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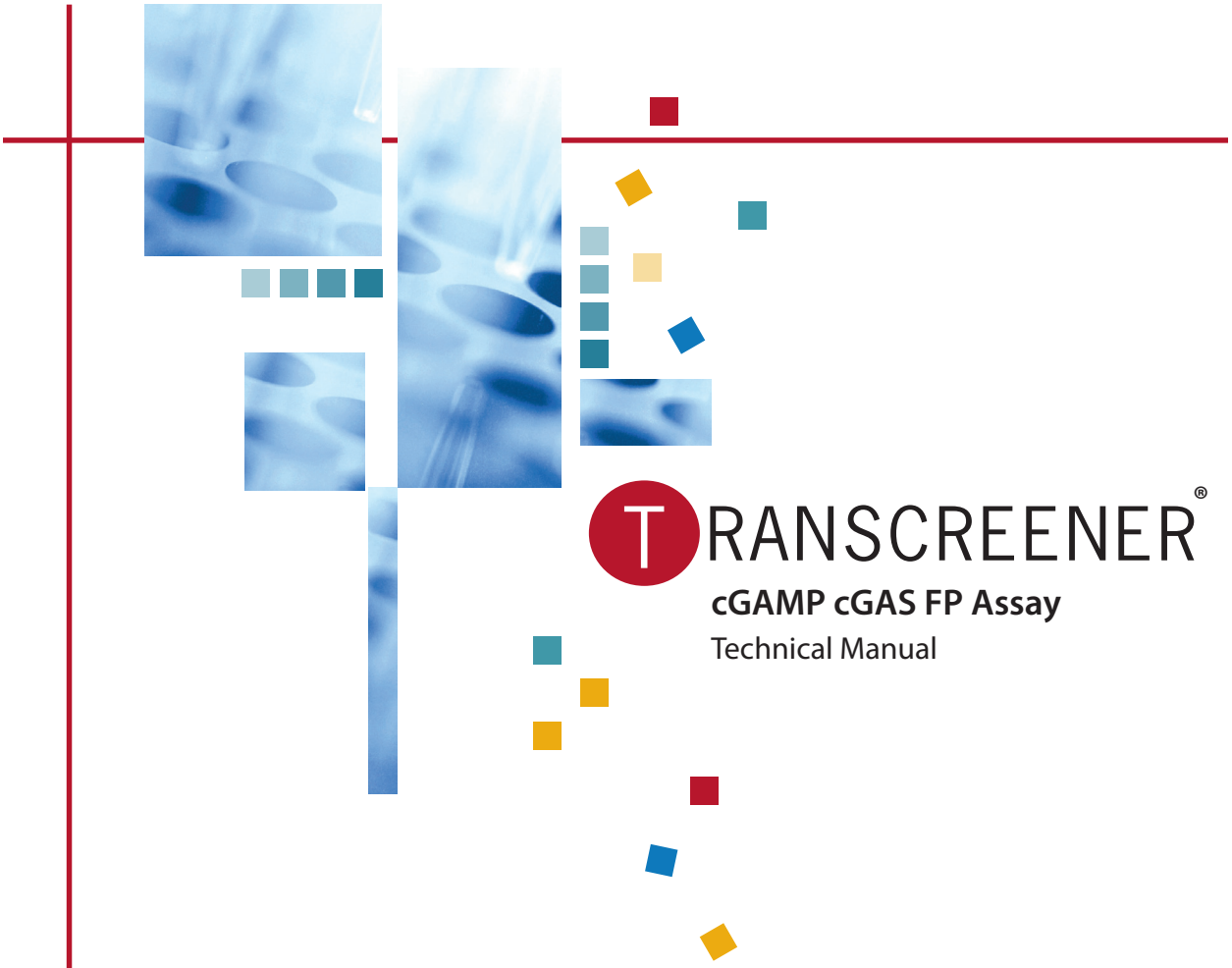

TRANSCREENER®

cGAMP cGAS FP Assay

Technical Manual

Instructions for Part Numbers 3024-1K and 3024-10K

Rev 2018-012

Transcreener® cGAMP cGAS FP Assay Technical Manual

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1.0 Introduction

The Transcreener® cGAMP cGAS FP Assay is a far-red, competitive fluorescence polarization (FP) assay (**Figure 1**). Because the antibody is highly selective for cGAMP, the assay can be used to measure activity of the cyclic GMP-AMP synthase (cGAS) enzyme which converts ATP and GTP, to cGAMP. cGAS is a recently discovered enzyme that acts as a foreign DNA sensor that induces an immune response via activation of the stimulator of interferon genes (STING) receptor. By directly measuring cGAMP with a highly selective antibody, it is possible to assay the activity of cGAS while screening large compound libraries for inhibitors.

