# Rapid and Accessible ELISA-Based Enzyme Kinetics Assay for Identifying Regulators of cGAS Activity

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## Abstract

Cyclic GMP–AMP synthase (cGAS) is a key double-stranded DNA sensor that activates innate immune signaling, and evaluating how specific molecules modulate its enzymatic activity is essential for mechanistic studies. Here, we describe a rapid and accessible ELISA-based protocol designed to quantify cGAS enzyme kinetics and determine how the addition of candidate factors influences its enzymatic activity. Reactions containing cGAS, with ATP and GTP in excess, are initiated by the addition of defined DNA ligands and quenched at multiple time points. The resulting time course of 2′3′-cGAMP formation is quantified using a commercially available ELISA to identify the steady-state linear phase of product accumulation. A suitable time point within this linear range is then used to perform reactions across a series of DNA ligand concentrations, allowing the initial reaction rate ($V_0$, 2′3′-cGAMP formation in $nM \cdot min^{-1}$) to be plotted against DNA concentration to generate Michaelis–Menten curves and corresponding kinetic parameters in the presence or absence of a test molecule. Overall, this approach provides direct, product-specific quantification of cGAS activity using only an absorbance plate reader and offers a sensitive, broadly accessible method for characterizing molecular regulators of cGAS even in laboratories without specialized instrumentation.

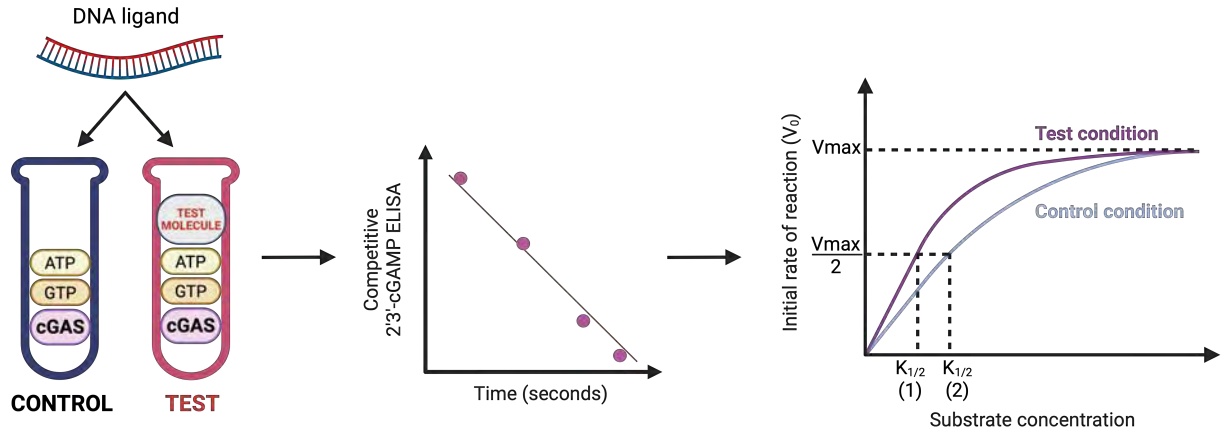
## Key features

- Developed to evaluate how cofactors or regulatory proteins modulate cGAS activity *in vitro*.
- Suitable for assessing cGAS kinetics with diverse nucleic acid substrates under defined reaction conditions.
- Determination of the initial reaction rate ($V_0$), maximum reaction rate ($V_{max}$), the catalytic turnover number ($K_{cat}$) and $K_{1/2}$ constant representing the DNA concentration at which cGAS reaches half of the $V_{max}$.
- Yields reproducible kinetic results within ~4 hours from reaction setup to data analysis.

