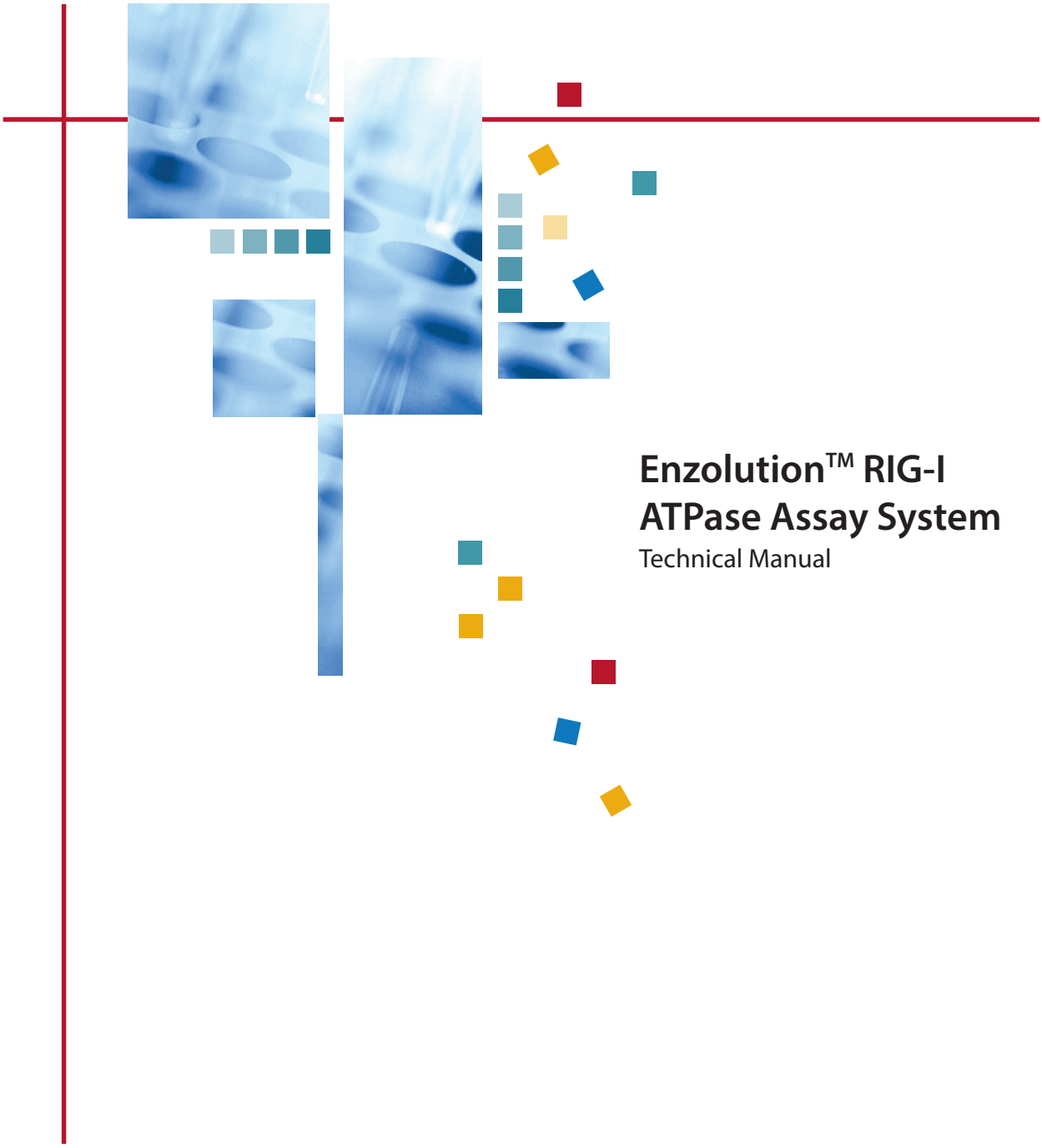
BellBrook Labs

RIG-I ATPase Assay System



Enzolution™ RIG-I ATPase Assay System

Technical Manual

Instructions for Part Numbers 3035-1K and 3035-10K

Rev 2024-04

Enzolution™ RIG-I ATPase Assay System Technical Manual

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U.S. Patent 7,332,278, 7,355,010 and 7,378,505 issued. U.S. Patent Application Nos. 11/353,500, 11/958,515 and 11/958,965, U.S. Divisional Application 12/029,932, and International Patent Application Nos. PCT/US07/088111, European Application Nos. 04706975.2 and 05785285.7, Canadian Application 2,514,877, and Japanese Application 2006-503179 applied. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes other than use of the product or its components to provide a service, information, or data. Commercial Purposes means any activity by a party for consideration other than use of the product or its components to provide a service, information, or data and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (3) resale of the product or its components, whether or not such product or its components are resold for use in research. BellBrook Labs LLC will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use, or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, BellBrook Labs LLC is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, BellBrook Labs LLC, 1232 Fourier Dr. Suite 115, Madison, Wisconsin 53717. Phone (608)443-2400. Fax (608)441-2967.

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1.0 Introduction

The Enzolution™ RIG-I ATPase Assay System is intended for use with the Transcreener® ADP² Assay Kits to measure the RNA-dependent ATPase activity of RIG-I. RIG-I (retinoic acid-inducible gene I), is a cytosolic pattern recognition receptor (PRR) that induces type-I interferon (IFN1) in response to dsRNA. ADP formation by the ATPase activity of RIG-I can be easily measured by the Transcreener ADP² assay, a far-red, competitive fluorescence assay that enables single addition, mix-and-read detection in a continuous or endpoint format. The assay has been optimized and extensively validated for high throughput screening (HTS) and inhibitor dose response measurements using most multimode plate readers.

