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In vitro STING Activation with the cGAMP-STINGΔTM Signaling Complex

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[Abstract] Activating the STING (stimulator of interferon genes) signaling pathway via administration of STING agonist cyclic GMP-AMP (cGAMP) has shown great promise in cancer immunotherapy. While state-of-the-art approaches have predominantly focused on the encapsulation of cGAMP into liposomes or polymersomes for cellular delivery, we discovered that the recombinant STING protein lacking the transmembrane domain (STINGΔTM) could be used as a functional carrier for cGAMP delivery and elicit type I IFN expression in STING-deficient cell lines. Using this approach, we generated anti-tumoral immunity in mouse melanoma and colon cancer models, providing a potential translatable platform for STING agonist-based immunotherapy. Here, we report the detailed *in vitro* STING activation protocols with cGAMP-STINGΔTM complex to assist researchers in further development of this approach. This protocol can also be easily expanded to other applications related to STING activation, such as control of various types of infections.

Keyword: STING pathway, cGAMP delivery, Protein purification, Ribonucleoprotein complex, Interferon stimulation *in vitro*

[Background] Over the past two decades, the STING (stimulator of interferon genes) signaling pathway has emerged as a crucial feature of the immune system and a promising therapeutic target against viral and bacterial infections, autoimmune disorders, and cancers. As such, the delivery of STING agonists to boost the immune response has become an area of great interest in both academic institutions and pharmaceutical companies (Ohkuri *et al.*, 2017). While existing efforts have focused mostly on developing synthetic delivery vehicles (Shae *et al.*, 2019), this assumes the presence of fully functional STING in cells. STING signaling has not only been shown to be frequently impaired in cancer cells due to epigenetic silencing of the protein (Ahn *et al.*, 2015; Xia *et al.*, 2016); there is also an ongoing debate on whether the general population is responsive to agonist-only therapies, since 19% of humans carry a mutated STING variant (R71H-G230A-R293Q, HAQ STING) reported to exhibit impaired function (Jin *et al.*, 2011; Fu *et al.*, 2015; Patel *et al.*, 2017; Sivick *et al.*, 2017).

To address these concerns, we engineered a truncated portion of the original STING protein to pre-assemble with STING agonists, acting as a functional carrier that can effectively trigger STING signaling

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even in the absence of STING proteins in mammalian cells. Our *in vivo* vaccination studies with this platform has shown efficient activation of B cells, cytotoxic T cells and memory precursor T cells, as well as robust anti-tumoral immunity against melanoma and colon cancer mouse models (He *et al.*, 2020).

Here, we report the detailed protocols of our *in vitro* STING activation assays (Table 1) with cGAMP-STINGΔTM ribonucleoprotein complex in three cell lines: human embryonic kidney (HEK293T) cell, mouse macrophage (RAW264.7) and mouse dendritic cell (DC2.4). The purification protocol of STINGΔTM is also included to ensure the reproducibility of our work. We believe this protocol may assist further mechanistic discoveries in the signaling pathway and more engineering applications of this platform in vaccinology and cancer immunotherapy.

Table 1. Summary of *in vitro* STING activation assays

Cell line	Medium	Assay	Performed with
HEK293T-luc2p/ISRE/Hygro	DMEM + 10% FBS + 1% P/S	IFN-luciferase assay	Cells 24 h post treatment
DC2.4	RPMI + 10% FBS + 1% P/S	CXCL10 ELISA	Medium 48 h post treatment
		mIFN-β qPCR	Cells 24 h post treatment
RAW264.7	DMEM + 10% FBS + 1% P/S	CXCL10 ELISA	Medium 48 h post treatment
		mIFN-β qPCR	Cells 24 h post treatment
RAW-Blue ISG	DMEM + 10% heat inactivated FBS + 1% P/S	IFN-SEAP assay	Medium 12-48 h post treatment

