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Assay for Human Rad51-Mediated DNA Displacement Loop Formation

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INTRODUCTION

Homologous recombination is an important mechanism for the repair of damaged chromosomes, for preventing the demise of damaged replication forks, and for several other aspects of chromosome metabolism and maintenance. The homologous recombination reaction is mediated by the Rad51 recombinase. In the presence of ATP, Rad51 polymerizes on single-stranded DNA (ssDNA) to form a nucleoprotein filament that is commonly referred to as the "presynaptic filament." The presynaptic filament is capable of locating a homologous duplex DNA molecule and catalyzing invasion of the duplex to form a DNA displacement loop called the "D-loop." This protocol describes an in vitro D-loop assay that uses a radiolabeled ssDNA oligonucleotide and a nonlabeled homologous supercoiled duplex DNA as substrates, and agarose gel electrophoresis together with PhosphorImaging for product analysis. To enhance the efficiency of the D-loop reaction, an ancillary factor (the Hop2-Mnd1 complex or Rad54) is included in the reaction. This reconstituted system provides researchers a biochemical means to dissect the mechanisms of the homologous recombination machinery.

MATERIALS

Reagents

It is imperative that highly purified proteins are used to avoid artifacts arising from contaminating nuclease, DNA helicase, or topoisomerase activities. For optimal activity of the purified homologous recombination proteins, avoid repeated freeze-thaw cycles.

[γ-^{32}P]ATP (10 mCi/mL, 6000 Ci/mmoL; Amersham Bioscience)

Agarose gel (0.9%)

Agarose gel loading buffer

D-loop reaction buffer (5X)

Oligonucleotide D1 (5′-AAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTT-3′)

The oligonucleotide is complementary to positions 1932–2022 of pBluescript SK DNA.

pBluescript SK plasmid DNA (Stratagene)

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Plasmid Midi kit (QIAGEN)

Proteinase K (Roche Applied Science)

Recombinant Hop2-Mnd1 complex, purified (5 μM) or recombinant human Rad54, purified (5 μM) (see Step 5)

Purify Hop2-Mnd1 complex as described by Chi et al. (2007).

Purify Rad54 as described by Sigurdsson et al. (2002).

Recombinant human Rad51, purified (5–10 μM)

Purify as described by Sigurdsson et al. (2001).

SDS (Sodium dodecyl sulfate; 10%)

T4 polynucleotide kinase (New England Biolabs)

T4 polynucleotide kinase buffer (10X; New England Biolabs)

TAE

Dilute the stock solution to 1X before use.

TE buffer, 10X

Dilute to 1X before use.

Equipment

Chromatography paper, DE81 (Whatman)

Equipment for agarose gel electrophoresis

Gel dryer

Heating blocks preset to 25°C, 37°C, and 65°C

Ice

Lead weight

MERmaid Spin Kit (Bio 101)

Paper towels

PhosphorImager

Plate, Plexiglas (or similar material) or glass

Radioactive safety and waste handling equipment

Spectrophotometer

Tubes, microcentrifuge (1.5-mL)

METHOD

Preparation of DNA Substrates

1. 5′-End-label the oligonucleotide D1 with [[γ-^{32}P]ATP:

i. Mix in a 1.5-mL microcentrifuge tube:

Oligonucleotide D1 (100 μM)	1.5 μL
T4 polynucleotide kinase buffer (10X)	10 μL

T4 polynucleotide kinase (10 U/μL)	4 μL
[γ-^{32}P]ATP, 10 mCi/mL	10 μL
H_2O	74.5 μL

ii. Incubate for 30 min at 37°C.

iii. Terminate the reaction by incubating for 20 min at 65°C.

iv. Remove unincorporated [γ-^{32}P]ATP using the MERmaid Spin kit according to the manufacturer's instructions.

v. Measure the DNA concentration spectrophotometrically. Store the labeled substrate at −20°C until use.

2. Prepare pBluescript SK DNA using QIAGEN Plasmid Midi kit (or equivalent). Store in TE buffer at −20°C until use.

Reaction efficiency is sensitive to the topological state of the duplex. Take care to minimize nicking of the supercoiled DNA during plasmid isolation.

D-Loop Assay

3. Prepare a 1.5-mL microcentrifuge tube on ice for each reaction:

i. Add 2.5 μL of 5X D-loop reaction buffer to each tube.

ii. Add purified Rad51 to a final concentration of 1 μM.

iii. Add ^{32}P-labeled oligonucleotide D1 prepared in Step 1 to a final concentration of 3 μM nucleotide.

iv. Add H_2O to a final volume of 11 μL.

4. Incubate the reactions for 5 min at 37°C to assemble the Rad51-ssDNA presynaptic filament.

5. For each reaction:

Option I

i. Add 0.5 μL of Hop2-Mnd1 (250 nM final concentration).

ii. Incubate for 1 min at 37°C.

iii. Initiate the reaction by adding 1 μL of pBluescript SK DNA (600 μM of base pairs). Incubate at 37°C.

Option II

iv. Add 0.5 μL of Rad54 (250 nM final concentration).

v. Incubate for 1 min at 25°C.

Rad54 rapidly becomes inactivated at 37°C.

vi. Initiate the reaction by adding 1 μL of pBluescript SK DNA (600 μM of base pairs). Incubate at 25°C.

*For optimal efficiency, human Rad54 necessitates the use of the rad51 K133R mutant (*Sigurdsson et al. 2002*) or specialized conditions that stabilize the Rad51 presynaptic filament (*Mazina and Mazin 2004*). Hop2-Mnd1 is much more versatile in this regard.*

6. Prepare a solution that contains 10% SDS and 10 mg/mL of proteinase K. Withdraw 6 μL of the reactions at the desired times (typically 2 min and 5 min). Mix each aliquot with 0.8 μL of the SDS/proteinase K solution.

7. Incubate the reaction mixtures for 5 min at 37°C.

8. Mix the deproteinized reaction mixtures with 2 μL of agarose gel loading buffer.

9. Resolve the mixtures in a 0.9% agarose gel in TAE buffer at 130 mA for 90 min at 25° C.

10. Sandwich the gel between two stacks of 20 paper towels. Place a Plexiglas or glass plate and a heavy lead weight on top of the stack for 5 min.

11. Dry the compressed gel onto Whatman DE81 chromatography paper using a gel dryer for 1 h at 80°C.

12. Expose the dried gel to a phosphor screen for the appropriate length of time (typically 5 h). Analyze using a PhosphorImager.

The D-loop product appears as a band that migrates much more slowly than the radiolabeled oligonucleotide substrate.

See Troubleshooting.

TROUBLESHOOTING

Problem: D-loop product formation is poor.

Solution: Consider the following:

1. The protein concentrations may not be optimal. Titrate the amount of Rad51 from 0.6 μM to 1.4 μM, and the amount of Hop2-Mnd1 or Rad54 from 100 nM to 300 nM to determine the concentrations of proteins that give the most robust D-loop reaction.
2. The proteins may be inactive. Purified proteins should be stored at −80°C. Once thawed, Rad51 and Hop2-Mnd1 are stable for at least 1 wk and Rad54 is stable for at least 3 d when stored on ice in their concentrated form.
3. The proteins may be contaminated with nuclease or topoisomerase activity. Such proteins will require further purification to eliminate the contaminating activity.
4. The duplex DNA may be nicked or relaxed. D-loop formation is most efficient when the duplex DNA is negatively supercoiled.

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