Development and Validation of a Sensitive Immunoassay for Monitoring Second Messenger 2’3’-cGAMP

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Abstract

The objective of this study was to develop an immunoassay for specific detection of 2’3’-cGAMP and enable sensitive, accurate quantification of 2’3’-cGAMP levels in cells and other biological matrices in order to assess effective manipulations of the cyclic GMP-AMP synthase (cGAS) signaling pathway.

2’3’-cGAMP acts as a second messenger during host defense and may also have roles in autoimmune or inflammatory diseases, including cancer. It is produced when the DNA sensor, cGAS, detects the presence of nucleic acids in the cytosol of mammalian cells as an indicator of bacterial or viral infection. Self-nucleic acids (RNA and DNA) or damaged nucleic acids can also trigger cGAS to produce 2’3’-cGAMP, which binds tightly to the adaptor protein STING (stimulator of interferon genes). This initiates the recruitment and activation of downstream proteins, TANK-binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3), that induce the transcription and translation of type I interferons responsible for triggering a localized immune response. Therefore, modulation of cGAS activity with subsequent induction or inhibition of 2’3’-cGAMP formation, in association with its upstream regulators and downstream effectors, make it a highly investigated and intriguing pharmacological target in cancer immunotherapy and autoimmune disease.

We report the development of a sensitive immunoassay for the quantification of 2’3’-cGAMP using a colorimetric 96-well microtiter plate format. The assay has a lower limit of detection of 9.6 pg/ml and allows for accurate readings in complex sample matrices without the need for sample purification, utilizing a sample size of ≤50 microliters. This assay allows for monitoring 2’3’-cGAMP formation, metabolism, and/or reduction in relevant biological samples.

Introduction

The innate immune system has historically been studied primarily in response to bacterial and viral infections. More recently, innate immunity is being identified and researched in tumorigenesis, autoimmune disease, and senescence, where 'self'-nucleic acids (RNA and DNA) escape into the cytosol trigger an immune response.

Two proteins, cGAS and STING, are the sensors of these escaped nucleic acids in immunity response mechanisms. Emerging data and exploration have found that under some conditions (*e.g.*, autoimmune disease) inhibition of this pathway may lead to desirable therapeutic effects. However, in cases such as tumor modulation, stimulation of cytokine production is desired such that induction of the pathway may be beneficial to disease outcome. As the biological roles of cGAS and STING continue to unfold, monitoring their use as a potential therapeutic target is a growing area of research investigation with respect to this unique pathway.

Therefore, development of a highly sensitive tool to measure secondary messenger 2’3’-cGAMP levels in human study samples as well as the effects of inhibitors/activators will be imperative in determining how modulation of cGAS activity influences specific disease outcomes.

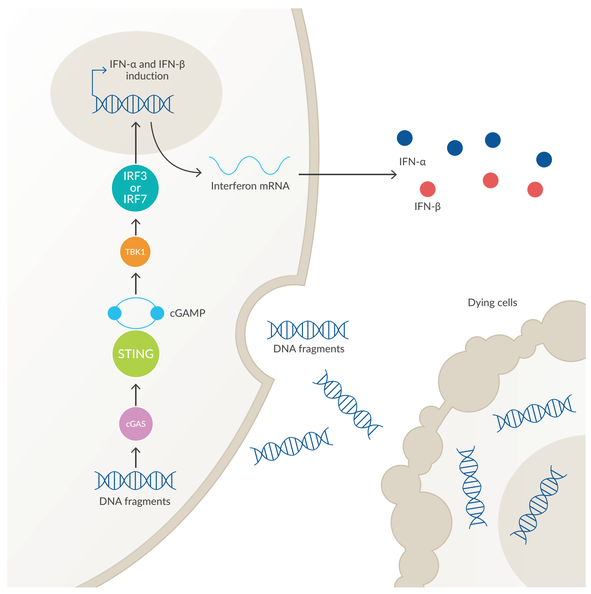

Figure 1. Cyclic GMP-AMP synthase (cGAS) signaling pathway. Figure adapted from Borden, E.C. *Nat. Rev. Drug Discov.* **18(3)**, 219-234 (2019).

Materials and Methods

2’3’-cGAMP immunogens were derived by conjugation of 2’3’-cGAMP to carrier proteins and rabbits were subsequently immunized. Antisera from all rabbits were tested, and rabbits with positive titer for reactivity against free 2’3’-cGAMP were identified and screened in subsequent antisera collections. 2’3’-cGAMP was directly conjugated to horseradish peroxidase (HRP) to make a tracer for a competitive ELISA format. All synthetic 2'3'-cGAMP derivatives were supplied by BioLog Life Science Institute.