**Keywords:** cGAS, Enzyme kinetics, ELISA, Accessible, Michaelis-Menten, $V_{max}$, $K_{1/2}$, $K_{cat}$

**This protocol is used in:** Communications Biology (2025), DOI: TBD
*Fréreux C., et al. PCBP1 Binding to Single-Stranded Poly-Cytosine Motifs Enhances cGAS Sensing and Impairs Breast Cancer Development. Commun. Biol. (In press)* [4]

## Graphical overview




**1) Enzymatic Reaction** **2) Steady-state phase confirmation** **3) Michaelis-Menten plot**

# Background

Cyclic GMP–AMP synthase (cGAS) is a cytosolic DNA sensor that catalyzes the synthesis of the second messenger 2′3′-cGAMP from ATP and GTP. Activation of cGAS triggers the STING pathway, leading to type I interferon production and downstream immune responses. Because of its central role in innate immunity, autoimmunity, infection, and cancer biology [1], there is strong interest in understanding how cGAS activity is regulated and how cofactors, binding partners, or small molecules influence its enzymatic function.

Several methods have been described to measure cGAS activity. The most direct approaches rely on HPLC, LC–MS/MS, or radiolabeled nucleotide assays, which allow high sensitivity and precise quantification of 2′3′-cGAMP but require specialized instrumentation, radioisotope handling, or technically demanding protocols. Here, we describe a more accessible alternative using an ELISA-based detection kit that exploits the high specificity of antibodies against 2′3′-cGAMP. ELISAs offer simplicity, scalability, and compatibility with standard laboratory equipment, making them well suited for kinetic analyses in laboratories without access to advanced analytical platforms.

The protocol described here provides a streamlined workflow to determine Michaelis–Menten kinetic parameters (Vmax, kcat, $K_{1/2}$) [2], [3] of cGAS using an endpoint ELISA readout. Compared to continuous real-time methods, this approach requires only standard molecular biology equipment and commercially available reagents, making it broadly applicable. While endpoint ELISA measurements rely on the assumption of linear product accumulation within the chosen incubation window, this can be validated with short time-course experiments. Once established, the protocol enables reproducible assessment of how test molecules (e.g., protein cofactors or small molecules) alter cGAS catalytic activity and substrate affinity.

Beyond evaluating candidate regulators of cGAS, the protocol can be adapted to study mutant cGAS variants, viral or cellular proteins that interact with cGAS, or pharmacological inhibitors and activators. Together, this method provides an accessible and adaptable framework for interrogating DNA-sensing enzymatic activity and modulators of innate immune signaling.

# Materials and reagents

## Reagents

1. Nuclease free, molecular biology grade water (Thermo Scientific, catalog number: J71786.XCR)
2. Tris-HCl pH 7.5 (1M)
3. $MgCl_2$ (1M)
4. Tween-20 (10%)
5. Adenosine Triphosphate (ATP, 5mM) (Cayman chemical, catalog number: 40182). Store in aliquots at -20°C.
6. Guanosine Triphosphate (GTP, 5mM) (Cayman chemical, catalog number: 16060). Store in aliquots at -20°C.
7. cGAS DNA ligand, Forward and Reverse sequences ordered from Eurofins [4]
8. His-cGAS human recombinant protein (1µM) (Cayman chemical, catalog number: 22810)
The enzyme is extremely temperature sensitive. Store in ~5µL aliquots at -80°C. Use each aliquot only one time to ensure best reproducibility.
9. Ethylenediaminetetraacetic acid (EDTA, 55mM) (Sigma Aldrich, catalog number: ED-100G)
10. 2’3’-cGAMP ELISA kit (Cayman chemical, catalog number: 501700). Store at 4°C.

## Solutions

1. Reaction buffer 4X (see Recipes). Store at 4°C for short-term or prepare fresh before assay.

## Recipes

**1. Reaction buffer 4X**

| Reagent | Final concentration | Quantity or Volume |
|---|---|---|
| Tris-HCl, pH 7.5 (1M) | 40mM | 200µL |
| MgCl2 (1M) | 10mM | 50µL |
| Tween-20 (10%) | 0.02% | 10µL |
| Nuclease free water | | 4.87mL |
| Total | | 5mL |

## Laboratory supplies

1. 0.2mL PCR tubes (Thermo Scientific, catalog number: AB0620)

