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Reconstituted System for the Examination of DNA Repair Synthesis in Homologous Recombination

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Abstract

In homologous recombination (HR), DNA polymerase δ -mediated DNA synthesis occurs within the displacement loop (D-loop) that is made by the recombinase Rad51 in conjunction with accessory factors. We describe in this article the reconstitution of the D-loop and repair DNA synthesis reactions using purified *S. cerevisiae* HR (Rad51, RPA, Rad54) and DNA replication (PCNA, RFC, and DNA polymerase δ) proteins and document the role of the Pif1 helicase in DNA synthesis via a migrating DNA bubble intermediate. These reconstituted systems are particularly valuable for understanding the conserved mechanism of repair DNA synthesis dependent on DNA polymerase δ and its cognate helicase in eukaryotic organisms.

Keywords

Homologous Recombination; BIR; D-loop; DNA synthesis; DNA Polymerase δ ; Pif1

1. Introduction

HR is a conserved pathway for the removal of DNA double strand breaks (DSBs) and the repair of injured DNA replication forks. Herein, a homologous DNA sequence is engaged by the processed lesion and serves as the template for DNA synthesis to initiate a usually error-free repair process (Jasin & Rothstein, 2013; Symington, Rothstein, & Lisby, 2014). Lesion processing entails nucleolytic resection of the 5' DNA strand associated with a break end to generate a 3'-ended DNA tail of a considerable length (Daley, Niu, Miller, & Sung, 2015). The ssDNA tail is bound by the conserved recombinase enzyme Rad51 and its accessory factors, and the resulting nucleoprotein ensemble then searches for and engages the homologous locale either in the sister chromatid or homologous chromosome, followed by invasion of the latter to form a hybrid DNA joint called the displacement loop, or D-loop (Kowalczykowski, 2015; San Filippo, Sung, & Klein, 2008). It should be noted that in the vast majority of eukaryotes, an additional, meiosis-specific recombinase called Dmc1 is needed for optimal inter-homolog recombination (Brown & Bishop, 2014; Hunter, 2015), but here we focus on Rad51 only.

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Rad51 catalyzes D-loop formation within the context of a right-handed nucleoprotein filament known as the presynaptic filament, whose assembly requires ATP binding by Rad51, although ATP hydrolysis prompts the dissociation of Rad51 protomers from the DNA ligand (Sung 1994). D-loop formation by the presynaptic filament is enhanced by associated factors including the Swi2/Snf2 family proteins Rad54 and Rdh54 and the ssDNA binding protein RPA (Daley, Gaines, Kwon, & Sung, 2014; Heyer, 2007). Following strand invasion, de novo DNA synthesis occurs within the D-loop. Several DNA polymerases (Pols), namely, Pol δ and Pol ϵ in yeast (Deem et al., 2011; Hicks, Kim, & Haber, 2010; Maloisel, Fabre, & Gangloff, 2008) and Pol η in vertebrates, (Kawamoto, Araki, Sonoda, Yamashita, Harada, Kikuchi, et al., 2005; McIlwraith, Vaisman, Liu, Fanning, Woodgate, et al., 2005; Buisson, Niraj, Pauty, Maity, Zhao, Coulombe, et al., 2014) have been proposed to contribute to DNA synthesis, and among them, Pol δ has emerged as the major player (Lydeard, Jain, Yamaguchi, & Haber, 2007; Maloisel, Fabre, & Gangloff, 2008; Wilson, Kwon, Chung, Chi, Niu, et al., 2013). Following repair DNA synthesis, the extended D-loop is resolved via one of several mechanistically distinct pathways to yield mature recombinants of different classes (Sung & Klein, 2006; Symington, Rothstein, & Lisby, 2014). The involvement of Pol δ in repair DNA synthesis has been studied most extensively within the context of a HR pathway called break-induced DNA replication (BIR), in which an invading DNA strand primes DNA synthesis capable of copying an entire arm of the donor chromatid (Figure 1) (Donnianni & Symington, 2013; Lydeard et al., 2007; Saini, Ramakrishnan, Elango, Ayyar, Zhang, Deem, et al., 2013; Wilson et al., 2013; Costantino, Sotiriou, Rantala, Magin, Mladenov, Helleday, et al., 2014; Dilley, Verma, Cho, Winters, Wondisford, & Greenberg, 2016)

