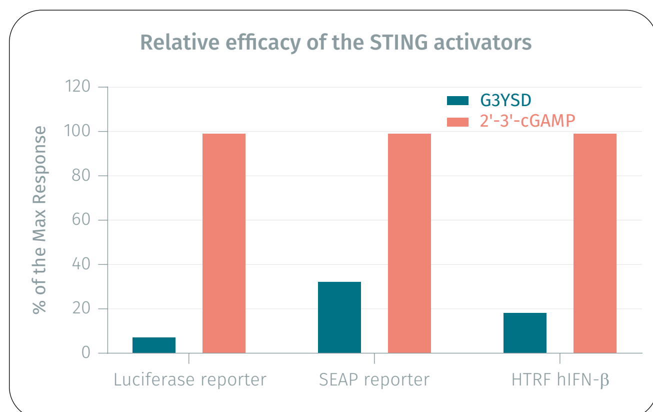


Figure 6: Comparison of G3YSD (blue) 2′-3′-cGAMP (orange) to activate the STING pathway.

HTRF IFN-β assay is well correlated with elisa assay

THP1 cells were seeded at 400,000 cells per well in 100µL RPMI supplemented with 10% FCS.

After incubation at 37°C, 5% CO2, the cells were stimulated with 50µL LPS or 2′-3′ cGAMP as indicated on the graphs.

Then 14µL or 50µL of different dilutions of cell supernatants were assessed for IFN-β secretion, using either HTRF or ELISA assay.

The graph represents IFN-β concentrations interpolated from a 32 fold dilution of cell supernatants.

As can be seen, the quantification of hIFN-β secreted either upon LPS or 2′-3′ cGAMP was similar between the two immunoassays.

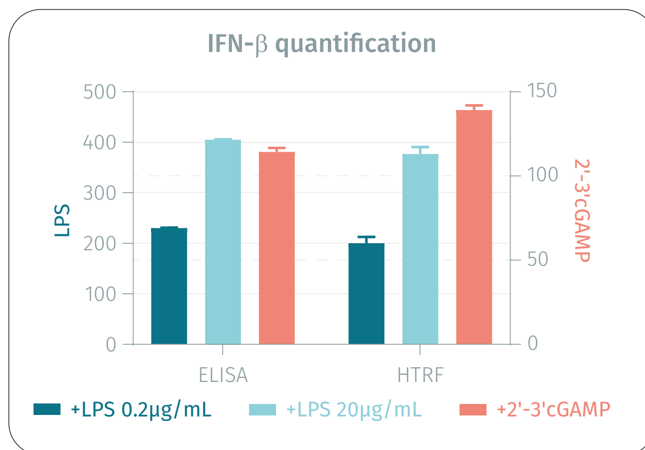


Figure 7: Quantification of hIFN-β secreted either upon LPS at 0.2ug/mL (dark blue), LPS at 20ug/mL (light blue) or 2′-3′ cGAMP (orange).

HIFN-β is a reliable readout of sting pathway activation in physiological cell models

Next, the activation of the STING pathway was studied in physiological cell models expressing endogenous levels of non genetically modified proteins.

To do this, THP1 WT were seeded at 50,000 cells per well, in 90µL RPMI supplemented with 10% FCS. After 24h incubation at 37°C, 5% CO2, the cells were stimulated with increasing concentrations of 2′3′ cGAMP under 10µL for 4h.

PBMC were seeded at 1,000,000 cells per well, in 275µL RPMI supplemented with 10% FCS. After 24h incubation at 37°C, 5% CO2, the cells were stimulated with increasing concentrations of 2′3′ cGAMP under 30µL, for 4h.

Then IFN-β secretion was quantified with HTRF using 14µL of cell supernatants, thus corresponding to 500 THP1 cells/µL and 5000 PBMC/µL.

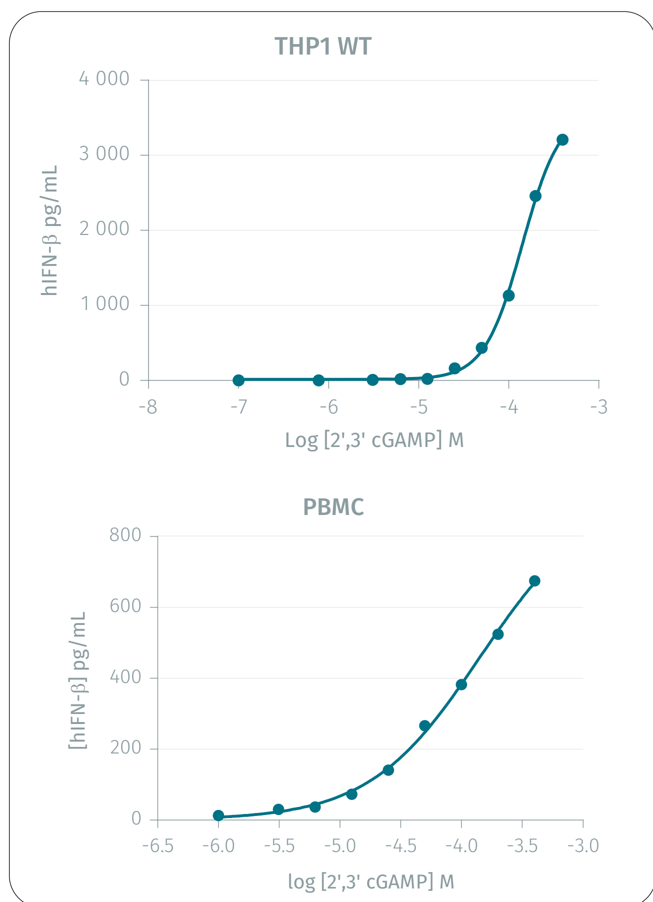


Figure 8: IFN-β release in THP1 WT and PBMC cells. Cells were stimulated with increasing concentrations of 2'3' cGAMP. IFN-β secretion was quantified with HTRF.

	THP1	PBMC
EC_{50} (µM)	142	141

As shown on the graphs, whereas THP1 cells release higher levels of IFN-β compared to PBMC, the EC50 of 2'-3' cGAMP was identical in both models, and correlated with the literature (3,4). These results demonstrated that the HTRF IFN-β represents a reliable readout to monitor STING pathway activation.

Conclusions

This study has shown that the secretion of IFN-β quantified by HTRF is well correlated with reporter gene approach, as well as ELISA.

Besides low sample consumption, ease of use and reliability, HTRF IFN-β can be applied to various cellular models from recombinant reporter cell lines to physiologically relevant cells such as PBMC. Thus, by combining technical performance and flexibility, this mix-and-read HTRF IFN-β assay is a powerful solution for investigating STING pathway and more generally innate immunity.

Finally, Revvity offers a comprehensive HTRF platform, including biochemical WT, 232H, and AQ STING binding assays, along with phospho-total STING and hIFN-β cell-based assays.

References

Literature

1. *Chen et al, Cur Top Medl Chem, 2019*
2. *Corrales, Cell Rep, 2016*
3. *Zhang et al, Mol cell, 2013*
4. *Ramanjulu et al, Nature, 2018*

Reagents

- HTRF kits: 62HIFNBPEG, 62HTNFAPEG, 62HIL1BPEG (Revvity)
- ELISA : Human IFN-β Quantikine ELISA Kit (R&D system DIFNB0)
- THP1 reporter cell line: THP1-Dual™ Cells (Invivogen, thpd-nfis)
- Compounds: 2'3'cGAMP (Biolog C161)
- G3-YSD (Invivogen, tlrl-ydna)

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