The Transcreener® assay is designed specifically for high throughput screening (HTS), with a single-addition, mix-and-read format. It offers reagent stability and compatibility with commonly used multimode plate readers.

The Transcreener® cGAMP cGAS FP Assay provides the following benefits:

- A simple single addition cGAS activity assay capable of HTS.
- Excellent data quality (Z' ≥ 0.7) and signal (≥85 mP polarization shift) at cGAMP ranges between 0.5 µM and 5 µM.
- Far-red tracer further minimizes interference from fluorescent compounds and light scattering.

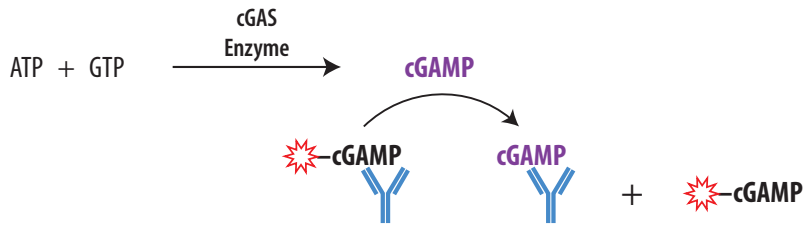

Figure 1. Schematic overview of the Transcreener® cGAMP cGAS FP Assay. The Transcreener® cGAMP Detection Mixture contains an cGAMP ATTO 633 tracer bound to an cGAMP antibody. cGAMP produced by the cGAS enzyme displaces the tracer, which rotates freely, causing a decrease in FP.

2.0 Product Specifications

Product	Quantity	Part #
Transcreener® cGAMP cGAS FP Assay	1,000 assays*	3024-1K
	10,000 assays*	3024-10K
	10 X 10,000 assays*	3024-100K

*The exact number of assays depends on enzyme reaction conditions. The kits are designed for use with 384-well plates, using 20 µL reaction volumes.

Storage

Store all reagents at –20°C upon receipt.

Please recommend avoiding freeze thaw cycles for the best result. The assay has exhibited little or no signal change with up to 5 freeze thaw cycles.

Use the reagents provided in this kit within 1 year from date of receipt.

2.1 Materials Provided

Component	Composition	Notes
cGAMP Antibody	1.8 mg/mL solution in PBS with 10% glycerol*	Sufficient antibody is included in the kit to complete 1,000 assays (Part # 3024-1K) or 10,000 assays (Part # 3024-10K).
cGAMP ATTO 633 Tracer	800 nM solution in 2 mM HEPES (pH 7.5) containing 0.01% Brij-35	The final tracer concentration in the 20 µL reaction is 4 nM.
Stop & Detect Buffer B, 10X	200 mM HEPES (pH 7.5), 400 mM EDTA, and 0.2% Brij-35	The Stop & Detect Buffer B components will stop enzyme reactions that require Mg^{2+}. To ensure that the enzyme reaction is stopped completely, confirm that the EDTA concentration is at least equimolar to the magnesium ion concentration in the reaction. The final concentration of Stop & Detect Buffer B at the time of FP measurement is 0.5X.
ATP	5 mM	The ATP supplied in this kit can be used for the enzyme reaction and standard curve.
GTP	5 mM	The GTP supplied in this kit can be used for the enzyme reaction and standard curve
cGAMP	500 µM	The cGAMP supplied in this kit can be used for a standard curve.
Interferon Stimulatory DNA	25 µM	The double stranded interferon stimulatory DNA (ISD) is a 45-bp oligomer used to activate the cGAS enzyme

*The exact concentration may vary from batch to batch. Please refer to the Certificate of Analysis for an accurate concentration.

