

## Molecular Beacon based helicase assays

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- Helicase catalysed separation of fluorescently labelled DNA was monitored
- The BMG LABTECH microplate reader was used to monitor fluorescence and inject ATP
- The MARS data analysis software was used to calculate IC<sub>50</sub> values

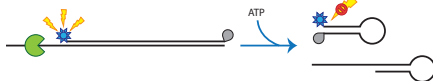
### Introduction

Helicases are motor proteins that bind duplex nucleic acids in order to separate and/or rearrange them. The reaction is fuelled by adenosine tri-phosphate (ATP). Helicases encoded by the genomes of bacteria, viruses, and mammals have diverse functions in processes including DNA replication and repair, RNA transcription, and translation. The importance of helicases in the maintenance of viral and pathogen infection makes helicases attractive drug targets. Small molecule inhibitors of helicases need to be identified. For that purpose the Molecular Beacon Helicase Assay (MBHA) was developed as an improved helicase assay.

This application note describes how to carry out an MBHA to determine the effect of potential inhibitors on the activity of the helicase encoded by the hepatitis C virus (HCV) NS3 helicase (a widely studied model helicase and drug target). A fluorescent double-stranded DNA substrate is used in combination with the BMG LABTECH microplate reader, and the MARS Data Analysis software.

### Assay Principle

In the MBHA, the two nucleotide strands of the annealed substrate are molecular beacons that, upon separation, form stem loop structures. One of the nucleic acid strands has both a quencher and a fluorophore at either end so that the two are brought together as the stem loop is formed after strand separation. Thus, a high fluorescence signal is obtained at the beginning of the assay and decreases as the helicase unwinds the substrate. If an inhibiting compound is present, the fluorescence is maintained.



**Fig. 1: MBHA assay principle.** The helicase (green) unwinds the doublestranded DNA substrate in a reaction fuelled by ATP (blue arrows). This causes the fluorescent signal from Cy5 (blue star) to be quenched as the IAbRQ (grey) is brought near Cy5 by the formation of molecular hairpins.

The MBHA can also detect small molecules that interfere in the assay either by absorbing or emitting light at wavelengths similar to Cy5. Interfering compounds either enhance fluorescence or decrease fluorescence relative to negative controls (e.g. DMSO-only). Thus, the MBHA is an ideal tool to determine the inhibitory effect of small molecules on helicase catalysed DNA unwinding.

### Materials & Methods

- White, 96-well, half-area microplates (Corning)
- Microplate reader equipped with injectors from BMG LABTECH
- DNA nucleotides with one strand carrying cyanine 5 at the 5' end and IAbRQ quencher at the 3' end were purchased from Integrated DNA Technologies

The expression and purification of the HCV helicase was carried out as previously described.

The DNA strands were annealed to create the Cy5-MBHA substrate. A working stock of 100 nM Cy5-MBHA substrate was prepared in 25 mM MOPS, pH 6.5. Potential helicase inhibitors (compounds B, C and D) were dissolved in DMSO and 8-point, 2-fold dilution series were created.

Reactions contained 25 mM MOPS pH 6.5, 1.25 mM MgCl<sub>2</sub>, 12.5 nM NS3 helicase, 5 µg/mL BSA, 0.001% (v/v) Tween20, 50 µM DTT, 5 nM Cy5-MBHA substrate, 5% DMSO, various concentrations of compounds, and 1 mM ATP in a final volume of 60 µL. A reaction mix containing all components except inhibitor and ATP was added to the microplate. Inhibitors were added subsequently in a 1:20 ratio to result in a final DMSO concentration of 5%. Quadruplicate negative and positive controls with 5% DMSO and 100 µM primuline (MP Biomedicals) were included. The plate was read using the BMG LABTECH fluorescence plate reader.

#### Instrument Settings

Measurement Method:	Fluorescence Intensity, top optic
Reading Mode:	Plate Mode Kinetic
Filters:	640-10 / 680-10
No. of cycles:	90-250
Cycle time:	5-20 sec
Injection:	after 10-20 cycles

The gain was set to 85% using a negative control well (DMSO added). The plate was read for approximately 2 minutes before the machine injected ATP at 1:10 ratio (1 mM final, smart dispensing used), then reading continued for approximately a half hour total. Note that cycle time, number of flashes, and number of cycles varies. Efforts should be made to collect many, quality data points. These settings should be adjusted to maintain assay time when different numbers of wells are read. Also note that these buffer conditions are optimized for this particular enzyme. Assay conditions should be optimized for other enzymes.



## Results & Discussion

Several MBHA's were conducted in 96-well using the BMG LABTECH microplate reader to determine the  $IC_{50}$  of inhibition of three known HCV helicase inhibitors (compounds B, C, and D).

The potency with which each compound inhibits helicase catalysed DNA unwinding was then evaluated, by first defining a "linear range" in the signal curve (Range 2 in Fig. 2, blue dashed boxes).

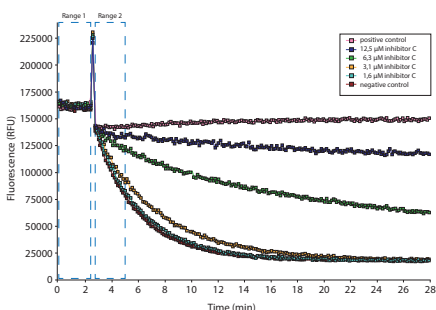


Fig. 2: Raw fluorescence data over time for one inhibitor (compound C) at different concentrations. Note the blue dashed boxes defining data ranges for analysis. Figure was taken directly from the MARS evaluation software.

The kinetic calculation feature of MARS was used to determine the slope of the linear range, which approximates the initial rate of enzyme unwinding. Using the re-calculated concentrations function in MARS, 4-Parameter fits of the slopes to log inhibitor concentration were done to create standard curves (Fig. 3). From these curves the  $IC_{50}$  values that represent the compound concentration needed to inhibit 50 % of the helicase activity were calculated.

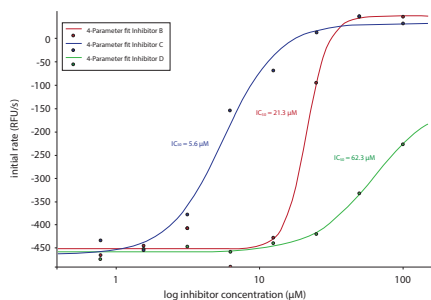


Fig. 3: 4-Parameter fit standard curves based on slope rates and inhibitors concentrations for compounds B, C, and D. The scale of the initial rate is negative because the fluorescence signal is decreasing over time [Fig. 2]

To judge compound interference, average fluorescence of each reaction before ATP addition (Range 1 figure 2) was compared to average fluorescence of reactions performed in the presence of vehicle (DMSO) alone. None of the compounds shown here interfered in the assay. Examples of compounds that interfere with the MBHA, such as the DNA binding compound thiazole orange, have been described elsewhere.

## Conclusion

This report demonstrates that the fluorescence mode of the BMG LABTECH microplate reader can be used to follow the unwinding of a double-stranded DNA substrate by helicase enzymes. We also show how the BMG LABTECH MARS data analysis software can be used to calculate the  $IC_{50}$  of inhibition.



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