7

Coupling of DNA Helicase Function to DNA Strand Exchange Activity

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1. Introduction

DNA repair can occur by a variety of mechanistically distinct pathways [for review, *see* ***(1)***]. Recombinational DNA repair is one such pathway, and it requires the coordinated action of many different enzymes. In the best studied organism, *Escherichia coli*, more than 20 different proteins are involved [for review, *see* ***(2)***]. The recombinational repair of a double-stranded DNA (dsDNA) break requires four general steps: (1) processing; (2) homologous pairing; (3) DNA heteroduplex extension; and (4) resolution. Here we describe assays to study aspects of the first two steps.

One of the most important proteins for DNA repair by homologous recombination is the RecA protein [for review, *see* ***(3–5)***]. RecA-like proteins are highly conserved and are present in all organisms thus far examined ***(6)***. Loss of RecA protein function renders *E. coli* cells highly sensitive to DNA damaging agents ***(7)***, and reduces recombination frequency to 0.1% of wild-type levels ***(8)***. In vitro, RecA protein promotes both the pairing and exchange of DNA strands between two homologous DNA molecules, provided that one of them contains a region of single-stranded DNA (ssDNA). RecA protein assembles on this ssDNA to form a nucleoprotein filament, referred to as the presynaptic complex. It is this nucleoprotein filament that is active for homologous pairing and DNA strand exchange ***(9)***.

In vivo, most DNA breaks do not directly produce a ssDNA substrate for RecA protein, thus necessitating processing of the lesion by other enzymes ***(1)***. In most cases, damage-specific DNA helicases process the DNA break to produce a region of ssDNA sufficient for binding by RecA protein ***(2)***. In *E. coli*, dsDNA breaks are acted upon by RecBCD enzyme, a DNA helicase/nuclease

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(2,10–13). If, instead, the lesion produces a ssDNA break or a ssDNA gap, processing is provided by the RecQ helicase *(14–16)*. Thus, the initial steps of recombinational DNA repair require that the unwinding activity of a DNA helicase is coupled to the homologous pairing and DNA strand exchange activity of the pairing protein, RecA protein.

The coupling of RecA protein and a DNA helicase to promote homologous pairing between homologous dsDNA has been achieved in vitro for both RecBCD enzyme *(17,18)* and RecQ helicase *(16)*. The general approach is to use DNA components that are substrates for the DNA helicase, but not for RecA protein, unless the DNA is first processed by the helicase. The assays use ^{32}P-labeled, linear dsDNA as the ssDNA-donor and a homologous supercoiled DNA (scDNA) as the recipient for homologous pairing (**Fig. 1A**). Because RecA protein alone is unable to pair the two fully dsDNA substrates *(19)*, this assay requires that the linear dsDNA substrate be separated into its constituent ssDNA strands so that RecA protein can form the presynaptic complex that is necessary for homologous pairing and DNA strand exchange. RecA protein-promoted homologous pairing between the linear ssDNA and its scDNA homolog produces a paired DNA species known as a joint molecule. The joint molecule consists of the linear ssDNA molecule (produced by dsDNA unwinding) basepaired to a complementary region within the recipient scDNA molecule (**Fig. 1A**). In the joint molecule, homologous pairing between the ssDNA and scDNA molecules results in the production and displacement of an unpaired DNA strand; therefore, this type of joint molecule is referred to as a displacement-loop or D-loop.

We describe two reaction protocols for the formation of joint molecules: one designed to test an uncharacterized RecA-like protein for homologous pairing activity (partial reaction) and the second designed to couple this activity to the unwinding activity of a DNA helicase (coupled reaction). In both cases, the two DNA substrates are pUC19 scDNA and ^{32}P-labeled, linear pUC19 dsDNA. The latter is produced by digestion of pUC19 scDNA with a restriction enzyme, followed by 5'-end labeling with (γ-[^{32}P]) ATP using T4 polynucleotide kinase. The two protocols differ only in the technique used to produce the linear ssDNA substrate for RecA protein. In the partial reaction, RecA protein is provided with linear ssDNA, which is generated by heat-denaturation to obviate the need for a DNA helicase. The coupled reaction, having linear dsDNA and scDNA as the DNA substrates, requires a DNA helicase to unwind the linear dsDNA to furnish RecA protein with a ssDNA substrate. Included in the protocol are three separate buffer conditions to optimize the reaction for uncharacterized proteins. The products of these reactions are analyzed by agarose gel electrophoresis, where D-loops appear as ^{32}P-labeled DNA species with a retarded mobility relative to the ^{32}P-labeled, linear ss- or dsDNA sub-