2.2 Materials Required but Not Provided

- ***Ultrapure Water***—Some deionized water systems are contaminated with nucleases that can degrade both nucleotide substrates and products, reducing assay performance. Careful handling and use of ultrapure water eliminates this potential problem.
- ***Enzyme***—Transcreener® cGAMP cGAS assays are designed for use with purified cGAS enzyme preparations. Contaminating enzymes, such as phosphatases or nucleotidases, can produce background signal and reduce the assay window.
- ***Enzyme Buffer Components***—User-supplied enzyme buffer components include enzyme, buffer, $MgCl_2$, Brij-35, and test compounds.
- ***Plate Reader***—A multidetection microplate reader configured to measure FP of the cGAMP ATTO 633 tracer is required. Transcreener FP Assays have been successfully used on the following instruments: BioTek Synergy™2 and Synergy™4; BMG Labtech PHERAstar and PHERAstar Plus; Molecular Devices Analyst GT; Perkin Elmer EnVision® and ViewLux; and Tecan Infinite® F500, Safire2™, and M1000.
- ***Assay Plates***—It is important to use assay plates that are entirely black with a nonbinding surface. We recommend Corning® 384-well plates (Cat. # 4514). The suggested plate has a square well top that enables easier robotic pipetting and a round bottom that allows good Z' factors. It has a recommended working volume of 15–20 µL.
- ***Liquid Handling Devices***—Use liquid handling devices that can accurately dispense a minimum volume of 2.5 µL into 384-well plates.

Note: Contact BellBrook Labs Technical Service for suppliers and catalog numbers for buffer components, and additional information regarding setup of FP instruments.

3.0 Before You Begin

1. Read the entire protocol and note any reagents or equipment needed (see **Section 2.2**).
2. Check the FP instrument and verify that it is compatible with the assay being performed (see **Section 4.1**).

4.0 Protocol

The Transcreener® cGAMP cGAS FP Assay protocol consists of 3 steps (**Figure 2**). The protocol was developed for a 384-well format, using a 10 µL enzyme reaction and 20 µL final volume when the plates are read. The use of different densities or reaction volumes will require changes in reagent quantities (see **Section 7.2** for example reaction volumes). Once the instrument parameters and enzyme optimization are complete, the assay itself consists of a single step, simply add detection reagents to your enzyme reaction and read the plate.

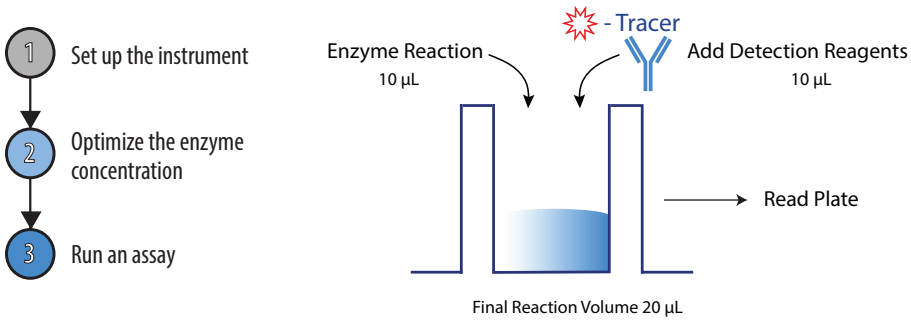

Figure 2. An outline of the procedure. The procedure consists of 3 main steps with a mix-and-read assay format.

4.1 Set Up the Instrument

Becoming familiar with ideal instrument settings for FP is essential to the success of the Transcreener® cGAMP cGAS FP Assay.

4.1.1 Verify That the Instrument Measures FP

Ensure that the instrument is capable of measuring FP (not simply fluorescence intensity) of cGAMP ATTO 633 Tracer.

Note: A complete list of instruments and instrument-specific application notes can be found online at: https://www.bellbrooklabs.com/technical-resources/instrument-compatibility Contact BellBrook Labs Technical Service if you have questions about settings and filter sets for a specific instrument.

4.1.2 Define the Maximum mP Window for the Instrument

Measuring high (tracer + antibody) and low (free tracer) FP will define the maximum assay window of your specific instrument. Prepare High and Low FP Mixtures in quantities sufficient to perform at least 6 replicates for each condition.

Use cGAMP ATTO 633 Tracer at 4 nM and Stop & Detect Buffer B at 0.5X concentration in a 20 µL final reaction volume. This mimics the 2-fold dilution when adding an equal volume of detection mixture to an enzyme reaction. As an example, the 1X detection mixture may contain 8 nM tracer. After adding this to the enzyme reaction, the concentration in the final 20 µL reaction volume would be 4 nM.

High FP Mixture

Prepare the following solution:

Component	Stock Concentration	Final Concentration	Example: 25 Assays	Your Numbers
cGAMP Antibody	1.8 mg/mL	1.0 µg/mL	0.28 µL*	
10X Stop & Detect Buffer B	10X	0.5X	25 µL	
cGAMP ATTO 633 Tracer	800 nM	4 nM	2.5 µL	
Water			472.22 µL	
Total			**500.0 µL**	

*Pipetting small sample volumes accurately requires the correct equipment and proper technique. An extra dilution step may be required to ensure accuracy.

