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Enzolution™ DDX3 ATPase Assay System Technical Manual

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info¹

¹BellBrook Labs LLC

BellBrook Labs

Tech. support phone: +1 (608) 443-2400 email: techsupport@bellbrooklabs.com



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We use this protocol and it's working

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Abstract

The Enzolution™ DDX3 Assay System is intended for use with the Transcreener® ADP² Assay Kits to measure the ATP-dependent RNA helicase activity of DDX3. DDX3, a DEAD Box helicase protein characterized by the conserved motif Asp-Glu-Ala-Asp (DEAD), is an ATP-dependent RNA helicase that unwinds RNA-RNA and RNA-DNA duplexes. ADP formation by the RNA helicase activity of DDX3 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™ DDX3 Assay System provides all reagents required to screen and profile DDX3 inhibitors, including the purified human DDX3 (aa 135-583) 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.

Introduction

- 1 The Enzolution™ DDX3 Assay System is intended for use with the Transcreener® ADP² Assay Kits to measure the ATP-dependent RNA helicase activity of DDX3. DDX3, a DEAD Box helicase protein characterized by the conserved motif Asp-Glu-Ala-Asp (DEAD), is an ATP-dependent RNA helicase that unwinds RNA-RNA and RNA-DNA duplexes. ADP formation by the RNA helicase activity of DDX3 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.

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Key Applications:

- Screening for DDX3 inhibitors (or activators)
- Generating dose response curves and IC₅₀ values for DDX3 inhibitors
- Kinetic and mechanistic analyses

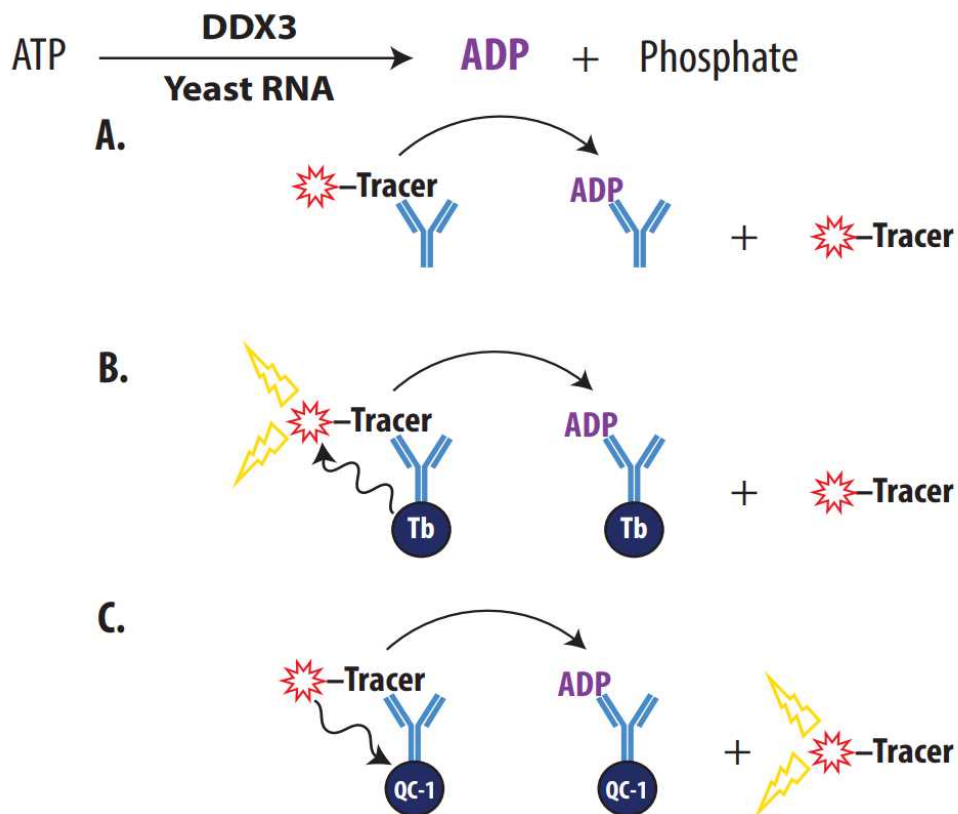


Figure 1. Schematic Overview of the Enzolution™ DDX3 Assay System with the Transcreeper ADP² Assays.