Importantly, in genetic studies done *S. cerevisiae*, Pif1, a member of the SF1 family of helicases, has been implicated in BIR in conjunction with Pol δ (Chung, Zhu, Papusha, Malkova, & Ira, 2010; Saini et al., 2013; Wilson et al., 2013). Using highly purified *S. cerevisiae* proteins, a system that permits dissection of the mechanistic underpinnings of D-loop-primed DNA synthesis reaction has been developed (Li et al., 2009; Sebesta et al., 2011; Wilson et al., 2013). Using this system, we have shown that Pif1 greatly stimulates Pol δ -mediated DNA extension within the context of the Rad51-made D-loop. Importantly, we have furnished evidence that Pif1 fulfills two distinct functions in the DNA synthesis reaction, namely, (i) it enhances the ability of the polymerase ensemble to catalyze DNA strand displacement synthesis via an interaction with the proliferating cell nuclear antigen (PCNA), the polymerase processivity clamp, and (ii) concomitant with DNA synthesis, Pif1 dissociates the invading strand to establish a migrating DNA bubble structure (Figure 1) (Wilson et al., 2013). This chapter describes the materials and experimental procedures for reconstituting the repair DNA synthesis reaction using a ssDNA oligonucleotide as the invading strand and supercoiled dsDNA as the information donor. Our method utilizes Rad51, RPA, and Rad54 to generate D-loops and PCNA, the multi-subunit PCNA loader Replication Factor C (RFC), the trimeric Pol δ , and Pif1 helicase in the DNA synthesis phase of the reaction. The methods for product analyses are also described.

2. Assembling and analysis of the D-loop reaction

This protocol describes the procedures for forming the Rad51 presynaptic filament on ^{32}P -labeled 90-mer ssDNA (Figure 2A) and the generation of D-loops using supercoiled pBluescript plasmid DNA as recipient and the ssDNA binding protein RPA and the dsDNA translocase Rad54 as accessory factors (Petukhova, Stratton, & Sung, 1998; Raschle, Van Komen, Chi, Ellenberger, & Sung, 2004; Sugiyama, Zaitseva, & Kowalczykowski, 1997). Since Rad54 hydrolyzes a large quantity of ATP, an ATP regenerating system consisting of creatine phosphate (CP) and creatine kinase (CK) should be included to avoid ATP depletion (Petukhova et al., 1998). Note that RPA, but not Rad54, is also needed for the efficiency of the subsequent DNA synthesis reaction (Yuzhakov, Kelman, Hurwitz, & O'Donnell, 1999).

2.1 Purification of ssDNA by denaturing polyacrylamide gel electrophoresis

2.1.1 Equipment

- Standard polyacrylamide gel electrophoresis (PAGE) equipment (e.g. PROTEAN II xi cell (Bio-Rad))
- Standard agarose gel electrophoresis equipment
- Circulating water bath with cooling and heating functions
- Dialysis membrane (e.g. FlexTube (IBI Scientific))
- Handheld UV lamp
- Amicon Ultra-4 microconcentrators (Millipore)
- Standard centrifuge (e.g. Sorvall Evolution RC (Thermo Scientific)), Rotor (e.g. SLA 600TC (Thermo Scientific)) and conical tube adaptor

2.1.2 Reagents and buffers

- Oligo-D (90-mer): synthetic DNA oligonucleotide that is homologous to residues 1932–2022 of pBluescript SK plasmid DNA (5'-AAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTT-3') (Raschle et al., 2004; Raynard & Sung, 2009)
- TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA)
- Gel-loading buffer (94% formamide, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.05% bromophenol blue)
- TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA)
- 10% polyacrylamide gel (20 cm × 16 cm × 1 mm) made in TAE buffer containing 7M urea

2.1.3 Procedure

- Dissolve Oligo-D in TE buffer to 10 µg/µl.