# Equipment

1. T100 thermal cycler (BioRad, catalog number/model: 1861096)
Alternatively, can use any equipment incubating at 37°C. The enzyme reactions can also be performed at room temperature, but it may reduce the efficiency of the enzyme.
2. SpectraMax iD5 Microplate reader (Molecular Devices, catalog number/model: ID5-STD)
Alternatively, any plate reader able to read the absorbance of 96 well plates at 450nm can be used.
3. Multichannel pipette (1 to 10µL) (Thermo Scientific, catalog number: 4661000N)

# Software and datasets

1. GraphPad Prism (Version 10.5.0)

# Procedure

## A. Overview of Kinetic Assay Design

This protocol allows investigators to test the effects of a molecule on cGAS enzymatic activity. From the determination of $V_{max}$ and $K_{cat}$, one can assess whether the molecule alters the intrinsic catalytic activity of cGAS, while the determination of $K_{1/2}$ indicates whether the molecule influences the apparent affinity of cGAS for its DNA ligand.

The substrates of cGAS are ATP and GTP, which are catalyzed to form 2′3′-cGAMP. However, cGAS requires binding to a DNA ligand as an allosteric activator to adopt the correct conformation for ATP and GTP binding and subsequent catalysis. For this reason, the DNA ligand concentration is varied and plotted on the X-axis to generate the Michaelis–Menten curve[2], [3].

The following procedure illustrates how to set up a single enzymatic reaction in parallel test and control tubes: a test condition containing the molecule of interest and a control condition without it, at a defined reaction time and DNA ligand concentration. To derive kinetic parameters ($V_{max}$, $k_{cat}$, $K_{1/2}$), multiple reactions must be performed varying both the incubation time and the DNA ligand concentration.

As a first step, short time-course experiments should be conducted for both test and control conditions (e.g., 30 s, 2 min, 5 min) at ~10 nM DNA and at a saturating DNA concentration (e.g., 500 nM), using 30 nM cGAS. This verifies that product accumulation is linear over the selected time window, ensuring that substrate is not depleted and that neither product buildup nor other factors inhibit the enzyme. Although linearity should be confirmed for each specific experimental setup, it has been validated under the conditions described in this protocol.
After establishing linearity, select a fixed incubation time within the linear range (we recommend around 30sec under these conditions). Next, perform reactions across a series of DNA ligand concentrations for both test and control reactions (e.g., 2.5, 5, 7.5, 15, 50, and 500 nM). Plotting the initial velocity ($V_0$, expressed as nM 2′3′-cGAMP produced per minute) versus DNA concentration will yield Michaelis–Menten curves from which $V_{max}$, $K_{1/2}$, and $k_{cat}$ can be calculated in the presence or absence of the test molecule. These parameters directly reveal how the molecule modulates cGAS activity.

## B. Enzymatic Reaction

1. Anneal cGAS DNA ligand by putting 1:1 ratio of Forward and Reverse oligos at 100µM each and incubate in a beaker of boiling water for several hours or overnight, until the water temperature is back to room temperature. The final double-stranded DNA concentration is 50µM and can be diluted to perform the reactions (Step 6).

2. Pre-equilibrate all following reagents to room temperature, except for recombinant proteins (cGAS, or other potential co-factors to test).

3. Pre-heat a thermocycler or any other tube incubator at 37°C.

4. Prepare quench tubes by filling 0.2 mL PCR tubes (one per reaction) with 55mM of EDTA solution. These tubes will be used to stop the enzymatic reactions (Step 9).

5. Prepare the Reaction Master Mix:
In a 0.2 mL PCR tube, combine the following (example for 2 reactions = control + test; scale proportionally for additional reactions):
    - 2.2µL of ATP 5mM (200µM final concentration)
    - 2.2µL of GTP 5mM (200µM final concentration)
    - 27.5µL of reaction buffer 4X (2X final concentration)
    - 19.8µL of nuclease free water

- 3.3µL of recombinant His-cGAS 1µM (60nM final concentration)
Total volume is 55µL.

**Critical:** Thaw a single 5µL aliquot of recombinant His-cGAS immediately before use and add it as the final component to the master mix. Do not refreeze; discard any remaining enzyme after use. Prolonged exposure of the enzyme at room temperature or repeated freeze-thaw cycles reduces activity.