The Enzolution™ RIG-I ATPase Assay System provides all reagents required to screen and profile RIG-I inhibitors, including the purified human RIG-I (Full-length, C-terminal 6xHis) and 5'ppp dsRNA, when used with the Transcreener ADP² Assay Kits, which are available with FP, FI and TR-FRET readouts. The protocol is configured for 384-well plates; use of different multi-well plate formats will require adjustment of reagent concentrations utilized in the assay.

Key Applications:

- Screening for RIG-I inhibitors (or activators)
- Generating dose response curves and IC_{50} values for RIG-I inhibitors
- Kinetic and mechanistic analyses

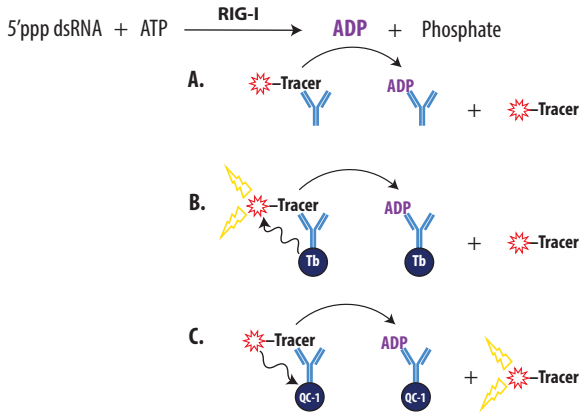


Figure 1. Schematic Overview of the Enzolution™ RIG-I ATPase Assay System with the Transcreener® ADP² Assays. For FP readout (A): ADP produced by the ATPase activity of RIG-I displaces an Alexa Fluor® 633 Tracer from the ADP² antibody, resulting in decreased fluorescence polarization. **For TR-FRET readout (B):** ADP produced by the ATPase activity of RIG-I displaces a HiLyte647 Tracer from the ADP² Antibody conjugated to terbium (Tb), resulting in a decrease in TR-FRET. **For FI readout (C):** ADP produced by the ATPase activity of RIG-I displaces an Alexa Fluor® 594 Tracer from the ADP² Antibody conjugated to an IRDye® QC-1 quencher, resulting in an increase in fluorescence intensity.

2.0 Product Specifications

Product	Quantity	Part #	
Enzolution™ RIG-I ATPase Assay System	1,000 assays*	FP	3035-1K-FP
		FI	3035-1K-FI
		TR-FRET	3035-1K-TR
	10,000 assays*	FP	3035-10K-FP
		FI	3035-10K-FI
		TR-FRET	3035-10K-TR

*The exact number of assays depends on the enzyme reaction conditions. The kits are designed for use with 384-well plates, using a 10 µL Enzyme Reaction and a 20 µL Complete Assay volume.

