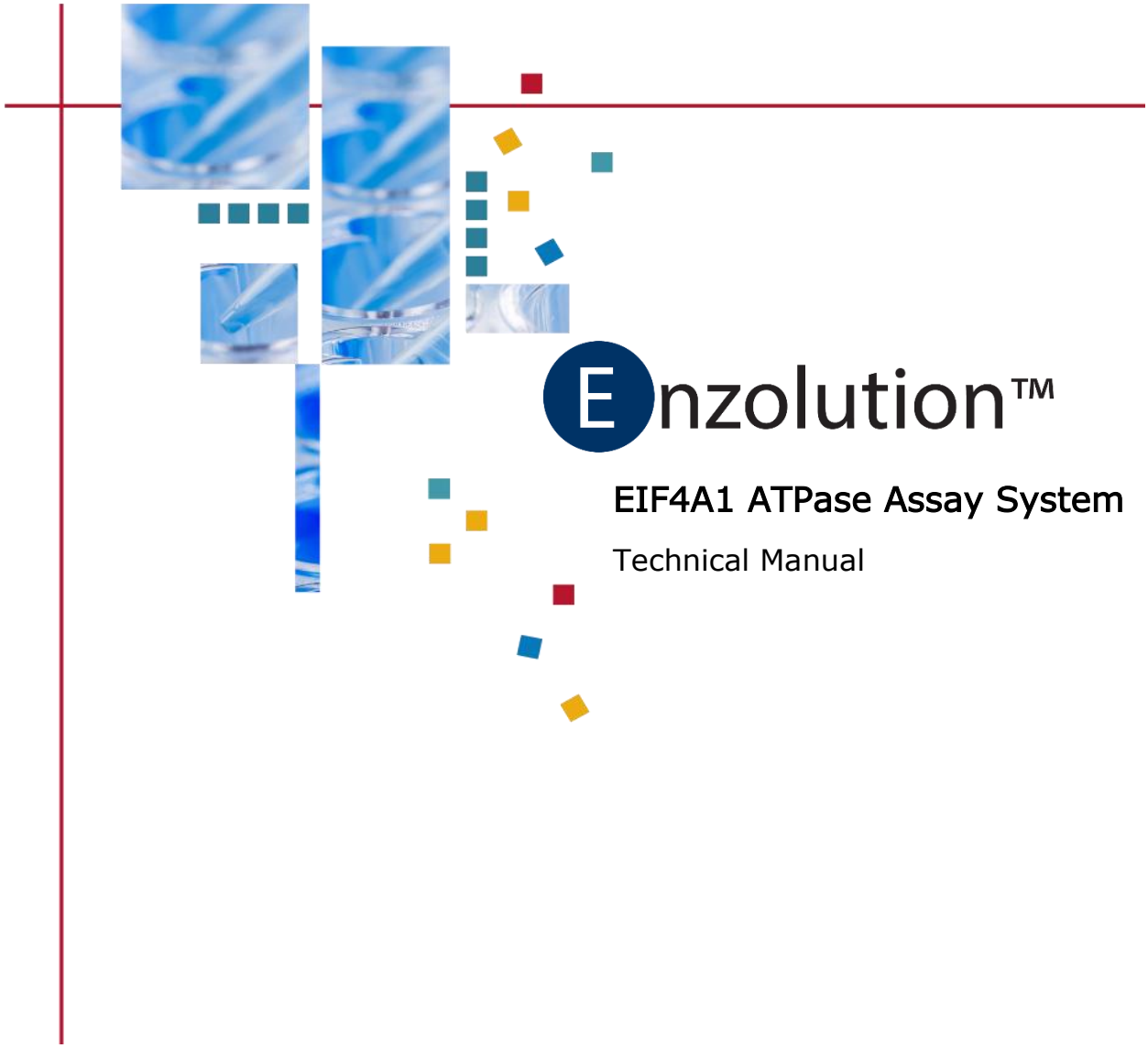
Advion Interchim scientific

BellBrook Labs



Enzolution™

EIF4A1 ATPase Assay System

Technical Manual

Instructions for Part Numbers 3055-1K and 3055-10K

Rev 2026-04

Enzolution™ EIF4A1 Assay System Technical Manual

Contents

1.0 Introduction

The Enzolution EIF4A1 ATPase Assay System is intended for use with the Transcreener® ADP² Assay Kits to measure the RNA-dependent ATPase activity of EIF4A1 (eukaryotic initiation factor 4A-I), a member of the DEAD-box family of RNA helicase family (DDX2A). EIF4A1 functions as a core component of the EIF4F translation initiation complex in eukaryotic cells and is upregulated in diverse cancers. ADP formation by EIF4A1 can be easily measured with 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 EIF4A1 ATPase Assay System provides all reagents required to screen and profile EIF4A1 inhibitors, including purified, full-length human EIF4A1 and yeast RNA, 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 reagents concentrations utilized in the assay.