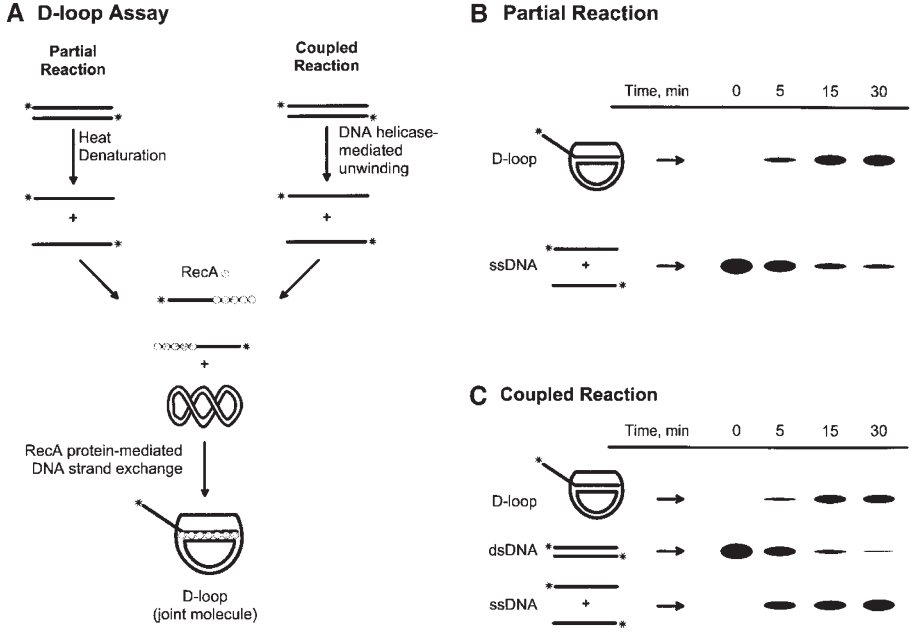

Fig. 1. Schematic diagram of the homologous pairing assays. See text for details. **(A)** D-loop assay for both the partial (i.e., RecA protein alone) and coupled (i.e., both RecA protein and a DNA helicase) assays. **(B)** Typical time-course of D-loop formation mediated by RecA protein in the partial reaction. **(C)** Typical time-course for a coupled reaction having both RecA protein and a DNA helicase.

strates (representations of typical autoradiograms are shown in **Fig. 1B and C**). In addition, we provide an agarose gel electrophoresis-based helicase assay, first described by Matson et al. ***(20)***, to determine the specific activity of an uncharacterized DNA helicase (**Fig. 2**). The specific activity calculated from this assay can then be used to determine the amount of the DNA helicase to use in the coupled reaction. As with the homologous pairing assays, the helicase assay protocol includes three separate buffer conditions to optimize unwinding for an uncharacterized DNA helicase.

2. Materials

2.1. Stock Solutions

All reagents were from Sigma-Aldrich (Boston, MA), except where noted.

1. 1 *M* Tris acetate, pH 7.5.
2. 0.1 *M* magnesium acetate, pH 7.5.
3. 0.1 *M* dithiotheritol (DTT) in water. Store aliquots at –20°C.
4. 0.5 *M* ethylenediaminetetraacetic acid (EDTA), pH 8.0.

