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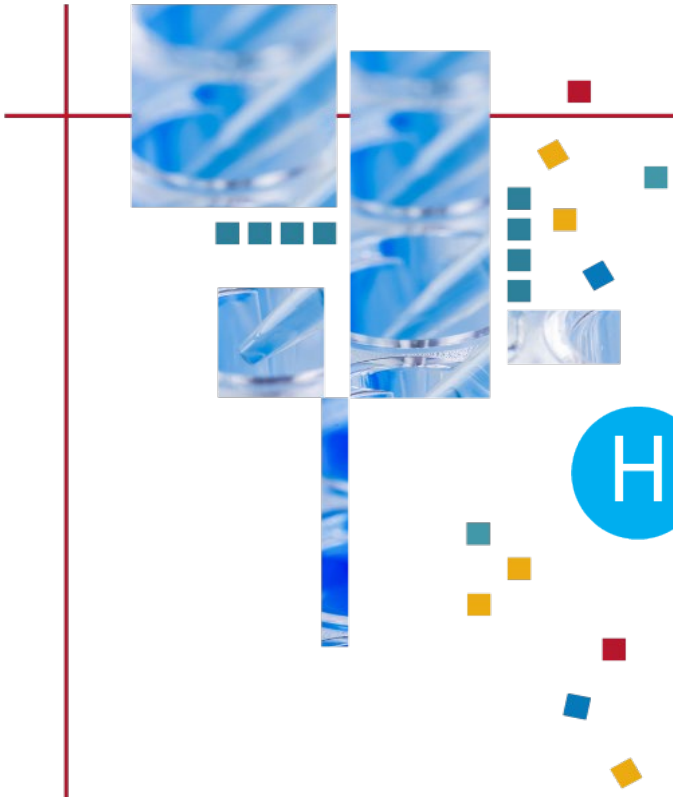

Heliscreener™

RNA Unwinding Assay

Technical Manual

Instructions for Part Numbers 3051-1K and 3051-10K

Heliscreener RNA Unwinding Assay Technical Manual

Contents

1.0 Introduction

The Heliscreener RNA Unwinding Assay is a biochemical assay designed to measure the RNA unwinding activity of RNA helicases in the DExH/D-box family. The assay utilizes a labeled double-stranded RNA (dsRNA) substrate that is quenched when intact and emits far-red fluorescence following unwinding (see **Figure 1**).

The assay can be performed in kinetic (continuous monitoring) or endpoint mode. Kinetic mode is recommended for initial velocity measurements, allowing for accurate enzyme kinetics and inhibitor IC_{50} determinations. Alternatively, for screening, the enzyme reaction can be stopped by adding the Stop & Detect Buffer B, and the quenched reactions can be read in endpoint mode.

Key Specifications:

- Single-addition, mix-and-read format
- Excellent data quality (Z' ≥ 0.7)
- Far red tracer minimizes interference from fluorescent compounds and light scattering

Key Applications:

- Screening for enzyme inhibitors/activators
- Generating dose-response curves and IC_{50} values for inhibitors
- Kinetic and mechanistic analyses

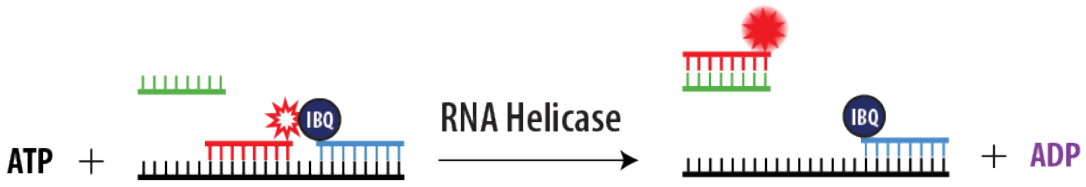

Figure 1. Schematic Overview of the Heliscreener RNA Unwinding Assay. Fluorescence is quenched when the Reporter RNA is intact. RNA helicases unwind the Reporter RNA, releasing the far-red fluorescence (Cy5) from the quencher (Iowa Black® Quencher). A Capture RNA (green) hybridizes to the fluorescent strand, preventing it from re-annealing with the quenching strand.

