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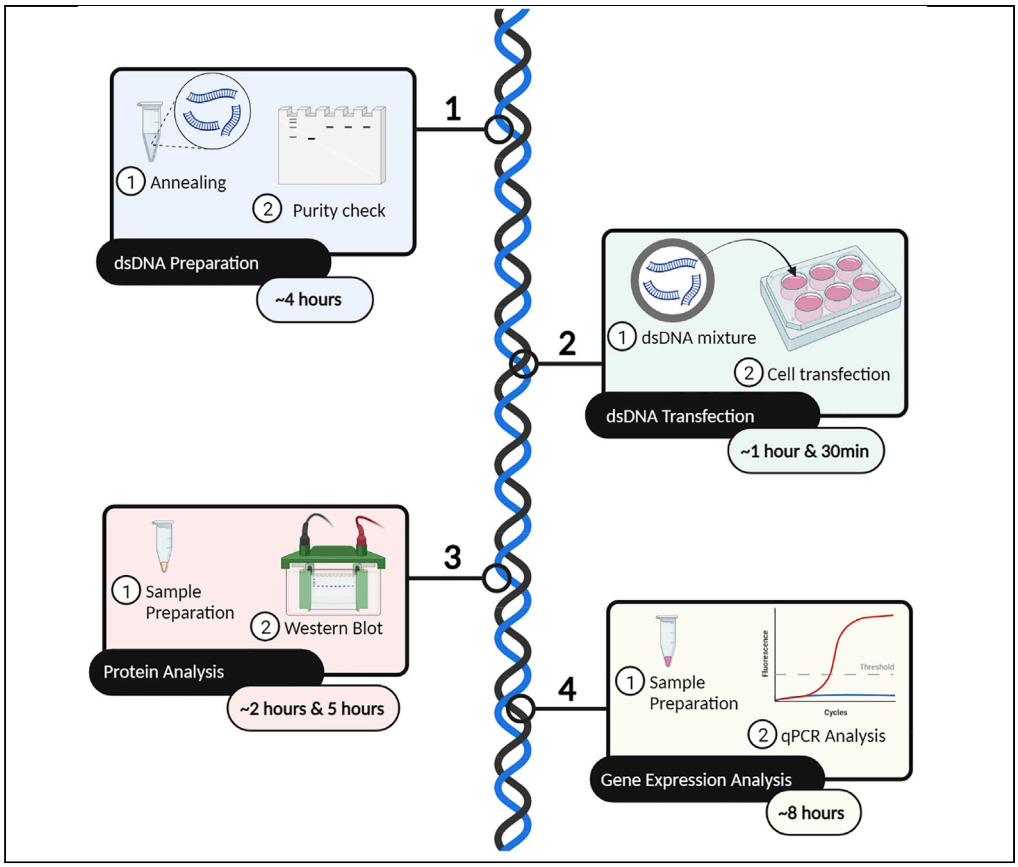
Protocol to induce and assess cGAS-STING pathway activation *in vitro*



The cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway plays a pivotal role in several cellular processes including pathogen recognition and inflammatory responses. We describe a protocol to activate the cGAS-STING pathway in murine cells using nucleic acids transfection. We describe how to prepare the nucleic acid probes and validate activation of the pathway by western blot and gene expression analysis. The protocol can be applied to investigate cGAS-STING signaling in both murine and human cell lines.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for inducing cGAS-STING pathway activation *in vitro*

Generation of dsDNA probes for transfection in cultured cells

Details to assess cGAS-STING pathway activation by western blot and RT-qPCR

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Protocol to induce and assess cGAS-STING pathway activation *in vitro*

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SUMMARY

The cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway plays a pivotal role in several cellular processes including pathogen recognition and inflammatory responses. We describe a protocol to activate the cGAS-STING pathway in murine cells using nucleic acids transfection. We describe how to prepare the nucleic acid probes and validate activation of the pathway by western blot and gene expression analysis. The protocol can be applied to investigate cGAS-STING signaling in both murine and human cell lines. For complete details on the use and execution of this protocol, please refer to Vila et al. (2022).

BEFORE YOU BEGIN

The cGAS-STING pathway operates as a first line of host immune defense against pathogens. cGAS, an innate immune sensor (Sun et al., 2013), interacts with cytosolic nucleic acids including ssDNA (Herzner et al., 2015), dsDNA (Ishikawa and Barber, 2008), and DNA:RNA hybrids (Guerra et al., 2020), producing the cyclic GMP-AMP (cGAMP) second messenger. cGAMP binds the STING scaffold protein (Ishikawa and Barber, 2008), promoting the recruitment of tank binding kinase 1 (TBK1), together with transcription factors, such as the interferon regulatory factor 3 (IRF3). TBK1 catalyzes phosphorylation of STING, along with phosphorylation and activation of IRF3 (Liu et al., 2015). Subsequently, phosphorylated IRF3 promotes the transcription of an array of inflammatory cytokines and type I interferons (IFNs) (Ishikawa et al., 2009). Emerging evidence indicates that this pathway may not be operational in all cell types (Qiao et al., 2021; Schadt et al., 2019), calling for standardized protocols allowing the verification of its functionality. This protocol below, used in Vila et al. (Vila et al., 2022) describes the following steps: (i) preparation of dsDNA probes from synthetic ssDNA (Stetson and Medzhitov, 2006), (ii) transfection of dsDNA in murine mouse embryonic fibroblasts (MEFs), and (iii) visualization of the activation of the cGAS-STING pathway by western blot and RT-qPCR. This protocol is the standard protocol used to verify cGAS-STING pathway activation in murine and human cell lines in our laboratory.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal cGAS (D3080) [dilution (1:1000)]	Cell Signaling Technology	Cat# 31659, RRID: AB_2799008
Rabbit monoclonal pTBK1 (Ser172) (D52C2) [dilution (1:500)]	Cell Signaling Technology	Cat# 5483, RRID: AB_10693472