- Mix 500 µg Oligo-D and 100 µl gel-loading buffer and heat the sample at 55°C for 5 min.
- With the use of the circulating bath, preheat the urea-containing polyacrylamide gel to 55°C (Sambrook, Fritsch, & Maniatis, 1989).
- Load the oligonucleotide solution onto the polyacrylamide gel and carry out electrophoresis at 150V for 2 hr (or until the bromphenol blue dye migrates through ~70% of the gel).
- Remove the top glass plate from the gel and identify the major DNA band with the UV lamp.
- Excise the gel slice containing the major DNA species and recover the DNA by electroelution at 100V for 2 hr in a dialysis membrane in the gel box normally used for agarose gel electrophoresis.
- Concentrate the eluted DNA in the microconcentrator to ~0.9 µg/µl (3 mM nucleotides) and store it at -20°C.

2.2 5' ³²P-end labeling of ssDNA

2.2.1 Equipment

- Standard PAGE equipment (e.g. Mini-PROTEAN vertical electrophoresis cell (Bio-Rad))
- Micro Bio-Spin 6 column (Bio-Rad)
- Vacuum gel dryer
- Phosphorimager (e.g. Personal Molecular Imager System (Bio-Rad))
- Quantity One software (Bio-Rad) for phosphorimage analysis.
- Heating block
- Microcentrifuge
- Amersham Hybond-N+ membrane (GE Healthcare)
- Whatman 3MM chromatography paper (GE Healthcare)

2.2.2 Reagents and buffers

- Gel-purified Oligo-D (3 mM nucleotides)
- T4 Polynucleotide Kinase (PNK, 10 U/µl) and 10X buffer (NEB)
- [γ -³²P]ATP (6,000Ci/mmol, 10 µCi/µl) (Perkin Elmer Life Science)
- TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA)
- TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA)
- Gel loading buffer (15 mM Tris-HCl, pH 7.5, 25% glycerol, 0.05% Orange G)
- 8% polyacrylamide gel made in TAE buffer

2.2.3 Procedure

- Mix the following and incubate at 37°C for 60 min.
 - Oligo-D, 3 µl
 - PNK buffer (10X), 5 µl
 - PNK, 2 µl
 - [γ -³²P]ATP (10 µCi/µl), 5 µl
 - H₂O, to 50 µl
- Heat the reaction mixture at 65°C for 20 min to inactivate PNK.
- Set aside 0.5 µl of the reaction mixture (Sample A) for PAGE analysis later
- Remove the unincorporated [γ -³²P]ATP using the Micro Bio-Spin 6 column (Bio-Rad) at 1,000Xg for 4 min in a microcentrifuge, and then adjust the volume to 100 µl with H₂O (Sample B).
- Mix separately Sample A and 1 µl Sample B with 10 µl of gel loading buffer and resolve them in the polyacrylamide gel at 100V for 30 min with TAE as the running buffer (Sambrook et al., 1989).
- Dry the gel onto Amersham Hybond-N+ membrane layered on Whatman 3MM Chromatography paper in the vacuum drier at 80°C.
- Expose the dried gel to a phosphor screen and quantify the signals in the phosphorimager using the Quantity One software; this serves to calculate the yield of ³²P-labeled Oligo-D.
- Store the labeled DNA in small aliquots at -20°C.

2.3 D-loop reaction

2.3.1 Equipment

- Heating block
- Microcentrifuge

2.3.2 Reagents and buffers

- ³²P-labeled Oligo-D (90 µM nucleotides)
- pBluescript SK supercoiled DNA*¹ (440 µM base pairs, prepared as described in (Raynard & Sung, 2009))
- Buffer A (5X stock) (135 mM Tris-HCl, pH 7.5, 5 mM DTT)
- Protein storage buffer (25 mM Tris-HCl, pH 7.5, 150 mM KCl, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal CA-630 (Sigma), 1 mM DTT)
- MgCl₂ (100 mM)
- BSA (10 mg/ml in TE buffer)

- ATP*² (100 mM, pH 7.5)
- Creatine phosphate*² (CP, purchased from Roche Diagnostics, 1M in H₂O)
- Creatine kinase*² (CK, purchased from Roche Diagnostics, 1.5 mg/ml in H₂O)
- Solution of dATP, dCTP, dGTP, TTP*³ (25 mM each in H₂O).
- Rad51 (23 μM, expressed and purified as described in (Van Komen, Macris, Sehorn, & Sung, 2006))
- Rad54 (2.5 μM, expressed and purified as described in (Raschle et al., 2004))
- RPA (5 μM, expressed and purified as described in (Van Komen et al., 2006))
- SDS (10%)
- Proteinase K (PK, purchased from Roche Diagnostics, 10 mg/ml in H₂O)

Note *1: D-loop formation is enhanced by supercoiling in the dsDNA (Van Komen, Petukhova, Sigurdsson, Stratton, & Sung, 2000). Analyze the pBluescript plasmid DNA by native gel electrophoresis in a 0.9% agarose gel and by ethidium bromide staining (Sambrook et al., 1989). The majority (90%) of the DNA should be in the supercoiled form.