Storage

RIG-I Enzyme and RIG-I dsRNA should be stored at -80°C; other reagents can be stored at –20°C. Though we have confirmed that the RIG-I Enzyme and the RIG-I dsRNA are stable and maintain greater than 80% activity up to 5 freeze-thaw cycles, we recommend aliquoting the reagents and snap-freezing for multiple uses to minimize loss of activity.

Use the reagents provided in this kit within 6 months from date of receipt.

2.1 Materials Provided

Component	Composition	Notes
RIG-I Enzyme	0.1 mg/mL (0.93 µM)* in 50 mM Tris (pH 8), 500 mM NaCl, 10% glycerol	Full-length (Amino acids 1-925), C-terminal 6xHis, 107 kDa. Sufficient enzyme is included in the kit to complete at least 1,000 assays (Part # 3035-1K) or 10,000 assays (Part #3035-10K).
RIG-I dsDNA	8.1 µM in H_2O	Supplied as a 19-bp 5'ppp dsRNA oligomer.
Enzyme Assay Buffer C, 10X	500 mM TRIS (pH 7.5), 500 mM NaCl, 25 mM $MgCl_2$, 0.1% Brij-35	Use Enzyme Assay Buffer C in the Enzyme Reaction and for preincubation with inhibitors. Changes to the assay buffer could affect enzyme activity and/or detection of ADP.
1 M DTT	1 M DTT	DTT should be added to the Enzyme Assay Buffer C before use. Prepare only enough DTT-containing buffer as required for the assay.
384-Well Low Volume Assay Plates	Corning #4514 - FP and FI Only Corning #4513 - TR-FRET Only	Polystyrene non-binding surface assay plates in either a 3-pack (1,000+ Assays) or a 30-pack (10,000+ Assays). We strongly recommend the use of these plates as inconsistent results have been observed with other plates.

*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

Component	Notes
Ultrapure Nuclease Free Water	Some deionized water systems are contaminated with nucleases that can degrade both nucleotide substrates and products, reducing assay performance. Use nuclease free water such as: Invitrogen Part # AM9930
Plate Reader	A multimode microplate reader configured to measure FP, FI, or TR-FRET is required. Transcreener® Assays have been validated 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, Safire2™, and M1000. Full list of compatible plate readers and settings.
Liquid Handling Devices	Use liquid handling devices that can accurately dispense submicroliter volumes into 384-well plates.
Laboratory Incubator	An incubator model that is capable of maintaining temperature stability at 30°C is required.

Transcreener® ADP² FP Assay - SOLD SEPARATELY		
Component	**Composition**	**Notes**
ADP² Antibody	3.4 mg/mL solution in PBS with 10% glycerol*	1,000 assays (Part #3010-1K) or 10,000 assays (Part #3010-10K).
ADP² Alexa Fluor® 633 Tracer	400 nM solution in 2 mM HEPES (pH 7.5) containing 0.01% Brij-35	1,000 assays (Part #3010-1K) or 10,000 assays (Part #3010-10K).
Stop & Detect Buffer B, 10X	200 mM HEPES (pH 7.5), 400 mM EDTA, and 0.2% Brij-35	The EDTA in the Stop & Detect Buffer B quenches the RIG-I Enzyme Reaction by chelating Mg^{2+}. The final concentrations of Mg^{2+} and EDTA in the Complete Assay are 2.5 mM and 20 mM, respectively.
ATP	5 mM	The ATP supplied in this kit can be used for the RIG-I ATPase Enzyme Reaction and the ATP/ADP standard curve.
ADP	5 mM	The ADP is used for the ATP/ADP standard curve.

Transcreener® ADP² FI Assay - SOLD SEPARATELY		
Component	**Composition**	**Notes**
ADP² Antibody-IRDye® QC-1	1.4 mg/mL solution in 100 mM KH_2PO_4 (pH 8.5)*	1,000 assays (Part #3013-1K) or 10,000 assays (Part #3013-10K).
ADP² Alexa Fluor® 594 Tracer	800 nM solution in 2 mM HEPES (pH 7.5) containing 0.01% Brij-35	1,000 assays (Part #3013-1K) or 10,000 assays (Part #3013-10K).
Stop & Detect Buffer B, 10X	200 mM HEPES (pH 7.5), 400 mM EDTA, and 0.2% Brij-35	The EDTA in the Stop & Detect Buffer B quenches the RIG-I Enzyme Reaction by chelating Mg^{2+}. The final concentrations of Mg^{2+} and EDTA in the Complete Assay are 2.5 mM and 20 mM, respectively.
ATP	5 mM	The ATP supplied in this kit can be used for the RIG-I ATPase Enzyme Reaction and the ATP/ADP standard curve.
ADP	5 mM	The ADP is used for the ATP/ADP standard curve.