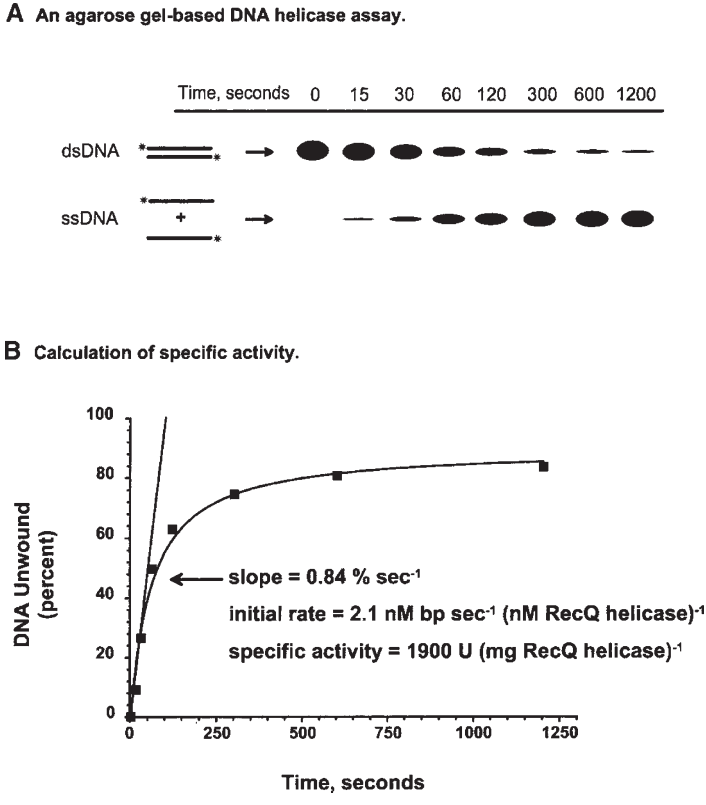

Fig. 2. DNA helicase activity assay. **(A)** Typical time-course for the unwinding of 10 µM (nucleotides) linear, pUC19 dsDNA by 20 n*M* RecQ helicase using agarose gel electrophoresis to detect strand separation. **(B)** Calculation of the specific activity of RecQ helicase from the data in A. *See* **Subheading 3.2.** for details.

5. 10% sodium dodecyl sulfate (SDS).
6. 0.1 *M* ATP in water, adjusted to pH 7.0 with NaOH. Store aliquots at –20°C.
7. 0.1 *M* phoshpoenol pyruvate (PEP) adjusted to pH 7.0 with NaOH. Store aliquots at –20°C.
8. Pyruvate kinase: Pipet 100 U of pyruvate kinase solution (homogeneous $(NH_4)_2SO_4$ suspension from rabbit muscle) into a 1.5-mL microcentrifuge tube, pellet protein by centrifugation in microcentrifuge (Micromax, IEC, Needham Heights, MA) at 20,000*g* for 2 min at 4°C, remove the supernatant, and dissolve the protein pellet in 50 µL of 1X pairing buffer. Final concentration is 2000 U/mL. Store at 4°C for up to 2 wk.
9. 40 % (w/v) polyethylene glycol (PEG): Dissolve 40 g of dry PEG (8000 molecular weight) in 40 mL water and mix well. Bring volume to 100 mL with water. Protect from light and store at room temperature.

10. 5X stop buffer: 250 m*M* EDTA, and 5% SDS. Store at room temperature for up to 1 wk.
11. 5X sample loading buffer: 15 % (w/v) Ficoll (Amersham-Pharmacia Biotech, Piscataway, NJ), in water with 0.25 % (w/v) each of xylene cyanol and bromophenol blue. Store at room temperature.
12. Stop solution: Mix equal volumes of 5X stop buffer and 5X sample loading buffer, and add proteinase K (Roche-Boehringer Mannheim) to 4.5 mg/mL. Prepare immediately before use and discard unused portion.
13. 1X TAE buffer for agarose electrophoresis: 40 m*M* Tris acetate, pH 8.0, 1 m*M* EDTA. Prepared as 50X concentrated solution: 2 *M* Tris base, 1 *M* acetic acid and 50 m*M* EDTA.
14. Deionized water using NANOpure reagent grade water system (Barnstead-Thermolyne, Dubuque, IA).
15. Agarose: type II, low EEO.
16. DE81 anion exchange paper (Whatman).

2.2. Preparation of 5'-End-Labeled Linear DNA Substrate

1. DNA: 2–10 µg of pUC19 scDNA. This DNA can be purchased from a commercial source or purified as described ***(21)***.
2. Restriction endonuclease (*Hind*III) and T4 polynucleotide kinase (New England Biolabs).
3. Shrimp alkaline phosphatase (United States Biochemical).
4. 10X restriction buffer: 100 m*M* Tris-HCl, pH 7.5, 100 m*M* $MgCl_2$, 1000 m*M* NaCl, 10 m*M* DTT. Store aliquots at –20°C.
5. MicroSpin G-25 columns (Amersham Pharmacia Biotech).
6. γ^{32}P ATP, 1000 µCi/mL.

2.3. DNA Helicase Assay

1. DNA helicase of interest. Purification of *E. coli* RecQ helicase and *E. coli* RecBCD enzyme have been described previously ***(11,16)***.
2. SSB protein (*see* **Note 1**). *E. coli* protein is commercially available: Promega and United States Biochemical. Purification of *E. coli* SSB protein has been described previously ***(22)***.
3. DNA: 2 µg 5'-end-labeled linear pUC19 dsDNA at a stock concentration of 250 µM (nucleotides) (*see* **Note 2**).
4. 10X helicase buffer: 250 m*M* Tris acetate, pH 7.5, 10 m*M* ATP, 10 m*M* PEP, and 10 m*M* DTT. Store aliquots at –20°C.