For FP readout (A): ADP produced by the RNA helicase activity of DDX3 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 DDX3 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 DDX3 displaces an Alexa Fluor® 594 Tracer from the ADP² Antibody conjugated to an IRDye® QC-1 quencher, resulting in an increase in fluorescence intensity.

Product Specifications

Product	Quantity	Part #	Part #
Enzolution™ DDX3 ATPase Assay System	1,000 assays*	FP	3038-1K-FP
		FI	3038-1K-FI
		TR-FRET	3038-1K-TR
	10,000 assays*	FP	3038-10K-FP
		FI	3038-10K-FI
		TR-FRET	3038-10K-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

DDX3 Enzyme and yeast RNA should be stored at -80°C; other reagents can be stored at -20°C. Though we have confirmed that the DDX3 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.

Materials Provided

2.1

Component	Composition	Notes
DDX3 Enzyme	1.0 mg/mL (19.1 µM)* in 50 mM Tris-HCl, 150 mM NaCl, 10% glycerol, pH 8.0	Truncated (amino acids 135-583), N-Terminal 6xHis, 52.4 kDa. Sufficient enzyme is included in the kit to complete at least 1,000 assays (Part # 3038-1K) or 10,000 assays (Part # 3038-10K).
Yeast RNA	10 mg/mL in H ₂ O	Yeast RNA purified from <i>Torulla</i> . Storage solution is nuclease-free water.
Enzyme Assay Buffer D, 10X	500 mM TRIS (pH 7.5), 20 mM MgCl ₂ , 0.1% Triton X-100	Use Enzyme Assay Buffer D in the Enzyme Reaction and for preincubation with inhibitors. Changes to the assay buffer could affect enzyme activity and/or detection of ADP.



Component	Composition	Notes
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.

Materials Provided But Not Required

2.2

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 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
ADP ² Antibody	4 mg/mL solution in PBS with 10% glycerol*	1,000 assays (Part # 3010-1K) or 10,000 assays (Part # 3010-10K).



Component	Composition	Notes
ADP ² 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 DDX3 Enzyme Reaction by chelating Mg ²⁺ . The final concentrations of Mg ²⁺ and EDTA in the Complete Assay are 2 mM and 20 mM, respectively.
ATP	5 mM	The ATP supplied in this kit can be used for the DDX3 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
ADP ² Antibody-IRDye® QC-1	1.4 mg/mL solution in 100 mM KH ₂ PO ₄ (pH 8.5)*	1,000 assays (Part # 3013-1K) or 10,000 assays (Part # 3013-10K).
ADP ² 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 DDX3 Enzyme Reaction by chelating Mg ²⁺ . The final concentrations of Mg ²⁺ and EDTA in the Complete Assay are 2 mM and 20 mM, respectively.
ATP	5 mM	The ATP supplied in this kit can be used for the DDX3 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
ADP ² 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 C quenches the DDX3 Enzyme Reaction by chelating Mg ²⁺ . The final concentrations of Mg ²⁺ and EDTA in the Complete Assay are 2 mM and 10 mM, respectively.
ATP	5 mM	The ATP supplied in this kit can be used for the DDX3 Enzyme Reaction and the ATP/ADP standard curve.
ADP	5 mM	The ADP is used for the ATP/ADP standard curve.