Note *2: Keep the stocks of ATP, CP, and CK in small aliquots at –80°C and use only freshly thawed reagents for the reaction.

Note *3: dNTPs are not needed for D-loop formation, but are essential for the DNA synthesis reaction to be described later.

2.3.3 Procedure

- For each 20 μl reaction, mix the following reagents in a 1.5 ml microcentrifuge tube:
 - 4 μl Buffer A (5X stock)
 - 0.4 μl ATP
 - 0.4 μl CP
 - 0.4 μl CK
 - 1.4 μl MgCl₂
 - 0.53 μl ³²P-labeled Oligo-D (2 μM nucleotides final concentration)
 - 0.08 μl dNTP mixture (100 μM final concentration)

An appropriate amount of H₂O to adjust the final reaction volume (see below) to 20 μl after protein additions

- Add Rad51*¹ (0.6 μM final concentration) to the above and incubate at 37°C for 10 min.

- Add RPA*¹ (0.4 μ M final concentration) to the above and incubate at 30°C for 5 min.
- Add Rad54*¹ (0.3 μ M final concentration) to the above and incubate at 23°C for 2 min.
- Add pBluescript (35 μ M base pairs final concentration) and incubate at 30°C for 2 min.
- Leave the tube on ice and then proceed to the steps in Section 3 or terminate and deproteinize the reaction by adding 1 μ l SDS and 1 μ l PK followed by a 5-min incubation at 37°C.

Note *1: The optimal amount of each protein can vary depending on the specific activity of protein preparations. In our experience, when stored on ice, Rad51 and RPA remain stable for at least one week, while Rad54 begins to lose its enzymatic activity after 2 days. Once thawed from -80°C storage, the protein preparations should be used immediately and not be refrozen.

3. Repair DNA synthesis reaction

This protocol is for assembling the DNA synthesis reaction using D-loops generated as described in section 2.3.3. The protein species needed for this reaction are the three-subunit Pol δ , the five-subunit RFC, homotrimeric PCNA and the Pif1 helicase. As shown in Figure 2B, Pol δ together with RFC and PCNA can efficiently extend the ³²P-labeled invading strand, while the addition of Pif1 leads to a strong stimulation of the DNA synthesis track length and the formation of a migrating DNA bubble structure with a growing 5' ssDNA tail produced as a result of Pif1-mediated dissociation of the extended invading strand. Methods for DNA synthesis product analysis are described in Section 4 and Section 5.

3.1 Equipment

- Heating block
- Microcentrifuge
- Water bath

3.2 Reagents and buffers

- Protein storage buffer (25 mM Tris-HCl, pH 7.5, 150 mM KCl, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal CA-630 (Sigma), 1 mM DTT)
- Pol δ (2 μ M*¹, purified as described in (Wilson et al., 2013))
- RFC (4 μ M*¹, expressed and purified as described in (Wilson et al., 2013))
- PCNA (4 μ M*¹, expressed and purified as described in (Sebesta et al., 2011))
- Pif1 (800 nM supplemented with 100 ng/ μ l BSA, expressed and purified as described in (Wilson et al., 2013))
- SDS (10%)

- Proteinase K (PK, 10 mg/ml)

Note *1: The optimal amount of each protein can vary depending on the specific activity of protein preparations. In our experience, when stored on ice, PCNA is stable for at least one week, while RFC, Pol δ and Pif1 lose activity after two days. Once thawed from -80°C storage, the protein preparations should be used immediately and not be refrozen.

