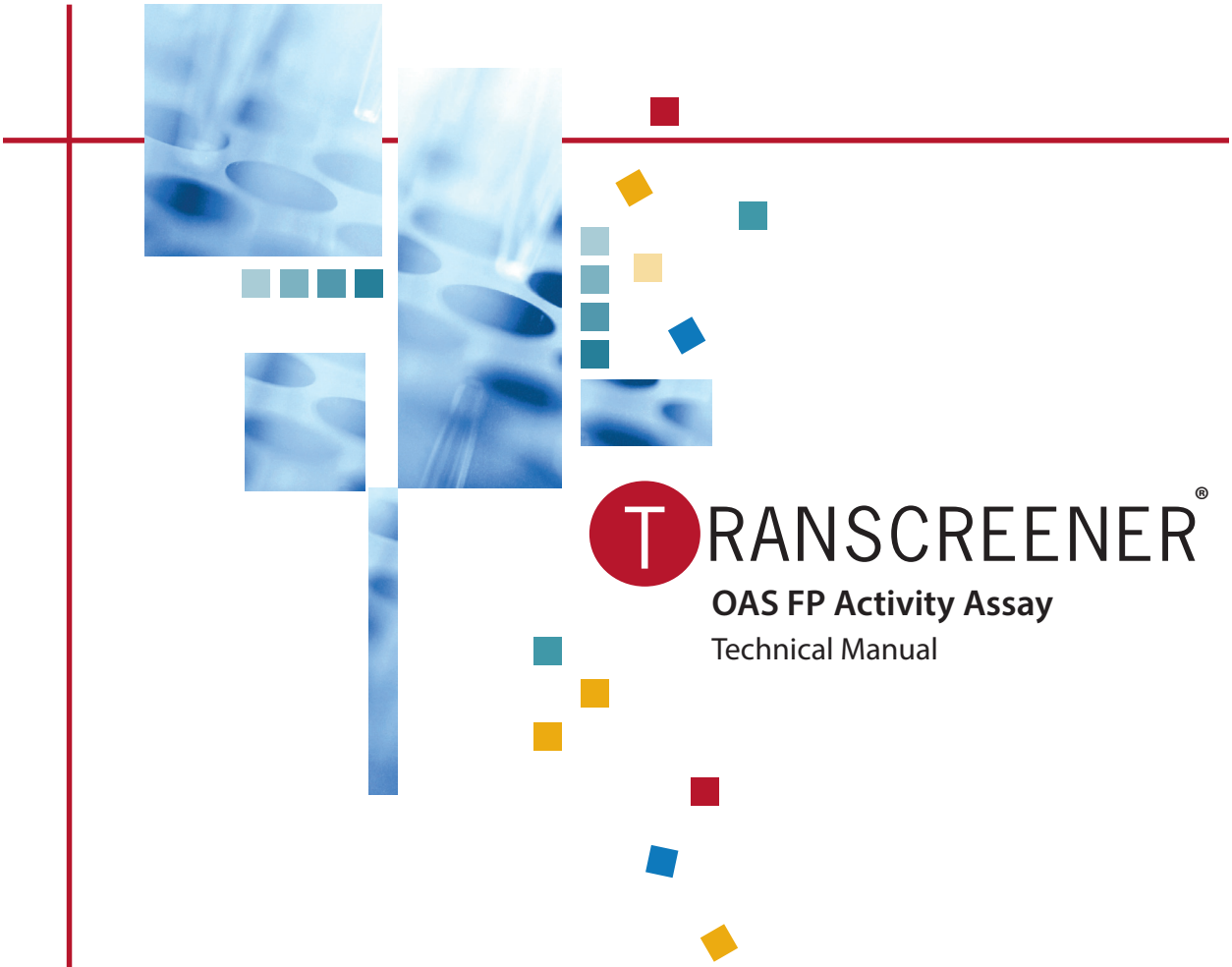
BellBrook Labs

FAR RED FP



TRANSCREENER®

OAS FP Activity Assay

Technical Manual

Instructions for Part Numbers 3027-1K and 3027-10K

Rev 2023-05

Transcreener® OAS FP Assay Technical Manual

Contents

1.0 Introduction

The Transcreener® OAS FP Assay is a far-red, competitive fluorescence polarization (FP) assay (**Figure 1**). The assay is designed to be used with the 2'-5'-oligoadenylate synthase (OAS) family of enzymes such as OAS1, OAS2, and OAS3), that produce the product 2-5A. The Transcreener OAS FP Assay is simple biochemical assay for measuring OAS activity based on the Transcreener AMP^2 Assay. The assay uses a coupling enzyme to convert 2-5A into AMP in the presence of ATP and dsRNA.

