

Use of a restriction enzyme-digested PCR product as substrate for helicase assays

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Received November 1, 2004; Revised December 1, 2004; Accepted December 14, 2004

ABSTRACT

DNA helicases play essential roles in many cellular processes. The currently available techniques to generate substrates for helicase assays are fairly complicated and need some expertise not available in all laboratories. Here, a PCR-based method to generate a substrate for a helicase assay is described, and its application for several archaeal, bacterial and viral enzymes is demonstrated.

INTRODUCTION

Many biological processes, including DNA replication, recombination, repair and transcription, require the transient unwinding of duplex DNA. This task is handled by a group of enzymes, DNA helicases, which catalyze the unwinding of duplex DNA using energy derived from nucleoside triphosphate hydrolysis to melt the duplex. Helicases translocate along one strand of DNA and displace the complementary strand (1,2) and have a specific polarity depending upon the single-stranded DNA (ssDNA) strand on which they move, either in the 3'→5' or in the 5'→3' direction.

To date, the most commonly used substrate for helicase assays use a short ³²P-labeled oligonucleotide annealed to a longer ssDNA molecule, either single-stranded plasmid DNA (M13 or φX174) (3,4) or to a long oligonucleotide (5). The helicase unwinds the duplex region presented in these partial duplex substrates, yielding two ssDNA molecule with different sizes that can be resolved from the starting duplex substrate by electrophoresis followed by autoradiography [summarized in (6,7)].

This approach has several disadvantages for routine work, especially for laboratories that do not routinely perform such assays. When oligonucleotides are used, they have to be labeled, annealed to each other and then purified from an acrylamide gel to remove unincorporated nucleosides and any excess unannealed oligonucleotides. In addition, the length of

the substrate is limited by the length of oligonucleotide that can be synthesized. When single-stranded plasmid DNA is used, a column (G-25 or G-50) is used to separate the labeled oligonucleotides from the substrate. To make a long substrate, an additional elongation step using DNA polymerase is needed.

Here, a new way to generate helicase substrates is described. This, a PCR-based approach, circumvents some of the problems encountered by the other conventional ways to make helicase substrates. This approach may be especially useful for laboratories that do not routinely perform helicase assays.

MATERIALS AND METHODS

Oligonucleotide-based helicase substrate preparation

Oligonucleotide DF54 (5'-GGGACGCGTCGGCCTGGCAC-GTCGGCCGCTGCGGCCAGGCACCCGATGGCGTTT-3') was labeled using [γ -³²P]ATP and T4 polynucleotide kinase. Labeling reactions were stopped by adding EDTA to a final concentration of 25 mM. The labeled DF54 oligonucleotide was hybridized to DF50c oligonucleotide (5'-GCCATCGGG-TGCCTGGCCGCAGCGGCCGACGTGCCAGGCCGACGC-GTCCC-3') at 1:2 molar ratio in a buffer containing 40 mM Hepes-NaOH (pH = 7.5) and 50 mM NaCl by heating to 100°C for 3 min followed by slow cooling to 25°C. Unincorporated [γ -³²P]ATP and unannealed oligonucleotides were removed using the following procedure. After hybridization, a 6× DNA loading buffer (0.1% xylene cyanol, 0.1% bromophenol blue and 50% glycerol) was added to a final concentration of 1×, and the mixture was fractionated through an 8% native polyacrylamide gel for 1 h at 100 V in 0.5× TBE (45 mM Tris, 4.5 mM boric acid and 0.5 mM EDTA). The substrate was located by autoradiography, the product was excised from the gel and sliced into small pieces and eluted in 3 vol of an elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH = 8.0) by incubating at 37°C for 2 h. After centrifugation, the supernatant was collected, and the insoluble material was extracted once more with elution buffer. Following centrifugation, both supernatant fractions were

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combined, and the DNA was ethanol precipitated and dissolved in TE (10 mM Tris-HCl, pH = 7.5, 1 mM EDTA). Specific activity of substrate was determined by liquid scintillation counting.