Low FP Mixture

Prepare the following solution:

Component	Stock Concentration	Final Concentration	Example: 25 Assays	Your Numbers
10X Stop & Detect Buffer B	10X	0.5X	25 µL	
cGAMP ATTO 633 Tracer	800 nM	4 nM	2.5 µL	
Water			472.5 µL	
Total			**500.0 µL**	

Caution: Contact BellBrook Labs Technical Service for assistance if the assay window is <100 mP.

4.1.3 Measure the FP

Subtract the Low FP Mixture readings from the corresponding High FP Mixture readings. The difference between the low and high FP values should be >100 mP.

4.2 Optimize the Enzyme Concentration

Perform an enzyme titration to identify the optimal enzyme concentration for the Transcreener® cGAMP cGAS FP Assay. Use enzyme buffer conditions, substrate, and DNA concentrations that are optimal for your enzyme and experimental goals. If a compound screen is planned, you should include the library solvent at its final assay concentration. We routinely use enzyme buffer containing 20 mM TRIS (pH 7.5), 5 mM $MgCl_2$, 0.01% Brij-35, and 1% DMSO (test compound solvent). Run your enzymatic reaction at its requisite temperature and time period. Refer to **Section 7.3** for the tolerance of different components for your buffer conditions.

4.2.1 Enzyme Titration Steps

To achieve the most robust assay and a high signal, the quantity of enzyme required to produce a 50–80% change in FP signal is ideal (EC_{50} to EC_{80}) for screening of large compound libraries and generating inhibitor dose-response curves (see **Figure 3**). It is recommended to have at least a 100 mP shift to achieve a good assay window. Typically, an EC_{70} to EC_{80} has been used with the cGAS enzyme. To determine the EC_{80} enzyme concentration, use the following equation:

$$EC_{80} = (80 \div (100 - 80))^{(1 \div \text{hillslope})} \times EC_{50}$$

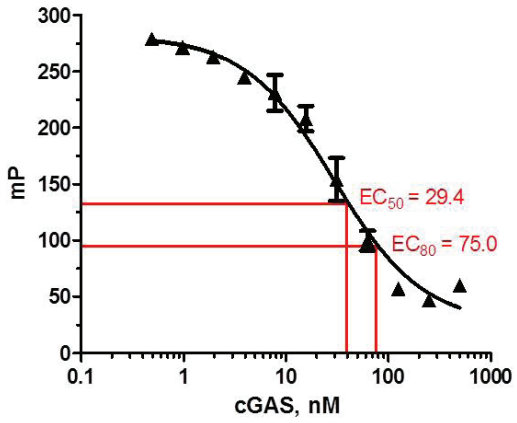

Figure 3. Enzyme titration curve. The ideal range of enzyme concentrations is shown in red.

4.2.2 Enzyme Assay Controls

The enzyme reaction controls define the limits of the enzyme assay.

Component	Notes
0 µM cGAMP Control	This control consists of the cGAMP Detection Mixture, the enzyme reaction components (without enzyme), 100 µM ATP, 100 µM GTP, and 0 µM cGAMP. It defines the upper limit of the assay window.
100 µM cGAMP Control	This control consists of the cGAMP Detection Mixture, the enzyme reaction components (without enzyme), 100 µM ATP, 100 µM GTP, and 100 µM cGAMP. It defines the lower limit of the assay window.
Minus-Nucleotide Control and Minus-DNA Control	To verify that the enzyme does not interfere with the detection module, perform an enzyme titration in the absence of nucleotide (i.e., ATP, GTP, or DNA).
cGAMP Standard Curve	Although optional, an cGAMP standard curve can be useful to ensure day-to-day reproducibility and that the assay conditions were performed using initial rates. It can also be used to calculate product formed and inhibitor IC_{50} values. See **Section 7.1** for a description of how to run the standard curve.
Background Control	Use only 0.5X enzyme reaction conditions and Stop & Detect Buffer B.

4.3 Run an Assay

4.3.1 Experimental Samples

Note: This is an example of running an assay for HTS or to obtain a dose response. Your volumes and concentrations may vary. It is important to have a 1:1 ratio of enzyme mix and detection mix for the final assay readout.