Key Applications:

- Screening for EIF4A1 inhibitors (or activators)
- Generating dose response curves and IC_{50} values for EIF4A1 inhibitors
- Kinetic and mechanistic analyses

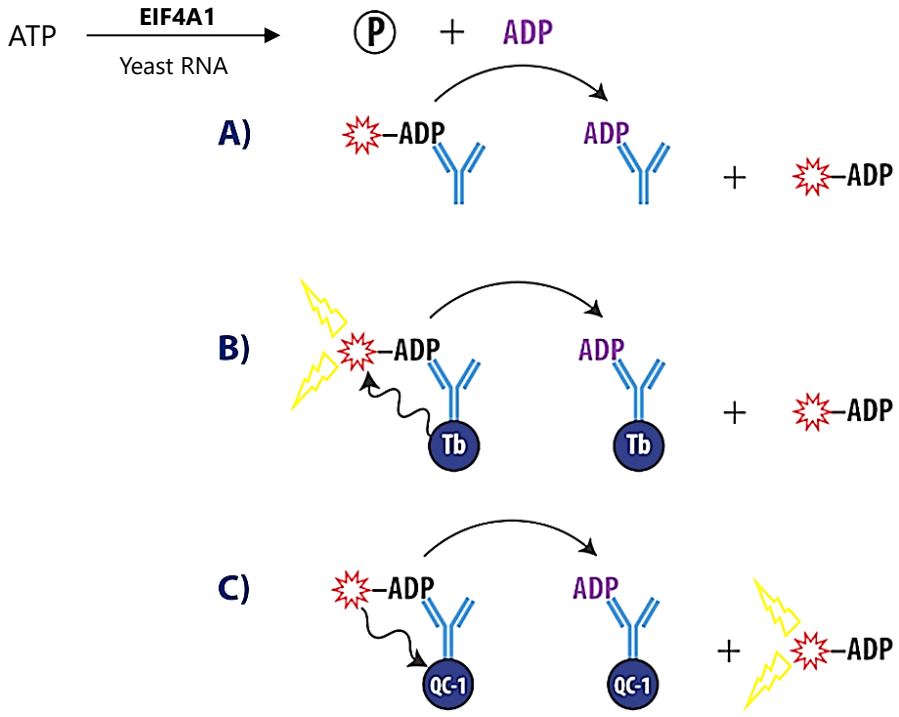


Figure 1. Schematic Overview of the Enzolution EIF4A1 ATPase Assay System with the Transcreener ADP² Assays. For FP readout (A): ADP produced by the RNA helicase activity of EIF4A1 displaces an Alexa Fluor® 633 Tracer from the ADP² antibody, resulting in decreased fluorescence polarization. **For TR-FRET readout (B):** ADP produced by the RNA helicase activity of EIF4A1 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 RNA helicase activity of EIF4A1 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 EIF4A1 ATPase Assay System	1,000 assays*	3055-1K-FP
		3055-1K-FI
		3055-1K-TR
	10,000 assays*	3055-10K-FP
		3055-10K-FI
		3055-10K-TR

*The exact number of assays depends on 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

