Unlock the sting polymorphism with a comprehensive HTRF binding platform.

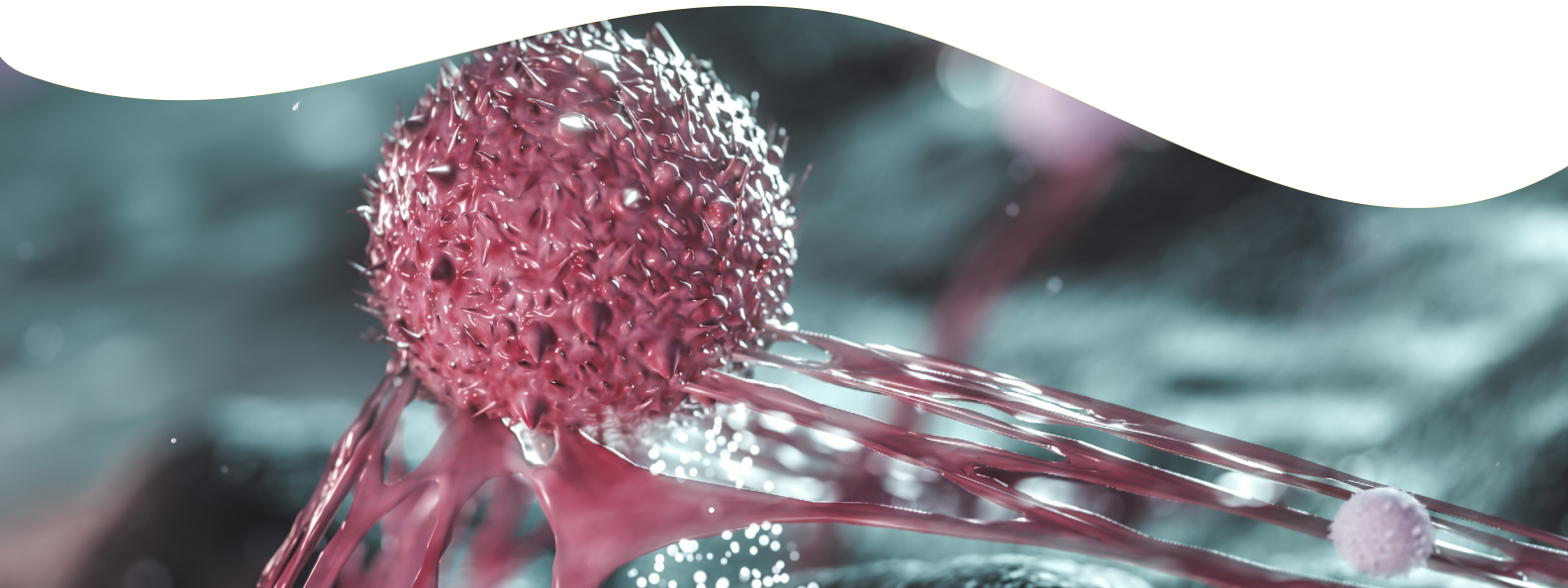
This application note demonstrates how the HTRF® STING binding assay platform represents a powerful new tool to identify and characterize compounds which bind to the STING protein.

Abstract

The STimulator of INterferon Genes (STING) is a four-transmembrane endoplasmic reticulum protein encoded by the TMEM 173 gene. The STING protein is activated by bacterial Cyclic Di-Nucleotides (CDNs) such as c di-GMP or c di-AMP, as well as by the mammalian non canonical 2′,3′cGAMP produced by a dsDNA sensor, cGAS, a cyclic GMP-AMP synthase. STING activation leads to the production of type 1 interferon (IFNα/β) and proinflammatory cytokines like IL6 or TNFα (1,2,3).

Because it is a key signalling hub in innate immunity, STING has become a promising target for cancer, infectious, and auto-immune diseases. Synthetic CDNs or small molecules modulating its activity have been recently identified (4,5). In particular, several on-going clinical trials include STING agonists to enhance anticancer immune responses (6).