1. Add the enzyme to the test compounds at the desired concentration. The total volume of this mixture is 5 µL. Mix on a plate shaker. Incubate the enzyme inhibitor mixture for the desired time (typically at least 30 minutes).
2. Start the enzyme reaction by adding 5 µL of ATP, GTP, and DNA, then mix. It is recommended to use concentrations of 100 µM ATP, 100 µM GTP, and 60 nM DNA in the 10 µL final enzyme reaction mixture. Concentrations may vary based on your experiment.
   Note: The final volume of the enzyme reaction mixture should be 10 µL for 384 well plates. Use 2X ATP, GTP, and DNA in 5 µL to achieve the appropriate final concentration. See **Section 7.2** for a list of other plate formats.
3. It is recommended to incubate the enzyme reaction for 1 hour at room temperature. Please incubate at a temperature and time ideal for your experiment.
4. Prepare 1X cGAMP Detection Mixture as follows:

	1X cGAMP Detection Mixture			
Component	**Stock**	**Detection Mix Conc.**	**Example Volume**	**Your Numbers**
cGAMP Antibody	1.8 mg/mL	2.0 µg/mL	11.1 µL	
cGAMP 633 Tracer	800 nM	8 nM	100 µL	
10X Stop & Detect Buffer B	10X	1X	1,000 µL	
Water	-	-	8,888.9 µL	
Total			**10,000 µL**	

5. Add 10 µL of 1X cGAMP Detection Mixture to 10 µL of the enzyme reaction. Mix using a plate shaker.
   Note: After detection mixture is added to enzyme reaction the final concentration of components in a 20 µL will be 1/2 the Detection Mixture (4 nM tracer, 0.5X Stop & Detect Buffer B, and 1 µg/mL cGAMP Antibody).
6. Incubate at room temperature (20–25°C) for 1 hour and measure FP.

4.3.2 cGAMP Detection Controls

These controls are used to calibrate the FP plate reader and are added to wells that do not contain enzyme.

Component	Notes
Minus Antibody (Free Tracer) Control	This control contains the cGAMP 633 Tracer without the cGAMP Antibody and is set to low mP, typically between 20-50 mP depending on the instrument.
Minus Tracer Control	This control contains the cGAMP Antibody without the cGAMP 633 Tracer and is used as a sample blank for all wells. It contains the same cGAMP Antibody concentration in all wells.

5.0 General Considerations

5.1 Assay Types

5.1.1. Endpoint Assay

The Transcreener® cGAMP cGAS FP Assay is designed for endpoint readout. The Stop & Detect Buffer B contains EDTA to stop Mg^{2+}-dependent enzyme reactions by chelating available Mg^{2+}.

5.1.2 Real-Time Assay

You can perform real-time experiments by substituting the Stop & Detect Buffer B, 10X (provided) with a detection buffer that does not contain EDTA. However, the equilibration time for the tracer and cGAMP Antibody is greater than 5 minutes, making it difficult to quantitate cGAMP produced during short-term enzyme reactions. Note that the optimal cGAMP Antibody concentration may change when EDTA is omitted.

5.2 Reagent and Signal Stability

Transcreener® technology provides a robust and stable assay method to detect cGAMP.

5.2.1 Signal Stability

The stability of the FP assay window at 5 µM cGAMP was determined after the addition of the cGAMP Detection Mixture to the standard samples. The mP value at 5 µM cGAMP remained constant (<10% change) for at least 24 hours at room temperature (20–25°C). If you plan to read FP on the following day, seal the plates to prevent evaporation.

5.2.2 cGAMP Detection Mixture Stability

The cGAMP Detection Mixture is stable for at least 8 hours at room temperature (20–25°C) before addition to the enzyme reaction (i.e., when stored on the liquid handling deck).