The final ELISA format utilized mouse anti-rabbit plates on which the 2’3’-cGAMP rabbit polyclonal antibody can bind. 2’3’-cGAMP conjugated to HRP was applied to the assay to compete for binding with standard or native 2’3’-cGAMP in the samples/standards. The signal demonstrated was inversely proportional to the amount of standard or native 2’3’-cGAMP present (**Figure 2**).

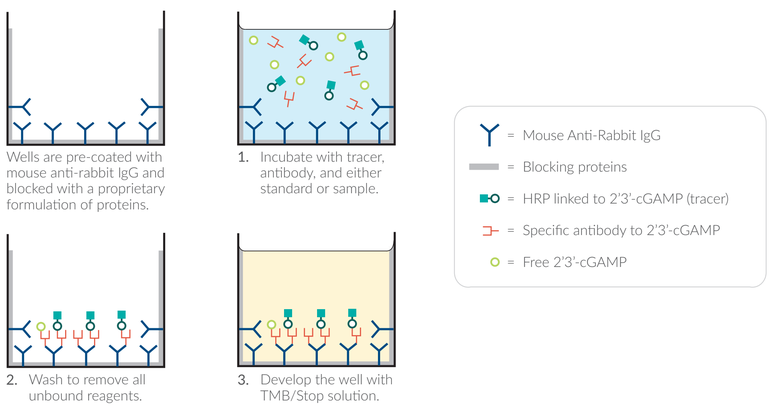

Figure 2. Schematic of the 2’3’-cGAMP ELISA.

Performance Characteristics

This sensitive 2’3’-cGAMP ELISA was fully validated for use in cell lysate preparations. The assay has a range of 6.1 pg/ml - 100 ng/ml with a lower limit of detection (LLOD) of 9.6 pg/ml and reliable, reproducible sensitivity of 85.3 pg/ml (80% B/B_0) (**Figure 3**). Specificity and selectivity of the assay was tested by running a panel of potential cross reactants in the assay. Cross reactivity was calculated by comparing the mid-point (50% B/B_0) value of the tested cross-reactant to the mid-point (50% B/B_0) value of the primary analyte, 2'3'-cGAMP (**Table 3**). Cross-reactivity values of tested compounds were all under 0.8% which supports high specificity to the 2'3'-cGAMP assay target.

Matrix effects of common lysis buffer M-PER™ were tested using a spike and recovery experiment where known amounts of synthetic 2’3’-cGAMP were spiked into M-PER™ and read off the standard curve, which was diluted with assay buffer. A linear response with a slope of 1.035 (**Figure 4**) is observed and indicative of nominal matrix interference from this lysis buffer.

Intra- and inter-assay precision (**Tables 1 and 2**, respectively) were determined using synthetic matrix controls comprised of spiked synthetic 2’3’-cGAMP in lysis buffer (M-PER™). Robustness and reproducibility were tested in the ELISA utilizing intra- and inter-assay precision testing. The low variability observed in the dynamic range of the assay is indicative of a robust and reproducible assay system.

Common lysis buffer components were tested in the assay to determine limit of detergent or buffer component that the assay could withstand without impact to calculated results (**Figure 5**). Tween-20, Triton X-100, sodium dodecyl sulfate (SDS), and IGEPAL® were tested utilizing a 25 ng/ml spike of 2’3’-cGAMP in assay buffer containing commonly utilized detergent concentrations to determine tolerance thresholds. Percent recoveries nearing 100% are the most ideal ranges for detergent thresholds in the assay. The assay is compatible with the following: Tween-20 below 1%, Triton X-100 below 1%, SDS below 0.125%, and IGEPAL below 0.25% in the sample going into the well. Higher concentrations of detergents can be used in lysis buffers, but samples will then need to be diluted to within these thresholds going into the assay to allow for accurate data.

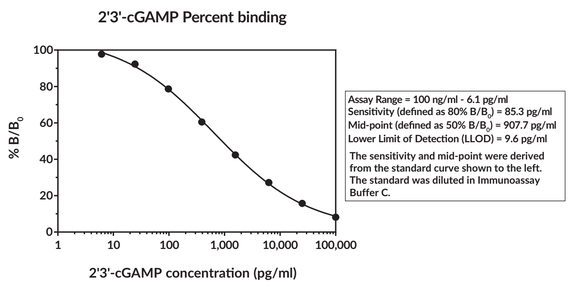

Figure 3. 2’3’-cGAMP standard curve and sensitivity.