2.4. RecA Protein-Mediated D-Loop Formation—Partial Reaction

1. RecA-like protein of interest. Purification of *E. coli* RecA protein has been described previously ***(23,24)***. RecA protein is also commercially available: Promega, United States Biochemical, New England Biolabs.
2. SSB protein (*see* **Notes 1** and **3**). *E. coli* protein is commercially available: Promega and United States Biochemical. Purification of *E. coli* SSB protein has been described previously ***(22)***.

3. DNA: 2 µg 5'-end-labeled linear pUC19 ssDNA and 4 µg pUC19 scDNA, at a stock concentration of 250 and 500 µM (nucleotides), respectively (*see* **Note 2**).
4. 10X pairing buffer: 250 m*M* Tris acetate, pH 7.5, 10 m*M* ATP, 10 m*M* PEP, and 10 m*M* DTT. Store aliquots at –20°C.

2.5. Coupling DNA Strand Exchange and a DNA Helicase to Form D-Loops—Coupled Reaction

1. RecA-like protein of interest. Purification of *E. coli* RecA protein has been described previously ***(23,24)***. RecA protein is also commercially available: Promega, United States Biochemical, New England Biolabs.
2. DNA helicase of interest, with activity defined in helicase units (*see* **Subheading 3.2.**).
3. SSB protein (*see* **Notes 1** and **3**). *E. coli* protein is commercially available: Promega and United States Biochemical. Purification of *E. coli* SSB protein has been described previously ***(22)***.
4. DNA: 2 µg 5'-end-labeled linear pUC19 duplex DNA and 4 µg pUC19 scDNA, at stock concentration of 250 and 500 µ*M* (nucleotides), respectively (*see* **Note 2**).
5. 10X coupled reaction buffer: 250 m*M* Tris acetate, pH 7.5, 10 m*M* ATP, 10 m*M* PEP and 10 m*M* DTT. Store aliquots at –20°C.

3. Methods

3.1. Preparation of 5'-End-Labeled Linear DNA Substrate

For the best results, prepare the 5'-end-labeled linear duplex DNA substrate just prior to its use. The labeled DNA may be stored for approx 3 d at 4°C, but its quality rapidly degrades over time because of nicking of the DNA.

1. Digest and dephosphorylate 2 µg of pUC19 scDNA by incubation in 1X restriction buffer with 10 U of *Hind*III and 1 U shrimp alkaline phosphatase at 37°C for 1 h in a final volume of 20 µL.
2. Stop the reaction by heating at 70°C for 15 min to inactivate the enzymes. Cool to room temperature. Remove condensate from the lid of the tube by centrigugation in a microcentrifuge at 20,000*g* for 30 s. After this step the DNA can be stored at –20°C indefinitely.
3. To label the DNA with ^{32}P at the 5'-ends, add 20 µCi (γ-[^{32}P]) ATP and 10 U T4 polynucleotide kinase, and incubate at 37°C for 30 min.
4. Terminate the reaction by heating at 70°C for 15 min to inactivate T4 polynucleotide kinase. Cool to room temperature. Remove condensate from the lid of the tube by centrifugation in a microcentrifuge at 20,000*g* for 30 s.
5. Remove unincorporated label and exchange the restriction buffer for water using a MicroSpin G-25 column as per the supplier's instructions (*see* **Note 4**). Determine the volume of the recovered solution using a micropipettor, and calculate the concentration of DNA in µg/mL, assuming no loss of DNA (*see* **Note 5**). To calculate the approximate molar concentration of the DNA in µ*M* nucleotides,

divide the number of μg/mL by 0.325. This procedure typically yields a DNA solution having a concentration of between 200–250 μ*M* nucleotides.

3.2. DNA Helicase Assay

This section describes an agarose gel-based helicase assay to determine the specific activity of a DNA helicase (**Fig. 2**). In this general protocol, the DNA unwinding activity of the helicase can be assayed at any of the three different magnesium ion concentrations used in the coupled reaction (i.e., "high," 12 m*M* Mg-acetate; "intermediate", 6 m*M* Mg-acetate; and "low," 1 m*M* Mg-acetate) simply by altering the amount of 0.1 *M* Mg-acetate added to the reaction (*see* **Subheading 3.3.**). The substrate for unwinding is 5'-end-labeled linear pUC19 dsDNA having nearly blunt dsDNA ends, because this is the substrate for the helicase in the coupled reaction (*see* **Note 6**). After determining the rate of DNA unwinding for a known amount of DNA helicase, the specific activity of the preparation is calculated in terms of helicase units per milligram of protein. In this case, one helicase unit is defined as the amount of helicase needed to unwind 1 nmol basepairs in 1 min under each of the conditions described here ***(11)***. The specific activity of the DNA helicase should be measured at several different protein concentrations to obtain an accurate specific activity.

