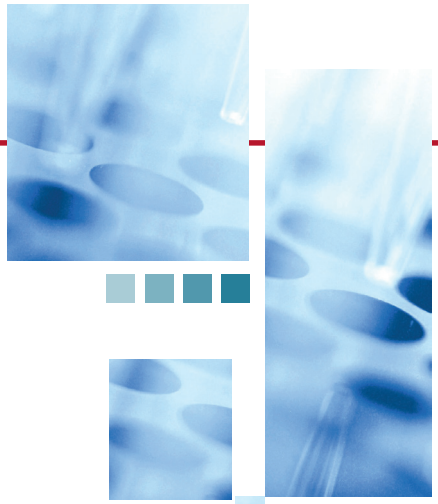
BellBrook Labs

FAR RED TR-FRET



# TRANSCREENER®

## cGAMP cGAS TR-FRET Assay

Technical Manual

Instructions for Part Numbers 3025-1K and 3025-10K

Rev 2023-05

# Transcreener® cGAMP cGAS TR-FRET Assay Technical Manual

## Contents

## 1.0 Introduction

The Transcreener cGAMP cGAS TR-FRET Assay is a competitive immunoassay for cGAMP with a far-red, time resolved Förster-resonance-energy-transfer readout (**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 compatibility with commonly used multimode plate readers.

In this manual, we describe optimal conditions for measuring cGAS initial velocity (1-10% substrate conversion) using sub-saturating ATP and GTP concentrations (100 µM), as is typical for HTS with an enzyme. The dynamic range of the assay can be increased or decreased by adjusting the tracer concentration, however this protocol covers most cGAS enzyme assays.

The Transcreener cGAMP cGAS TR-FRET Assay provides the following benefits:

- A simple single addition cGAS activity assay capable of HTS.
- Excellent data quality (Z′ ≥ 0.7) at cGAMP ranges between 0.5 µM and 100 µM.
- Time-gated method largely eliminates interference that can result from prompt fluorescence of test compounds.
- Far-red tracer further minimizes interference from fluorescent compounds and light scattering.

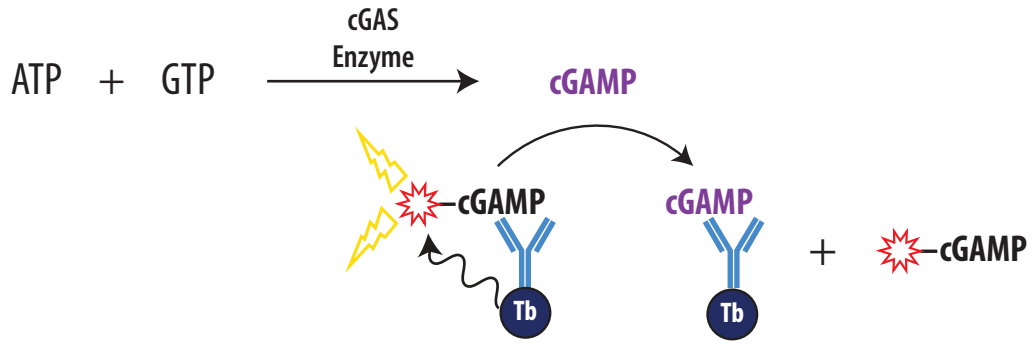



**Figure 1. Schematic overview of the Transcreener cGAMP cGAS TR-FRET Assay.** The Transcreener® cGAMP Detection Mixture contains an cGAMP ATTO 647 tracer bound to an cGAMP antibody conjugated to terbium(Tb).Excitation of the Tb complex in the UV range (~330nm) results in energy transfer to the tracer and emission at a higher wavelength (665 nm) after a time delay. cGAMP produced by the cGAS enzyme displaces the tracer, causing a decrease in TR-FRET

## 2.0 Product Specifications

| Product | Quantity | Part # |
|---|---|---|
| Transcreener cGAMP cGAS TR-FRET Assay | 1,000 assays* | 3025-1K |
| | 10,000 assays* | 3025-10K |
| | 10 X 10,000 assays* | 3025-100K |

*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.