Materials and Reagents

Protein Purification

1. Poly-Prep chromatography column (Bio-Rad, catalog number: 7311550)
2. Zeba™ Spin Desalting Columns 40k MWCO 10 ml (Thermo Fisher Scientific, catalog number: 87772)
3. BL21 (DE3) competent *E. coli* (NEB, catalog number: C2527I)
4. Rosetta™ (DE3) competent *E. coli* (Millipore Sigma, catalog number: 70954)
5. Isopropyl-β-D-thiogalactopyranoside (IPTG, Millipore Sigma, catalog number: I6758-10G)
6. Phosphate Buffered Saline (PBS, Lonza, catalog number: 17-516F)
7. Imidazole (Millipore Sigma, catalog number: I5513)
8. Lysozyme from chicken egg white (Millipore Sigma, catalog number: 62971-10G-F)
9. Triton™ X-100 (Millipore Sigma, catalog number: T8787-250ml)
10. Triton™ X-114 (Millipore Sigma, catalog number: 93422-250ml)
11. Phenylmethylsulfonyl fluoride (PMSF, Millipore Sigma, catalog number: P7626-5G)
12. cOmplete™ Mini, EDTA free (Roche, catalog number: 11836170001)
13. Bovine Serum Albumin (BSA, Millipore Sigma, catalog number: A3608)
14. HisPur™ Cobalt Resin (Thermo Fisher Scientific, catalog number: 89964)
15. 1,4-Dithiothreitol (DTT, Millipore Sigma, catalog number: 10197777001)

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16. Sodium Phosphate Monobasic (Millipore Sigma, catalog number: S3139)
17. Sodium Phosphate Dibasic (Millipore Sigma, catalog number: S3264)
18. Sodium Chloride (Millipore Sigma, catalog number: S9888)
19. HEPES (Millipore Sigma, catalog number: H3375)
20. Glycerol (Millipore Sigma, catalog number: G5516)
21. Protein Binding Buffer (see Recipes)
22. Protein Elution Buffer (see Recipes)
23. Protein Storage Buffer (see Recipes)

Cells and cell culture media

1. HEK293T and RAW264.7 cells were obtained from the American Type Culture Collection (ATCC)
2. DC2.4 cells were obtained from Rock lab, University of Massachusetts Medical School, MA, USA
3. RAW-Blue ISG cells were obtained from Invivogen
4. Dulbecco's modified Eagle's medium (DMEM, Corning, catalog number: 10-041-CV)
5. Roswell Park Memorial Institute (RPMI) medium (Corning, catalog number: 10-013-CV)
6. 0.25% Trypsin-EDTA (Gibco, catalog number: 25200-056)
7. Fetal bovine serum (FBS, Gibco, catalog number: 10437-028)
8. Penicillin-Streptomycin Solution, 100x (Corning, catalog number: 30-002-CI)

IFN-luciferase assay

1. 96-well clear bottom white plate (Millipore Sigma, catalog number: CLS3610)
2. pGL4.45[luc2p/ISRE/Hygro] Vector (Promega)
3. Firefly Luciferase Assay Kit (Biotium, catalog number: 30075-2)
4. TransIT-X2® Transfection Reagent (Mirus, catalog number: MIR 6004)

RNA extraction, Reverse Transcription, and qPCR

1. LightCycler™ 480 Multiwell Plate 96 clear with Sealing Foils (Roche, catalog number: 05102413001)
2. RNeasy™ micro kit (Qiagen, catalog number: 74004)
3. Beta-Mercaptoethanol (βME, Millipore Sigma, catalog number: M6250-10ML)
4. Reverse transcription kit (Thermo Fisher Scientific, catalog number: 4374966)
5. SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, catalog number: 4309155)
6. qPCR primers used for detection: mIFN-β-F: 5'-GCCTTTGCCATCCAAGAGATGC-3', mIFN-β-R: 5'-ACACTGTCTGCTGGTGGAGTTC-3', mActin-F: 5'-CATTGCTGACAGGATGCAGAAGG-3', and mActin-R: 5'-TGCTGGAAGGTGGACAGTGAGG-3' (ordered from IDT as custom oligo DNA)

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mCXCL10 ELISA

1. Greiner Bio-One MICROLON™ 600 High Binding 96-Well ELISA Assay Microplates (Fisher Scientific, catalog number: 07-000-627)
2. Mouse CXCL10 ELISA kit (R&D, catalog number: DY466)
3. TMB Substrate Set (BioLegend, catalog number: 421101)
4. TWEEN 20 (Millipore Sigma, catalog number: P9416-100ml)