6. Prepare two 0.2mL PCR tubes for your control and test condition containing the following volumes of Reaction Master Mix, test molecule, nuclease-free water and DNA ligand. It is important to add the DNA ligand last to both tubes simultaneously using a multichannel pipette as this will start the enzymatic reaction ($t_0$).
**Note:** This reaction will have to be performed with varying amounts of DNA ligands to plot a Michaelis-Menten curve. See Table 2 for suggested amounts of DNA ligand.

| | **CONTROL CONDITION** | **TEST CONDITION** |
|---|---|---|
| **REACTION MASTER MIX (STEP 4)** | 25µL | 25µL |
| **TEST MOLECULE** | NA | To optimize<br>e.g. 3µL of test protein at 1µM (= 60nM final concentration) |
| **DNA LIGAND 0.5µM VARIABLE (ADD LAST)** | 1.5µL | 1.5µL (15nM final concentration) |
| **NUCLEASE-FREE WATER** | Volume for 50µL | Volume for 50µL |
| **TOTAL** | 50µL | 50µL |

**Note:** The final enzymatic reaction contains: 100µM of ATP, 100µM of GTP, 10mM Tris-HCl pH 7.5, 2.5mM MgCl2, 0.005% Tween-20, 30nM of recombinant His-cGAS and variable amounts of test molecule (here example with 60nM of test protein), and DNA ligand (here example with 15nM but will be variable across enzymatic reactions).

7. Immediately flick the tubes and briefly centrifuge in a tabletop PCR tube centrifuge.

8. Quickly place tubes in a thermocycler or tube-incubator pre-heated at 37°C.
**Note:** Alternatively, the reaction can be performed at room temperature, but it may reduce the enzyme activity. Keep the temperature consistent between replicates.

9. Incubate for the desired amount of time.
**Note:** Incubation between 30s and 2min typically yields 2'3'-cGAMP concentrations within the linear range of the ELISA standard curve, avoiding the need for sample dilution.

10. Using a multichannel pipette, add 5µL of 55mM EDTA to each 50µL reaction immediately after incubation to quench the reaction. Mix by flicking the tubes and briefly centrifuge. The final EDTA concentration is 5mM and the final volume per tube is now 55µL.
**Note:** Add EDTA immediately at the end of the incubation period to stop the reaction consistently across samples and ensure reproducible replicates.
**Pause point:** Samples can be stored at -20°C short-term or proceed directly with the ELISA.

11. Perform this enzymatic reaction (step to 1 to 10), at several different time points (e.g. 30s, 1min, 1:30min, 2min) with a lower (e.g. 10nM) and higher DNA ligand concentration (500nM) to confirm linearity of product formation (see section A. of Data Analysis for an example of linearity curve)

12. After confirming linearity of product formation, choose a time within the window tested (e.g. 30s), and perform the same enzymatic reactions at different DNA ligand concentrations (e.g. 2.5, 5, 7.5, 15 and 500nM) to generate the Michaelis-Menten plot (see section B. of Data Analysis).
**Note:** It is recommended to perform each enzymatic reaction at least twice, ideally three times independently to generate the Michaelis-Menten curve with good confidence intervals.

## C. 2’3’-cGAMP ELISA

13. Follow the manufacturer’s instructions for the 2′3′-cGAMP ELISA (Cayman Chemical, catalog number: 501700). Load 50µL of each enzymatic reaction per well. The kit protocol allows incubation for 2h at room temperature or overnight at 4°C; we performed all incubations for 2h at room temperature, which yielded consistent results.
    **Note:** The samples should not require any dilution to perform the ELISA.