PCR-based helicase substrate preparation

Two PCR primers flanking a part of the pBluescript SK⁺ multiple cloning site were used to generate the DNA fragments used for the preparation of the helicase substrate. Using the primers SAC (5'-GAGCTCCACCGCGGTGGC-3', map position 743-760) and primer KPN (5'-GGTACCGGGCCCCCCTC-3', map position 653-670) resulted in a PCR fragment of 108 bp. Prior to the PCR reaction, 10 pmol of the SAC primer was ³²P-labeled in a 10 µl reaction mixture containing 1× T4 polynucleotide kinase buffer, 16.6 pmol of [γ -³²P]ATP (3000 Ci/mmol, GE Biosciences) and 5 U of enzyme (Fermentas). The mixture was incubated at 37°C for 30 min and directly added to the PCR reaction. The PCR reaction (50 µl) was performed with *Pyrococcus furiosus* (*Pfu*) polymerase (Stratagene) in 1× *Pfu* buffer in the presence of 5 ng pBluescript SK⁺ as template and 10 pmol of the labeled SAC primer and 10 pmol of KPN primer. For experiments using a labeled nucleotides in lieu of the labeled primer, 50 pmol of [α -³²P]dCTP (6000 Ci/mmol, GE Biosciences) was added to PCR reaction containing 0.05 mM dNTPs. For both PCR reactions, the products were purified by a QIAprep PCR purification kit (Qiagen) in order to remove excess primer and nucleotides. Specific activity of substrate was determined by liquid scintillation counting.

DNA helicase assay

Methanothermobacter thermautotrophicus minichromosome maintenance (MCM) helicase activity was measured as described previously (8,9) in reaction mixtures (15 µl) containing 20 mM Tris-HCl (pH = 8.5), 10 mM MgCl₂, 2 mM DTT, 100 µg/ml BSA, 5 mM ATP, 10 fmol of substrate and proteins as indicated in Figure 1. After incubation at 60°C for 30 min, reactions were stopped by adding 5 µl of 5× loading buffer (100 mM EDTA, 1% SDS, 0.1% xylene cyanol, 0.1% bromophenol blue and 50% glycerol), and aliquots were fractionated on an 8% native polyacrylamide gel in 0.5× TBE and electrophoresed for 1.5 h at 150 V at 4°C. The helicase activity was visualized and quantitated by phosphorimaging.

Helicase activities of the mesophilic enzymes (SV40 Large T-antigen, PriA, Rep, RecG, UvrD, RecQ) were measured in reaction mixtures (15 µl) containing 20 mM Tris-HCl (pH = 7.5), 10 mM MgCl₂, 2 mM DTT, 100 µg/ml BSA, 5 mM ATP, 10 fmol of substrate and 1.2 pmol of enzyme. Mixtures were incubated at 37°C for 30 min and analyzed as described for the *M.thermautotrophicus* MCM.

RESULTS AND DISCUSSION

A number of DNA helicases were shown to require only a short ssDNA overhang to initiate DNA unwinding. Examples are given in Table 1 and in Figure 1, which show that the *M.thermautotrophicus* MCM helicase required a minimum of 4 bases of 3' overhang for helicase activity. Therefore, one may suggest that a helicase substrate could be generated using restriction digest of DNA molecules to generate a 4 nt 3' or 5'

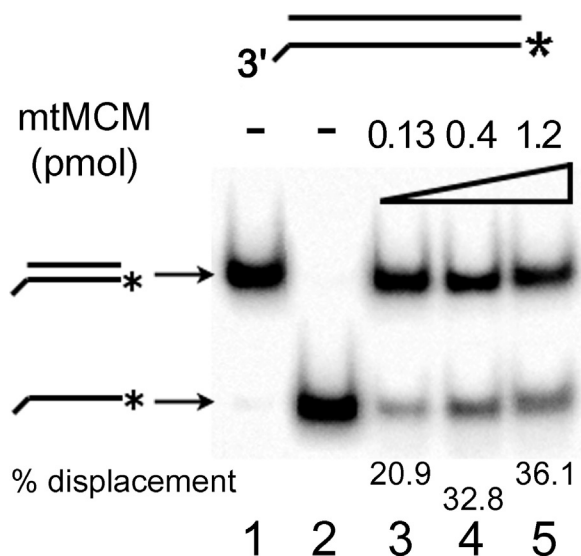


Figure 1. *M.thermautotrophicus* MCM can initiate duplex DNA unwinding from a short 3' single-stranded overhang. Two oligonucleotides were used to generate a helicase substrate with a 4-base ssDNA overhang. Helicase assays were performed as described in Material and Methods in 15 µl reactions with the indicated concentrations of protein and 10 fmol substrate. The ³²P-labeled oligonucleotide is marked with an asterisk. Lane 1, substrate only; lane 2, boiled substrate; lanes 3-5 contain 0.13, 0.40 and 1.2 pmol of proteins (as monomer), respectively. The percent displacement of the labeled oligonucleotide is indicated as %.