2.0 Product Specifications

Product	Quantity	Part#
Heliscreener RNA Unwinding Assay	1,000 assays*	3051-1K
	10,000 assays*	3051-10K

***NOTE: The exact number of assays depends on enzyme reaction conditions. The kits are designed for use with 384-well plates, using a 20 µL complete assay volume.**

Storage

Reporter RNAs and Capture RNA should be aliquoted for multiple uses and stored at -80°C.

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

2.1 Materials Provided

Component	Composition	Notes
Reporter RNA	4 µM in 50 mM Tris, 50 mM NaCl, and 1 mM EDTA	dsRNA with a 3' overhang. The fluorescent strand is labeled with Cy5 and the quenching strand is labeled with Iowa Black Quencher.
Capture RNA	4 µM in 50 mM Tris, 50 mM NaCl, and 1 mM EDTA	ssRNA complementary to the fluorescent strand in the Reporter RNA.
ATP, 100 mM	100 mM in Nuclease-free water	
Enzyme Assay Buffer D, 10X	500 mM Tris (pH 7.5), 20 mM $MgCl_2$, and 0.1% Triton X-100	Enzyme Assay Buffer D has been optimized to support activity of DDX3, DDX5, DDX17, and DHX9.
Unwound RNA Control	4 µM RNAs in 50 mM Tris, 50 mM NaCl, and 1 mM EDTA	A mixture of RNAs that mimics a completed unwinding reaction: the fluorescent strand annealed to the Capture RNA, and the quenching strand by itself. It serves as a control for the maximum assay window.
Stop & Detect Buffer B, 10X	200 mM HEPES (pH 7.5), 400 mM EDTA, and 0.2% Brij-35	The Stop & Detect Buffer B components quench the Enzyme Reaction by chelating metals required for activity.

2.2 Materials Required or Recommended but Not Provided

Component	Notes
Enzyme	The Heliscreener RNA Unwinding Assay is validated for use with purified DDX3, DDX5, DDX17, and DHX9, which are available from BellBrook as stand-alone products (Part# 2251, 2307, 2309, 2317).
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
RNase Inhibitor (recommended)	RNase inhibitors, such as RNaseOUT™ or RNasin®, provide protection against RNase contamination, ensuring that the detected fluorescent signal originates from the helicase enzyme reaction rather than from RNA degradation. A concentration greater than 0.005 U/µL of RNaseOUT or RNasin or equivalent is recommended in the enzyme reaction. If the RNase inhibitor is omitted, it is essential to include a no-ATP control to assess any RNase-related fluorescence.
Assay Plate	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.

3.0 Before You Begin

- Read the entire protocol and note any reagents or equipment needed (see **Section 2.2).**
- Ensure that your plate reader is capable of a) measuring Cy5 fluorescence (excitation peak: 649 nm, emission peak: 670 nm), b) maintaining a temperature of 30°C, and c) performing kinetic readings at an interval of one to three minutes. Running the assay at room temperature is possible but may require more enzyme, while a larger reading interval may result in missing the initial velocity phase of the enzyme reaction.

- Define the Maximum Signal Window for Your Plate Reader: The RFUs for the Unwound RNA Control (20 µL of 8 nM Unwound RNA Control in 0.5X Enzyme Assay Buffer D) represents the High signal, and the RFUs for the Reporter RNA (20 µL of 8 nM Reporter RNA in 0.5X Enzyme Assay Buffer D) represents the Low signal; Maximum Signal = $RFU_{High} - RFU_{Low}$. Prepare High and Low RFU Controls in quantities sufficient to perform at least 12 replicates for each condition. Calculate the Z' factor using the equation below; values greater than 0.7 are acceptable.

$$Z' = 1 - \frac{[(3 \times SD_{High}) + (3 \times SD_{Low})]}{|(Mean_{High}) - (Mean_{Low})|}$$