Importantly, the TMEM 173 gene encoding the STING protein is heterogeneous in the human population (7). Main STING variants are R232, HAQ (R71H-G230A-R293Q), AQ (G230A-R293Q), Q293, and H232 (7). Since this polymorphism has profound effects on both STING expression and functions (7), it has become essential to characterize the binding properties of newly identified compounds toward the different STING alleles. The STING agonist, DMXAA has failed in clinical trials for non small-cell lung cancer and was later reported as a murine-selective agonist (8), which highlights the need for characterization of compounds on both human and murine forms of STING in early discovery phases.



To do so, we have designed a platform of biochemical STING binding assays enabling compound identification and characterization towards the main human STING variants, R232 (WT), AQ, and H232, as well as towards the mouse form of STING (Table 1). This platform of assays, based on our proprietary no-wash HTRF® technology, is robust and cost-effective. The validation performed with a relevant set of STING binders shows that it can recapitulate the main binding characteristics of CDNs as well as those of small molecules recently identified as promising STING therapeutic agents.

Design of the recombinant STING proteins

The same strategy was used to produce each STING subtype (Fig. 1).

A 6His Tag and a short linker were added to the N-terminal part of amino acids 153-379 (human) or 152-378 (mouse). The construct includes the CDN binding domain and the c-terminal tail of STING. Recombinant proteins were produced in E. coli.

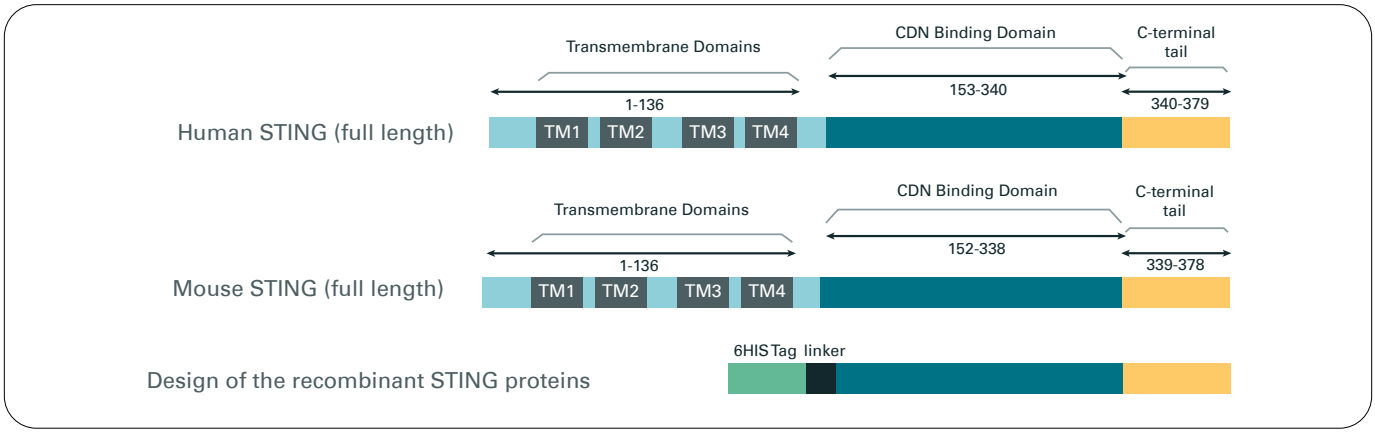


Figure 1. Strategy used to produce the STING proteins

The different variants of the human STING were obtained by introducing mutations in CDN binding domains at positions 230, 232, and 293 respectively (Table 1).