Table 1. Examples of DNA helicases that are capable of initiating DNA unwinding from a short (≤4 bases) ssDNA overhang

Helicase	Polarity
Calf DNA helicase E	3'→5'
<i>E.coli</i> DNA helicase II	3'→5'
<i>E.coli</i> RecB	3'→5'
<i>E.coli</i> RecQ	5'→3'
<i>E.coli</i> Rep helicase	3'→5'
<i>M.thermautotrophicus</i> MCM	3'→5'
SV-40 large T-antigen	3'→5'

ssDNA overhang. Thus, PCR can be used to generate a labeled product that upon digestion can serve as helicase substrate. This would make substrate preparation simpler in comparison with other techniques and ease preparation of long DNA substrates.

To determine whether a restriction enzyme-digested PCR product can serve as a helicase substrate, a protocol, summarized in Figure 2, was developed. A PCR reaction was performed with either ³²P-labeled primer (only one primer labeled) or in the presence of ³²P-dNTPs (see Materials and Methods). *Pfu* polymerase was used for the reaction, as this enzyme produces a blunt-ended product. On the other hand, *Thermus aquaticus* (*Taq*) DNA polymerase resulted in the addition of an adenine residue at the 3' end of the DNA and thus may serve as a substrate for helicases requiring only a single base 3' overhang. Following PCR, the product was purified using QIAquick PCR purification kit (Qiagen) and digested with restriction enzymes that generate either a 3' or a 5' 4-base ssDNA overhang. The restriction sites can be located in the middle of the fragment or in the primers.

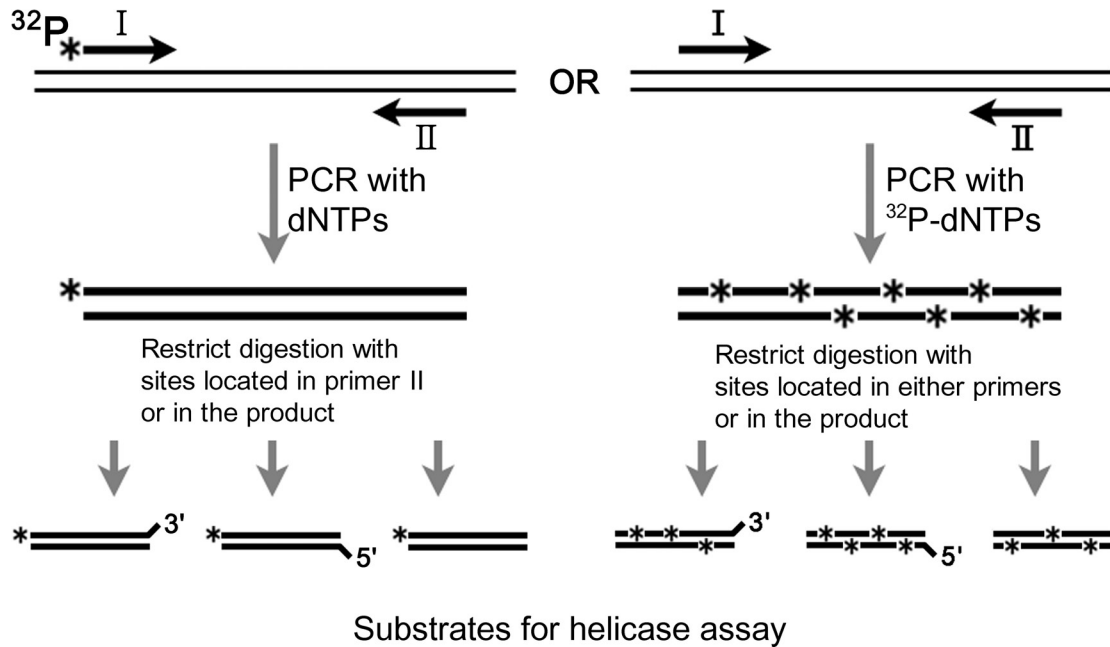


Figure 2. Schematic diagram for the procedures developed to generate a PCR-based substrate for helicase assays. Bold arrows are the PCR primers and ^{32}P is depicted by asterisks.

Following digestion, the DNA can be used directly, without any further purification, in a helicase assay. The protocol was tested using a number of different restriction enzymes, locations (in the primers versus within the PCR products) and different PCR product sizes. One set of these products is shown here.