Double stranded RNA enters the cell from viral infection. As a first line of defense in the innate immune system, OAS is activated by viral double-stranded RNA. Upon activation, OAS synthesizes 2-5A from ATP which in turn activates RNase L. The result of this signaling event cleaves both viral and host RNA and in turn inhibition of protein synthesis in the cell, thus ending the replication of invading RNA viruses. Mutation or inadvertent stimulation can produce autoimmune conditions by activating the pathway inconsistently even while not in the presence of viral invaders.

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 OAS FP Assay provides the following benefits:

- A simple single addition OAS activity assay capable of HTS.
- Excellent data quality (Z' ≥ 0.7) and signal (≥100 mP polarization shift) for determining OAS activity by measuring 2-5A production.
- Far-red tracer further minimizes interference from fluorescent compounds and light scattering.

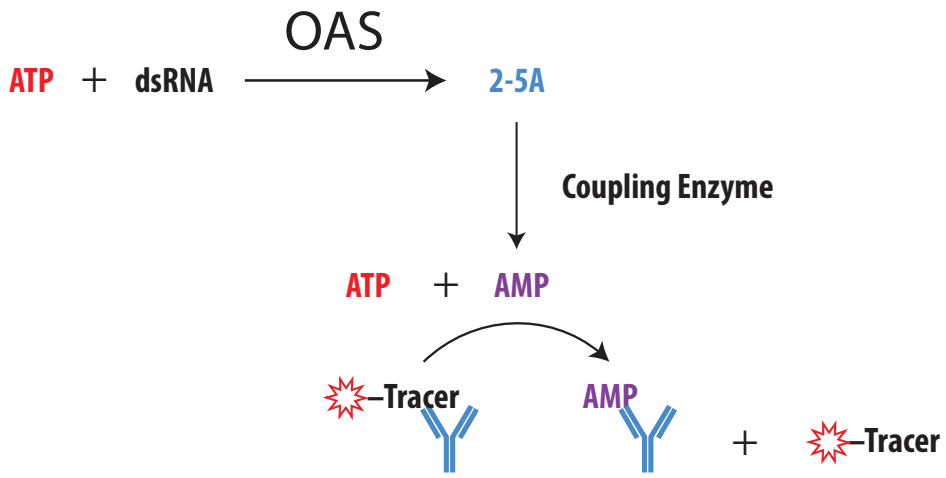


Figure 1. Schematic overview of the Transcreener OAS FP Assay. The Transcreener 2-5A Detection Mixture contains a coupling enzyme that generates AMP and ATP from 2-5A, and an AMP AlexaFluor® 633 tracer bound to an AMP antibody. AMP produced by the coupling enzyme displaces the tracer, which rotates freely, causing a decrease in FP. Note that the coupling enzyme regenerates ATP, preventing OAS substrate depletion.

2.0 Product Specifications

Product	Quantity	Part #
Transcreener® OAS FP Assay	1,000 assays*	3027-1K
	10,000 assays*	3027-10K

*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. Please aliquot and store if not using multiple reagents at one time. A maximum of 3 freeze-thaw cycles have shown no effect on assay results.

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

2.1 Materials Provided

Component	Composition	Notes
AMP²/GMP² Antibody	1.26 mg/mL solution in PBS with 10% glycerol*	Sufficient antibody is included in the kit to complete 1,000 assays (Part # 3027-1K) or 10,000 assays (Part # 3027-10K).
AMP²/GMP² Alexa Fluor® 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. Sufficient tracer is included in the kit to complete 1,000 assays (Part # 3027-1K) or 10,000 assays (Part # 3027-10K).
2-5A–AMP Coupling Enzyme	1000 nM 2-5A-AMP Coupling Enzyme in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM glutathione, 0.1 mM EDTA, 0.25 mM DTT, 0.1 mM PMSF, 25% glycerol	Sufficient coupling enzyme present in excess to ensure 2-5A is converted to AMP for detection by detection reagents.
ATP	5 mM ATP in deionized water, pH 7.0	The ATP is the substrate for OAS and is regenerated by the coupling enzyme in the detection mixture
AMP	5 mM AMP in deionized water, pH 7.0	AMP is used to create a standard curve.
dsRNA	10 mg/mL dsRNA in RNAse free water.	dsRNA is required to activate OAS enzymes.
OAS Buffer, 10X	250 mM Tris (pH7.5), 100 mM $MgCl_2$ and 0.1% Brij-35	Buffer prepared for use with OAS enzymes. Buffer changes may be necessary depending on experimental design.