4.0 Protocol

4.1 Performing a Kinetic Enzyme Assay

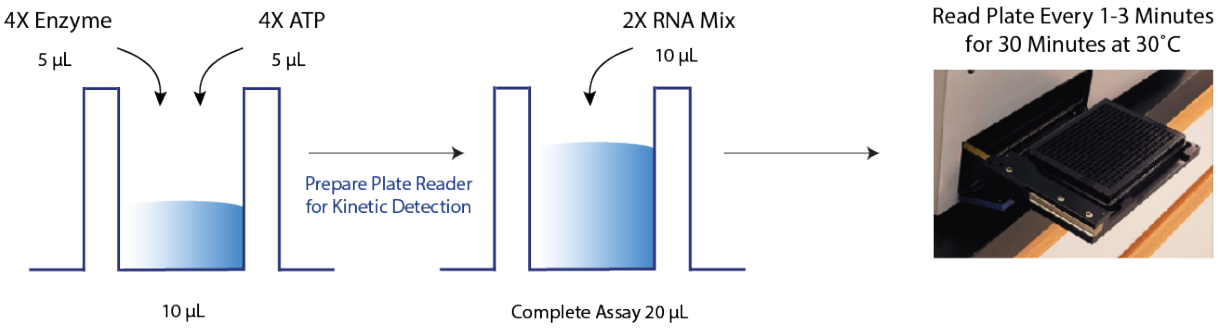

Figure 2. Simple Mix-and-Read Format. The plate is read every one to three minutes for a total of 30 minutes to capture the initial velocity and maximize the assay window.

The following assay protocol is for 384-well format, using 20 µL Complete Assay volume when the plates are read. All the reagent mixes can be prepared ahead of time and stored on ice for at least 2 hours before use.

1. **Prepare Working Stocks (see Table 1)**
   a. Prepare Complete Assay Buffer containing 0.5X Enzyme Assay Buffer D and 0.005 U/µL of RNaseOUT (or equivalent of other RNase inhibitors, recommended) in Ultrapure Nuclease-Free Water.
   b. Dilute your enzyme to 4X the desired concentration in Complete Assay Buffer.
   c. Dilute 100 mM ATP to 4X the desired concentration in Complete Assay Buffer.
   d. Prepare 2X RNA Mix by combining 16 nM Reporter RNA, and 16 nM Capture RNA in Complete Assay Buffer.

Component	Stock	Working Concentration	Final Concentration in 20 µL Enzyme Reaction
Enzyme*		4X	1X
ATP*	100 mM	4X	1X
Reporter RNA	4 µM	16 nM	8 nM
Capture RNA	4 µM	16 nM	8 nM
Enzyme Assay Buffer D	10X	0.5X	0.5X
RNaseOUT (recommended)		0.005 U/µL	0.005 U/µL

Table 1. Kinetic Assay Components. *Typical Enzyme and ATP concentrations shown in Table 2.

2. **Run Enzyme Reaction**
   a. Add 5 µL of 4X Enzyme to each well.
   b. Add 5 µL of 4X ATP to each well.
   c. Get the plate reader ready. Then, add 10 µL of 2X RNA Mix. Mix briefly on a plate shaker. Read every one to three minutes for a total of 30 minutes at 30°C.

	Concentration in 20 µL Complete Assay			
Component	**DDX3**	**DDX5**	**DDX17**	**DHX9**
Reporter RNA	8 nM	8 nM	8 nM	8 nM
Capture RNA	8 nM	8 nM	8 nM	8 nM
ATP*	0.5 mM	1.5 mM	0.5 mM	0.14 mM
Enzyme Assay Buffer D	0.5X	0.5X	0.5X	0.5X
RNaseOUT™ (recommended)	0.005 U/µL	0.005 U/µL	0.005 U/µL	0.005 U/µL

*ATP is near the K_m concentration for DDX3, DDX5, DDX17, and 2*K_m concentration for DHX9