Table 1. hSTING mutations introduced in the CDN binding domain

Name	Residue 230	Residue 232	Residue 293
hSTING WT	G	R	R
hSTING AQ	A	R	Q
hSTING H232	G	H	R

Principle of The HTRF STING binding assays

All STING binding assays share the same principle. The STING ligand is detected in a competitive assay format using a specific Anti 6His antibody labeled with Terbium Cryptate (donor) which binds to a 6His-tagged STING protein and a STING ligand labelled with d2 (acceptor). The detection principle is based on HTRF® technology. When the dyes are in close proximity, the excitation of the donor with a light source (laser or flash lamp) triggers a Fluorescence Resonance Energy Transfer (FRET) towards the acceptor, which in turn fluoresces at a specific wavelength (665 nM). A STING compound competes with the d2- labelled STING ligand, and thereby prevents FRET from occurring. The specific signal is inversely proportional to the compound concentration (Fig. 2).

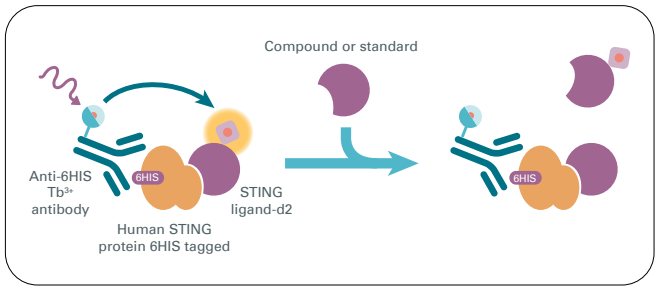


Figure 2. Principle of the HTRF STING binding assays

Table 2. List of the STING binding assays available

Htrf Sting Binding Assays	Catalog References
Human STING WT binding kit - 500 tests	64BDSTGPEG
Human AQ STING binding kit - 500 tests	64BDSTGQPEG
Human H232 STING binding kit - 500 tests	64BDSTGHPEG
Mouse STING WT binding kit - 500 tests	64BDSTGMPEG

A unique mix and read assay protocol

The same mix and read protocol was established for all the assays of the STING binding platform (Fig. 3). Therefore experiments are easily parallelized and automated to identify and/or characterize compounds of interest.

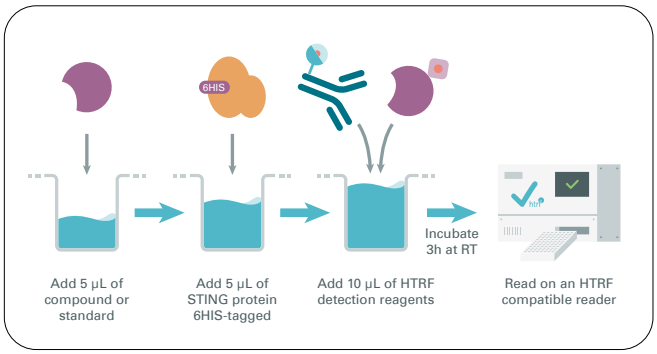


Figure 3. The mix and read protocol of the binding assays

Fluorescent ligand displays a high affinity for all sting proteins

We successfully developed a fluorescent ligand displaying a high affinity for the different variants of the human STING protein, as well as for the mouse STING.

The dissociation constant (Kd) was determined by running the different HTRF STING binding assays with fluorescent ligand concentrations ranging from 0 to 200 nM in the absence (Total Signal) or in the presence of 20 µM of the standard (unlabeled competitor to determine the unspecific signal).

The specific assay signal was then calculated (Total signal – Unspecific signal) and fitted to determine the Kd of the fluorescent ligand (Table 3).

Representative data for each STING subtype are shown in figure 4 below.