*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

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

- ***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 OAS assays are designed for use with purified OAS enzyme preparations. Contaminating enzymes, such as phosphatases or nucleotidases, can produce background signal and reduce the assay window.
- ***Plate Reader***—A multidetection microplate reader configured to measure FP of the AMP Alexa Fluor 633 tracer is required. Transcreener FP Assays have been successfully used on the following instruments: BioTek Synergy™2 and Synergy™4; BMG Labtech PHERAstar Plus and CLARIOstar Plus; Molecular Devices SpectraMax™ Paradigm; Perkin Elmer EnVision and ViewLux; and Tecan Infinite F500, Safire 2™, 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.

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 OAS 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.

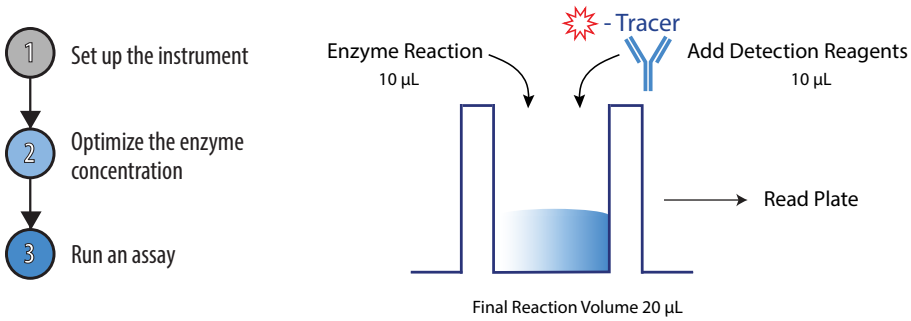


Figure 2. An outline of the procedure. The procedure consists of 3 main steps with a mix-and-read assay format.

4.1 Set Up the Instrument

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

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.

4.1.1 Verify That the Instrument Measures FP

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

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 AMP Alexa Fluor 633 Tracer at 4 nM in your enzyme buffer in a 20 µL complete assay. This mimics the 2-fold dilution when adding an equal volume of detection mixture to an enzyme reaction. As an example, the 1X AMP Detection Mixture may contain 8 nM tracer. After adding this to the enzyme reaction, the concentration in the final 0.5X, 20 µL complete assay would be 4 nM.

High FP Mixture

Prepare the following solution.

Component	Stock Concentration	Complete Assay Concentration	Example: 25 Assays	Your Numbers
AMP² Antibody	1.26 mg/mL*	16.0 µg/mL	6.3 µL**	
AMP Alexa Flour 633 Tracer	800 nM	4 nM	2.5 µL	
OAS Enzyme Buffer, 10X	10 X	1 X	50.0 µL	
Water			441.2 µL	
Total			**500.0 µL**	

*Please note AMP² Antibody concentration varies by lot number. This is an example and should be adjusted based on stock concentration accordingly.

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

Note: The complete assay concentrations with the Stop & Detect Buffers are based on a 20 µL final volume.

Low FP Mixture

Prepare the following solution.

Component	Stock Concentration	Complete Assay Concentration	Example: 25 Assays	Your Numbers
AMP Alexa Fluor 633 Tracer	800 nM	4 nM	2.5 µL	
OAS Enzyme Buffer, 10X		1 X	50.0 µL	
Water			447.5 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 OAS FP Assay. Use enzyme buffer conditions and substrate 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. Run your enzymatic reaction at its requisite temperature and time period.