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Rabbit monoclonal STING (D2P2F) [dilution (1:1000)]	Cell Signaling Technology	Cat# 13647, RRID: AB_2732796
Rabbit monoclonal phospho-STING (Ser366) (D7C3S) [dilution (1:1000)]	Cell Signaling Technology	Cat# 19781, RRID: AB_2737062
Mouse monoclonal GAPDH [dilution (1:5000)]	ProteinTech	Cat# 60004-1-Ig, RRID: AB_2107436
Rabbit monoclonal phospho-IRF3 (Ser396) (4D4G) [dilution (1:500)]	Cell Signaling Technology	Cat# 4947, RRID: AB_823547
Rabbit monoclonal TBK1/NAK (D1B4) [dilution (1:1000)]	Cell Signaling Technology	Cat# 3504, RRID: AB_2255663
Rabbit monoclonal HSP90 (C45G5) [dilution (1:1000)]	Cell Signaling Technology	Cat# 4877, RRID: AB_2233307
Anti-IRF3 Rabbit Mab (D83B9) [dilution (1:1000)]	Cell Signaling Technology	Cat# 4302, RRID: AB_1904036
Anti-rabbit IgG, HRP-linked [dilution (1:1000)]	Cell Signaling Technology	Cat# 7074, RRID: AB_2099233
Anti-mouse IgG, HRP-linked [dilution (1:1000)]	Cell Signaling Technology	Cat# 7076, RRID: AB_330924
Oligonucleotides		
Sense 80 bp probe	(Stetson and Medzhitov, 2006)	ACATCTAGTACATGTCTAGTCAGTA TCTAGTGATTATCTAGACATACATG ATCTATGACATATATAGTGGATAAG TGTGG
Anti-sense 80 bp probe	(Stetson and Medzhitov, 2006)	CCACACTTATCCACTATATATGT CATAGATCATGTATGTCTAGATA ATCACTAGATACTGACTAGACAT GTACTAGATGT
Ifnβ Forward primer	(Vila et al., 2022)	See Table 1
Ifnβ Reverse primer	(Vila et al., 2022)	See Table 1
Cxcl10 Forward primer	(Vila et al., 2022)	See Table 1
Cxcl10 Reverse primer	(Vila et al., 2022)	See Table 1
Isg15 Forward primer	(Vila et al., 2022)	See Table 1
Isg15 Reverse primer	(Vila et al., 2022)	See Table 1
Oas1 Forward primer	Self-designed primers	See Table 1
Oas1 Reverse primer	Self-designed primers	See Table 1
Hsp90 Forward primer	(Vila et al., 2022)	See Table 1
Hsp90 Reverse primer	(Vila et al., 2022)	See Table 1
Chemicals, peptides, and recombinant proteins		
Chloroform	VWR Chemicals	22711.290
Isopropanol	VWR Chemicals	20842.298
Ethanol Absolute	VWR Chemicals	20821.296
Ethylenediamine tetraacetic acid (EDTA)	Sigma-Aldrich	139-33-3
Bromophenol Blue Solution	Sigma-Aldrich	B8026-5G
Sodium Chloride Solution (NaCl)	Sigma-Aldrich	71386-1L
Magnesium Chloride Solution ($MgCl_2$)	Sigma-Aldrich	63069-100ML
Potassium Chloride Solution (KCl)	Fluka	60135-250ML
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich	93482-50ML-F
β-Mercaptoethanol	Sigma-Aldrich	M3148-2ML
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A2153-100G
TWEEN® 20	Sigma-Aldrich	P7949-500ML
Triton™ X-100	Sigma-Aldrich	T8787-100ML
TRIzol™	Ambion, Inc.	15596018
SDS 20%	Biosolve	0019812323BS
Protein Assay Dye Reagent Concentrate	Bio-Rad	5000006
Tris Base	Euromedex	200923-A
Glycine	Euromedex	26-128-6405-C
Glycerol 100%	Fisher Scientific	BP229-1
Gibco™ Trypsin EDTA 0.25%	Thermo Fisher Scientific	25200-056
Penicillin-Streptomycin (PEN-STREP)	Lonza	DE17-602E
L-Glutamine	Lonza	BE17-605E
Fetal Bovine Serum	Eurobio Scientific	CVFSVF00-01
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	Thermo Scientific	34580
SuperSignal™ West Femto Maximum Sensitivity substrate	Thermo Scientific	34095
Restore™ PLUS Western Blot Stripping Buffer	Thermo Scientific	46430

