Helicases are essential enzymes that unwind DNA-DNA, DNA-RNA, or RNA-RNA duplexes by disruption of the hydrogen bonds that hold the strands together (1). This unwinding activity is coupled to hydrolysis of nucleoside 5'-triphosphates (NTP's). Helicases are present in most organisms and are critical to numerous fundamental biological processes. For example, unwinding and generation of a single stranded DNA substrate is required for DNA polymerase to perform DNA replication and repair. Bacteria and viruses must unwind the host DNA duplexes for recombination and bacterial conjugation to occur. Compounds that inhibit helicases of pathogenic viruses and bacteria may be important therapeutic molecules for treatment of many infectious diseases (2). We describe here a sensitive, homogeneous electrochemiluminescence assay format to detect and quantitate helicase activity using the bacterial helicase IV as a model enzyme (Figure 1). Helicase IV, MW 75,000, is one of at least 8 helicases that have been identified in *E. coli* (1,3). This helicase, like many, utilizes ATP as the preferred NTP substrate and unwinds unidirectionally in the 3' to 5' direction. The assay’s modular design allows customers to optimize the assay design for other helicases.

**PRODUCT USE**

A Helicase Substrate DNA was prepared by synthesizing a 47mer containing a poly-T 3' tail that is labeled with BV-TAG on the 5' end. This labeled oligonucleotide was annealed to a complementary (17 bp of duplex) unlabeled 32mer. A Helicase Biotin Capture Solution was made with a 32mer identical to the unlabeled strand but labeled with biotin on the 5' end. Both of these oligonucleotide preparations were stored in 1X TE buffer at concentrations of 2.5 pmol/μl for the substrate DNA and 25 pmol/μl for the Biotin Capture Solution. A Helicase Assay Buffer was also prepared containing 12.5 mM MOPS buffer, pH 7.0, 1.5 mM MgCl₂, 1.25 mM DTT, 2.5% (v/v) glycerol, 0.05% (v/v) Triton-X® 100, and 0.5% (w/v) BSA. Dynabeads® M280 Streptavidin, 10 mg/ml, is available from BioVeris Corporation (catalog #110028 with 2 ml, or #110029 with 10 ml).

*E. coli* helicase IV was kindly provided by Dr. S.W. Matson. A stock solution of 100 mM ATP (Sigma Chemical Co.) was prepared in Helicase Assay Buffer. Since helicases are Mg²⁺-dependent, a chelating stop solution (300 mM NaCl, 480 mM EDTA, pH 8.0) was prepared and used to dilute the 250X Helicase Biotin Capture Solution to 1X (0.1 μM). The inhibitor mitoxantrone (Sigma Chemical Co.) was diluted to working concentrations in 50% DMSO.

**Figure 1**

Format for BV helicase assay.

The Substrate DNA is a 5'-BV-TAG-labeled 47mer with a 3' poly(T) tail that is annealed to an unlabeled 32mer (17 bp of duplex). The Biotin Capture Solution contains a capture DNA, a 5' biotinylated 32mer complementary to the BV-TAG-labeled DNA strand, that can anneal to unwind substrate. Streptavidin-coated paramagnetic particles capture the labeled annealed complexes via the biotin moiety on the capture oligonucleotide.
All components were kept on ice. The Helicase Substrate DNA was diluted to 25 nM (1:100) with Helicase Assay Buffer. The diluted Substrate DNA, Helicase Assay Buffer, and diluted helicase enzyme were combined in a 1:24:10 ratio, respectively. The ATP stock solution was diluted to 5 mM in BV Helicase Assay Buffer prior to conducting the assay. A final concentration of 1 mM ATP was used for enzyme titration and inhibition experiments. The user may substitute other concentrations of ATP or other (d)NTP’s as needed for alternative helicases. Thirty-five µl of vortexed Enzyme master mix or a negative control (no enzyme) was pipetted into wells of a round bottom polypropylene plate (Corning/Costar catalog #3365) and 5 µl of inhibitor or vehicle was added to each well, then 10 µl of 5 mM ATP was added to each well. The plate was covered and incubated at room temperature (RT) without shaking. For this bacterial helicase IV demonstration, the final component concentrations during the enzyme reaction (50 µl reaction volume and a 15 minute RT incubation) were: 0.5 nM Helicase Substrate DNA, 1 mM ATP, and 0.2 to 40 pM helicase IV. Ten microliters of Biotin Capture Solution diluted to 0.1 µM was added to each well for 15 minutes at RT. Dynabeads M280 Streptavidin were diluted to a working concentration of 0.067 mg/ml in PBS, pH 7.8, and then 150 µl was added to each well and the plates were incubated at RT for 30 minutes. Helicase unwinding activity was then quantitated on an M-SERIES® Analyzer. A parallel "100% unwound" positive control, prepared identically but without enzyme, was boiled for 3 minutes followed by snap-cooling on ice for 2 minutes for comparison to those wells containing enzyme. For the inhibition experiments, 5 µl of various concentrations of mitoxantrone were added to the reaction mixture (helicase IV at 20 pM), followed by ATP and incubated for 15 minutes at RT. For the inhibition experiment, positive control wells (no inhibitor) also contained a 5% (v/v) final concentration of DMSO.

**EXPERIMENTAL FINDINGS**

Helicase IV activity was quantitated over an enzyme concentration range of 0.080 to 40 pM using 1 mM ATP substrate and a 60 minute incubation (Figure 2). Under these conditions, the signal with respect to enzyme concentration was linear from approximately 0.3 to 5 pM. Signal to background ratios of >20:1 were obtained using an enzyme concentration of only 2.5 pM. We also performed an ATP titration to determine the K_m for this assay. Our experiments (Figure 3) yielded a K_m of 82 µM, similar to the value reported in the literature of 160 µM (1). We tested the ability of the BV assay to identify a known helicase inhibitor, mitoxantrone (4). We found that mitoxantrone effectively and reproducibly inhibited bacterial helicase IV at an enzyme concentration of 20 pM, yielding almost identical IC_{50}’s of 0.2 and 0.18 µM in two independent experiments (data not shown). These results were also consistent with the literature (4). In addition, this experiment contained 5% (v/v) DMSO which had no apparent effect on the assay signal. Furthermore, final DMSO concentrations up to 10% do not affect assay results (data not shown).

**Figure 2**

_E. coli_ helicase IV titration.

Helicase unwinding activity was determined as described in Methods. Data are plotted as mean signal (triangles and right hand axis) or % unwinding (squares and left hand axis) ± SD (n=6). Percent unwinding was calculated relative to a "100% Unwound" control sample prepared as described in the Methods.

**Figure 3**

ATP substrate titration.

Using helicase IV, the reaction was incubated for 15 minutes at RT under the conditions described in Methods. Data are plotted as mean ± SD (n=3). The inset graph is a Lineweaver-Burk plot of the same data.
PRODUCT SUMMARY

The BV helicase assay provides excellent signal to background ratios using much less enzyme than many other methods (2, 5). This feature of the assay is particularly useful for lead optimization. As more potent leads are produced, enzyme concentrations are often reduced to more accurately assess structure activity relationships (5). The BV Helicase assay format has the sensitivity, precision, and lack of interferences needed for these demanding studies, while also having the ease of assay set-up and minimal reagents required for a screening lab. The assay is readily adapted to other helicase types. To date, this assay has been successfully used (with no or only slight modification) to quantify activity of the bacterial helicases II, IV, and PcrA, and the viral HCV and SV40 helicases. BioVeris provides custom synthesis and labeling of oligonucleotides for this assay and other nucleic acid-based methods.

REFERENCES


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