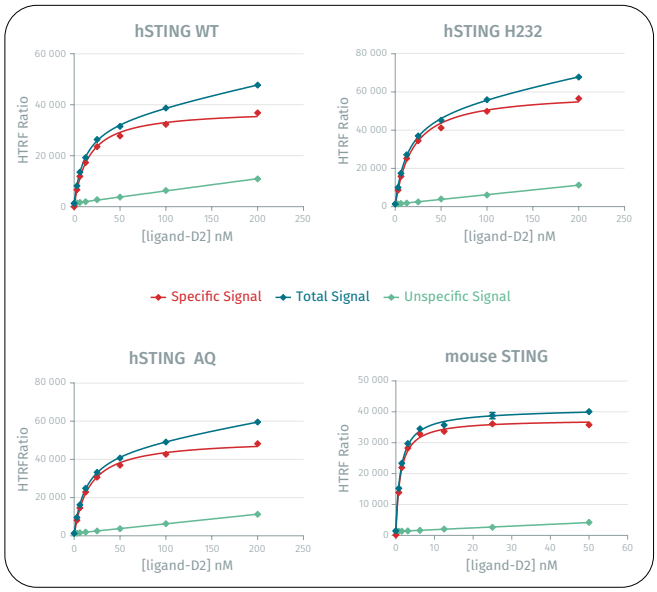


Figure 4. Titration of the d2 fluorescent ligand against the different STING subtypes.

Table 3. Affinity of the fluorescent ligand for the different STING subtypes

STING subtype	Kd (nM) of the fluorescent ligand
Human STING WT	15.3
Human STING AQ	16
Human STING H232	18.5
Mouse STING	1.1

Performances of the STING binding assays

When using a ligand concentration between its EC_{50} and its EC_{80}, each STING binding assay displays a robust S/B (>14) at T=3H (Fig.5).

It will enable reproducible data to be obtained when compounds of interest are tested in a competitive assay format.

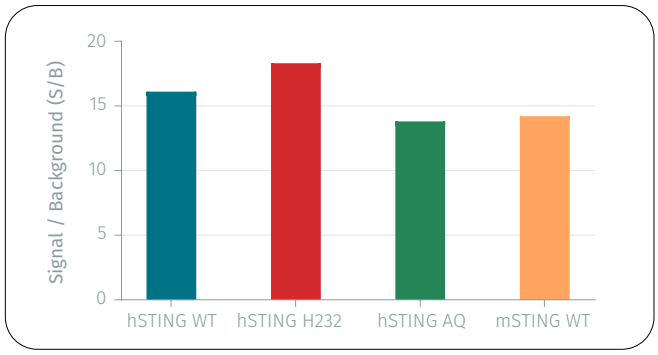


Figure 5. S/B obtained with an optimized fluorescent ligand concentration in each STING binding assay

Kinetic of the STING binding assays

The kinetic of the each STING binding assay was investigated between 3H and 24H (Fig. 6). All human STING binding assays were near equilibrium (>90%) after an incubation of 3H at room temperature, while the mouse STING binding assay required an incubation of 24H to reach equilibrium.

However, since all the assays already displayed both a robust S/B at 3H (Fig.5) and a similar Ki value for the reference compound (standard provided in each kit) regardless of the incubation time (Table 4), an incubation time of 3H is recommended for the entire STING binding platform.

The excellent signal stability at 24H makes these assays suitable for a use in high throughput screening (HTS), where a good signal stability facilitates assay implementation to test large compound librairies.

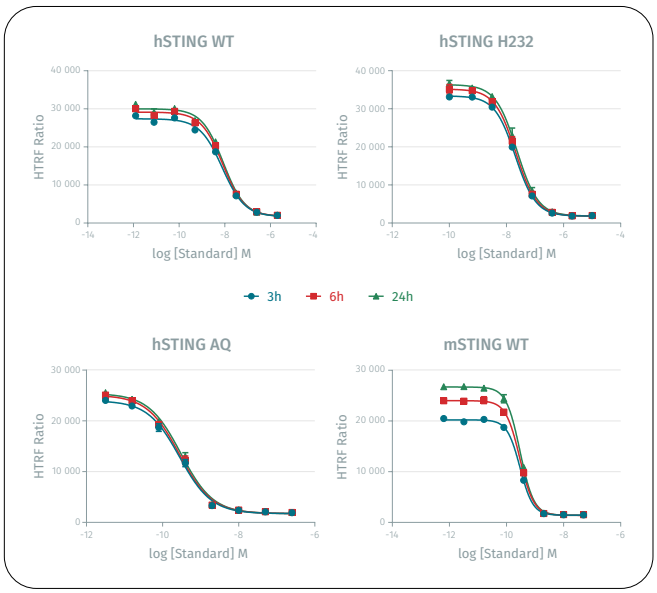


Figure 6. Determination of the equilibrium kinetic of the different STING binding assays