SEAP-IFN assay

1. QUANTI-Blue™ Solution (Invivogen, catalog code: rep-qbs)

Equipment

1. Misonix sonicator 3000
2. Real-time PCR system (Roche, model: LightCycler 480)
3. Nanodrop spectrophotometer (Thermo Fisher, model: ND-1000)
4. Thermal cycler (Bio-Rad, model: T100)
5. Plate reader (Tecan, model: Infinite M200)

Software

1. GraphPad Prism

Procedure

A. STINGΔTM protein purification

The DNA sequences (sources: Tmem173 NM_028261 Mouse Tagged ORF Clone, Origene Catalog number: MR227544, STING TMEM173 NM_198282 Human Tagged ORF Clone, Origene Catalog number: RC208418) of STINGΔTM protein (138 to 378 amino acids for mouse STING, 139 to 379 amino acids for human STING) were synthesized as gBlock DNA fragments (Integrated DNA Technologies) and cloned into the pSH200 expression vector (linearized via Nco I and Not I restriction enzymes) with a hexa-histidine-tag at the N-terminus (Figure 1). Plasmids for STINGΔTM mutants such as S365A were then generated via site-specific mutagenesis. DE3 *Escherichia coli (E. coli)* was used to express the proteins (mouse STINGΔTM in BL21 DE3, human STINGΔTM in Rosetta DE3). Lysogeny Broth (LB) containing the antibiotic ampicillin (100 mg/L) was used for bacteria culture, shaker conditions were 37 °C 220 rpm for growth and 18 °C 220 rpm for induction.

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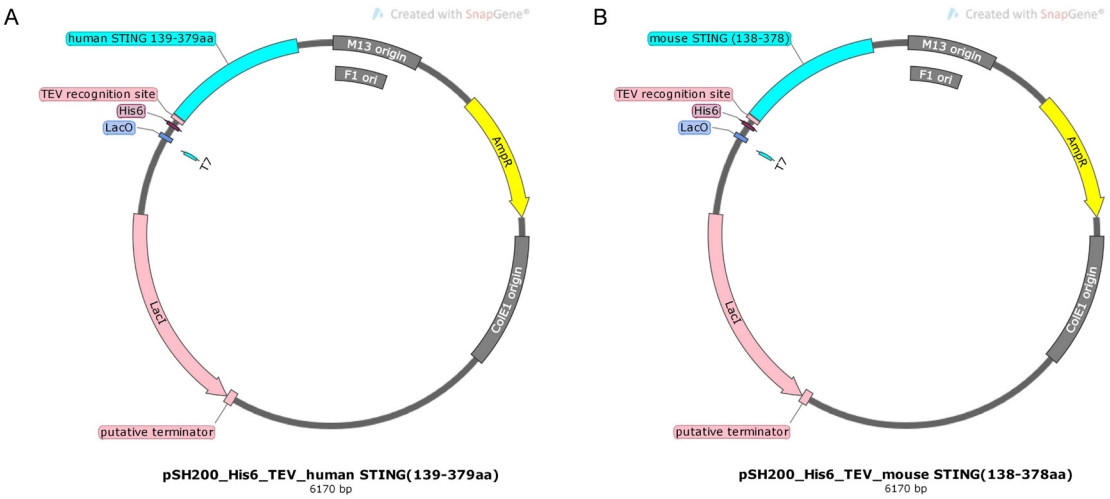

Figure 1. Plasmid maps of (A) pSH200_His6_TEV_human STING (139-379aa) and (B) pSH200_His6_TEV_mouse STING (138-378aa)

Day 1: Bacteria culture

1. Pick DE3 *E. coli* from glycerol stock and culture in 50 ml of LB with ampicillin at 37 °C overnight.

Day 2: Induction

1. Transfer 50 ml of overnight culture into a flask containing 1 L LB with ampicillin and culture for approximately 2 h at 37 °C until the culture's OD_{600} reaches 0.4. This OD value is critical for expressing human STINGΔTM in Rosetta DE3, as we have found that higher concentration can compromise protein purity; for the expression of mouse STINGΔTM in BL21 DE3, the OD600 can be in the range of 0.4-0.8 without compromising yield and purity.
2. Cool the 1 L culture on ice and add 0.5 ml 1 M IPTG, then shake at 18 °C for approximately 20 h.