EIF4A1 Enzyme and yeast RNA should be stored at -80°C; other reagents can be stored at –20°C. Though we have confirmed that the EIF4A1 Enzyme and the yeast RNA 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
EIF4A1 Enzyme	0.54 mg/mL (11.49 µM)* in 50 mM Tris-HCl, 500 mM NaCl, 10% Glycerol, pH 8.0	Full-length (amino acids 1- 406), 46.99 kDa. Sufficient enzyme is included in the kit to complete at least 1,000 assays (Part # 3055-1K) or 10,000 assays (Part # 3055-10K).
Yeast RNA	10 mg/µL in H_2O	Yeast RNA purified from *Torulla*. Storage solution in Ultrapure Nuclease Free Water.
Enzyme Assay Buffer E, 10X	500 mM TRIS (pH 7.5), 2.5 mM $MgCl_2$, 0.1% Triton x-100	Use Enzyme Assay Buffer E in the Enzyme Reaction and for preincubation with inhibitors. Changes to the assay buffer could affect enzyme activity and/or detection of ADP.
TCEP, 0.5 M	Aqueous TCEP solution, Neutral pH	TCEP (Tris (2-carboxyethyl) phosphine) solution, neutral pH
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 enzymes 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. Full list of compatible plate readers and settings.
Liquid Handling Devices	Use liquid handling devices that can accurately dispense sub-microliter 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 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 EIF4A1 Enzyme Reaction by chelating Mg^{2+}. The final concentrations of EDTA in the Complete Assay is 20 mM.
ATP	5 mM	The ATP supplied in this kit can be used for the EIF4A1 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	1.7 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 m EDTA, and 0.2% Brij-35	The EDTA in the Stop & Detect Buffer B quenches the EIF4A1 Enzyme Reaction by chelating Mg^{2+}. The final concentrations of EDTA in the Complete Assay is 20mM.
ATP	5 mM	The ATP supplied in this kit can be used for the EIF4A1 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 m EDTA, and 0.2% Brij-35	The EDTA in the Stop & Detect Buffer C quenches the EIF4A1 Enzyme Reaction by chelating Mg^{2+}. The final concentrations EDTA in the Complete Assay is 10 mM.
ATP	5 mM	The ATP supplied in this kit can be used for the EIF4A1 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

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

4.0 Protocol

The methods described below are for endpoint detection of ADP formation by EIF4A1 under initial velocity conditions (≤ 10% conversion of ATP to ADP) with a sub-saturating concentration of ATP (30 µM) and saturating yeast RNA (1 mg/mL). These conditions will ensure sensitive detection of inhibitors that compete with ATP while minimizing the effects of compounds that inhibit EIF4A1 via non-specific interactions with RNA. Significant changes in the ATP concentration may require adjustment of the dynamic range, as described in the **Transcreener ADP^2 Assay** Tech Manuals (**Section 4.2**).

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.

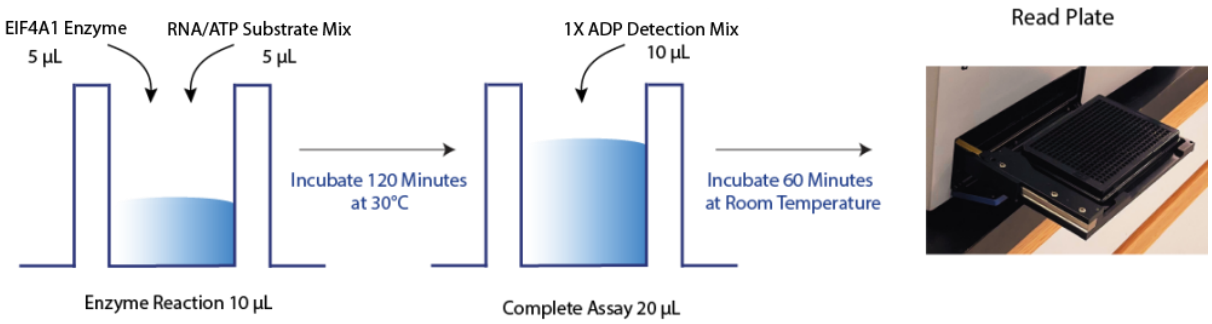


Figure 2. Simple Mix-and-Read Format. The EIF4A1 Enzyme Reaction is initiated by the addition of yeast RNA and ATP. After the Enzyme Reaction incubation is completed, 1X ADP Detection Mix is added, which contains EDTA to quench the Enzyme Reaction. Plates are allowed to sit for 60 min at room temperature before reading to allow the detection reaction to reach equilibrium.

	10 µL Enzyme Reaction Components	
Component	**Working Stock**	**Final Concentration in 10 µL**
Complete Assay Buffer	1X Enzyme Assay Buffer E, 1 mM TCEP, in Nuclease Free Water	50 mM Tris-HCl, pH 7.5, 0.25 mM $MgCl_2$, 1 mM TCEP, 0.01% Triton X-100
EIF4A1 Enzyme, 0.54 mg/mL (11.49 µM)	2X in Complete Assay Buffer	20 nM – 30 nM* (See **Section 4.1**)
ATP, 5 mM	60 µM in Complete Assay Buffer (with 2 mg/mL Yeast RNA)	30 µM
Yeast RNA, 10 mg/mL	2 mg/mL in Complete Assay Buffer (with 60 µM ATP)	1 mg/mL

Table 1. EIF4A1 Enzyme Reaction Components. Concentrations are provided for the standard protocol using 5 µL of EIF4A1 Enzyme Mix and 5 µL of RNA/ATP Substrate Mix for the Enzyme Reaction.