PCR-based substrates were generated using the polylinker of pBluescript SK⁺ (Stratagene) as template and two primers, one of which was ^{32}P -labeled, encompassing a part of the polylinker resulting in a 108 bp fragment (Figure 3, lane 1). The product was digested with either PstI restriction enzyme, resulting in a 50 bp fragment containing 4 bases of 3' overhang (lane 2); EcoRI, yielding a 56 bp fragment containing 4 bases of 5' overhang (lane 3) or SmaI restriction enzyme, resulting in a 44 bp blunt-ended fragment (lane 4). In each case, there is an additional fragment produced with similar overhang that is not labeled. These substrates were used in a helicase assay for the *M.thermautotrophicus* MCM helicase (Figure 4A). As shown in Figure 4A, the enzyme efficiently unwinds the substrate containing a 3' overhang (lanes 3–5) but not the substrate containing a 5' overhang (lanes 8–10) or the blunt-ended substrate (lanes 13–15). This is similar to the observations made with substrates containing longer ssDNA regions, either when oligonucleotides were annealed to ssM13 (10,11) or to a longer oligonucleotide (12). The helicase was as efficient in unwinding the oligonucleotide-based substrate as the PCR-based substrate (cf. lanes 3–5 in Figures 1 and 4A). Other restriction enzymes have also been used with similar results (data not shown). These results demonstrate that a PCR-based helicase substrate is applicable for helicase studies.

M.thermautotrophicus MCM is active at high temperature (60°C). Therefore, in order to determine whether the approach is applicable to helicases that are active at lower temperature and with different polarities, a number of additional helicases

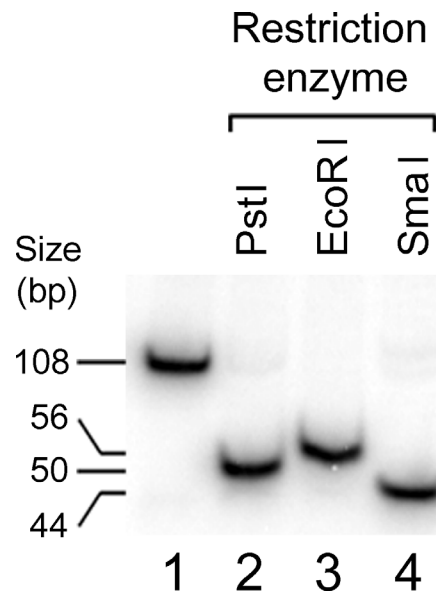


Figure 3. Substrates for helicase assays generated by PCR. A PCR product and its restriction fragment derivatives are shown. PCR reactions were performed as described in Materials and Methods with ^{32}P -labeled primer, and products were purified using the QIAquick PCR purification kit (Qiagen) (lane 1) and digest with PstI (lane 2), EcoRI (lane 3) and SmaI (lane 4) restriction enzymes.

were analyzed. As shown in Figure 4B, several helicases with 3'→5' or 5'→3' polarity (7) are active on the PCR-based substrate. However, as is evident from the results presented in Figure 4B, this is by no means an approach suitable for all helicases. It is known that some helicases require a longer ssDNA region or even are active only on a fork-like DNA substrate.