Day 3: Protein purification

1. After induction, centrifuge bacteria cultures at 6,000 × *g* for 20 min.
2. Collect pellets and wash once with 30 ml PBS, then lyse in 20 ml protein binding buffer (Recipe 1) with 20 mg lysozyme, 200 µl Triton X-100, 1 mM PMSF (replenished every 30 min until Cobalt binding) and one tablet of cOmplete protease inhibitor cocktail tablets at room temperature for 20 min with gentle rotation at 20 rpm. After cells are lysed, the proteins should be kept ice-cold throughout the purification to minimize degradation (using cold protein binding buffer, protein elution buffer, and protein storage buffer).
3. Cool cell lysate on ice water and sonicate with Misonix sonicator 3000 at 18 W (with 3-s on and 5-s off intervals to prevent heat-deactivation of the protein) for a total of 5 min.
4. After sonication, centrifuge cell lysate at 14,000 × *g* at 4 °C for 30 min.
5. Wash 0.5 ml HisPur Cobalt Resin with 5 ml protein binding buffer, then add to the bacteria supernatant separated from the centrifugation, along with 20 µl Triton X-114 (to eliminate

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endotoxin) for Cobalt-HisTag binding (4 °C for 1 h with gentle rotation at 20 rpm).

6. After binding, carefully aspirate the supernatant and wash the Cobalt resin twice (4 °C for 30 min each time with gentle rotation at 20 rpm) with 5 ml protein binding buffer containing 5 µl Triton X-114.
7. Transfer washed Cobalt resin into a Poly-Prep chromatography column with 5 ml of protein binding buffer. After all the protein binding buffer has drained out under gravity, add 1.5 ml protein elution buffer (Recipe 2) and collect the elution.
8. Desalt the protein elution with Zeba™ Spin Desalting Columns (40k MWCO 10 ml). Add 10 ml of protein storage buffer (Recipe 3) into the column and allow it to completely drain under gravity (until no liquid remains above the resin surface). Add the 1.5 ml protein elution dropwise onto the middle of the resin and allow it to drain completely. Finally, collect five 1 ml fractions with protein storage buffer (add 1 ml of storage buffer then collect the flow-through as “Fraction 1”, then add another 1 ml of storage buffer and collect “Fraction 2” … all the way till “Fraction 5”). Combine Fractions 3 and 4 (which contains the majority of the desalted protein) for SDS-PAGE characterization and BCA quantification of protein concentration. Fractions 1, 2 may contain minimal amounts of protein, fraction 5 may contain protein with imidazole. The concentrations of each fraction may also be quantified through the BCA assay.
9. For storage, 1 mM DTT was added and protein solution was aliquoted then stored in -80 °C. Protein function can be maintained for years under this storage condition, but can be compromised by multiple freeze-thaw cycles.

B. Cell lines used for *in vitro* STING activation

Human embryonic kidney 293T (HEK293T) cells are deficient in cGAMP synthase (cGAS) and STING proteins, but express other essential proteins in downstream STING signaling, including TANK-binding kinase 1 (TBK1) and Interferon regulatory factor 3 (IRF3). Therefore, it provides a good model for studying the function of cGAMP-STINGΔTM without interactions with endogenous cGAMP or STING proteins (Figure 2). Additionally, it can be transfected to overexpress full-length mutant STING proteins (for example HAQ STING). To detect STING activation, we generated an interferon (IFN) reporter cell line by integrating an IFN-stimulated response element (ISRE) that drives luciferase expression through the stable transfection of pGL4.45[luc2p/ISRE/Hygro] plasmid selected in hygromycin (200 µg/ml). HEK293T cells are not capable of uptaking cGAMP-STINGΔTM complex directly, so commercial transfection reagents (we primarily used TransIT-X2, though others like Lipofectamine also work) are required in order to deliver cGAMP-STINGΔTM complex into the cells. HEK293T cells are cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