## D. Other formatting notes

**Table 2. Special symbols and units**

| Symbol name | Symbol |
|---|---|
| Degree Celsius | X °C (with space between unit and numeral) |
| Micro | µ |
| Nano | n |
| Pico | p (pmole) |
| Molar | M (mM, µM, nM) |
| Prime | ′ |
| Liter | L (mL, µL) |
| Exponentials | $Y^x$ |
| Scientific notation | $Y \times 10^x$ |
| Hyphen indicating range (en dash) | 1–3 |

# Data analysis

## E. Confirm linearity of 2’3’-cGAMP production at early time-points.

1. Plot the %B/B0 over time (from step B.11) to confirm linearity of product formation. The $R^2$ value should be ~1.

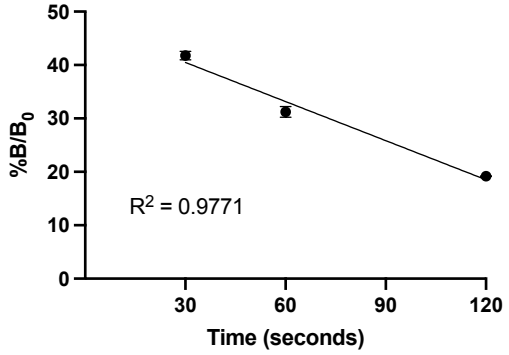



**Figure 2: Linearity of 2′3′-cGAMP production at different time points.** Reactions were performed with 30nM recombinant cGAS and 500nM DNA ligand (saturating conditions). Product accumulation was quantified by competitive ELISA at the indicated time points. The amount of 2′3′-cGAMP increased linearly with time ($R^2 = 0.9771$), confirming that the chosen incubation window reflects the initial velocity ($V_0$) under saturating substrate conditions.

## F. Michaelis-Menten plot: Determination of Vmax, Kcat and $K_{1/2}$ in presence or absence of the test molecule.

2. After completing step B.12 and the ELISA, convert the %B/B0 values into 2′3′-cGAMP concentrations (pg/mL) using the ELISA standard curve, then convert to nanomolar (nM). Normalize to the incubation time to obtain the rate of product formation in $nM.min^{-1}$. For example, if x nM of 2′3′-cGAMP is produced in 30s, multiply that value by 2 to express the rate per minute.

3. Use GraphPad Prism to create an XY table with the DNA ligand concentrations entered in the X column and the corresponding initial reaction rates ($V_0$, in $nM.min^{-1}$) entered in side-by-side replicate columns for the Y axis.

4. Enter three independent replicate values for each DNA ligand concentration under both the control condition and the test condition.

**Table 3: Example of dataset entered in GraphPad Prism to generate a Michaelis-Menten curve.** The initial rate of reaction $V_0$ obtained for each DNA ligand concentration (2.5mM, 7.5nM, 15nM, 50nM and 500nM) was provided in absence (control condition) or presence (test condition) of a protein suspected to be a co-sensor of cGAS.

| DNA LIGAND (nM) | $V_0$ CONTROL CONDITION ($nM.min^{-1}$) | | | $V_0$ TEST CONDITION ($nM.min^{-1}$) | | |
|---|---|---|---|---|---|---|
| **2.5** | 0.11 | 0.11 | | 0.1 | 0.13 | |
| **7.5** | 4.32 | 3.74 | | 6.36 | 7.23 | |
| **15** | 4.83 | 5.14 | 5.72 | 8.41 | 9.23 | 8.57 |
| **50** | 7.84 | 9.71 | | 9.82 | 11.9 | |
| **500** | 8.74 | 8.16 | 9.33 | 8.87 | 8.32 | 10.41 |

5. Click the '*Analyze*' tab > '*Regression and Curves'* > '*Nonlinear Regression (Curve Fit)'*. Select your dataset. Then under the '*Enzyme kinetics – Velocity as a function of substrate*' drop-down menu, select '*Michaelis-Menten'*.

6. In the '*Results'* section, the Vmax and $K_{1/2}$ values for both the control and test conditions will be reported along with their associated 95% confidence intervals (CI). Increasing the number of biological replicates will further narrow these intervals and strengthen the reliability of the parameter estimates.
In the '*Graphs'* section, non-linear regression curves will be shown for both the control and test conditions. Data points can be displayed with error bars representing either the standard deviation (SD) or the standard error of the mean (SEM).