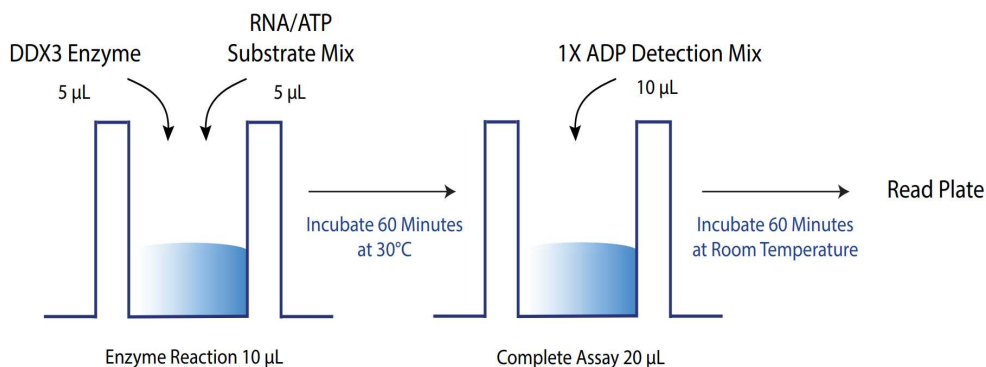
*The exact concentration may vary from batch to batch. Please refer to the Certificate of Analysis for an accurate concentration

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 Assays. **Full list of compatible plate readers and settings.**
3. Please read and understand the Transcreener ADP² Assay Technical Manuals prior to use with this kit.

Protocol

4



The methods described below are for single-addition, endpoint detection: the DDX3 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 μ L DDX3 Enzyme Reaction and 10 μ L of detection/quench reagents (final volume 20 μ L 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 ($\leq 20\%$ conversion of ATP to ADP) by DDX3 with a sub-Km concentration of ATP (100 μ M) and a saturating concentration of 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 DDX3 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 Transcreeper ADP² Assay Manual.

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

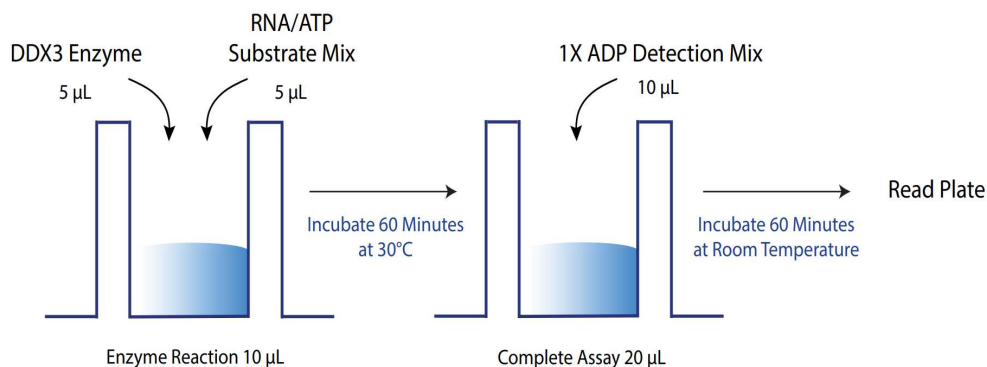


Figure 2. An Outline of the Procedure. The DDX3 Enzyme Reaction is initiated by the addition of yeast RNA and ATP. After the Enzyme Reaction incubation is completed, ADP detection reagents are added (Transcreeper ADP² Antibody and Tracer) along with EDTA to quench the DDX3 reaction.

10 µL Enzyme Reaction Components

Component	Working Stock	Final Concentration in 10 µL
Enzyme Assay Buffer D, 10X	1X in Nuclease Free Water	1X (50 mM Tris-HCl (pH 7.5), 2 mM MgCl ₂ , 0.01% Triton X-100)
DDX3 Enzyme, 1.0 mg/mL (19.1 µM)	2X in 1X Enzyme Assay Buffer D	35 nM – 50 nM*
ATP, 5 mM	200 µM in 1X Enzyme Assay Buffer D (with 2 mg/mL Yeast RNA)	100 µM
Yeast RNA, 10 mg/mL	2 mg/mL in 1X Enzyme Assay Buffer D (with 200 µM ATP)	1 mg/mL

*See Section 4.1 for Determining the Optimal Enzyme Concentration.