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

## 2.1 Materials Provided

| Component | Composition | Notes |
|---|---|---|
| cGAMP Antibody-Terbium Conjugate | 800 nM solution in 25 mM HEPES buffered saline | Sufficient antibody is included in the kit to complete 1,000 assays (Part # 3025-1K) or 10,000 assays (Part # 3025-10K). The final antibody concentration in the 20 µL reaction is 4 nM. |
| cGAMP ATTO 647 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 8 nM. |
| Stop & Detect Buffer C, 10X | 500 mM HEPES (pH 7.5), 200 mM EDTA, and 0.2% Brij-35 | The Stop & Detect Buffer C 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 C at the time of FRET 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 |

## 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 TR-FRET of the Tb: cGAMP ATTO 647 donor : acceptor pair is required. This assay has been designed to provide high-quality data on any HTS-qualified instrument configured to measure TR-FRET using standard europium or terbium complexes with emission wavelengths at 615 nm and 665 nm. Validation was completed using PHERAstar Plus Ex337/Em620/Em665 (BMG LABTECH) and Envision Ex320/ Em615/ Em665 (Perkin Elmer).
- ***Assay Plates***—It is important to use assay plates that are entirely white with a nonbinding surface. We recommend Corning® 384-well plates (Cat. # 4513). 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 TR-FRET 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 instrument and verify that it is compatible with the assay being performed (see **Section 4.1**).

## 4.0 Protocol

The Transcreener cGAMP cGAS TR-FRET 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.

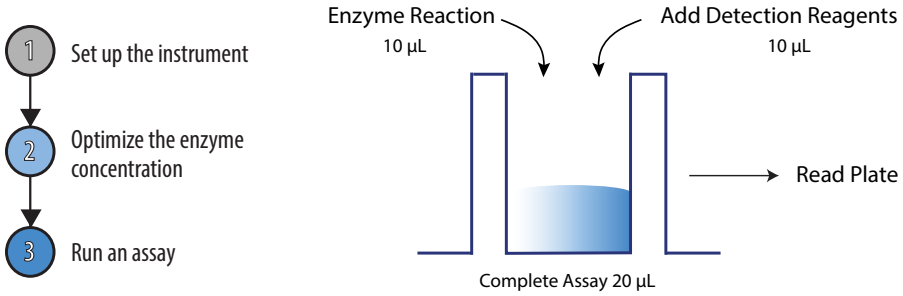



**Figure 2. An outline of the procedure.** The procedure consists of 3 main steps with a mix-and-read assay format.

### 4.1 Set Up the Instrument

Becoming familiar with ideal instrument settings for TR-FRET is essential to the success of the Transcreener cGAMP cGAS TR-FRET Assay.

#### 4.1.1 Verify That the Instrument Measures TR-FRET

Ensure that the instrument is capable of measuring TR-FRET (not simply fluorescence intensity) of the terbium : ATTO 647 TR-FRET pair ($Ex_{320}$ / $Em_{615}$ / $Em_{665}$).

**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 TR-FRET Window for the Instrument

Measuring high (0% conversion) and low (100% conversion) FRET will define the maximum assay window of your specific instrument. Prepare High and Low FRET Mixtures in quantities sufficient to perform at least 6 replicates for each condition.

Use cGAMP ATTO 647 Tracer at 8 nM and Stop & Detect Buffer C 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 16 nM tracer. After adding this to the enzyme reaction, the tracer concentration in the final 20 µL reaction volume would be 8 nM.

***High FRET Mixture***

Prepare the following High FRET Mixture as indicated in the table. Pipette 20 µL of the Total High FRET Mixture to each well (from the example: 20 µL from 240 µL). Do not further dilute.

| Component | Stock Concentration | Final Concentration | Example: 12 Assays | Your Numbers |
|---|---|---|---|---|
| cGAMP Antibody-Tb | 800 nM | 4 nM | 1.2 µL | |
| 10X Stop & Detect Buffer C | 10X | 0.5X | 12.0 µL | |
| cGAMP ATTO 647 Tracer | 800 nM | 8 nM | 2.4 µL | |
| ATP | 5 mM | 50 µM | 2.4 µL | |
| GTP | 5 mM | 50 µM | 2.4 µL | |
| Water | | | 219.6 µL | |
| **Total** | | | **240.0 µL** | |

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

***Low FRET Mixture***

Prepare the following Low FRET Mixture as indicated in the table. Pipette 20 µL of the Total Low FRET Mixture to each well (from the example: 20 µL from 240 µL). Do not further dilute.