Table 4. Ki of 2′-3′ cGAMP established at various detection timepoints

Sting Subtype	Ki Ref (nM)	Ki Ref (nM)	Ki Ref (nM)
	t=3H	t=6H	t=24H
hSTING WT	3.9	4.1	4.3
hSTING AQ	0.14	0.14	0.15
hSTING H232	10	11	12
Mouse STING	0.1	0.1	0.1

DMSO tolerance

The effect of DMSO on each STING binding assay was assessed by testing DMSO percentages ranging from 0 to 10%. Each DMSO condition was tested in absence (maximum HTRF signal) or in presence of 20 µM of standard (minimum signal). The S/B (Signal Max / Signal Min) obtained with the different DMSO percentages were then normalized to the signal obtained in absence of DMSO (Fig 7).

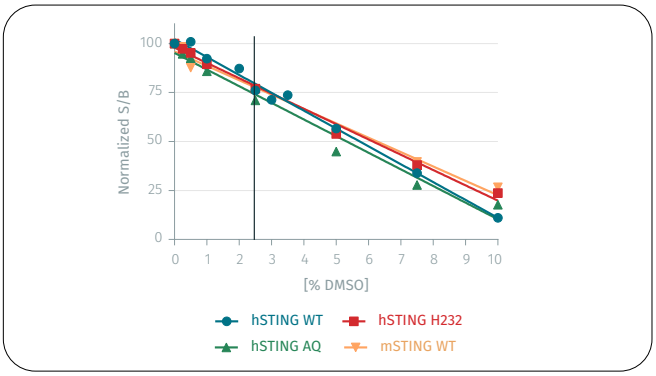


Figure 7. DMSO effect on S/B of each STING binding assay

All the STING binding assays were equally affected by increasing percentages of DMSO.

We suggest considering 2.5% DMSO as the maximum percentage of DMSO to use, since it limits negative impact on the S/B of the binding assays.

We then drew up the standard curves for each STING binding assay, and compared the Ki of 2′3′ cGAMP obtained for each kit standard in absence or in presence of DMSO 2.5% (Table 5).

The results indicate that the Ki values of the standard are not affected by DMSO percentages up to 2.5%.

Table 5. Ki values of standard determined in absence or presence of DMSO

% DMSO	hSTING Wt	hSTING H232	hSTING AQ	mSTING Wt
0%	3.1 nM	14 nM	0.1 nM	0.1 nM
2.50%	2.3 nM	11 nM	0.1 nM	0.08 nM

Validation of the STING binding platform using a relevant set of compounds

A relevant set of compounds was selected to validate the platform of STING binding assays.

It included 2′,3′ cGAMP and other CDNs known to activate STING (3′,3′ cGAMP, c diAMP, and c diGMP) as well as a synthetic CDN, ADU-S100, engineered to be resistant to phosphodiesterases. ADU-S100 was shown to activate all known human STING variants as well as mouse STING (4).

In addition to CDNs, three small molecules were selected. Two compounds, DMXAA and CMA, were selected for their selectivity towards the mouse STING (8). The third one is a recently published amidobenzimidazole compound 2 (diABZI #2), which activates all STING subtypes and shows promising anti-cancer effects (5).

The panel of compounds was tested in the different HTRF STING binding assays. The resulting Ki values are summarized in Table 6. Dose responses curves obtained with 2′,3′ cGAMP, ADU-S100, diABZI #2, and DMXAA are shown in figure 8.