Transcreener® ADP² TR-FRET Assay - SOLD SEPARATELY		
Component	**Composition**	**Notes**
ADP² Antibody - Terbium Conjugate	800 nM solution in HEPES-buffered saline	1,000 assays (Part #3011-1K) or 10,000 assays (Part #3011-10K).
ADP HiLyte647 Tracer	10 µM solution in 2 mM HEPES (pH 7.5) containing 0.01% Brij-35	1,000 assays (Part #3011-1K) or 10,000 assays (Part #3011-10K).
Stop & Detect Buffer C, 10X	500 mM HEPES (pH 7.5), 200 mM EDTA, and 0.2% Brij-35	The EDTA in the Stop & Detect Buffer B quenches the RIG-I Enzyme Reaction by chelating Mg^{2+}. The final concentrations of Mg^{2+} and EDTA in the Complete Assay are 2.5 mM and 10 mM, respectively.
ATP	5 mM	The ATP supplied in this kit can be used for the RIG-I ATPase Enzyme Reaction and the ATP/ADP standard curve.
ADP	5 mM	The ADP is used for the ATP/ADP standard curve.

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

3.0 Before You Begin

1. Read the entire protocol and note any reagents or equipment needed (see **Section 2.2**).
2. Check the plate reader and verify that it is compatible with the Transcreener® ADP² Assay for the respective readout: Full list of compatible plate readers and settings.
3. Please read and understand Transcreener® ADP² Assay Technical Manuals prior to use with this kit.

4.0 Protocol

The methods described below are for single-addition, endpoint detection: the RIG-I ATPase Enzyme Reaction is quenched by the addition of EDTA along with the detection reagents (see **Figure 2**). The methods were designed for 384-well plates using a 10 µL RIG-I ATPase Enzyme Reaction and 10 µL of detection/quench reagents (final volume 20 µL when the plates are read). The use of different plate densities or reaction volumes will require changes in reagent quantities (see **Section 5.1** for example reaction volumes).

The methods were optimized for initial velocity detection of ADP formation (≤ 20% conversion of ATP to ADP) by RIG-I with a sub-K_m concentration of ATP (100 µM) and a saturating concentration of RIG-I dsRNA (16.2 nM). These conditions will ensure sensitive detection of inhibitors that compete with ATP while minimizing the effects of compounds that inhibit RIG-I via non-specific interactions with RNA. Significant changes in the ATP concentration may require optimization of the ADP² Antibody concentration (in the case of the FP and FI readout modes) or the ADP Tracer (in the case of the TR-FRET readout mode) to adjust the dynamic range as described in the Transcreener ADP² Assay Manual.

Note: Antibody (TR-FRET) and Tracer (FP and FI) concentrations remain constant in the 20 µL Complete Assay regardless of changes to other reaction conditions.

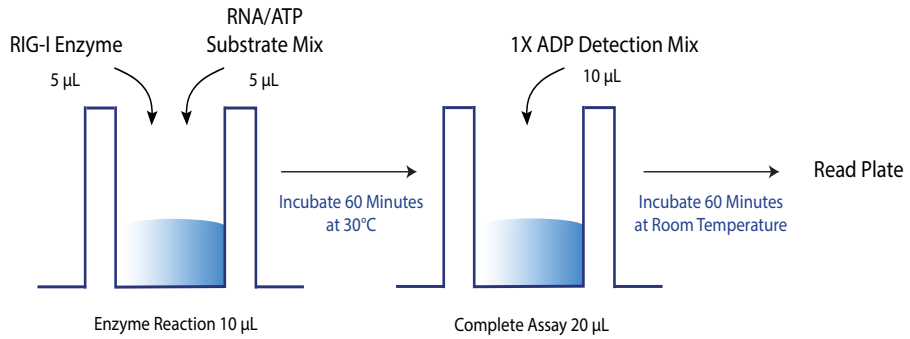


Figure 2. An Outline of the Procedure. The RIG-I ATPase Enzyme Reaction is initiated by the addition of RIG-I dsRNA and ATP. After the Enzyme Reaction incubation is completed, ADP detection reagents are added (Transcreener ADP^2 Antibody and Tracer) along with EDTA to quench the RIG-I ATPase reaction.