| Component | Stock Concentration | Final Concentration | Example: 12 Assays | Your Numbers |
|---|---|---|---|---|
| cGAMP Antibody-Tb | 800 nM | 4 nM | 1.2 µL | |
| 10X Stop & Detect Buffer C | 10X | 0.5X | 12.0 µL | |
| cGAMP ATTO 647 Tracer | 800 nM | 8 nM | 2.4 µL | |
| cGAMP | 500 µM | 50 µM | 24.0 µL | |
| Water | | | 200.4 µL | |
| **Total** | | | **240.0 µL** | |

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

### 4.1.3 Measure TR-FRET

Test the Z′ factor and assay window on your instrument by adding 20 µL of the Low FRET Mixture in 12 wells and 20 µL of High FRET Mixture in 12 wells. Calculate the Z′ factor using the equation below; values greater than 0.7 are acceptable.

**Caution:** Contact BellBrook Labs Technical Service for assistance if the calculated Z′ factor is less than 0.7.

$$Z' = 1 - \frac{[(3 \times SD_{\text{High FRET Mixture}}) + (3 \times SD_{\text{Low FRET Mixture}})]}{|(\text{mean of High FRET Mixture ratio 665:615}) - (\text{mean of Low FRET Mixture ratio 665:615})|}$$

## 4.2 Optimize the Enzyme Concentration

Perform an enzyme titration to identify the optimal enzyme concentration for the Transcreener cGAMP cGAS TR-FRET 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.

### 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 FRET signal is ideal ($EC_{50}$ to $EC_{80}$) for screening of large compound libraries and generating inhibitor dose-response curves (see **Figure 3**). Typically, an $EC_{80}$ has been used with the cGAS enzyme. To determine the $EC_{80}$ enzyme concentration, use the following equation:

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

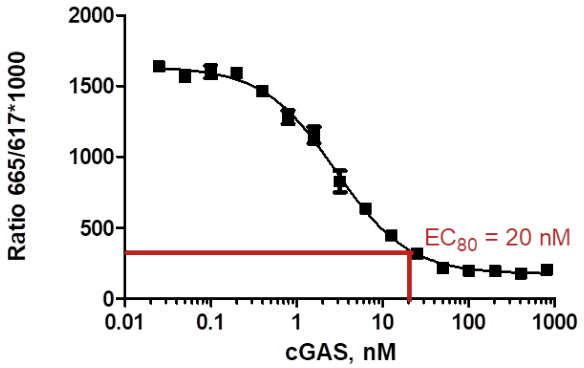



**Figure 3. Enzyme titration curve.** The ideal enzyme concentration is shown in red.

### 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), 0 µM ATP, 0 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-Tb | 800 nM | 8 nM | 100 µL | |
| cGAMP ATTO 647 Tracer | 800 nM | 16 nM | 200 µL | |
| 10X Stop & Detect Buffer C | 10X | 1X | 1,000 µL | |
| Water | - | - | 8,700 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 (8 nM tracer, 0.5X Stop & Detect Buffer C, and 4 nM cGAMP Antibody-Tb).
6. Incubate at room temperature (20–25°C) for 1 hour and measure TR-FRET.

# 5.0 General Considerations

## 5.1 Assay Types

### 5.1.1. Endpoint Assay

The Transcreener® cGAMP cGAS TR-FRET Assay is designed for endpoint readout. The Stop & Detect Buffer C 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 C, 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 ATTO 647 Tracer concentration may change when EDTA is omitted.

## 5.2 Reagent and Signal Stability

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

### 5.2.1 Signal Stability

The stability of the TR-FRET assay window at 5 µM cGAMP was determined after the addition of the cGAMP Detection Mixture to the standard samples. The ratio at 5 µM cGAMP remained constant (<10% change) for at least 2 hours at room temperature (20–25°C). If you do not plan to read TR-FRET on immediately, seal the plates to prevent evaporation.

### 5.2.2 cGAMP Detection Mixture Stability

The cGAMP Detection Mixture is stable for at least 2 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 tracer concentration. Is that something I need to do for the cGAMP TR-FRET Assay? | Unlike other Transcreener assays, the cGAMP TR-FRET Assay does not require adjustment to the tracer concentration. The cGAMP ATTO 647 Tracer 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 ratio 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 |

## 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.

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