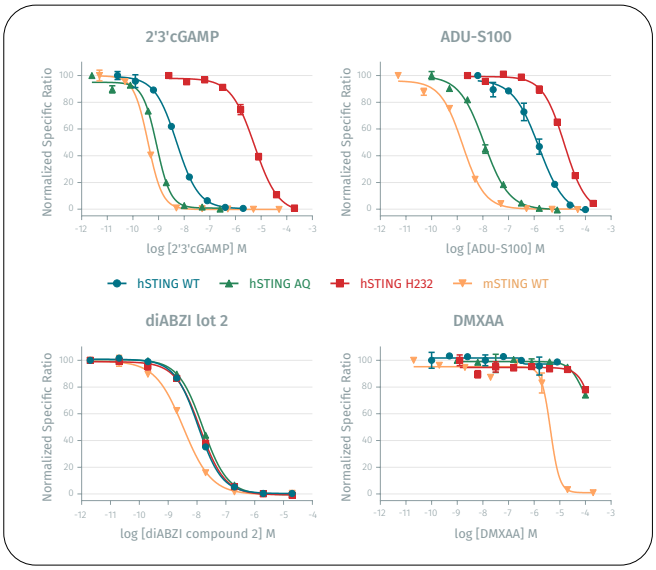


Figure 8. Dose response curves of 2′,3′ cGAMP, diABZI #2, ADU-S100, and DMXAA obtained on the different STING subtypes

The results obtained are in good agreement with published data.

DMXAA and CMA did not display any binding to the human STING variants but were able to bind mouse STING, as expected.

All CDNs showed a binding to all STING subtypes. However, again as expected, 2′,3′ cGAMP was found to be more potent than the other CDNs despite a relatively weak affinity toward the hSTING H232 variant. Moreover, its nanomolar affinity towards the human STING WT is in good agreement with the literature.

Table 6. Ki values of the compounds obtained from the different STING binding assays

	Ki of investigated compound (M)			
Compound	hSTING WT	hSTING H232	hSTING AQ	mSTING WT
2′,3′ cGAMP	2.60E-09	2.80E-06	4.20E-10	1.30E-10
3'3' cGAMP	2.30E-06	2.90E-06	1.40E-08	1.50E-09
c diAMP	1.10E-05	3.40E-05	1.30E-07	1.30E-08
c diGMP	1.30E-06	4.10E-06	6.50E-07	4.90E-08
ADU-S100	7.70E-07	7.70E-06	5.60E-09	5.40E-10
diABZI 2	5.50E-09	6.40E-09	7.90E-09	1.10E-09
DMXAA	No binding	No binding	No binding	1.40E-06
CMA	No binding	No binding	No binding	2.90E-06

As reported, ADU-S100 was able to bind all STING variants. Of note, Ki values related to the different variants were highly scattered, ranging from 0.5 nM for the mouse STING to 7.7 µM for the human STING H232.

Finally, di ABZI #2 displayed a high affinity (Ki<8 nM) for all STING proteins. Moreover its affinity for hSTING WT (Ki=5.5 nM) is in good agreement with the published data (IC_{50}=20 nM in a radioactive binding assay).

Taken together, these data suggest that our new STING binding platform represents a valuable tool to identify and characterize new STING binders.

Conclusion

The HTRF STING binding assay platform represents a powerful new tool to identify and characterize compounds which bind to the STING protein, at least for the CDN binding domain. Assay features like robust S/B, DMSO tolerance, and signal stability make this platform highly compatible either with HTS or compound profiling.

Data obtained with reference compounds are in good agreement with the literature and provide new insight into the binding characteristics of compounds like ADU-S100 or diABZI #2 currently used in clinical trials.

The combination of this STING binding platform with the available HTRF cell-based assays related to the STING pathway (e.g. phospho-STING or human IFNβ) represents a comprehensive HTRF solution in the early discovery phases for new drugs targeting the STING protein.

References

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