6.0 Frequently Asked Questions

Question	Possible Solutions
Other Transcreener Assays require adjustment of antibody concentration. Is that something I need to do for the cGAMP FP Assay?	Unlike other Transcreener assays, the cGAMP FP Assay does not require adjustment to the antibody concentration. The cGAMP antibody demonstrates no cross-reactivity with ATP and GTP (up to 1 mM), therefore one concentration of antibody (1 µg/mL) will cover a substrate range of 50 µM ATP/GTP to 1 mM ATP/GTP.
Will the assay work with any kind of DNA?	The preferred DNA is 45-bp dsDNA. Other dsDNA oligos or salmon sperm DNA will also activate cGAS. If using other DNA, perform a DNA titration determine the optimal concentration. When using longer DNA, interference may occur at higher concentrations. For example we achieve a larger assay window when using 5 ng/mL of salmon sperm DNA as opposed to 20 ng/mL ISD. We have not seen ssDNA or ssRNA activate the cGAS enzyme.
What is the equilibration time for the antibody?	Typically between 5-7 minutes, therefore the assay can be used in real-time, kinetic mode.
Is a standard curve required every time I run the cGAS reaction?	If you choose to convert your raw mP values into cGAMP formed, you will need a cGAMP standard curve. We do not recommend using a standard curve from previous experiments, rather generate a new curve with each experiment to achieve the most accurate result.
Do I need to add ATP, GTP, and DNA to my standard curve?	It is best to run a standard curve that mimics your enzyme reaction, to estimate the cGAMP more accurately. We routinely run the standard curves with ATP, GTP and dsDNA.
Can this assay be used with cell lysates?	In the presence of lysate, the signal diminishes and loses sensitivity, so unfortunately this assay cannot be used with lysates.

7.0 Appendix

7.1 cGAMP Standard Curve

The standard curve mimics an enzyme reaction in which cGAMP is formed. The concentration of ATP and GTP does not change to ensure correct cGAMP quantitation under varying enzymatic conditions . The standard curve allows calculation of the concentration of cGAMP produced in the enzyme reaction. In this example, a 12-point standard curve was prepared using the concentrations of cGAMP, ATP, and GTP shown in **Table 1**. Commonly, 8- to 12-point standard curves are used.

cGAMP (µM)	ATP (µM)	GTP (µM)	DNA (nM)
100	100	100	60
50	100	100	60
25	100	100	60
15	100	100	60
10	100	100	60
7.5	100	100	60
5.0	100	100	60
3.0	100	100	60
2.0	100	100	60
1.0	100	100	60
0.5	100	100	60
0	100	100	60

Use the following equations to calculate the Z' factor:

$$Z' = 1 - \frac{[(3 \times SD_{0\ \mu M\ cGAMP}) + (3 \times SD_{sample})]}{|(mP_{0\ \mu M\ cGAMP}) - (mP_{sample})|}$$

$$\Delta mP = mP_{0\ \mu M\ cGAMP} - mP_{sample}$$

7.2 Using the Assay with Different Volumes and Plate Format

Component	Total Volume	Enzyme Reaction Volume	cGAMP Detection Mix Volume
96 Well Low Volume Plate	50 µL	25 µL	25 µL
384 Well Low Volume Plate	20 µL	10 µL	10 µL
1536 Well Low Volume Plate	8 µL	4 µL	4 µL

Please check the working plate volumes from the manufacturer to ensure they are within the suggest volumes ranges of your plate.

7.3 Summary of Additive Effects on the Transcreener® cGAMP cGAS FP Assay

The assay window was determined to have limited effect with these components when used under the recommended concentrations. The results show tolerance in the presence of the component for 24 hours prior to reading the plate. Contact BellBrook Labs Technical Support for further reagent compatibility information.

Component	24-Hour Tolerance No Enzyme (0 to 5 µM cGAMP)[a]	24-Hour Tolerance with cGAS Enzyme (0 to 50 nM cGAS)[b]
Solvents		
DMSO	2.5%	0.6%
Ethanol	10%	10%
Methanol	10%	10%
Glycerol	5%	5%
Detergents		
Brij-35	1%	0.03%
CHAPS	0.06%	0.03%
NP40	0.25%	0.03%
Triton X-100	1%	0.03%
Metal chelates		
EDTA	20 mM	Not Recommended
EGTA	10 mM	0.3 mM
Reductants		
Dithiothreitol	5 mM	5 mM
Salts		
Magnesium acetate	50 mM	12.5 mM
Magnesium chloride	50 mM	12.5 mM
Magnesium sulfate	100 mM	3 mM
Manganese chloride	100 mM	Not Recommended
Potassium chloride	100 mM	Not Recommended
Sodium azide	100 mM	Not Recommended
Sodium chloride	100 mM	Not Recommended
Zinc chloride	25 mM	Not Recommended
Phosphatase Inhibitors		
Imidazole	6.2 mM	50 mM
Sodium orthovanadate	6.2 mM	Not Recommended
Carrier Proteins/Coactivators		
BSA	1 mg/mL	0.5 mg/mL
BGG	1 mg/mL	Not Recommended

a. mP at 0 or 5 µM increased or decreased <3 standard deviations of the plate controls at the listed concentration and below.

b. <10% drop in ΔmP observed at the listed concentration and below.

Not all combination of these components have been tested together. Results may vary depending on your assay conditions.

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