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Invitrogen™ Trypan Blue Stain 0.4%	Thermo Fisher Scientific	T10282
Sodium Azide (NaN3)	Sigma-Aldrich	S2002
Dulbecco's Phosphate Buffered Saline (10×)	Sigma-Aldrich	D1408-500ML
Dulbecco's Phosphate Buffered Saline (1×)	Sigma-Aldrich	D8537-500ML
Sterile-filtered Water	Sigma-Aldrich	W3500-1L
jetPRIME® Buffer	Polyplus-transfection	712-60
jetPRIME® Reagent	Polyplus-transfection	114-75
Acrylamide/Bis 19:1, 40% (w/v) solution	MP Biomedicals™	11BIAC2902-CF
Ammonium Persulfate (APS)	Sigma-Aldrich	A3678-25G
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich	T7024-50ML
TBE Buffer (5×)	PanReac AppliChem	A4228,5000PE
TB Green Premix Ex Taq (2×)	Takara Bio	RR420L
Invitrogen™ 10× Turbo™ DNase Buffer	Thermo Fisher Scientific	8167G
Invitrogen™ Turbo™ DNase	Thermo Fisher Scientific	2238G
Invitrogen™ SuperScript™ IV Reverse Transcriptase	Thermo Fisher Scientific	18090010
Invitrogen™ SuperScript™ IV RT Reaction Buffer	Thermo Fisher Scientific	18090050B
Invitrogen™ 10 mM dNTP Mix	Thermo Fisher Scientific	18427-013
Invitrogen™ DNase Inactivation Reagent	Thermo Fisher Scientific	8174G
Oligo dT 50 µM (5′-/5Phos/TTT TTT TTT TTT TTT TTT TTVN-3′)	Integrated DNA Technologies IDT	230010100
Invitrogen™ RNaseOUT™ Recombinant Ribonuclease Inhibitor	Thermo Fisher Scientific	CA92008
Custom qPCR primers	Integrated DNA Technologies IDT	N/A
Experimental models: Cell lines		
Mouse Embryonic Fibroblasts (MEFs)	Gift from Soren R. Paludan Lab at Aarhus University, Aarhus	N/A
Software and algorithms		
Image Lab	Bio-Rad Laboratories	N/A
Prism 9	GraphPad	https://www.graphpad.com/scientific-software/prism/
Biovision	VILBER	https://www.vilber.com/bio-print/w
Biorender	Web application	https://biorender.com/
Microsoft Excel	Microsoft	N/A
Other		
Invitrogen™ Novex™ WedgeWell™ 12% Tris-Glycine Gel	Thermo Fisher Scientific	XP00125BOX
Trans-Blot Turbo Transfer Pack	Bio-Rad	1704159
Safe-Lock Tubes 1.5 mL	Eppendorf	0030121872
Safe-Lock Tubes 2.0 mL	Eppendorf	0030121880
Falcon® 15 mL Polystyrene Centrifuge Tube	Corning Inc.	352095
Falcon® 50 mL High Clarity PP Centrifuge Tube	Corning Inc.	352098
Semi Micro Cuvette 1.5 mL	Kartell	01938-00
Falcon® Multiwell 6 Well Plate	Corning Inc.	353046
Full-cream Milk Powder	Régilait France	N/A
Bioruptor® Sonicator	Diagenode	N/A
Thermocycler	N/A	N/A
ChemiDoc Imaging System	Bio-Rad	N/A
LightCycler®	Roche Life Science	N/A
Trans-Blot® Turbo™ Transfer System	Bio-Rad	N/A
Quantum Gel Imager	VILBER	N/A
Spectrophotometer®	Eppendorf	N/A

Note: DNA probes and qPCR primers were purchased from Integrated DNA Technologies.

MATERIALS AND EQUIPMENT

Sense 80 bp= ACATCTAGTACATGTCTAGTCAGTATCTAGTGATTATCTAGACATACATGATCTA TGACATATATAGTGGATAAGTGTGG.

Anti-sense 80 bp= CCACACTTATCCACTATATATGTCATAGATCATGTATGTCTAGATAATCACTA GATACTGACTAGACATGTACTAGATGT.

Note: cGAS-dependent detection of dsDNA is sequence-independent. Alternative dsDNA sequences may be used.

5× annealing buffer (50 mL)			
Reagent	Stock concentration	Final concentration	Amount
NaCl	5 M	300 mM	3 mL
Tris-HCl (pH 7.4)	1 M	50 mM	2.5 mL
EDTA	0.5 M	1 mM	0.1 mL
ddH_2O	n/a	n/a	44.4 mL
Annealing buffer should be stored at room temperature (RT) i.e., (20°C–25°C) up to 1 year.			

Lysis buffer (50 mL)			
Reagent	Stock concentration	Final concentration	Amount
Tris-HCl (pH 7.4)	1 M	20 mM	1 mL
NaCl	5 M	150 mM	1.5 mL
KCl	1 M	10 mM	500 µL
EDTA	0.5 M	0.5 mM	50 µL
Triton X-100	20% (v/v)	0.5% (v/v)	1.25 mL
Glycerol	50% (v/v)	10% (v/v)	10 mL
$MgCl_2$	1 M	1.5 mM	75 µL
ddH_2O	n/a	n/a	35.625 mL
Lysis buffer should be stored at 4°C up to 1 month.			

Note: immediately before use, add 0.7 µL of β-Mercaptoethanol (10 mM final) and 5 µL of Phenylmethylsulfonyl fluoride (PMSF) (0.5 mM final) per mL to cold (4°C) buffer. β-Mercaptoethanol can be replaced by DTT at 2 mM (final concentration).

10× Running Buffer (1 L)			
Reagent	Stock concentration	Final concentration	Amount
Tris Base	n/a	250 mM	15 g
Glycine	n/a	1.92 M	72 g
Sodium dodecyl sulfate (SDS)	20%	1%	25 mL
ddH2O	n/a	n/a	qs 1 L
Running buffer should be stored at RT (20°C–25°C) up to 3 months.			

Note: Prepare 1× Running Buffer in ddH_2O before use.