Table 1. RIG-I ATPase Enzyme Reaction Components. Concentrations are provided for the standard protocol using 5 µL of RIG-I Enzyme Mix and 5 µL of RNA/ATP Substrate Mix for the Enzyme Reaction.

	10 µL Enzyme Reaction Components	
Component	**Working Stock**	**Final Concentration in 10 µL**
Enzyme Assay Buffer C, 10X 1 M DTT	1X in Nuclease Free Water with 5 mM DTT	1X (50 mM TRIS pH 7.5, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.01% Brij-35) with 5 mM DTT
RIG-I Enzyme, 0.1 mg/mL (0.93 µM)	2X in 1X Enzyme Assay Buffer C with 5 mM DTT	0.5 nM – 2 nM*
ATP, 5 mM	200 µM in 1X Enzyme Assay Buffer C with 5 mM DTT (with 32.4 nM RIG-I dsRNA)	100 µM
RIG-I dsRNA, 8.1 µM	32.4 nM in 1X Enzyme Assay Buffer C with 5 mM DTT (with 200 µM ATP)	16.2 nM

*See Section 4.1 for Determining the Optimal Enzyme Concentration.

Table 2. 1X ADP Detection Mix Components. The optimal concentrations for each of the detection reagents based on the preferred readout mode are shown. Changes to the concentrations may require re-optimization of the assay.

	1X ADP Detection Mix - Add 10 µL Per Well					
	FP		FI		TR-FRET	
Component	**Detection Mix Concentration**	**Complete Assay**	**Detection Mix Concentration**	**Complete Assay**	**Detection Mix Concentration**	**Complete Assay**
Stop & Detect Buffer	1X	0.5X	1X	0.5X	1X	0.5X
ADP Tracer	4 nM	2 nM	8 nM	4 nM	197.8 nM	98.9 nM
ADP Antibody	82 µg/mL	41 µg/mL	93.7 µg/mL	46.85 µg/mL	8 nM	4 nM

4.1 Determining the Optimal Enzyme Concentration

Using the enzyme concentration suggested in the RIG-I Enzyme Certificate of Analysis should provide a robust signal that is within the linear range for ADP formation. However, for best results, we suggest performing an enzyme titration to identify the optimal enzyme concentration (EC_{50} to EC_{80}), especially when running the assay in a different buffer system or with a different ATP or RIG-I dsRNA concentration. This example uses a 2X serial dilution; it should be performed at least in duplicate. If a compound screen is planned, you should include the solvent (e.g., DMSO) at its final assay concentration.

4.1.1 Enzyme Titration Steps

1. Prepare 1000 µL 1X Enzyme Assay Buffer C with 5 mM DTT: dilute 100 µL of 10X Enzyme Assay Buffer C and 5 µL of 1M DTT in 895 µL Ultrapure Nuclease Free Water.
2. Prepare 40 µL of 100 nM RIG-I Enzyme: dilute 4.3 µL of 0.93 µM RIG-I Enzyme in 35.7 µL 1X Enzyme Assay Buffer C with 5 mM DTT.
3. Add 10 µL of the RIG-I Enzyme to well 1 (including replicates).
4. Add 5 µL of 1X Enzyme Assay Buffer C with 5 mM DTT to wells 2-16, DO NOT add the Assay Buffer to well 1.
5. Transfer 5 µL from well 1 to well 2 and mix by pipetting, then transfer 5 µL from well 2 to well 3 and mix by pipetting; repeat this serial dilution process until well 15 has received RIG-I Enzyme. Well 16 is to be used as a blank and should not include enzyme.
   IMPORTANT: After mixing the last well (15) in the dilution series, remove 5 µL from that well only and discard, so that all the wells contain 5 µL final volume.