4.2.1 Enzyme Titration Steps

For a robust assay, it is recommended to use an OAS concentration that produces at least a 100 mP shift (see **Figure 3**). This is usually achieved using an enzyme concentration that results in 50-80% of the maximum polarization shift (EC_{50} to EC_{80}). To determine the EC_{80} enzyme concentration, use the following equation:

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

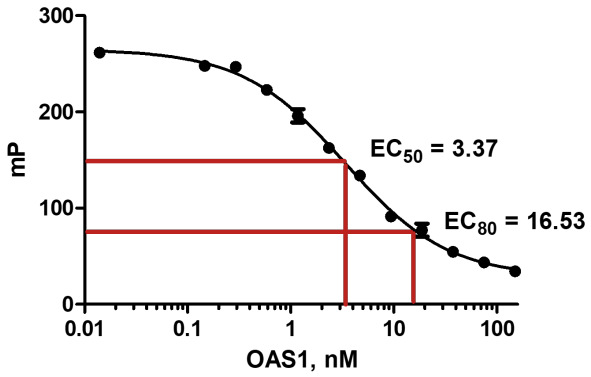


Figure 3. Enzyme titration curve. Titration with the EC_{80} concentration indicated. The EC_{80} may vary based on enzyme lot. Please use C of A for the recommended EC_{80} for your assay.

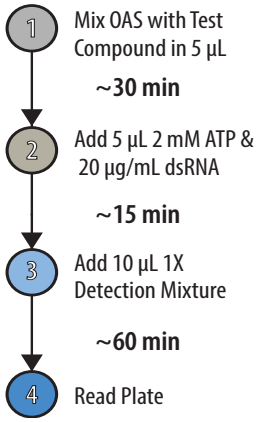
4.2.2 Enzyme Assay Controls

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

Component	Notes
0% 2-5A Conversion Control	This control consists of the 2-5A Detection Mixture, the enzyme reaction components (without OAS enzyme, 0 mM AMP). It defines the upper limit of the assay window.
100% 2-5A Conversion Control	This control consists of the ADP Detection Mixture, the enzyme reaction components (without OAS enzyme, 1 mM AMP). It defines the lower limit of the assay window.
Minus-Nucleotide Control	To verify that the enzyme does not interfere with the detection module, perform an enzyme titration in the absence of ATP.
ATP/AMP Standard Curve	Although optional, a standard curve can be useful to ensure day-to-day reproducibility and that assay conditions were performed using initial rates. It can also be used to calculate product formed. See **Section 7.1** for a description of how to run the standard curve.
Background Control	Use only enzyme reaction conditions including 1X OAS buffer.

4.3 Run an Assay

4.3.1 Experimental Samples



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).
1. Prepare both the substrates and 1X 2-5A Detection Mixture prior to starting the enzymatic assay. Since the assay is to be run in continuous mode, we recommend the 2-5A Detection Mixture will be added directly after the substrates in order to effectively measure enzymatic activity.
2. Start the enzyme reaction by adding 5 µL of ATP and dsRNA, then mix. It is recommended to use concentrations of 1 mM ATP and 10 µg/mL dsRNA, in the 10 µL 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 (2 mM) and 2X dsRNA (20 µg/mL), in 5 µL to achieve the appropriate final concentration. See **Section 7.2** for a list of other plate formats
3. Prepare 1X 2-5A Detection Mixture as follows: **The detection mixture should be made as fresh as possible. It has a deck stability up to 2 hours after being prepared.**

	1X 2-5A Detection Mixture			
Component	**Stock**	**Detection Mix Conc.**	**Example Volume**	**Your Numbers**
AMP² Antibody	1.26 mg/mL	32.0 µg/mL	254 µL	
AMP Alexa Fluor 633 Tracer	800 nM	8 nM	100 µL	
Coupling Enzyme	1,000 nM	62.5 nM	625 µL	
OAS Enzyme Buffer	10X	1X	1,000 µL	
Water			8,021 µL	
Total			**10,000 µL**	

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.

4. Add 10 µL of 1X 2-5A 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 complete assay will be 0.5X the Detection Mixture (4 nM tracer, 16 µg/mL AMP² Antibody 31.25 nM Coupling Enzyme, and 0.5 mM ATP).
5. Mix gently on a plate shaker(~1 minute). Incubate at 30°C for 1 hour and measure FP.

4.3.2 2-5A 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 AMP Tracer without the AMP^2 Antibody and is set to low mP, typically between 20-50 mP depending on the instrument.
Minus Tracer Control	This control contains the AMP^2 Antibody without the AMP Tracer and is used as a sample blank for all wells. It contains the same AMP^2 16 µg/mL Antibody concentration in all wells.

5.0 General Considerations

5.1 Continuous Mode Assay

The Transcreener OAS Assay Kit does not have a stop solution currently available. The assay has to be performed in continuous mode to determine enzymatic activity. The equilibration time for the tracer and AMP^2 Antibody can be greater than 15 minutes, making it difficult to quantitate AMP produced during short-term enzyme reactions. We recommend reading the plate after 60 minutes for the best results.