The catalytic turnover number ($K_{cat}$) can be readily calculated using the relationship:

$$K_{cat} = \frac{V_{max}}{[E]_{total}}$$

where $[E]_{total}$ is the concentration of enzyme in the reaction.
Since the enzymatic reactions were performed with 30nM cGAS, kcat ($min^{-1}$) can be obtained as:

$$K_{cat} = \frac{V_{max}\ (\text{nM}.min^{-1})}{30\ (\text{nM})}$$

Here, $K_{cat}$ represents the number of molecules of 2′3′-cGAMP produced per minute per molecule of cGAS, whereas Vmax reflects the total concentration of product formed per minute by the entire 30nM enzyme population present in the assay.

A change in $K_{cat}$ indicates a corresponding change in the intrinsic catalytic activity of cGAS, independent of enzyme concentration. Therefore, a higher $K_{cat}$ in presence of a test molecule would represents an increase in cGAS catalytic activity.

Lastly, the $K_{1/2}$ value represents the DNA concentration at which the reaction rate reaches half of $V_{max}$ and is interpreted as an indicator of cGAS's apparent affinity for DNA. A lower $K_{1/2}$ indicates that cGAS achieves half-maximal velocity at a lower DNA concentration, reflecting higher apparent affinity, whereas a higher $K_{1/2}$ reflects reduced apparent affinity (see General note 3.)

| Nonlin fit<br>Table of results | A<br>- PCBP1 | B<br>+ PCBP1 |
|---|---|---|
| | Y | Y |
| **Michaelis-Menten** | | |
| **Best-fit values** | | |
| Vmax | 9.462 | 10.63 |
| $K_{1/2}$ | 11.84 | 5.870 |
| **95% CI (profile likelihood)** | | |
| Vmax | 8.194 to 10.80 | 8.657 to 12.76 |
| $K_{1/2}$ | 7.056 to 19.03 | 2.480 to 11.91 |
| **Goodness of Fit** | | |
| Degrees of Freedom | 10 | 10 |
| R squared | 0.9046 | 0.7503 |
| Sum of Squares | 11.30 | 37.74 |
| Sy.x | 1.063 | 1.943 |
| **Constraints** | | |
| $K_{1/2}$ | $K_{1/2} > 0$ | $K_{1/2} > 0$ |
| **Number of points** | | |
| # of X values | 15 | 15 |
| # Y values analyzed | 12 | 12 |

**Figure 3: Example of Michaelis-Menten analysis generated with GraphPad Prism.** The quality of the nonlinear regression was assessed using the coefficient of determination ($R^2$) and 95% confidence intervals (CI) for the fitted parameters. $R^2$ indicates the proportion of variance in the observed data explained by the Michaelis–Menten model, with values closer to 1.0 reflecting a stronger fit. The 95% CI provides an estimate of the precision of Vmax and $K_{1/2}$, with narrower intervals indicating greater reliability.

An example of the Michaelis-Menten plot generated with the data in Table 3, followed by $V_{max}$ and $K_{1/2}$ calculations can be found in the following article: Fréreux C., et al. (2025). PCBP1 Binding to Single-Stranded Poly-Cytosine Motifs Enhances cGAS Sensing and Impairs Breast Cancer Development. Commun. Biol. (Figure 7L) [4].

# Validation of protocol

This protocol has been used and validated in the following research article (Open access):

- *Fréreux C., et al. (2025). PCBP1 Binding to Single-Stranded Poly-Cytosine Motifs Enhances cGAS Sensing and Impairs Breast Cancer Development. Commun. Biol. (Figure 7, panels G to L and supplementary tables S3)* [4].

# General notes and troubleshooting

## General notes

1. Each test condition should always be performed in parallel with its corresponding control within the same batch (for example +/− test molecule). However, independent biological replicates or assays with different DNA ligand concentrations do not need to be carried out all at once; they can be performed on separate days, provided the cGAS enzyme has been properly aliquoted and stored at −80 °C to preserve activity.

2. This protocol is not suitable for pre-steady-state kinetics. The method relies on endpoint ELISA measurements after incubation, which cannot capture very short time scales (milliseconds to seconds). Therefore, it cannot resolve rapid pre–steady-state events such as burst phases or conformational transitions.