6. Prepare 300 µL of RNA/ATP Substrate Mix: dilute 1.2 µL 8.1 µM RIG-I dsRNA and 12 µL 5 mM ATP in 286.8 µL 1X Enzyme Assay Buffer C with 5 mM DTT.
7. Start the Enzyme Reaction by adding 5 µL of the RNA/ATP Substrate Mix to every well (1-16). Gently mix for 40 to 60 seconds on a plate shaker. Incubate at 30°C for 60 minutes.
8. Prepare 600 µL 1X ADP Detection Mix based on the concentrations provided in **Table 2**: 60 µL 10X Stop & Detect Buffer B, 6 µL ADP[2] Alexa Fluor 633 Tracer and 82 µg/mL ADP[2] Antibody in Ultrapure Nuclease Free Water.
   Note: The 1X ADP Detection Mix varies for the readout mode used. Table 2 lists the 1X ADP Detection Mix for each readout.
9. Add 10 µL of 1X ADP Detection mix to every well (1-16), in replicate.
10. Gently mix on a plate shaker for 40 to 60 seconds and then allow it to incubate at room temperature for 60 minutes before reading.
    Note: The reagent volumes indicated above are sufficient for running the enzyme titration in duplicate plus excess for pipetting dead volume. Scaling of volumes can be performed if necessary.

For detection of inhibitors at single concentration or in dose response mode, we recommend selecting an enzyme concentration that produces a 50–80% change in signal (EC_{50} to EC_{80}) (see **Figure 3**). This will result in initial velocity conditions, which correspond to the linear phase of the reaction after conversion of values to ADP formation (see **Figure 6**). The EC_{50} is provided by common graphing programs; the EC_{80} enzyme concentration can be calculated from the EC_{50}, as follows:

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

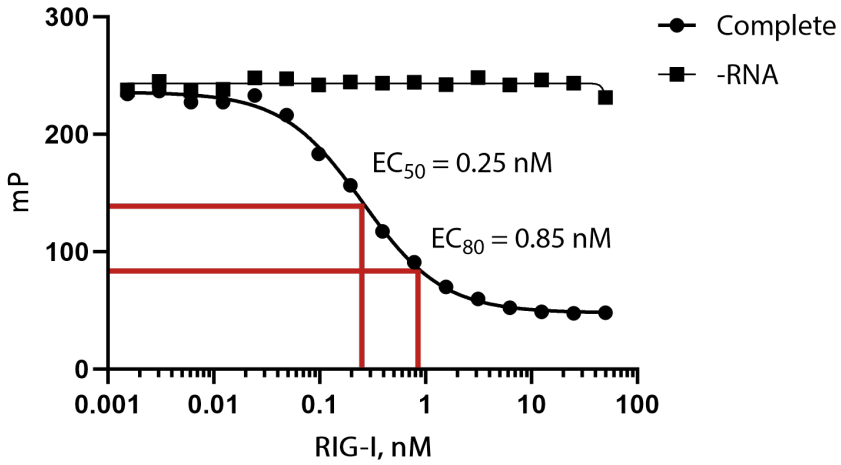
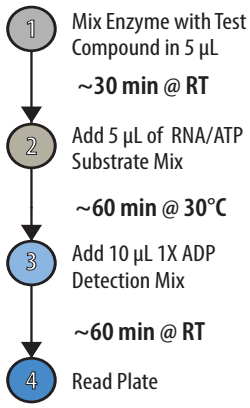


Figure 3. Enzyme Titration Curve. Example RIG-I Enzyme titration. The ideal range of enzyme concentrations is between EC_{50} and EC_{80}; the specific concentration may vary depending on the enzyme lot.

4.2 Performing Single Compound Screening and Dose-Response Assays

4.2.1 Experimental Samples

1. Perform a serial dilution of test compounds with your method of choice. Add the enzyme to the test compounds at the desired concentration so that the total volume of this mixture is 5 µL. Mix gently on a plate shaker for 40 to 60 seconds. Preincubate the Enzyme Inhibitor Mix for the desired time (typically at least 15 minutes) at room temperature to allow equilibration of the E-I complex.
   Note: Final concentration of test compounds should be based on the volume of the Enzyme Reaction.
2. Start the Enzyme Reaction by adding 5 µL of the RNA/ATP Substrate Mix. It is recommended to incubate the Enzyme Reaction at 30°C for 60 minutes.
   Note: The final volume of the Enzyme Reaction mixture should be 10 µL for 384 well plates. See Section 5.1 for a list of other plate formats.
3. After the incubation, add 10 µL of 1X ADP Detection Mix to the 10 µL Enzyme Reaction and mix the 20 µL Complete Assays using a plate shaker.
   Note: The 1X ADP Detection Mix varies for the readout mode used. Table 2 lists the 1X ADP Detection Mix for each readout.
4. Incubate at room temperature (20–25°C) for 60 minutes and measure FP.