4× Laemmli Buffer (10 mL)			
Reagent	Stock concentration	Final concentration	Amount
Tris-HCl (pH 6.8)	1 M	200 mM	2 mL
SDS	n/a	8%	0.8 g
Bromophenol Blue	n/a	0.4%	16 mg
Glycerol	100%	40% (v/v)	4 mL
β-Mercaptoethanol	14.3 M	400 mM	280 µL
ddH_2O	n/a	n/a	qs 10 mL
Laemmli buffer aliquots should be stored at −20°C up to a year.			

Phosphate Buffered Saline-Tween 20 (PBST) (1 L)			
Reagent	Stock concentration	Final concentration	Amount
Tween 20	20%	0.1%	5 mL
PBS	10×	1×	100 mL
ddH_2O	n/a	n/a	895 mL
PBST should be stored at RT (20°C–25°C) up to 1 month.			

Sodium azide/ Bovine Serum Albumin (BSA) 5% (100 mL)			
Reagent	Stock concentration	Final concentration	Amount
BSA	100%	5%	5 g
Sodium Azide (NaN_3)	10%	0.05%	0.5 mL
PBST	n/a	n/a	qs 100 mL
Sodium azide/ Bovine Serum Albumin (BSA) should be stored at 4°C up to a year.			

Ammonium persulphate solution (APS)

Prepare 10% APS by dissolving in dH_2O (aliquots should be stored at −20°C) up to a year.

Alternatives: Gibco™ Penicillin-streptomycin (10,000 U/mL) (Thermo Fisher Scientific, 15140122) can be used as an alternative to Penicillin-Streptomycin (PEN-STREP).

Alternatives: Gibco™ L-glutamine (200 mM) (Thermo Fisher Scientific, 25030081) can be used as an alternative to L-Glutamine.

Alternatives: cGAS human antibody (D1D3G) (Cell signaling, 15102S) can be used to probe cGAS in human cell-lines.

STEP-BY-STEP METHOD DETAILS

The protocol herein highlights the three major steps for assessing the functionality of the cGAS-STING pathway in mammalian cells, through evaluation of the downstream signaling pathway.

Preparation of dsDNA and purity check

Timing: 4 h

This section describes the steps involved in the preparation of dsDNA probes from ssDNA and the method of verifying the integrity of the annealed probes.

1. Prepare annealing reaction in PCR tubes as per below:

Reagent(s)	Volume per reaction
5× annealing buffer	20 µL
Anti-sense probe 80 bp [100 µm]	5 µL
Sense probe 80 bp [100 µm]	5 µL
PCR grade water	70 µL
Total volume	100 µL

Note: a 100 µL reaction should provide sufficient annealed probes to stimulate approximately 10 wells of a 6-well plate.

2. Insert the tubes in the PCR machine and use the following program for annealing:

Temperature	Time	Cycles
95°C	4 min	1
85°C	4 min	1
82°C	4 min	1
78°C	4 min	1
75°C	4 min	1
72°C	4 min	1
70°C	10 min	1
69°C* Ramp of −1°C per cycle	1 min	58
10°C	1 min	1
4°C	∞	

3. dsDNA probes purity check: Prepare a 10% Acrylamide gel using an acrylamide gel casting system (e.g., Biorad gel casting system):

Reagent(s)	Volume per reaction
Acrylamide Bis 40% (19:1)	2.5 mL
Tris-Borate-EDTA (TBE) 5×	2 mL
ddH_2O	5.428 mL
Ammonium persulphate solution (APS) 10%	65 µL
TEMED	7 µL
Total volume	10 mL

a. After the annealing step, measure the concentration of the dsDNA preparation sample by UV spectrophotometry (e.g., Nanodrop).

Note: you should obtain a concentration close to 0.25 µg/µL.

b. Load in the 10% Acrylamide gel, 0.5 µg of each sample with 6× loading dye (e.g., orange/blue DNA gel loading dye) along with a 5 bp or 100 bp ladder. Load same amount of ssDNA as a control.
c. Run the gel in 1× TBE at 120 V for around 40 min.
d. Stain the gel with diluted Ethidium Bromide (1 µg/mL) or 1× SYBR™ safe solution in 1× TBE for 10 min under the chemical hood.
e. To verify the annealing and integrity of the dsDNA probes, trans-illumination with UV light can be used (gel imaging system UV light based e.g., Biovision) (Figure 1A).

MEFs seeding and dsDNA transfection

Timing: 1.5 h

This section describes the method of plating the cells followed by transfection using the already prepared dsDNA.

Day 1: 1 h.