Mouse macrophage RAW264.7 and dendritic cell DC2.4 both express cGAS and STING, as well as TBK1 and IRF3 (Figure 2). They are also capable of internalizing cGAMP-STINGΔTM complex without the help of a transfection reagent. To detect STING activation, we can measure the cell secreted CXCL10 concentration in the medium with enzyme-linked immunosorbent assay (ELISA)

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or quantify the interferon-β mRNA level with qPCR. The aforementioned media contains no cells – medium that contains cells is denoted simply as 'cells'. In addition, a reporter cell line derived from RAW264.7: RAW-Blue™ ISG cells can be used to study the kinetics of STING activation *in vitro*, and since the Secreted embryonic alkaline phosphatase (SEAP) – IFN assay only requires 20 µl of media, it can be performed at multiple time points post treatment. RAW264.7 cells are cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. For SEAP-IFN assay, RAW-Blue™ ISG cells are cultured in DMEM with 10% heat-inactivated (56 °C, 30 min) FBS and 1% penicillin/streptomycin. DC2.4 cells are cultured in Roswell Park Memorial Institute (RPMI) medium with 10% FBS and 1% penicillin/streptomycin.

All cells are cultured in a 37 °C, 5% CO_2 incubator, used at low passage number and tested negative for *Mycoplasma* contamination.

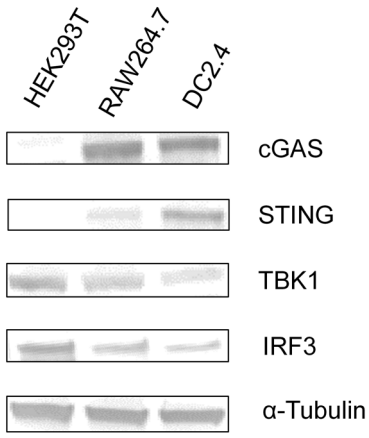

Figure 2. Immunoblotting of endogenous expression of cGAS, STING, TBK1, and IRF3 proteins from HEK293T, RAW264.7, and DC2.4 cells

C. IFN-luciferase assay with HEK293T-luc2p/ISRE/Hygro

Day 1: Preparation of Cells

1. Seed HEK293T-luc2p/ISRE/Hygro cells into 96-well plates (clear bottom flat white plates for luciferase assay) at a density of 3×10^5 cells/ml in 100 µl media per well.

Day 2: Cell treatment

1. For each well, mix 1 µg of STINGΔTM protein with 0.025 µg cGAMP, then add 1 µl of TransIT-X2 in OptiMEM media to a total volume of 20 µl and incubate at room temperature for 15 min.
2. Add the mixture to the cell medium without pipetting up and down. At least 3 replicates should be performed for each treatment.
3. Incubate treated cells for 24 h.

Day 3: Luminescence measurement

1. Remove plates from the incubator, aspirate medium and add 25 µl lysis buffer (five-fold diluted

with DI water from the provided 5× firefly luciferase lysis buffer) to each well, then incubate at room temperature for 15 min with orbital shaking.

2. Add 50 µl of luciferase assay buffer to each well (with D-luciferin freshly added at final concentration of 0.2 mg/ml), read plate for bioluminescence on a Tecan microplate reader.

D. mCXCL10 ELISA with RAW264.7 and DC2.4

Day 1: Preparation of Cells

1. Seed RAW264.7 or DC2.4 cells into 96-well plates at a density of 2 × 10^5 cells/ml in 100 µl medium per well.

Day 2: Cell treatment

1. For each well, mix 1 µg of STINGΔTM protein with 0.025 µg cGAMP and 1 µl of TransIT-X2 in 20 µl OptiMEM medium, then incubate at room temperature for 15 min. Alternatively, for vehicle-free treatment, mix 5 µg of STINGΔTM protein with 0.125 µg cGAMP in 20 µl OptiMEM medium and incubate at room temperature for 15 min.
2. Add the mixture to the cell medium without pipetting up and down. At least 3 replicates should be performed for each treatment.
3. Incubate treated cells for 48 h.

Day 3: Preparation of ELISA plates

ELISA was performed according to the manufacturer's protocol: Mouse CXCL10 ELISA kit (R&D, DY466)

1. Dilute CXCL-10 capture antibody in PBS to 2 µg/ml.
2. Add 100 µl capture antibody solution to each well of the ELISA assay plates. Incubate at 4 °C overnight.