1. Aliquot 10 μL of freshly prepared stop solution into eight 1.5-mL microcentrifuge tubes. Label the tubes as "0 min," "15 s," "30 s," "60 s," "120 s," "300 s," "600 s," and "1200 s."
2. Prepare the reaction mixture (which will have a final volume of 200 μL after adding all the components) by adding 20 μL of 10X pairing buffer and 4 μL of pyruvate kinase to 100 μL of water in 1.5-mL microcentrifuge tubes. Mix the solution in the tube thoroughly using a vortex mixer.
3. Add 0.1 *M* Mg-acetate to achieve the desired final magnesium concentration: 24 μL for 12 m*M* Mg-acetate, 12 μL for 6 m*M* Mg-acetate, or 2 μL for 1 m*M* Mg-acetate. Test each of these magnesium ion concentrations individually to determine the specific activity of the helicase under each of the conditions to be used below in the coupled reaction. Mix the solution thoroughly with a vortex mixer.
4. To each tube, add SSB protein and 5'-end-labeled linear pUC19 dsDNA to a final concentration of 1 μ*M* and 10 μ*M*, respectively (*see* **Note 7**). Bring the volume of each reaction mixture up to 200 μL with water. Mix gently using a vortex mixer.
5. Incubate the reaction mixtures for 5 min at 37°C, then remove a 20-μL aliquot from the reaction, and add to the corresponding tube containing stop solution (i.e., "0 min"). Continue incubation of the remainder of the reaction mixture at 37°C. Mix the stopped aliquot thoroughly with vortex mixer and place on ice (*see* **Note 8**).
6. Initiate the unwinding reaction by adding the DNA helicase to a final concentration of 20 nM (calculate for a reaction volume of 180 μL) (*see* **Note 9**), mix gently, and continue incubation at 37°C. Take 20 μL aliquots at 15 s, 30 s, 60 s,

120 s, 300 s, 600 s, and 1200 s. Stop the reaction in each aliquot by adding to the appropriate tube containing the stop solution. Mix each aliquot thoroughly using vortex mixer and place on ice.

7. Deproteinize the DNA in each of the samples by incubating the aliquots at 37°C for 5 min.
8. Load 20 µL of each sample onto a 0.8% (w/v) agarose gel (13 × 26 × 0.5 cm) with 12 (1 × 0.15 cm) wells and run the gel at 1.5 V/cm for 12 h with 1X TAE as the running buffer. To visualize the DNA, dry the gel onto Whatman DE81 paper (*see* **Note 10**) and expose to either X-ray film or a phosphorimager screen (*see* **Note 11**). **Fig. 2A** is a representation of an autoradiogram for a typical time-course of RecQ-helicase-mediated unwinding of linear 5'-end-labeled dsDNA.
9. Determine the amount of radioactivity in the band corresponding to intact dsDNA at each time point, and calculate the percentage of dsDNA unwound by the helicase at each time-point (*see* **Note 11**).
10. Plot percent dsDNA unwound vs time as indicated in **Fig. 2B**. The resultant unwinding curve should be hyperbolic (*see* **Note 9**). Calculate the initial rate of unwinding in nM bp s^{-1} (n*M* helicase)$^{-1}$ from the slope of the initial linear portion of the curve using the following equation:

$$\text{initial rate} = \text{slope} \times (C/[100\%]) \times (1/X)$$

where C is the concentration of basepairs in n*M* (i.e., 5000 n*M*), and X is the concentration of DNA helicase in n*M*.

11. Calculate the specific activity of the helicase preparation in helicase units (mg of protein)$^{-1}$ for each magnesium concentration using the following equation:

$$\text{specific activity} = \text{initial rate} \times ([60\text{ s}]/[1\text{ min}]) \times ([1.0E + 6]/Y)$$

where Y is the molecular weight of the helicase. Use the calculated specific activity to determine the amount to DNA helicase to use in the coupled reaction (*see* **Subheading 3.4.**).