3. In the Michaelis Menten model, the Michaelis constant, $K_m$, represents the substrate concentration at which the reaction rate reaches half of $V_{max}$ and is commonly interpreted as an indicator of the enzyme's apparent affinity for its substrate. A lower $K_m$ value indicates that the enzyme achieves half-maximal velocity at a lower substrate concentration, reflecting a higher apparent affinity, whereas a higher $K_m$ reflects reduced apparent affinity.

   However, the Michaelis-Menten model is based on the assumption that the substrate concentration needs to be in large excess over the enzyme so that the concentration of substrate bound to the enzyme ([ES]) is negligeable relative to the concentration of total substrate. Therefore, the kinetics parameters can be calculated using the total concentration of substrate rather than using [ES] which is unknown at low substrate concentrations. In this assay, it is sometimes necessary to use dsDNA concentrations that are close to or even below the concentration of cGAS in order to evaluate DNA-dependent activation. Under these conditions, a substantial fraction of the DNA becomes bound to cGAS,

violating the requirement that substrate binding does not significantly alter the concentration used for kinetic analysis. Consequently, fitting velocity versus total DNA yields an apparent DNA-dependence constant rather than a mechanistic $K_m$. For this reason, we report this parameter as $K_{1/2}$, defined as the total DNA concentration required to reach 50% of Vmax under the experimental conditions. Although $K_{1/2}$ is not a mechanistic $K_m$, it reliably captures the DNA sensitivity of cGAS activation and allows comparison between control and test conditions, as the same limitation applies uniformly across all curves. In addition, because dsDNA functions as an allosteric activator of cGAS rather than the true chemical substrates (ATP and GTP), the term $K_{1/2}$ is conceptually more appropriate for describing ligand-dependent activation.

Finally, $V_{max}$ is not affected by this limitation because, at high DNA concentrations where saturation is achieved, the condition $[S]_{free} \approx [S]_{total}$ becomes valid again. At these saturating ligand concentrations, the reaction rate reflects $K_{cat}\ x\ [E]_{total}$ , ensuring that $V_{max}$ can be reliably determined in this experimental format.

### Troubleshooting

**Problem 1:** High 2′3′-cGAMP ELISA absorbance values (comparable to B0 values).
Possible cause: Insufficient 2′3′-cGAMP production.
Solutions: - Include all recommended ELISA controls (Blank, NSB, B0, TA) to confirm proper assay performance.
- Verify that cGAS enzyme was correctly aliquoted and stored at −80 °C; do not refreeze or reuse aliquots.
- If necessary, increase the cGAS concentration per reaction (e.g., 60 nM instead of 30 nM).

**Problem 2:** Low 2′3′-cGAMP ELISA absorbance values.
Possible cause: Excessive accumulation of 2′3′-cGAMP beyond the linear range of the ELISA.
Solutions: - Shorten the reaction time, ensuring it remains within the validated linear range of product formation.
- Alternatively, decrease the cGAS concentration per reaction.

**Problem 3:** Inconsistent replicate values.
Possible cause: Variability in cGAS enzyme activity or handling.
Solutions: - Confirm that cGAS enzyme was properly aliquoted and stored at −80 °C; never reuse the same aliquot.
- Prepare reaction Master Mixes (Step B.5) to minimize pipetting variability between control and test conditions
- Use consistent and precise incubation times across all conditions and batches.

## Acknowledgments

Specific contributions of each author: Conceptualization, C.F.; Investigation, C.F.; Writing, C.F.; Funding acquisition, P.H.H.; Supervision, P.H.H.

This work was supported by the National Institutes of Health, National Cancer Institute grant [CA154663] to P.H.H.

This protocol was described and validated in the following article: Fréreux C., et al. (2025). *PCBP1 Binding to Single-Stranded Poly-Cytosine Motifs Enhances cGAS Sensing and Impairs Breast Cancer Development.* Commun. Biol. [4]

## Competing interests

The authors declare no competing interests.

## Ethical considerations

No animal or human subjects have been used in the elaboration of this protocol.

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