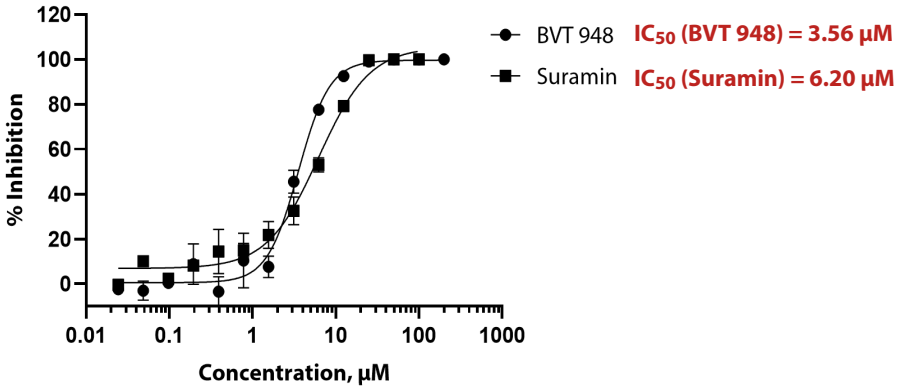


Figure 4. Dose-Response Curve. Example titration of probe inhibitors Suramin and BVT 948 in the presence of RIG-I Enzyme.

4.3 Setting Up a Standard Curve

Use of a standard curve for conversion of values to amount of ADP formed allows quantitative measurement of the enzyme activity and accurate IC_{50} determinations; it is not typically done for screening at single concentrations. The standard curve mimics the Enzyme Reaction (as ATP concentration decreases, ADP concentration increases); the adenine nucleotide concentration remains constant. The ADP/ATP standard curve allows calculation of the concentration of ADP produced in the Enzyme Reaction and, therefore, the percent ADP conversion.

In this example, a 12-point standard curve was prepared using the concentrations of ADP and ATP shown in the following Table. Commonly, 8- to 12-point standard curves are used. Here we describe preparation of a standard curve from 100 µM to 0.5 µM ADP, which encompasses the appropriate range for this assay.

Table 3. Concentration of ATP and ADP to prepare a 12-point standard curve

% Conversion	ATP (µM)	ADP (µM)
100	0	100
60	40	60
30	70	30
15	85	15
12	88	12
10	90	10
6	94	6
4	96	4
2	98	2
1	99	1
0.5	99.5	0.5
0	100	0

4.3.1 Preparing the Standard Curve

1. Prepare 1X Enzyme Assay Buffer C with 5 mM DTT.
2. Dilute 5 mM ATP and ADP to 100 µM in 1X Enzyme Assay Buffer C with 5 mM DTT. The volume for both dilutions should be based on the total amount of volume to be utilized during preparation of the ATP/ADP percent conversion solutions.
3. Starting from 100 µM of ADP, add proportional values of ATP and ADP with the method of choice (i.e., Automatic Dispenser). For manual preparations, add reagents in separate microcentrifuge tubes to generate the desired percent conversions utilized in the standard curve. An example of the dilution scheme can be found in **Table 3**. Once all dilutions are completed, add 10 µL of each solution to the respective wells.
4. Afterwards, add 10 µL of 1X ADP Detection Mix. The 1X ADP Detection Mix varies for the readout mode used. **Table 2** lists the 1X ADP Detection Mix for each readout.
5. Finally, mix for 40 to 60 seconds, before measuring in the instrument of choice.

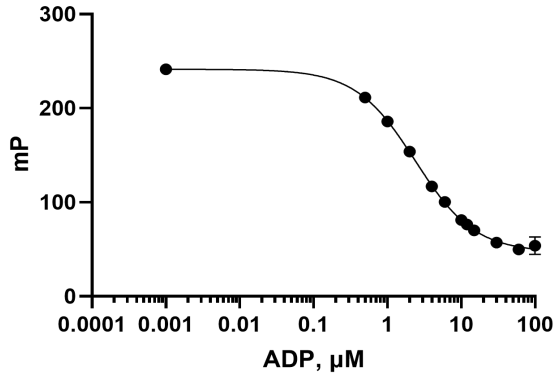


Figure 5. ADP Standard Curve. Standard curve using 1X ADP Detection Mix as shown in **Table 2**. (82 µg/mL ADP[2] antibody, 4 nM ADP[2] Tracer).