Table 2. Example Kinetic Assay Conditions for DDX3, DDX5, DDX17, and DHX9

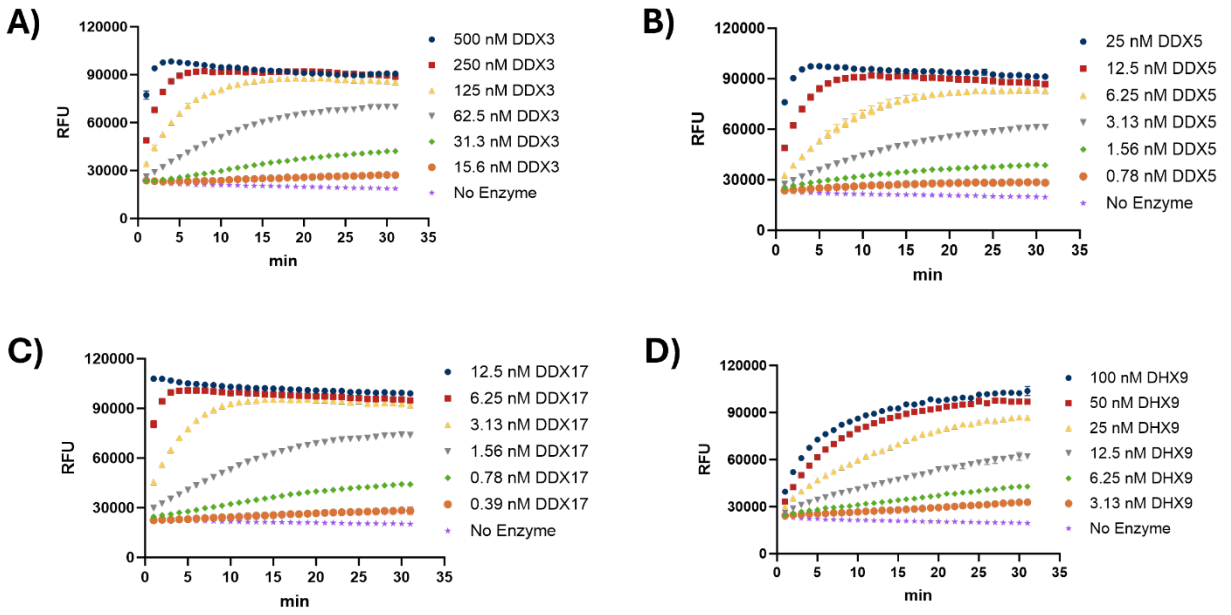

Figure 3. Example Enzyme Titration – Kinetic Assay. For screening and profiling inhibitors, choose an enzyme concentration that maximizes signal window while still in linear range at the 5 min time point. For example, the ideal enzyme concentration in this data set is 80 nM, 5 nM, 2 nM, and 40 nM for (A)DDX3, (B)DDX5, (C)DDX17, and (D)DHX9 respectively.

4.2 Performing an End-Point Enzyme Assay

The assay can also be conducted as an end-point assay. However, it is important to note that the initial velocity develops quickly, even at low enzyme concentrations; therefore, the reaction time should be determined carefully. We have found that a reaction time of 15 minutes is ideal for validated enzymes, providing a flexible workflow while remaining close to the initial velocity phase of the reaction. The following protocol is designed for a 384-well format, utilizing 15 µL of enzyme reaction solution and 5 µL of Stop & Detect Buffer B. All reagent mixtures can be prepared in advance and stored on ice for up to 2 hours before use.

1. **Prepare Working Stocks (see Table 3)**
   a. Prepare Complete Assay Buffer containing 0.5X Enzyme Assay Buffer D and 0.005 U/µL of RNaseOUT™ (or equivalent of other RNase inhibitors, recommended) in Ultrapure Nuclease-Free Water.
   b. Dilute your enzyme to 3X the desired concentration in Complete Assay Buffer.
   c. Dilute 100 mM ATP to 3X the desired concentration in Complete Assay Buffer.
   d. Prepare 3X RNA Mix by combining 24 nM Reporter RNA, and 24 nM Capture RNA in Complete Assay Buffer.
   e. Dilute 10X Stop & Detect Buffer B to 4X in Ultrapure Nuclease-Free Water.

Component	Stock	Working Concentration	Final Concentration in 15 µL Enzyme Reaction
Enzyme		3X	1X
ATP	100 mM	3X	1X
Reporter RNA	4 µM	24 nM	8 nM
Capture RNA	4 µM	24 nM	8 nM
Enzyme Assay Buffer D	10X	0.5X	0.5X
RNaseOUT™ (recommended)		0.005 U/µL	0.005 U/µL
Stop & Detect Buffer B	10X	4X	1X in 20 µL Complete Assay

Table 3. End-point Assay Components.

2. **Run Enzyme Reaction**
   a. Add 5 µL of 3X Enzyme to each well.
   b. Add 5 µL of 3X ATP to each well.
   c. Add 5 µL of 3X RNA Mix. Mix briefly on a plate shaker. Incubate at 30°C for 15 minutes.
   d. Add 5 µL of 4X Stop & Detect Buffer B. Mix on a plate shaker. Read the plate.