Table 1. DDX3 Enzyme Reaction Components. Concentrations are provided for the standard protocol using 5 µL of DDX3 Enzyme Mix and 5 µL of RNA/ATP Substrate Mix for the Enzyme Reaction.

1X ADP Detection Mix - Add 10 μ L Per Well						
Component	FP		FI		TR-FRET	
	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	500 nM	250 nM
ADP Antibody	109 μ g/mL	54.5 μ g/mL	101 μ g/mL	50.5 μ g/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.

Determining the Optimal Enzyme Concentration

- 4.1 Using the enzyme concentration suggested in the DDX3 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 Yeast RNA 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.

Enzyme Titration Steps

1. Prepare 1000 μ L 1X Enzyme Assay Buffer D: Dilute 100 μ L of 10X Enzyme Assay Buffer D in 900 μ L Ultrapure Nuclease Free Water.
 2. Prepare 60 μ L of 500 nM DDX3 Enzyme: dilute 1.57 μ L of 19.1 μ M DDX3 Enzyme in 58.43 μ L 1X Enzyme Assay Buffer D.
 3. Add 10 μ L of the DDX3 Enzyme to well 1 (including replicates).
 4. Add 5 μ L of 1X Enzyme Assay Buffer D to wells 2-11, DO NOT add the Assay Buffer to well 1.
 5. Transfer 5 μ L from well 1 to well 2 and mix by pipetting, then transfer 5 μ L from well 2 to well 3 and mix by pipetting; repeat this serial dilution process until well 11 has received DDX3 Enzyme. Well 12 is to be used as a blank and should not include enzyme.
- IMPORTANT: After mixing the last well (11) in the dilution series, remove 5 μ L from that well only and discard, so that all the wells contain 5 μ L final volume.**
6. Prepare 300 μ L of RNA/ATP Substrate Mix: mix 60 μ L of 10 mg/mL Yeast RNA and 12 μ L of 5 mM ATP in 228 μ L 1X Enzyme Assay Buffer D.
 7. Start the Enzyme Reaction by adding 5 μ L of the RNA/ATP Substrate Mix to every well (1-12). Gently mix for 40 to 60 seconds on a plate shaker. Incubate at 30°C for 60 minutes.
 8. Prepare 600 μ L 1X ADP Detection Mix based on the concentrations provided in Table 2: 60 μ L 10X Stop & Detect Buffer B, 6 μ L ADP² Alexa Fluor 633 Tracer and 109

µg/mL ADP² 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 (1-12), 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 measuring signals.

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 (EC₅₀ to EC₈₀) (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 EC₅₀ is provided by common graphing programs; the EC₈₀ enzyme concentration can be calculated from the EC₅₀, as follows:

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

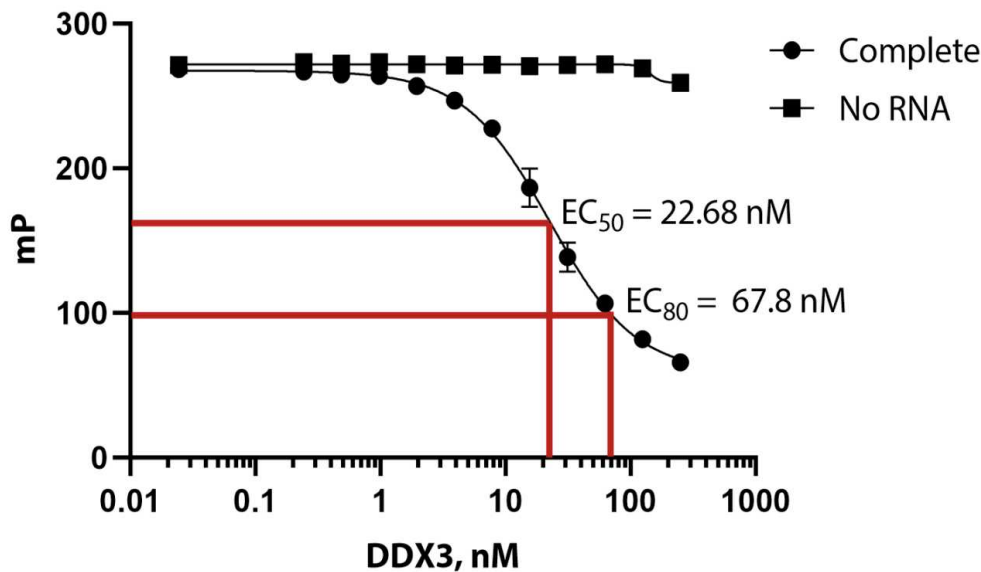


Figure 3. Enzyme Titration Curve. Example DDX3 Enzyme titration. The ideal range of enzyme concentrations is between EC₅₀ and EC₈₀; the specific concentration may vary depending on the enzyme lot.

Performing Single Compound Screening and Dose-Response Assays

4.2 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 μ L. 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.

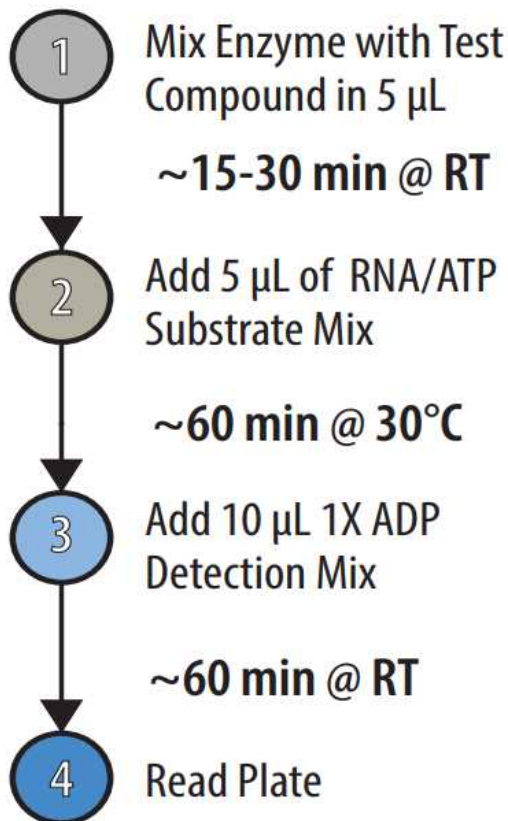
2. Start the Enzyme Reaction by adding 5 μ L of the RNA/ATP Substrate Mix in each well. It is recommended to incubate the Enzyme Reaction at 30°C for 60 minutes.

Note: The final volume of the Enzyme Reaction mixture should be 10 μ L for 384 well plates. See Section 5.1 for a list of other plate formats.

3. After the incubation, add 10 μ L of 1X ADP Detection Mix to the 10 μ L Enzyme Reaction and mix the 20 μ L Complete Assay 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 for 60 minutes before measuring signals.