Day 4: ELISA

1. Aspirate each well of the ELISA assay plates, wash three times with Wash Buffer (PBS with 0.05% Tween 20).
2. Add 300 µl Reagent Diluent (PBS with 1% BSA) to each well to block plates. Incubate at room temperature for 1 h with orbital shaking at 220 rpm.
3. Aspirate plates and wash three times with Wash Buffer.
4. Add 100 µl media of RAW264.7 or DC2.4 cells 48 h post-treatment without dilution. Incubate at room temperature for 2 h with orbital shaking at 220 rpm.
5. Aspirate plates and wash three times with Wash Buffer.
6. Add 100 µl of the Detection Antibody at 100 ng/ml (diluted in Reagent Diluent) to each well. Incubate at room temperature for 2 h with orbital shaking at 220 rpm.
7. Aspirate plates and wash three times with Wash Buffer.
8. Add 100 µl of the provided Streptavidin-HRP stock solution 40-fold diluted in Reagent Diluent

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to each well. Incubate at room temperature for 20 min avoiding light with orbital shaking at 220 rpm.

9. Aspirate plates and wash three times with Wash Buffer.
10. Add 100 µl of TMB Substrate Solution, freshly prepared from mixing equal volumes of TMB Substrate A with TMB Substrate B. Incubate at room temperature for 20 min with orbital shaking at 220 rpm, avoiding light.
11. Add 50 µl of Stop Solution (2 N H_2SO_4) to each well. Gently tap the plate for mixing. Measure absorbance at 450 nm with a spectrophotometer/plate reader.

E. Quantification of mIFN-β expression by qPCR with RAW264.7 and DC2.4

Day 1: Preparation of Cells

1. Seed RAW264.7 or DC2.4 cells into 24-well plates at a density of 3 × 10^5 cells/ml in 400 µl media per well.

Day 2: Cell treatment

1. For each well, mix 5 µg of STINGΔTM protein with 0.125 µg cGAMP and 5 µl of TransIT-X2 in 50 µl OptiMEM medium. Incubate at room temperature for 15 min. Alternatively, for vehicle-free treatment, mix 25 µg of STINGΔTM protein with 0.625 µg cGAMP in 50 µl OptiMEM medium and incubate at room temperature for 15 min.
2. Add the mixture to the cell medium without pipetting up and down.
3. Incubate treated cells for 24 h.

Day 3: RNA extraction, reverse transcription and qPCR

Perform RNA extraction following protocol provided by RNeasy Micro Kit:

1. Prepare fresh lysis buffer by adding 10 µl β-mercaptoethanol into 1 ml RLT buffer.
2. Aspirate medium, wash cells once with PBS, then add 350 µl lysis buffer per well. Incubate at room temperature for 5 min.
3. Transfer the cell lysate into Eppendorf tubes, vortex for 20 s, then add 350 µl 70% ethanol, pipette up and down, transfer to a spin column provided in the kit, and centrifuge for 15 s at 8,000 × *g*.
4. Discard flowthrough, add 350 µl RW1 buffer, and centrifuge for 15 s at 8,000 × *g*.
5. Prepare DNase buffer from DNase supplied in lyophilized form in glass vials. Use syringe to inject 500 µl water into the glass vial to dissolve the powder. Then open the vial and add 10 µl DNase solution + 70 µl RDD buffer to the column. Incubate at room temperature for 15 min. Then add 350 µl RW1 buffer to the spin column, centrifuge for 15 s at 8,000 × *g* and discard the collection tube.
6. Place the spin column in a new 2 ml collection tube as supplied. Add 500 µl RPE buffer, centrifuge for 15 s at 8,000 × *g*, and discard the flow through.

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7. Add 500 µl 80% ethanol to the spin column, centrifuge for 2 min at 8,000 × *g* and discard the collection tube.
8. Place the spin column in another new 2 ml collection tube as supplied. Centrifuge at 8,000 × *g* for 5 min, discard the collection tube.
9. Place the spin tube in a new 1.5 ml collection tube as supplied, add 14 µl RNase-free water to the center of the spin column, and centrifuge for 1 min at 8,000 × *g* to elute the RNA.
10. Measure RNA concentration of each cell sample with Nanodrop spectrophotometer.