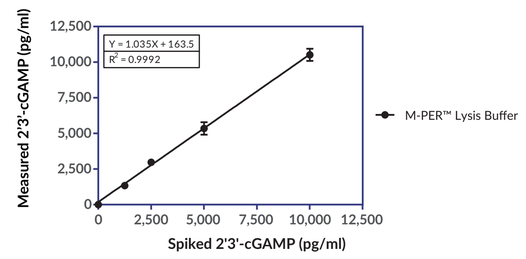

Figure 4. Spike and recovery of 2’3’-cGAMP in M-PER™ lysis buffer in the 2’3’-cGAMP ELISA.

Intra-assay precision

Table 1. Intra-assay precision was determined by analyzing 24 replicates of 3 synthetic matrix samples in a single assay.

Matrix Control (pg/ml)	%CV
7,165.1	8.4
828.6	14.5
102.5	21.3

Inter-assay precision

Table 2. Inter-assay precision was determined by analyzing replicates of 3 synthetic matrix samples in separate assays spanning across several days.

Matrix Control (pg/ml)	%CV
7,530.1	9.6
811.8	9.6
72.2	18.7

Cross Reactivity

Table 3. Cross reactivity of the 2’3’-cGAMP ELISA.

Compound	Cross Reactivity
2'3'-cGAMP	100%
2'2'-cGAMP	0.8%
3'3'-cGAMP	<0.01%
cyclic di-AMP	<0.01%
cyclic di-GMP	<0.01%
cGMP	<0.01%
cAMP	<0.01%
ATP	<0.01%
GTP	<0.01%

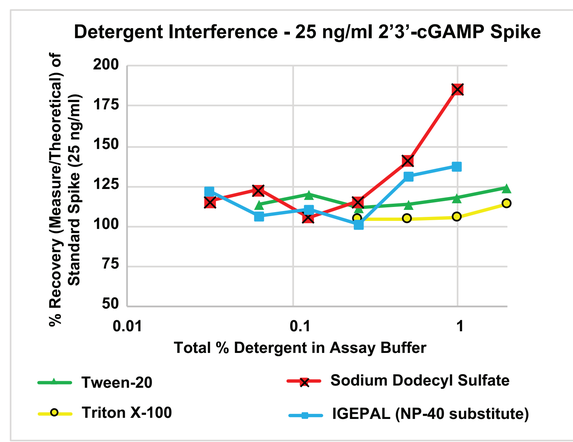

Figure 5. High spike of 2’3’-cGAMP in common lysis buffer detergents to determine assay tolerances.

Monitoring cGAS Activity utilizing 2'3'-cGAMP ELISA

The 2'3'-cGAMP ELISA can be used to monitor cGAS production and/or activity under various conditions and stimuli. To demonstrate this, the activity of recombinantly expressed and purified cGAS was monitored in correlation to activation with DNA induction. Recombinant purified cGAS was treated with a titration of Interferon Stimulatory DNA (ISD) (0-27 µg/ml) and supplemented with 2.5 mM ATP and 2.5 mM GTP. The production of 2'3'-cGAMP from this DNA induction event was measured using the 2'3'-cGAMP ELISA and found that at higher concentrations of DNA stimuli, higher concentrations of 2'3'-cGAMP were observed (**Figure 6**). This is indicative of increased cGAS activity as dosage of DNA stimuli increases allowing a higher turnover of ATP and GTP into 2'3'-cGAMP.

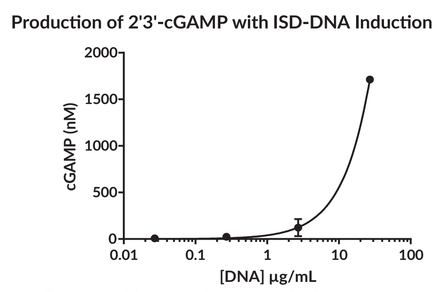

Figure 6. Purified cGAS was stimulated with increasing concentrations of ISD-DNA. Reaction mixtures were tested in the 2'3'-cGAMP ELISA for detection of 2'3'-cGAMP. Detected levels of 2'3'-cGAMP are plotted as a function of ISD-DNA stimulus concentration.

COLLABORATE WITH US

We are seeking cell lysate or tissue samples that are believed to have cGAS-cGAMP-STING upregulation to test for diversity of the 2'3'-cGAMP ELISA.

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