3.3 Procedure

- Mix RFC and PCNA (final concentration of 200 nM each) and leave on ice.
- Add 16 μl of the D-loop reaction from section 2.3.3 to the PCNA-RFC mixture and incubate on ice for 2 min.
- Add Pol δ (100 nM final concentration) and Pif1 (40 nM final concentration) and incubate at 15°C up to 8 min.
- Terminate and deproteinize the synthesis reaction by adding 1 μl SDS and 1 μl PK and a 10-min incubation at 37°C .

4. Analysis of D-loops and Extended D-loops

The products (D-loops, extended D-loops, and extended D-loops with a migrating bubble) from the D-loop and DNA synthesis reactions can be resolved by native gel electrophoresis, while the length of the DNA synthesis track can be more accurately determined by denaturing gel electrophoresis and 2-D gel electrophoresis.

4.1 Equipment

- Standard PAGE equipment (e.g. PROTEAN II xi cell (Bio-Rad))
- Standard agarose gel electrophoresis equipment and setup for phosphorimaging analysis as described earlier.

4.2 Reagents and buffers

- Native gel loading buffer (15 mM Tris-HCl, pH 7.5, 25% glycerol, 0.05% Orange G)
- Denaturing gel loading buffer (94% formamide, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.05% bromophenol blue)
- TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA)
- TBE buffer (90 mM Tris-HCl, pH 8.3, 90 mM boric acid, 2 mM EDTA)
- Alkaline solution 1 (0.5 M NaOH, 10 mM EDTA)
- Alkaline solution 2 (50 mM NaOH, 1 mM EDTA)
- Neutralization buffer (0.5 M Tris-HCl, pH 7.5)
- 0.8% agarose gel made in TAE buffer

- 4% polyacrylamide gel (20 cm × 16 cm × 1 mm) made in TBE buffer containing 7M urea
- 0.9% agarose gel (11 cm × 15 cm × 6 mm) made in alkaline solution 2

4.3 Native gel electrophoresis

- Mix 10 µl of the reaction mixture described in section 3.3 with 10 µl of native gel loading buffer.
- Resolve the mixture in the 0.8% agarose gel at 90 mA for 120 min at room temperature.
- Dry the gel as described in section 2.2.3.
- Subject the dried gel to phosphorimaging analysis as described in section 2.2.3. A schematic of the expected results and actual results from a typical experiment are shown in Figure 2C and 2D, respectively.

4.4 Denaturing gel electrophoresis

4.4.1—This protocol is appropriate for characterizing DNA synthesis products up to 1.5 kilobases in length.

- Mix 10 µl of the reaction from section 3.3 with 10 µl of denaturing gel loading buffer and incubate at 95°C for 5 min.
- Resolve the mixture in the denaturing polyacrylamide gel at 200 mA at 55°C for 90 min (Sambrook et al., 1989).
- Dry the gel and analyze the dried gel in the phosphorimager as described in section 2.2.3.

4.4.2—This protocol is for characterizing DNA synthesis products up to several kilobases in length.

- Mix 10 µl of the reaction from section 3.3 with 2 µl alkaline solution 1 and 8 µl native gel loading buffer
- Resolve the mixture in the agarose gel in alkaline solution 2 at 50 mA for 300 min (Sambrook et al., 1989).
- Soak the gel in neutralization buffer for 20 min.
- Dry the gel and analyze the dried gel in the phosphorimager as described in section 2.2.3.

4.5 2D-gel electrophoresis

The 2D-gel analytical protocol provides an independent means for determining the length of DNA synthesis products and also of the population of extended invading strand that has been dissociated from the D-loop by Pif1. Herein, the reaction products from section 3.3 are first separated by native agarose gel electrophoresis (Figure 3A, (i)) as described in section 4.3, followed by a second electrophoretic step in a denaturing agarose gel (Figure 3A, (ii)).

- Carry out native gel electrophoresis of the DNA synthesis products in duplicate following the procedure described in section 4.3.
- Dry down one of the two lanes as in section 2.2.3.
- Place the other lane on the leading edge of a gel tray (11 cm × 15 cm × 6 mm) and pour 100 ml of molten 0.9% agarose in alkaline solution 2 to imbed the lane.
- Develop the gel in alkaline solution 2 at 50 mA for 8 hr.
- Soak the gel in neutralization buffer for 20 min.
- Dry the gel and analyze the dried gel in the phosphorimager as described in section 2.2.3 (Figure 3B).