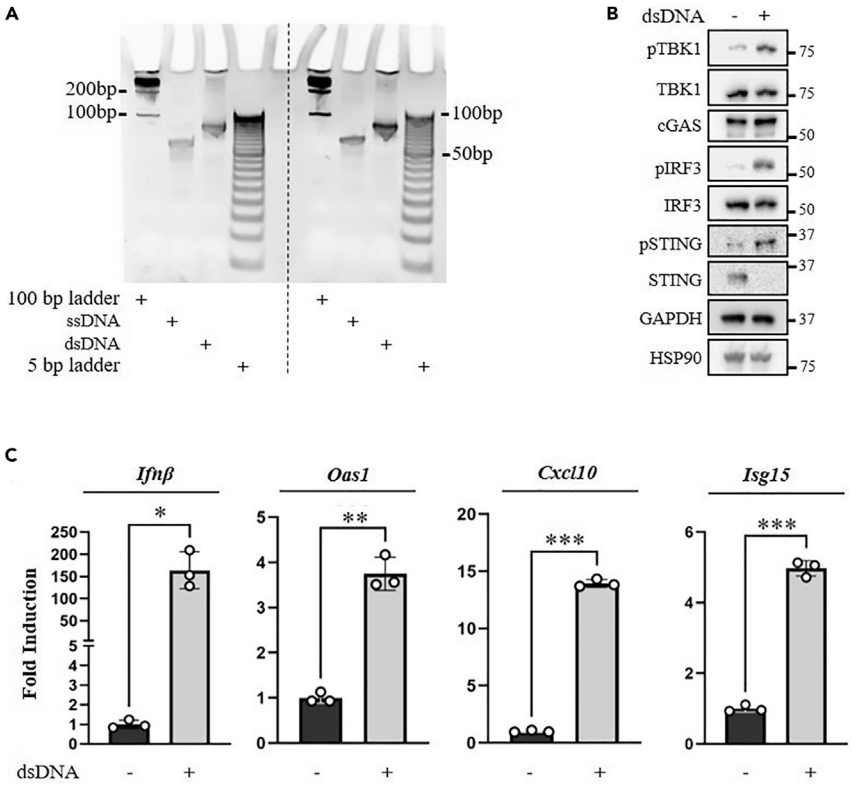

Figure 1. Outcome of dsDNA preparation and transfection in MEFs

(A) DNA samples (dsDNA and ssDNA) migrated in a 10% acrylamide gel were visualized using UV light after SYBR safe staining.

(B) Whole cell extracts prepared from MEFs following 6 h stimulation with dsDNA were analyzed by WB using indicated antibodies.

(C) mRNA levels of *Ifnβ*, *Oas1*, *Cxcl10* and *Isg15* mRNA levels in MEF, following dsDNA stimulation for 6 h (n = 3 biological replicates). All graphs present means ± SEM. p values were determined by paired Student's t test. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

4. Seed 6-well plates with 2 mL of complete media at a concentration of 125,000 cells per mL. Note that the number of cells seeded, and the growth medium depends on the cell line (Here, we used MEFs in DMEM supplemented with 10% FBS; 1% Penicillin/Streptomycin and 1% Glutamine). According to the manufacturer's recommendation (https://www.polyplus-transfection.com/products/jetprime/), cells have to reach 60%–80% confluency for dsDNA transfection with JetPRIME reagent.
5. Incubate the plate at 37°C with 5% CO_2 for 24 h before transfection.

Day 2: 30 min.

6. Replace media with 1 mL fresh complete media.
7. Transfection is performed using the JetPRIME reagent, according to the manufacturer's instructions (https://www.polyplus-transfection.com/products/jetprime/). For activation of the cGAS-STING pathway, 2 μg of dsDNA per well is sufficient. Calculate the volume containing 2 μg of dsDNA from your dsDNA probes sample. As a negative control, mock transfection is performed, using a volume of 1× annealing buffer corresponding to that used in dsDNA transfection. Prepare the dsDNA transfection and control mixtures (volumes for one well is shown). Multiply the volume of each material by the number of wells required and prepare the mixture accordingly.

dsDNATransfection Mixture (per condition)	
jetPRIME® buffer	Complete to 200 μL
dsDNA (2 μg)	8 μL
Total volume = 200 μL	

Control/non transfected Mixture (per condition)	
jetPRIME® buffer	Complete to 200 μL
1× Annealing buffer	8 μL
Total volume = 200 μL	

8. Add 4 μL of jetPRIME® reagent per point in each mixture, vortex for 1 s, and incubate for 10 min at RT (20°C–25°C).
   a. Add 200 μL of mixture into each well dropwise containing 1 mL of complete medium.
   b. Gently swirl the plate to ensure even distribution of the mixture.
9. Incubate the plates at 37°C with 5% CO_2 for 6 h.
10. After 6 h, aspirate the media and wash each well twice with 500 μL cold 1× PBS.
11. Aspirate PBS and dry the wells. You can then proceed with protein analysis (western blot) or gene expressing analysis (RT-qPCR).

Western blot

Timing: 7 h

This section describes a detailed stepwise method on how to perform a western blot. It also describes the process of stripping the membrane and reprobing for different proteins.

Day 1: 2 h.

12. Lyse cells with lysis buffer:
    a. Add 30 μL of lysis buffer (completed with β-Mercaptoethanol and PMSF) per well (plate on ice) and scrape the cells with buffer.
    b. Collect each point in Eppendorf tubes and incubate on ice at 4°C for 30 min.
    c. Centrifuge the tubes at 12,000 rcf for 30 min at 4°C.
    d. Discard the pellet and collect the supernatant i.e., the whole-cell lysate.
13. Measure protein concentration of lysates using Bradford method (other methods like bicinchoninic acid assay can also be used).
    a. Add 1 μL of cell lysate to a cuvette containing 1 mL of 1× Bio-Rad Protein Assay dye.
    b. For the blank measurement, add 1 μL of Lysis buffer to 1 mL of 1× Bio-Rad Protein Assay dye.
    c. Measure the optical density (OD) at 595 nm of the samples with a photometer.
    d. Measure the optical density (OD) at 595 nm of the standard curve samples (BSA at 2, 1.5, 1, 0.75, 0.5, 0.25, 0.125 μg/μL) as recommended by the manufacturer (https://www.bio-rad.com/webroot/web/pdf/lsr/literature/4110065A.pdf).
    e. Create a standard curve by plotting OD values (y-axis) versus their concentration in μg/μL (x-axis). Determine the unknown sample concentration using the standard curve.