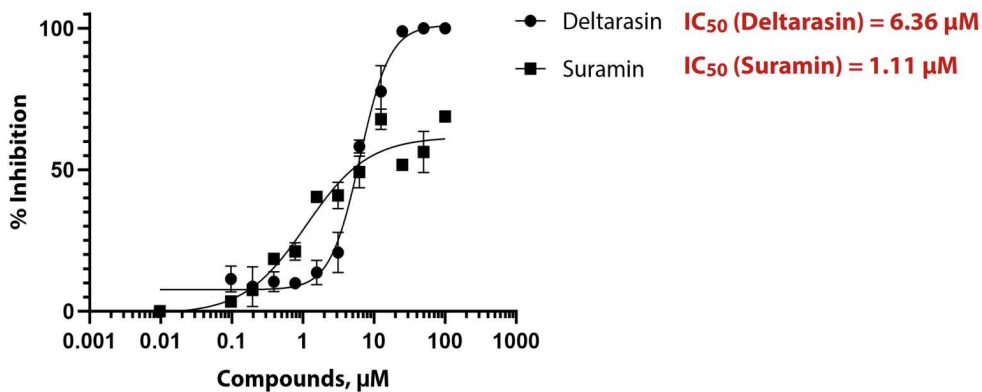


Figure 4. Dose-Response Curve. Example titration of probe inhibitors Suramin and Deltarasin in the presence of DDX3 Enzyme.

Setting Up a Standard Curve

- 4.3 Use of a standard curve for conversion of values to amount of ADP formed allows quantitative measurement of the enzyme activity and accurate IC₅₀ 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.

	% Conversion	ATP (μM)	ADP (μM)
	100	0	100
	50	50	50
	25	75	25
	15	85	15
	10	90	10
	8	92	8

	% Conversion	ATP (μM)	ADP (μM)
	4	96	4
	2	98	2
	1	99	1
	0.5	99.5	0.5
	0.2	99.8	0.2
	0	100	0

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

Preparing the Standard Curve

1. Prepare 1X Enzyme Assay Buffer D.
2. Dilute 5 mM ATP and ADP to 100 μM in 1X Enzyme Assay Buffer D. 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 , 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 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. Incubate at room temperature for 60 minutes before measuring signals.

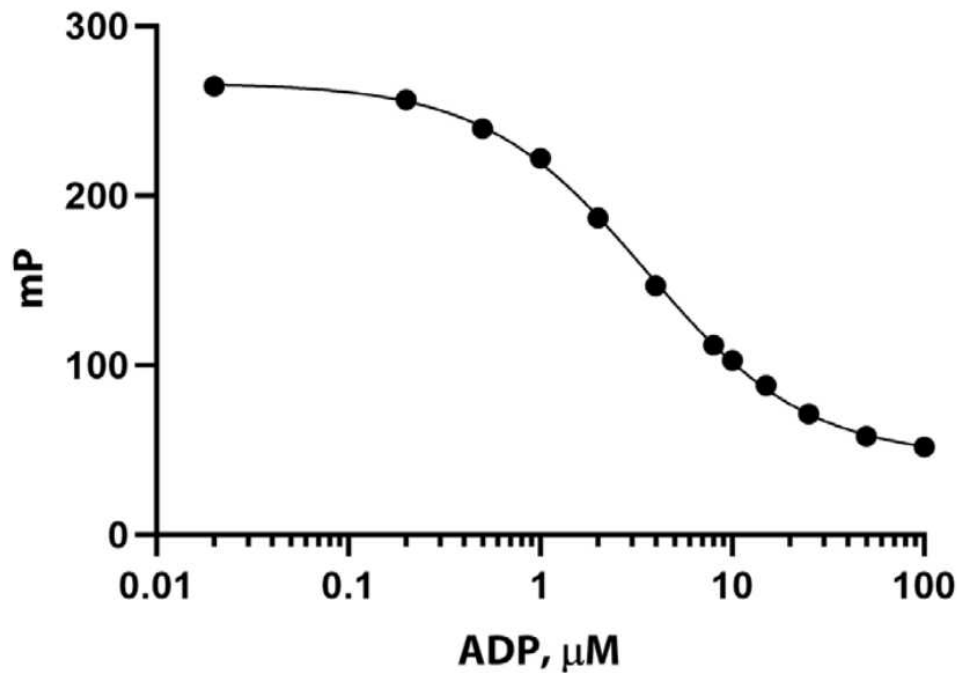


Figure 5. ADP Standard Curve. Standard curve using 1X ADP Detection Mix for FP readout as shown in Table 2 (109 $\mu\text{g/mL}$ ADP² antibody, 4 nM ADP² Tracer).