5. Analysis of DNA synthesis within a migrating D-loop

5.1 Equipment

- Standard PAGE equipment (e.g. PROTEAN II xi cell (Bio-Rad))
- Standard agarose gel electrophoresis equipment and setup for phosphorimaging analysis as described earlier.
- Heating block
- Microcentrifuge
- Gel documentation station fitted with a UV light source

5.2 Reagents and buffers

- Restriction enzymes *AhdI* and *XmnI* (NEB)
- Calf thymus Topoisomerase I (Invitrogen)
- SDS (10%)
- Proteinase K (PK, 10 mg/ml)
- BSA (10 mg/ml in TE buffer)
- Phenol-chloroform-isoamyl alcohol or PCI mix (25:24:1)
- NaOAc (3M, pH 5.2)
- Ethanol
- Chloroform
- Ethidium bromide (Sigma-Aldrich)
- Native gel loading buffer (15 mM Tris-HCl, pH 7.5, 25% glycerol, 0.05% Orange G)
- Denaturing gel loading buffer (94% formamide, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.05% bromophenol blue)

- TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA)
- TBE buffer (90 mM Tris-HCl, pH 8.3, 90 mM boric acid, 2 mM EDTA)
- Alkaline solution 1 (0.5 M NaOH, 10 mM EDTA)
- Alkaline solution 2 (50 mM NaOH, 1 mM EDTA)
- Neutralization buffer (0.5 M Tris-HCl pH 7.5)
- TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA)
- NEBuffer 4 (50 mM K-acetate, 20 mM Tris-acetate, pH 7.9, 10 mM Mg-acetate, 1 mM DTT)
- 0.8% agarose gel made in TAE buffer
- 4% polyacrylamide gel (20 cm × 16 cm × 1 mm) made in TBE buffer containing 7M urea

5.3 Verification of the supercoiled state of duplex DNA

It is crucial to verify that the pBluescript plasmid DNA retains its supercoiled state during DNA synthesis. The analysis provides assurance that extensive DNA synthesis is mediated via D-loop migration and not through a DNA relaxation or rolling circle mechanism that could result from a contaminating topoisomerase or endonuclease activity, respectively. The method described here helps verify that the plasmid DNA template remains intact.

- Mix 10 µl of the reaction from section 3.3 with 10 µl native gel loading buffer and incubate at 95°C for 2 min to dissociate the extended invading DNA strand from the supercoiled pBluescript plasmid DNA.
- Resolve the mixture along with supercoiled pBluescript marker DNA in the 0.8% agarose gel at 90 mA for 120 min using TAE as the electrophoresis buffer.
- Soak gel in H₂O with ethidium bromide (1 µl/ml final concentration) for 10 min to stain DNA and then in a large volume of H₂O for 1 h to reduce background staining.
- Record the stained DNA species in the gel documentation station.

5.4 Testing for dependence of DNA synthesis track length on topoisomerase

Topological stress that accumulates during extension of the invading strand would inhibit the movement of the polymerase ensemble within the D-loop. In this case, the addition of a topoisomerase can lead to an increase of the DNA synthesis track length (Li et al., 2009; Wilson et al., 2013). However, in the bubble-migration mode of DNA synthesis, topological stress in the plasmid DNA is relieved via dissociation of the extended invading strand mediated by Pif1 (Wilson et al., 2013). Therefore, the lack of any stimulatory effect of topoisomerase addition provides experimental support for the bubble migration mechanism (Wilson et al., 2013). Here, we describe the method to test whether DNA synthesis is responsive to topoisomerase addition.

- Perform the DNA synthesis reaction with RFC, PCNA and Pol δ as described in section 3.3.
- After a 4-min incubation, add Pif1 (40 nM final concentration) and 8 units of calf thymus topoisomerase I to the reaction mixture (20 μ l final volume) and incubate for 8 min.
- As controls, carry out the reaction in the same fashion but omit Pif1 and/or topoisomerase I.
- Terminate and deproteinize the reaction by adding 0.5 μ l SDS and 1 μ l PK and incubating it at 37°C for 10 min.
- Resolve the reaction mixtures in a denaturing polyacrylamide gel and analyze the DNA products in the phosphorimager as described in section 2.2.3.