5.2 Reagent and Signal Stability

5.2.1 Signal Stability

Since the assay is a continuous mode the signal will change as the enzyme produces more product. We recommend reading the assay at 60 minutes after addition of the 2-5A Detection Mixture for the best results. It is important to read plates at the same interval to avoid plate to plate variability when performing high throughput screens.

5.2.2 2-5A Detection Mixture Stability

The 2-5A Detection Mixture is stable for up to 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 antibody concentration. Is that something I need to do for the OAS FP Assay?	Unlike other Transcreener assays, the OAS FP Assay does not require adjustment to the antibody concentration. It is designed to be used within the working range of initial velocity for OAS enzymes.
No change in FP observed	Low antibody/tracer activity or Δ mP signal. • The tracer and antibody are stable for up to 6 freeze-thaw cycles. For frequent use aliquot the antibody and tracer and store the aliquots at -20°C. Use a minimum of 20 µL aliquots. • Other components of the detection mix should not have multiple freeze-thaw cycles. Aliquot reagents and store at -20°C for future use.
Is a standard curve required?	No, it is not required to run a standard curve. We recommend running the ATP/AMP standard curve, if you want to convert raw mP values to product formed. While designing a standard curve, make sure that most of the points are between 0% and 30% conversion (initial velocity). 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.
Can this assay be used with cell lysates?	The assay will only work with purified recombinant OAS protein. The presence of nucleases in the lysates prohibits the use of Transcreener assays with lysates.
High background signal or change in signal after incubation with detection mixture.	• Buffers should be made fresh with nuclease free water to prevent non-specific hydrolysis of nucleotides. • Be sure to not leave the plate in the instrument between the reads as this may cause evaporation leading to high background. • Use non-binding black plates. Medium binding or high binding plates will cause increased assay variability.
Why is my window with OAS very small?	Example reasons for a diminished window • Be sure that OAS enzyme has activity. We recommend using OAS from a commercial source as a positive control as needed. • Handle the coupling enzyme with care. We recommend storing the enzyme at -20°C and aliquoting to prevent loss of activity. • Since the equilibration time of the assay is 10-15 minutes, start reading the plate at 60 min. Earlier time points may lead to diminished signal.

7.0 Appendix

7.1 Standard Curve

The standard curve mimics any enzyme reaction where 2-5A is formed and then converted to AMP. In this example a 12-point standard curve was prepared using the concentration of ATP and AMP show in **Table 1**. Commonly, 8-12 point standard curves are used. In this case you may omit ATP or not run a standard curve depending on your experiment.

Table 1. AMP Standard Curve. Standard curve to help convert raw mP values to product formed.

% Conversion	AMP (µM)	ATP (µM)
100	1000.0	0
5.00	50.0	950.0
2.50	25.0	975.0
1.25	12.5	987.5
1.00	10.0	990.0
0.50	5.0	995.0
0.30	3.0	997.0
0.20	2.0	998.0
0.10	1.0	999.0
0.05	0.5	999.5
0.02	0.2	999.8
0.01	0.1	999.9
0	0	1000.0

Use the following equations to calculate the Z′ factor:

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

$$\Delta mP = mP_{0\ mM\ AMP} - mP_{sample}$$

7.2 Using the Assay with Different Volumes and Plate Formats

Component	Total Volume	Enzyme Reaction Volume	2-5A 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 OAS FP Assay

The assay window was determined to have limited effect with certain components when used under the recommended conditions. To determine the additive affects of a buffer component please test by titrating the component in the known concentration range. You can use only the detection mix and a standard curve to determine the effect on assay performance, however we would suggest use of the OAS enzyme to understand effects on the assay system with OAS enzyme. Below are common components used in enzyme buffers. The maximum tolerance is defined as less than 10% drop in mP observed at the listed concentration below. Tolerance listed includes human OAS1 enzyme.

Component	Maximum Tolerance
DMSO	10%
MeOH	1.25%
Brij-35	0.25%
Triton X-100	0.03%
EDTA	1.25%
1Mg Cl_2	6.25 mM
NaCl	12.5 mM
Imidazole	25 mM
BSA	0.125 mg/mL
BGG	0.125 mg/mL

1232 Fourier Drive, Suite 115
Madison, Wisconsin 53717 USA
Email: TechSupport@bellbrooklabs.com
Phone: 608.443.2400
Toll-Free: 866.313.7881
FAX: 608.441.2967