Pause point: At this step, you can either proceed to sample preparation or store the samples at −20°C for up to a month or at −80°C for up to a year.

14. Sample preparation for gel loading:
    a. Calculate the volume of lysate containing 20 μg of protein.

b. Add this volume of lysate into 3.75 µL 4× Laemmli buffer.
c. Increase the total volume to e.g., 15 µL by adding dH_2O.
d. Heat the mixtures at 95°C for 5 min.
e. Spin down the tubes for 2–3 s using a microcentrifuge (376 rcf).

Pause point: At this step, you can either proceed to load the gel or store the samples at −20°C for up to a month or at −80°C for up to a year.

15. Load the lysate samples and run a 10% SDS-PAGE gel.
    a. Set up a mini gel tank and fill it with 1× running buffer prepared from 10× running buffer.
    b. Position the gel inside the tank and load the sample in each well.
    c. Run the gel at 200 V until the blue dye front is out.
16. Perform dry transfer of proteins from the gel to nitrocellulose membrane using Trans-Blot Turbo transfer system (other transfer methods such as wet or semi-dry transfer can also be performed).
    a. Place the membrane with filter papers at the bottom, inside the cassette and position the gel above the membrane.
    b. Place filter papers on top of the gel and use the roller to remove trapped air bubbles from the 'transfer sandwich'.
    c. Insert the cassette into the blotting apparatus and transfer for 10 min at a constant current of 1.3A (using the 'mixed molecular weights' program).
17. After transfer, submerge the nitrocellulose membrane in Ponceau S stain for 1 min.
18. Rinse the membranes with demineralized water to destain the background and visualize protein bands.
19. Wash the membrane with PBST until it has completely destained (5–10 min).
20. Block the membrane in PBST containing 5% milk for 30 min at RT (20°C–25°C).
21. Wash the membrane 3 times with PBST (10 min each).
22. Incubate the membrane with primary antibody solution at appropriate dilution (e.g., Here, 1:1,000 and 1:500 dilutions of antibodies was used, in PBST supplemented with 5% BSA and overnight (12–16 h) incubation at 4°C).

Day 2: 5 h.

23. Remove the primary antibody; wash the membrane 3 times (10 min each) using PBST.
24. Incubate the membrane in secondary antibody solution (1:1,000 in PBST supplemented with 5% milk) at RT (20°C–25°C) for 1 h.
25. Wash the membrane 3 times (10 min each) using PBST.
26. Detect the protein bands by chemiluminescent visualization (e.g., Bio-Rad ChemiDoc imaging system).
    a. Prepare a solution by mixing equal volumes of SuperSignal™ West Pico PLUS. Luminol/Enhancer and SuperSignal™ West Pico PLUS Stable Peroxide.
    b. Put the solution on top of the membrane and incubate for 5 min at RT (20°C–25°C).
    c. If the signal strength is too low, wash the membrane in PBST for 5 min and use SuperSignal™ West Femto Maximum Sensitivity Substrate as in step a-b above to increase the signal strength (Figure 1B).
27. To probe for remaining protein bands, the membrane must be stripped and re-blocked.
    a. Submerge the membrane in Restore™ PLUS Stripping Buffer and incubate at RT (20°C–25°C) for 5–15 min on a rocker. The reagent can be reused up to 5 times.
    b. Wash the membrane with PBST 3 times for 10 min each.
    c. Block membrane with PBST-5% milk and probe for other bands as mentioned previously.

Real time-quantitative polymerase chain reaction (RT-qPCR)

Timing: 8 h

This section describes a detailed stepwise method on how to perform an RT-qPCR.

Principles of the procedure: It is a well-established technique used for the quantification of RNA in biological samples that involves amplification of target genes. For more information about key points and parameters of RT-qPCR, refer to Kralik et al. (Nolan et al., 2006). This method comprises three major steps:

RNA extraction and preparation

Timing: 2 h

28. Directly add TRIzol™ (acid-guanidinium-phenol based reagent designed for the extraction of RNA) on cells in every well (500 µL for a well in 6 well plate).
29. Put the plates on a plate rocker for 5 min at RT (20°C–25°C) (under a hood).
30. Resuspend up and down the lysed cells and collect them into 1.5 mL Eppendorf tubes.

Pause point: At this step, you can either proceed or store at −20°C for the following days or store at −80°C for up to a year.

⚠ CRITICAL: TRIzol™ is a harmful agent that is toxic if it contacts with skin (causing skin burns) and if swallowed. According to the safety sheet, it is suspected of causing damage to organs through repeated exposure so it is always recommended to use it only under a chemical hood. TRIzol™ RNA extraction can be replaced by RNA extraction column-based protocols not involving TRIzol and chloroform (e.g; GenElute™ Total RNA Purification Kit/ Sigma or RNeasy Kits/Qiagen).

31. Add 100 µL of chloroform per 500 µL of TRIzol™ reagent used for lysis in each RT-qPCR sample, then securely cap the tube.
32. Shake by inverting the tubes several times for 1 min.
33. Incubate for 2 min at RT (20°C–25°C).
34. Centrifuge for 15 min at 12,000 rcf at 4°C.

Note: The mixture separates into a lower red phenol-chloroform organic phase containing protein, DNA etc., an interphase (white layer), and a colorless upper aqueous phase containing RNA.

35. Collect 200 µL of the aqueous transparent phase containing RNA and transfer to a new Eppendorf tube.

Optional: Tilt the tube at 45° to collect the upper phase easily.

Note: Be careful to not touch the interface layer, if not, this generates DNA and proteins contamination.