***See Section 4.1 for Determining the Optimal Enzyme Concentration.**

Component	1X ADP Detection Mix – Add 10 µL Per Well					
	FP		FI		TR-FRET	
	Detection Mix Conc.	Complete Assay	Detection Mix Conc.	Complete Assay	Detection Mix Conc.	Complete Assay
Stop & Detect Buffer	1X	0.5X	1X	0.5X	1X	0.5X
ADP Tracer	4 nM	2 nM	8 nM	4 nM	64.8 nM	32.4 nM
ADP Antibody	33.4 µg/mL	16.7 µg/mL	28.6 µg/mL	14.3 µg/mL	8 nM	4 nM

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.

4.1 Performing an Enzyme Assay

The following assay protocol is for a 384-well format, using a 10 µL Enzyme Reaction and 20 µL Complete Assay volume when the plates are read. The use of different formats will require changes in reagent quantities (see **Section 5.1**). All the reagent mixes can be prepared ahead of time and stored on ice for 2 hours before use.

1. Prepare Working Stocks

a) Prepare Complete Assay Buffer by combining 1X Enzyme Assay Buffer E, and 1 mM TCEP in Ultrapure Nuclease-Free Water.
b) Dilute EIF4A1 enzyme to 2X the desired concentration* in Complete Assay Buffer.
c) Prepare 2X Substrate Mix by combining 60 µM ATP, and 2 mg/mL Yeast RNA in Complete Assay Buffer.

2. Run the EIF4A1 Enzyme Reaction

a) Add 5 µL of 2X EIF4A1 enzyme to each well, followed by 5 µL of 2X Substrate Mix to initiate the reaction. Mix gently on a plate shaker and incubate at 30°C for 120 minutes.

3. Prepare and Dispense ADP Detection Mix

b) Centrifuge ADP[2] Antibody at 10,000 x g for 10 minutes to remove any aggregates. This step is critical to ensure optimal assay performance.

c) Prepare 1X ADP Detection Mix by diluting Stop & Detect Buffer, ADP[2] Tracer, and ADP[2] Antibody in Ultrapure Nuclease-Free Water to the concentrations described in Table 2, according to the appropriate readout mode.

d) Add 10 µL of the 1X ADP Detection Mix to each well and mix gently on a plate shaker. Incubate at room temperature for 60 minutes to allow the detection reaction to reach equilibrium.

*Using the EC_{80} concentration suggested in the EIF4A1 Enzyme Certificate of Analysis should provide a robust signal that is within the linear range for ADP formation. However, for best results, it may be useful to perform an enzyme titration to identify the optimal enzyme concentration (EC_{50} to EC_{80}) **(see Figure 3a)**. The EC_{50} is provided by common graphing programs; the EC_{80} enzyme concentration can be calculated from the EC_{50}, as follows:

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

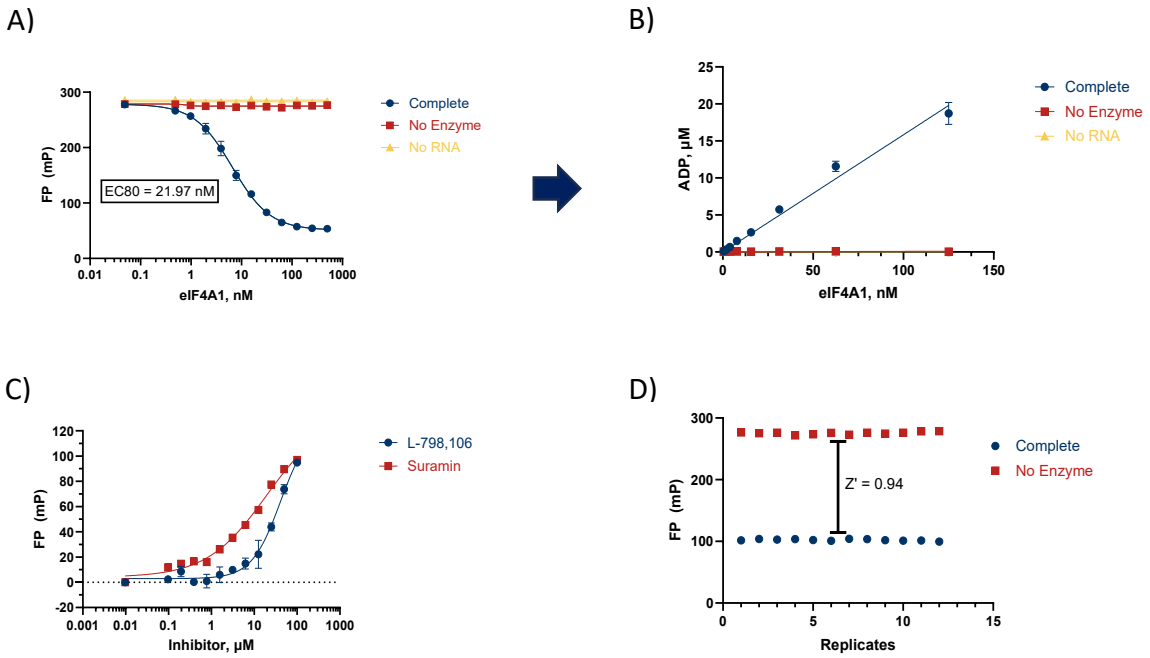


Figure 3. Sample Data for FP Readout Mode. (a) Enzyme titration curve. (b) Raw polarization in enzyme titration curve is converted to ADP formed using the standard curve in Section 4.3. Only the linear portion of the graph is shown; interpolation was performed using GraphPad Prism (see Section 5.2 for guidance). (c) Dose-Response Curves of probe inhibitors (see Section 4.2). (d) Z' Measurement (n=12) (see Section 4.4).

4.2 Performing Single Compound Screening and Dose-Response Assays

Single Compound Screening and Dose-Response Assays follow the protocol listed in Section 4.1. The target EIF4A1 Enzyme is added to the test compounds pre-dispensed in wells; the total mixture volume should be 5 µL and DMSO concentration should not exceed 1-2%. We recommend mixing gently on a plate shaker for 40 to 60 seconds and preincubating for 30 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

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.

4.3.1 Preparing the Standard Curve

1. Prepare 1X Complete Assay Buffer.
2. Dilute 5 mM ATP and ADP to 30 µM in 1X Complete Assay Buffer. 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 30 µM, 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 percentage conversions utilized in the standard curve. An

example of the dilution scheme can be found in **Figure 4**. 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. Incubate at room temperature for 60 minutes before measuring signals.

% Conversion	ATP (µM)	ADP (µM)
100	0	30
50	15	15
25	22.5	7.5
15	25.5	4.5
10	27	3
8	27.6	2.4
4	28.8	1.2
2	29.4	0.6
1	29.7	0.3
0.5	29.85	0.15
0.2	29.94	0.06
0	30	0

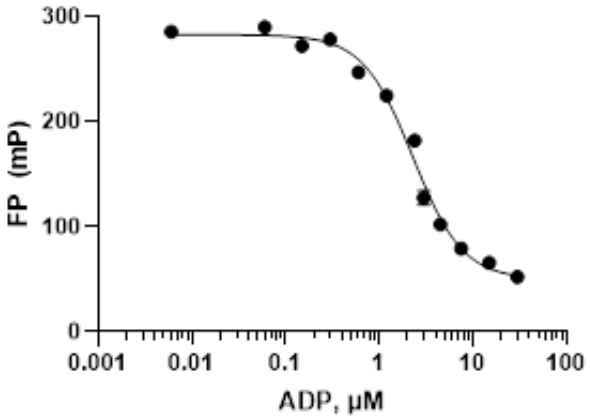


Figure 4: ADP Standard Curves. Example concentration and data for a standard curve corresponding to 30 µM ATP in EIF4A1 Enzyme Reaction.

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 EIF4A1 Assay, run the controls with and without enzyme (no test compound) to achieve final results. Use the following formula to determine Z'.

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

5.0 Appendix

5.1 Using the Assay with Different Volumes and Plate Formats

Component	Total Volume	Enzyme Reaction Volume	1X ADP 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 suggested 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
- List of Commonly Used Plate Readers and Settings
- Using GraphPad Prism to Interpolate Data from a Standard Curve to Generate a Dose-Response

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