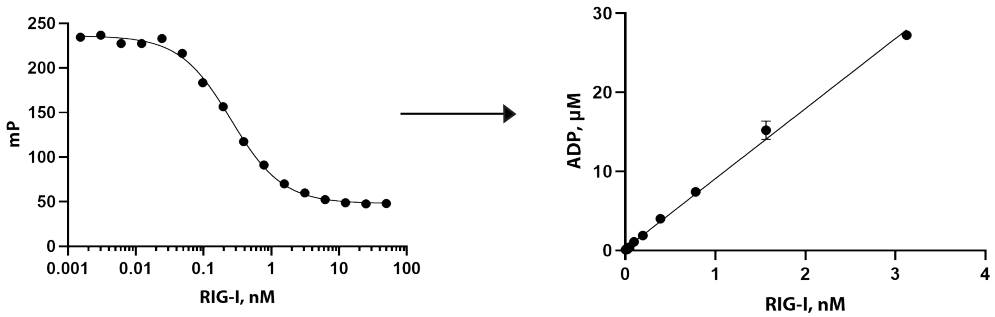


Figure 6. Enzyme Titration Curve Converted to ADP Formed. Raw polarization signal (mP) is converted to ADP formed using a standard curve as described in **Section 4.3**. Only the linear portion of the graph is shown; interpolation was performed using GraphPad Prism.

4.4 Measuring Assay Robustness with Z'

By taking into account both dynamic range and data variability at the high and low ranges of the assay, the Z' statistic provides a measure of what is of most interest when considering the suitability of an assay for HTS: the usable screening or "assay window." It is a dimensionless coefficient for the quality of the screening window that is relevant for any assay, regardless of detection method or readout, without the intervention of test compounds. As a guideline, a Z' value of 0.5 or greater is generally considered to be indicative of a very good screening window for a biochemical assay, thus the assay is an excellent assay. When running the RIG-I ATPase Assay, run the controls with and without enzyme (no test compound) to achieve final results. Use the following formula to determine Z':

$$Z' = 1 - \frac{[(3 \times SD_{\text{No Enzyme}}) + (3 \times SD_{\text{Complete Reaction}})]}{|(\text{Mean}_{\text{No Enzyme}}) - (\text{Mean}_{\text{Complete Reaction}})|}$$

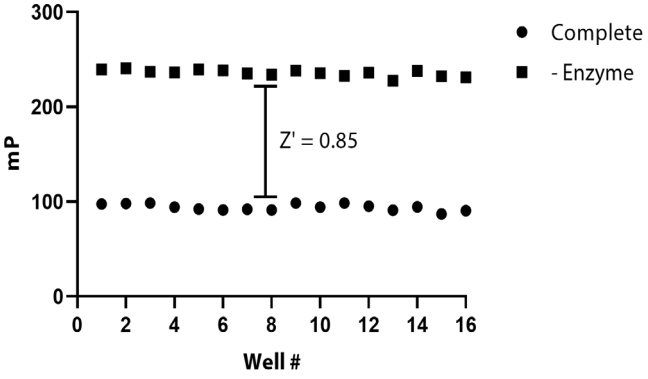


Figure 7. Z' Measurement. Complete Assay is performed with and without RIG-I Enzyme (n=16). Z' is then calculated based on the formula shown in **Section 4.4**.

5.0 Appendix

5.1 Using the Assay with Different Volumes and Plate Formats

Component	Total Volume	Enzyme Reaction Volume	1X ADP Detection Mixture 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.

5.2 Links to Applicable Application Notes

- A Guide to Navigating Hit Prioritization After Screening Using Biochemical Assays
- A Guide to Measuring Drug-Target Residence Times with Biochemical Assays
- Using GraphPad Prism to Interpolate Data from a Standard Curve to Generate a Dose-Response
- List of Commonly Used Plate Readers and Settings

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