36. Add 250 µL of isopropanol to the tube only containing aqueous phase to precipitate RNA.
37. Shake and incubate at RT (20°C–25°C) for 10 min.
38. Centrifuge for 10 min at 12,000 rcf at 4°C.
39. Discard the supernatant carefully.

Note: Total RNA precipitate forms a white gel-like pellet at the bottom of the tube.

40. Resuspend each RNA pellet in 500 µL of 70% ethanol (prepared with PCR grade water).

Note: The RNA can be stored in 70% ethanol for at least 1 year at −20°C, or at least 1 week at 4°C.

41. Vortex the sample briefly.
42. Centrifuge for 5 min at 7,500 rcf at 4°C.

Note: Heat nuclease free water to 65°C.

43. Discard the supernatant.
44. Centrifuge for 30 s at 7,500 rcf to remove any excess of ethanol.
45. Let the RNA pellet dry for 5–10 min under the hood (for ethanol evaporation).
46. Resuspend the pellet in 10 µL of nuclease free water (preferentially warmed up 65°C, as recommended above).
47. Incubate the samples for 10 min at RT (20°C–25°C).
48. Quantify the RNA using UV spectrophotometry (e.g., Nanodrop).

Note: The measurements will be in ng/µL with two ratios to take into consideration: A260/A280 which is a ratio for phenol contamination, and A230/A280 which is a ratio for protein contamination. If the ratios are close to 2; this means that the purity of RNA is high. Otherwise, this could affect RT-qPCR results (e.g., later Cycle Threshold (Ct)).

Pause point: At this step, you can either proceed or store at −20°C for the following days or store at −80°C for up to one year.

DNAse treatment and cDNA synthesis

Timing: 3 h

49. Perform DNAse treatment with 2 µg per sample. Add water to reach a final volume of 8.5 µL per sample.
50. Prepare a 'DNAse mix' for all your samples with 1 µL 10× TURBO DNAse Buffer and 0.5 µL of DNAse enzyme per sample.
51. Add the 1.5 µL of 'DNAse mix' into the sample.
52. Incubate at 37°C for 20 min.
53. Resuspend DNAse inactivation reagent and add 2 µL into each reaction.
54. Vortex and incubate for 5 min at RT (20°C–25°C) (mix occasionally by rotating the tubes).
55. Centrifuge at 10,000 rcf for 3 min at RT (20°C–25°C).
56. Transfer 9 µL of each reaction into a PCR tube.

Pause point: You can either proceed with cDNA synthesis or store samples at −20°C for next day use. For longer storage, keep the reaction at −80°C.

57. Perform cDNA synthesis using SuperScript IV system. Add Mix 1 of cDNA synthesis (listed the reagents below) to the RNA samples (9 µL RNA + 4.5 µL of Mix 1 per reaction).

Mix 1 of cDNA synthesis

Reagent(s)	Amount per reaction
oligodT 50 µM	0.5 µL
dNTPs 10 µM	1 µL
H_2O	3 µL
Total volume	4.5 µL

58. Start the PCR program as follows:
59. Add tubes in the PCR machine for the annealing step.

Reverse Transcription reaction		
Steps	Temperature	Time
Annealing	65°C	5 min
Hold 1	4°C	–
Reverse Transcription	55°C	10 min
Enzyme inactivation	80°C	10 min
Hold 2	10°C	–

60. When there is the "hold 1" at 4°C, add the mix 2 (6.5 μL per reaction, total will be 20 μL).

Mix 2 of cDNA synthesis	
Reagent(s)	Amount per reaction
5× buffer	4 μL
RNAse out™	1 μL
DTT 0,1 M	1 μL
SSIV	1.5 μL
Total volume	6.5 μL

61. Vortex, spin down the PCR tubes and resume the PCR program.

Pause point: You choose either to proceed with preparing the plate or to store the synthesized cDNA at −20°C if you continue to use it the day after. Otherwise, for longer storage, keep it at −80°C.

Gene expression analysis (qPCR)

Timing: 3 h

62. Prepare cDNA dilutions for qPCR: Adjust cDNA samples to 20 ng/μL using nuclease-free water (so that 2.5 μL provides 50 ng cDNA per reaction).
63. Choose the below tested genes for cGAS-STING pathway activation (Table 1) and add housekeeping genes (e.g., HSP90, HPRT). Prepare the primer master mix for each gene.

PCR reaction master mix	
Reagent(s)	Amount per well
Forward primer [10 μM]	0.2 μL
Reverse primer [10 μM]	0.2 μL
2× Takara mix	5 μL
H_2O	2.1 μL
Total volume	7.5 μL

64. Load 2.5 μL of diluted cDNA.
65. Load 7.5 μL of primer master mix into diluted cDNA.
66. Seal the plates with optical heat seals and spin down quickly for 3–5 s.

Note: This can be done in 96 or 384-well reaction plates.