4.3 Performing Single Compound Screening and Dose-Response Assays

Single Compound Screening and Dose-Response Assays follow the protocol listed in **Section 4.1** or **4.2**. The target enzyme is added to the test compounds pre-dispensed in wells; the total mixture volume should be 5 µL. The final concentration of the test compounds should be based on the volume of the enzyme reaction (20 µL for kinetic assay and 15 µL for end-point assay). 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.

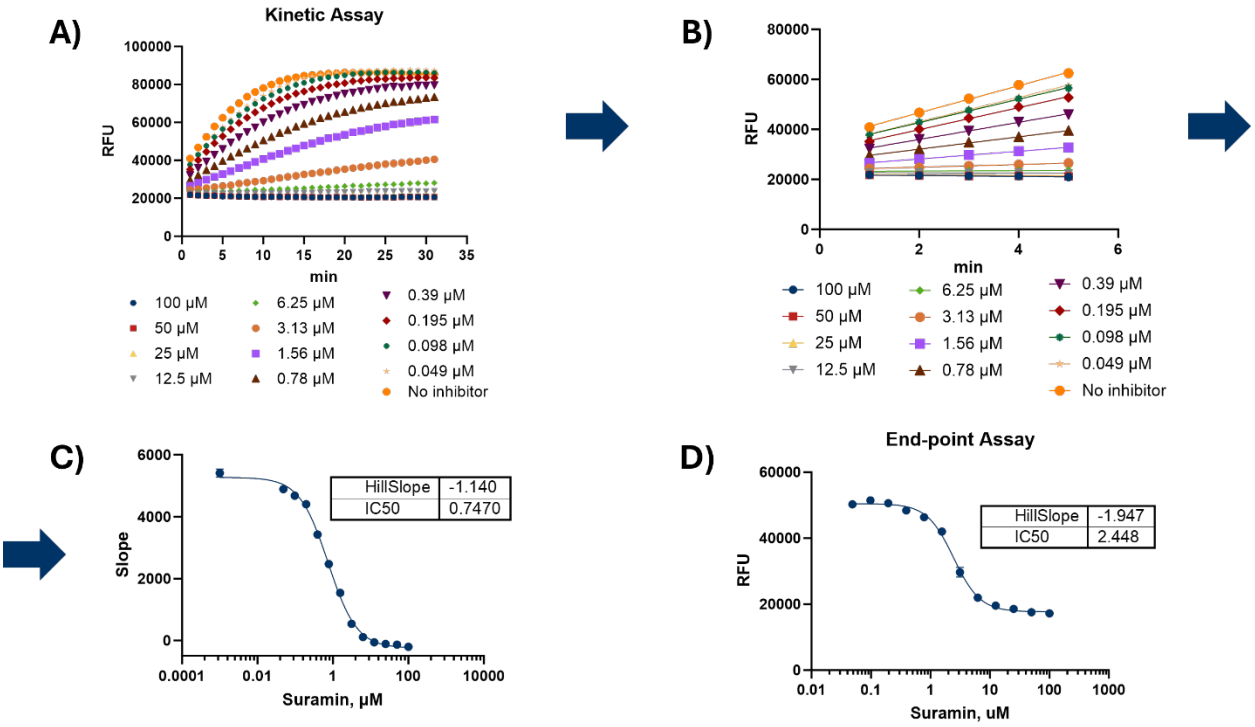

Figure 4. Example Dose Response Curve. (A) A kinetic assay using 2 nM DDX17 and varying amounts of the probe compound, Suramin. (B) The initial velocity phase of the progression curves was fitted linearly to obtain slopes that represent the enzyme's initial rate at each compound concentration. (C) These slopes are then plotted against compound concentration for accurate IC_{50} determination. (D) A dose-response curve was obtained with an endpoint assay using 2 nM DDX17 for 15 minutes; the higher value relative to the kinetic assay (C) is a result of exceeding initial velocity conditions in the endpoint assay.

5.0 Using the Assay with Different Volumes and Plate Formats

Please check the working plate volumes from the manufacturer to ensure they are within the suggested volume ranges of your plate.

Component	Total Volume	Enzyme Reaction Volume	2X RNA 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

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