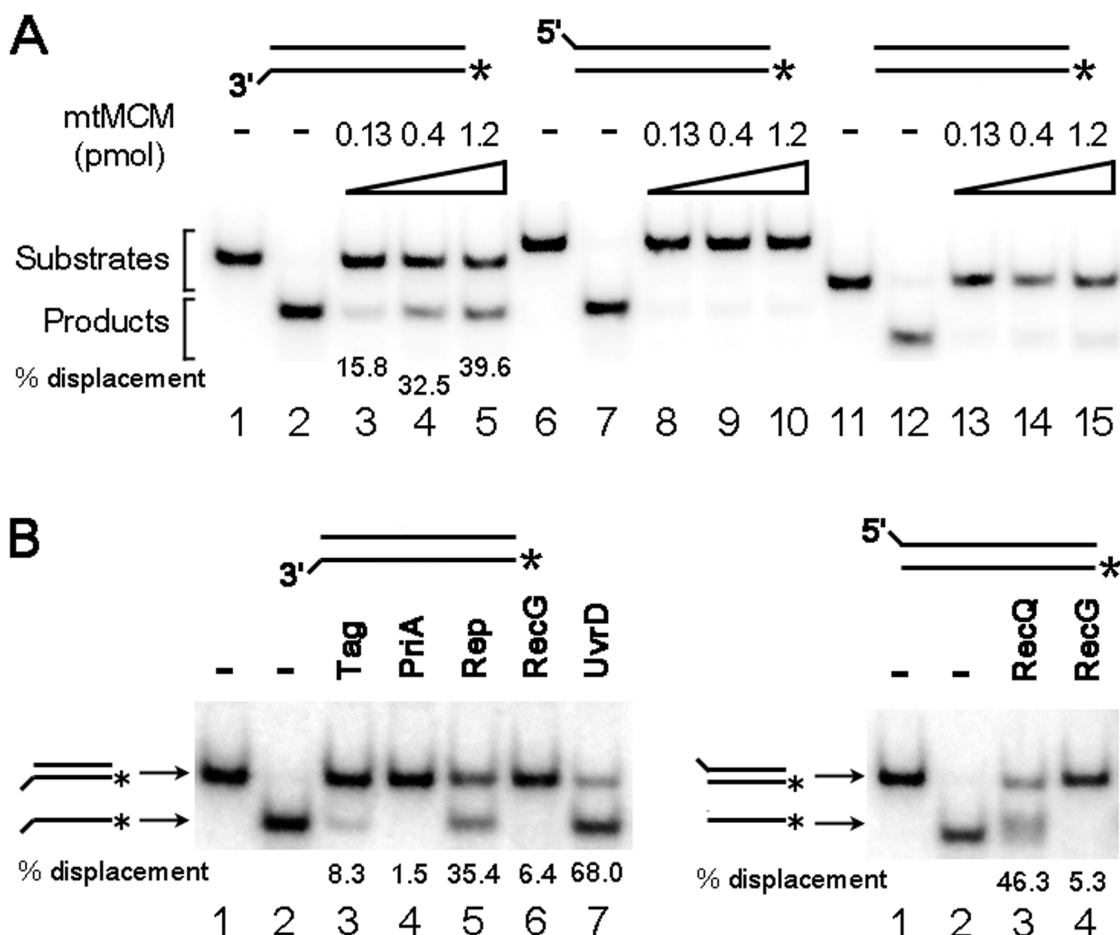


Figure 4. PCR-generated substrates can be used for helicase assays. (A) Helicase assays with the *M.thermautotrophicus* MCM helicase. Helicase assays were performed as described in Material and Methods with 10 fmol of substrate as indicated in the figure. Lanes 1, 6 and 11, substrate only; lanes 2, 7 and 12, boiled substrate; lanes 3–5, 8–10 and 13–15, containing 0.13, 0.40 and 1.2 pmol of proteins (as monomer), respectively. The ^{32}P -labeled strands are marked with an asterisk. The percent displacement of the labeled oligonucleotide is indicated as %. (B) PCR-generated substrates can be used for helicases with different polarities. Helicases with known 3'→5' polarity (left panel) or 5'→3' polarity (right panel). The helicase activity of several bacterial and viral helicases was determined using the PCR-based substrate containing either a 5' or a 3' single-stranded overhang. Helicase assays were performed as described in Material and Methods with 10 fmol of each substrate and 1.2 pmol of enzyme. ^{32}P -labeled strands are marked with an asterisk. Left panel: lane 1, substrate only; lane 2, boiled substrate; lane 3, SV-40 large T-antigen; lane 4, *E.coli* PriA; lane 5, *E.coli* Rep helicase; lane 6, *E.coli* RecG; lane 7, *E.coli* UvrD. Right panel: lane 1, substrate only; lane 2, boiled substrate; lane 3, *E.coli* RecQ; lane 4, *E.coli* RecG. The percent displacement of the labeled oligonucleotide is indicated as %.

In the past several years, a large number of new helicases have been identified in laboratories working on different aspects of nucleic acid enzymology. The technique described here may enable laboratories that do not routinely make helicase substrates to use readily available laboratory equipment and techniques to analyze putative helicases. It may also be used to generate helicase substrates containing specific DNA sequences, e.g. protein-binding sites, DNA sequences capable of forming Z-DNA, etc.

ACKNOWLEDGEMENTS

We thank Dr Jerard Hurwitz for providing the SV-40 large T-antigen and Dr Kenneth Marians for providing the *Escherichia coli* helicases. This work was supported by American Cancer Society Research Scholar Grant RSG-04-050-01-GMC, who also provided funding to pay the Open Access publication charges for this article.

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