Table 1. qPCR primers		
Mouse primers	Forward primer	Reverse primer
Ifnβ	F-CTGCGTTCCTGCTGTGCTTCTCCA	R-TTCTCCGTCATCTCCATAGGGATC
Cxcl10	F-ATGACGGGCCAGTGAGAATG	R-TCAACACGTGGGCAGGATAG
Isg15	F-GTGCTCCAGGACGGTCTTAC	R-CTCGCTGCAGTTCTGTACCA
Oas1	F- TGCATCAGGAGGTGGAGTTTG	R- ATAGATTCTGGGATCAGGCTTGC
Hsp90	F-GTCCGCCGTGTGTTCATCAT	R-GCACTTCTTGACGATGTTCTTGC

67. Start the PCR program as per below:

PCR cycling conditions				
Steps	Temperature	Time	Ramp rate (°C/s)	Cycles
Initial Denaturation	95°C	30 s	4.4	1
Denaturation	95°C	5 s	4.4	45 cycles
Annealing	60°C	30 s	2.2	
Extension	72°C	30 s	4.4	
Melting curve	95°C	5 s	4.4	1
	60°C	1 min	2.2	
	95°C	Continuous	0.11 (5 acquisitions per °C)	
Cooling	50°C	30 s	2.2	1
Hold	4°C		∞	

68. Using Ct values for the housekeeping gene and genes of interest, calculate the delta CT: ΔCt = Ct (gene of interest e.g., Cxcl10) – Ct (housekeeping e.g., Hsp90). Calculate $2^{-\Delta Ct}$ for each sample and normalize your value to "Control/non transfected" sample to obtain fold induction for dsDNA sample (Livak and Schmittgen, 2001) (Figure 1C).

EXPECTED OUTCOMES

The described method for preparation of dsDNA is applicable to other types of nucleic acids, including RNA:DNA hybrids or double-stranded RNA (dsRNA). In the case of dsDNA, upon visualization, ascertain that your dsDNA is of the correct size (i.e., 80 bp in our case) by referring to the molecular weight ladders. The bands of ssDNA and dsDNA should be sharp with no remaining ssDNA visible (Figure 1A). The western blot (WB) technique allows the verification of the phosphorylated and total protein levels. If the cGAS-STING pathway is activated, WB should reveal increased phosphorylation of TBK1, IRF3 and STING, with no change in total proteins for TBK1 and IRF3, but decreased STING levels (Figure 1B). Assessing the status of phosphorylation of those proteins in absence of dsDNA transfection may be relevant in contexts such as viral infection or chemotherapy treatment. Finally, if transfected dsDNA is detected by the cGAS-STING pathway, a typical type I IFN signature will be measurable by RT-qPCR analysis, comprising increased *Interferon beta* (*Ifnβ*) mRNA levels, accompanied by upregulation of interferon-stimulated genes (ISGs), such as *2′-5′-Oligoadenylate Synthetase 1* (*Oas1*), *C-X-C motif chemokine ligand 10* (*Cxcl10*), or *Interferon-stimulated gene 15* (*Isg15*) (Figure 1C).

QUANTIFICATION AND STATISTICAL ANALYSIS

We used the paired t-test to calculate the significance of the RT-qPCR results. (n = *3 biological replicates*) *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

LIMITATIONS

In the above-described protocol, we used murine cells (MEFs) that have both proteins (cGAS and STING). However, this protocol would be unsuccessful when using cell lines that do not express

cGAS as described in several cancer cell lines (Xia et al., 2016). In this context, the expression of cGAS and STING is an important criterion to take into consideration.

TROUBLESHOOTING

Problem 1

In the process of dsDNA preparation (see section preparation of dsDNA and purity check), the dsDNA probes sample could be contaminated by ssDNA or degraded, which would decrease recognition by cGAS and subsequent pathway activation.

Potential solution

If you encounter a contamination by ssDNA in the prepared dsDNA sample repeat the annealing reaction.

Problem 2

In the process of dsDNA transfection (see section MEFs seeding and dsDNA transfection), the transfection efficiency can be different based on cell type.

Potential solution

For e.g., floating cells can be refractory to transfection of dsDNA using the Jetprime reagent. One alternative is to force adhesion by plating cells on coated plates (e.g., poly-D-lysine and/or Laminin) before transfection. Alternatively, other transfection reagents (e.g., lipofectamine) may be used. Also, make sure that the cells are at appropriate confluency, since high confluency lowers transfection efficacy.

Problem 3

In the process of Western blot analysis (see section western blot), some phosphorylated proteins can be difficult to visualize in particular p-IRF3 and p-STING.

Potential solution

Digital imaging is a rapid and convenient technology. However, in our experience (and depending on the camera used), sensitivity is lower than X-ray film. If phospho-proteins are not detectable by digital imaging proceed with X-Ray film.

Problem 4

In the process of RNA extraction (see section RNA extraction and preparation), RNA concentration at the end of the experiment can be too low to proceed with 2 μg of total RNA for DNAse treatment and retrotranscription (RT).

Potential solution

Cell seeding number can be increased, 2 wells can be pooled together, or RT mixture can be prepared with less than 2 μg of RNA (e.g., 0.5 or 1 μg) if cDNA dilution is done accordingly (final: 20 ng/μL).

Problem 5

In the process of gene expression analysis (see section gene expression analysis (qPCR)), an increase in *Ifnβ* mRNA levels can be detected, no increase of ISGs mRNA levels is measured.

Potential solution

ISGs are genes induced in response to Interferon β production. As a result, ISGs are increased later than *Ifnβ*. Typically, mRNA levels of ISGs can be efficiently measured at 16–24 h after dsDNA transfection. To measure ISG mRNA levels, 6 h after dsDNA transfection replaced "dsDNA transfection mixture/media" by 2 mL of fresh complete media. Proceed with RNA extraction at 24 h using the procedure described for the 6 h dsDNA transfection time point.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Nadine Laguette (Nadine.laguette@igh.cnrs.fr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

No new code has been generated in this study.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

H.C. and S.G. performed the experiments, drafted the manuscript, and prepared the figures. N.L. and I.K.V. supervised the project and reviewed and edited the manuscript and figures.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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