Reverse transcription

1. Dilute 1 µg extracted RNA with water to a total volume of 10 µl.
2. For each RNA sample, prepare below mixture in a PCR tube:

Diluted RNA	10 µl
10× RT buffer	2 µl
dNTP	0.8 µl
Random primer	2 µl
RNase inhibitor	1 µl
Enzyme	1 µl
Water	3.2 µl
Total volume	20 µl

3. Treat the sample with thermal cycler with the program below:

25 °C	10 min
37°C	2 h
85 °C	5 min
4 °C	Infinite hold

qPCR

1. Dilute qPCR primers to 10 µM working concentration.
2. Add 80 µl water to 20 µl reverse transcription product from the previous step (this 5-fold dilution is denoted as ‘template’ in the following step)
3. Add the following to the qPCR 96-well plate for each reaction (add primer first, then add master mix):

Template	1 µl
2x SYBR mixture	10 µl
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
Water	8 µl
Total volume	20 µl

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F. IFN-SEAP assay with RAW-Blue ISG cells

Day 1: Preparation of Cells

1. Seed RAW264.7 or DC2.4 cells into 96-well plates at a density of 2 × 10^5 cells/ml in 100 µl media per well.

Day 2: Cell treatment

1. 2 h prior to treatment, remove the medium and replenish it with 100 µl DMEM with 10% heat-inactivated FBS and 1% penicillin/streptomycin pre-warmed to 37 °C in order to reduce the noise level in subsequent QUANTI-Blue assay.
2. For each well, mix 1 µg of STINGΔTM protein with 0.025 µg cGAMP then 1 µl of TransIT-X2 in 20 µl OptiMEM medium, incubated at room temperature for 15 min. Alternatively, for vehicle-free treatment, mix 5 µg of STINGΔTM protein with 0.125 µg cGAMP in 20 µl OptiMEM medium and incubate at room temperature for 15 min.
3. Add the mixture to the cell medium without pipetting up and down. At least 3 replicates should be performed for each treatment.
4. Incubate treated cells in 37 °C incubator.

Days 3-4: IFN-SEAP assay

1. Take 20 µl of medium from treated cell wells and mix with 180 µl of QUANTI-Blue assay buffer in a separate 96-well plate. (Multiple time points between 12 to 48 h can be taken to study the kinetics.)
2. Incubate the plate at 37 °C for 6 to 10 h until a visible color difference is observed (Figure 3).
3. Determine the IFN-SEAP activity by measuring the absorbance at 635 nm with a spectrophotometer/plate reader.

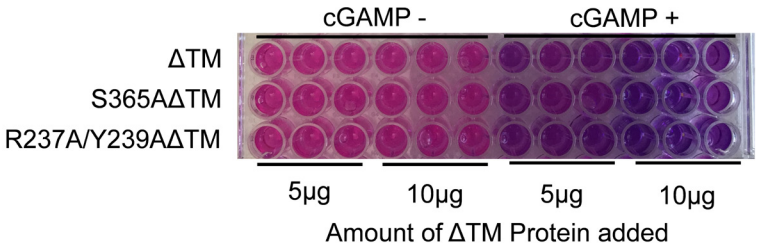

Figure 3. Example IFN-SEAP assay of mixing medium of treated RAW-Blue ISG cells with QUANTI-Blue assay buffer showing color differences due to STING signaling

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Data analysis

Data can be analyzed with GraphPad Prism and statistical analyses performed with one-way analysis of variance (ANOVA) followed by Student's *t*-test for statistical significance.

Recipes

1. Protein Binding Buffer
   50 mM sodium phosphate
   0.5 M NaCl
   10 mM imidazole
   pH 7.4
2. Protein Elution Buffer
   50 mM sodium phosphate
   0.5 M NaCl
   150 mM imidazole
   pH 7.4
3. Protein Storage Buffer
   20 mM HEPES
   150 mM NaCl
   10% glycerol
   pH 7.4

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Competing interests

The authors declare that they have no competing interests.

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