5.5 Analysis of the DNA synthesis products by restriction enzyme digests

This analysis is to provide definitive biochemical evidence for a migrating DNA bubble established during DNA synthesis. The conceptual basis for the method, which relies on monitoring the susceptibility of sites in the pBluescript plasmid molecule to restriction enzymes, is explained in Figure 4A.

- Perform the DNA synthesis reaction (20 μ l) with RFC, PCNA and Pol δ as described in section 3.3.
- After a 4-min incubation, add Pif1 (40 nM final concentration) and incubate for 8 min.
- As control, carry out the reaction in the same fashion but omit Pif1.
- Mix the reaction with 80 μ l TE and extract with 100 μ l PCI and subsequently with 100 μ l chloroform twice to deproteinize as described in (Sambrook et al., 1989).
- Add 10 μ l NaOAc (3M, pH 5.2) and 300 μ l absolute ethanol followed by a 1 h-incubation on ice to precipitate DNA (Sambrook et al., 1989).
- Collect DNA precipitate by centrifugation and wash the pellet with 300 μ l ice cold 70% ethanol twice and then air dry the pellet.
- Dissolve the pellet with 10 μ l of NEBuffer 4 supplemented with 0.1 mg/ml BSA.
- Add 2.5 U of *AhaI* or 10 U of *XmnI* (20 U/ μ l) and incubate at 37°C for 10 min.
- Terminate and deproteinize the reaction by adding 0.5 μ l SDS and 1 μ l PK and incubating it at 37°C for 10 min.
- Add 5 μ l of denaturing gel-loading buffer and incubate at 95°C for 5 min.
- Resolve the DNA species by electrophoresis in the denaturing polyacrylamide gel at 55°C and 200 mA for 90 min using TBE buffer as the electrophoresis buffer.

- Dry the gel and analyze the dried gel by phosphorimaging analysis as described in section 2.2.3 (Figure 4B).

5.6 Special Note

The presence of a migrating DNA bubble in the DNA synthesis reaction can also be verified by electron microscopy (Wilson et al., 2013). However, this requires highly specialized equipment and training and is therefore not covered in this article.

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Figure 1.

DNA intermediates and the role of Pif1 in repair DNA synthesis during HR. The 3' ssDNA tail resulting from DNA end resection is engaged by the Rad51 recombinase, which then invades a homologous sequence to form a D-loop. DNA synthesis within the D-loop is carried out by DNA Pol δ in conjunction with PCNA. In *S. cerevisiae*, the Pif1 helicase greatly stimulates the extent of DNA synthesis via a migrating D-loop. Note that PCNA is loaded onto the 3' terminus of the invading DNA strand by RFC, which is not depicted in the figure.

Figure 2. Schematics for the D-loop reaction (A) and DNA synthesis reaction primed from a D-loop (B). The reaction products can be separated by native gel electrophoresis as shown in the cartoon in (C) and as the gel image of an actual experiment (D). (C) & (D) Lane 1: D-loop product, lane 2: D-loop that has been extended by Pol δ -PCNA-RFC, and lane 3: D-loop that has been extended by Pol δ -PCNA-RFC in conjunction with Pif1. The bracket identifies the extended invading ssDNA strand that has been dissociated from the D-loop by Pif1.

Figure 3. 2-D gel electrophoresis of DNA synthesis products. (A) Cartoon representation of the DNA species first resolved by native gel electrophoresis (i) and then by a second electrophoretic step in an alkaline gel together with DNA size markers (MK) (ii). B. 2D gel analysis of DNA synthesis reactions mediated by Pol δ -PCNA-RFC (i) and Pol δ -PCNA-RFC-Pif1 (ii). The gel images are taken from our published study (Wilson et al., 2013)).

Figure 4.

Analysis of DNA extension products by restriction digests. (A) *AhdI* and *XmnI* incise dsDNA at 115 and 714 nucleotides from the 5' end of the non-extended ^{32}P -labeled invading strand, respectively. The extended DNA strand is normally susceptible to the action of either restriction enzyme (RE). However, upon its release from the D-loop by Pif1, the extended invading strand becomes resistant to the restriction enzymes. (B) Gel images from a typical experiment are shown (modified from our published study (Wilson et al., 2013)). The bracket identified extended DNA species resistant to the restriction enzymes.