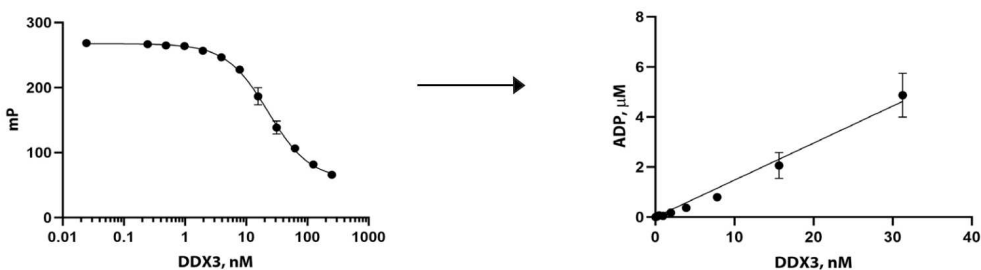


Figure 6. Enzyme Titration Curve Converted to ADP Formed. Raw polarization signal (mP) is converted to ADP formed using a standard curve as described in **Section 4.3**. Only the linear portion of the graph is shown; interpolation was performed using GraphPad Prism.

Measuring Assay Robustness with Z'

- 4.4 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 DDX3 Assay, run the controls with and without enzyme (no test compound) to achieve final results. Use the following formula to determine Z' .

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

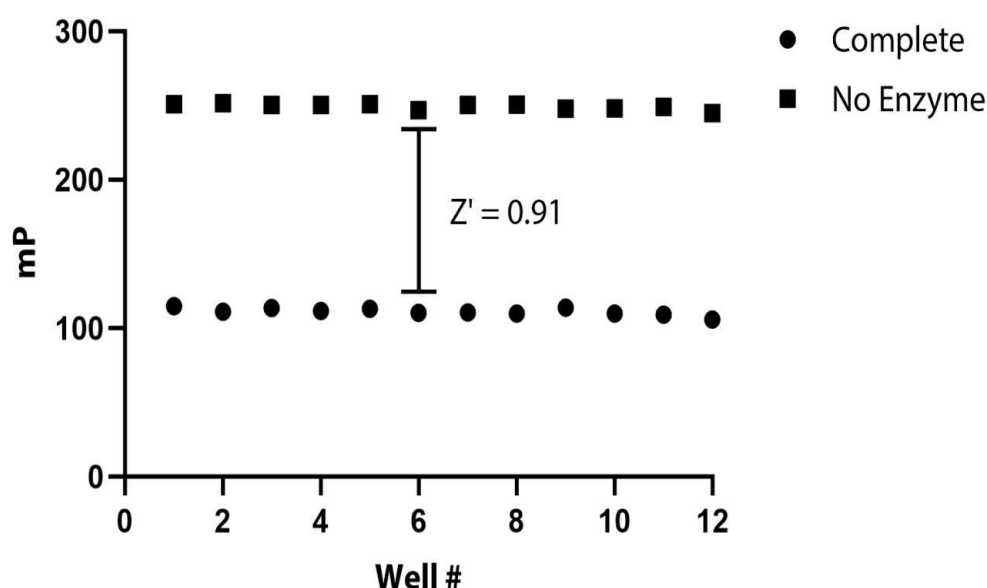


Figure 7. Z' Measurement. Complete Assay is performed with and without DDX3 Enzyme (n=12). Z' is then calculated based on the formula shown in **Section 4.4**.

Appendix

5

Using the Assay with Different Volumes and Plate Formats

5.1

	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.

Links to Applicable Application Notes

- 5.2
- [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](#)

Contact Information

6

Email: TechSupport@bellbrooklabs.com

Phone: 608.443.2400

Toll-Free: 866.313.7881

FAX: 608.441.2967