3.3. RecA Protein-Mediated D-Loop Formation—Partial Reaction

This section describes a protocol for optimizing the homologous pairing activity of an uncharacterized RecA-like protein by varying the magnesium ion concentration present in the buffer (*see* **Note 12**). The magnesium ion conditions are as follows: "high magnesium" (12 m*M* Mg-acetate), "intermediate magnesium" (6 m*M* Mg-acetate), and "low magnesium" (1 m*M* Mg-acetate with 10% PEG).

3.3.1. Preparation of the 5'-End-Labeled, Linear pUC19 ssDNA Substrate

Prepare this DNA substrate immediately before it is needed to ensure that the DNA added to the pairing reaction it is fully denatured.

1. Denature 2 µg of 5'-end-labeled linear pUC19 duplex DNA (*see* **Subheading 3.1.**) by heating at 95°C for 5 min.

2. Transfer the tube quickly to an ice-cold water bath and chill for 5 min. Remove condensate from the lid of the tube by centrifugation in microcentrifuge at 20,000*g* for 30 s at 4°C. Keep the denatured DNA on ice to prevent re-annealing of the ssDNA strands.

3.3.2. RecA Protein-Mediated D-Loop Assay

1. Aliquot 10 µL of freshly prepared stop solution into twelve 1.5-mL microcentrifuge tubes. Label sets of four tubes with "high," "intermediate," and "low." In each set, label a tube "0 min," "5 min," "15 min," and "30 min."
2. Prepare three reaction mixtures (which will have a final volume of 100 µL after adding all the components) by adding 10 µL of 10X pairing buffer and 2 µL of pyruvate kinase to 40 µL of water in 1.5-mL microcentrifuge tubes. Mix each tube well using a vortex mixer. Label one tube "high," the second "intermediate," and the last "low."
3. To the tube labeled "high," add 12 µL of 0.1 *M* Mg-acetate, to the tube labeled "intermediate," add 6 µL of 0.1 *M* Mg-acetate, and to the tube labeled "low," add 1 µL 0.1 *M* Mg-acetate. Also, add to the tube labeled "low," 25 µL of 40% PEG (*see* **Note 13**). Mix the solution in each tube well with a vortex mixer.
4. To each tube, add RecA protein and 5'-end labeled linear pUC19 heat-denatured ssDNA to a final concentration of 5 µ*M* and 10 µ*M*, respectively (*see* **Note 14**). Bring the volume of each reaction mixture up to 100 µL with water. Mix gently using a vortex mixer.
5. Incubate the reaction mixtures for 3 min at 37°C, then add SSB protein to a final concentration of 1 µ*M* (*see* **Note 14**) and return to incubation at 37°C. After an additional 2 min, remove a 20 µL aliquot from each reaction, and add to the corresponding tube containing stop solution (for example, "high, 0 min"). Continue incubation of the remainder of the reaction mixture at 37°C. Mix the stopped aliquots thoroughly with a vortex mixer and place on ice (*see* **Note 15**).
6. Initiate the homologous pairing reaction by adding pUC19 scDNA to a final concentration of 20 µ*M* (calculate for a reaction volume of 80 µL), mix gently, and continue incubation at 37°C. Take 20-µL aliquots at 5, 15, and 30 min. Stop each pairing reaction by adding each aliquot to the appropriate tube containing the stop solution. Mix each aliquot thoroughly using a vortex mixer and place on ice.
7. Deproteinize the DNA in each of the samples by incubating the aliquots at 37°C for 5 min.
8. Load 20 µL of each sample onto a 1% (w/v) agarose gel (13 × 26 × 0.5 cm) with 12 (1 × 0.15 cm) wells and run the gel at 1.5 V/cm for 12 h with 1X TAE as the running buffer. To visualize the DNA, dry the gel onto Whatman DE81 paper (*see* **Note 10**) and expose to either X-ray film or a phosphorimager screen. **Fig. 1B** is a representation of an autoradiogram for a typical time-course of the D-loop reaction mediated by RecA protein and 5'-end-labeled linear ssDNA.

3.4. Coupling the Activities of RecA Protein and a DNA Helicase to Form D Loops—Coupled Reaction

The protocol below describes a procedure to couple the activities of a DNA helicase and RecA protein to produce D loops. To optimize the reaction, D-loop formation is assayed under three different magnesium concentrations (*see* **Note 16**): "high magnesium" (12 m*M* Mg-acetate), "intermediate magnesium" (6 m*M* Mg-acetate) and "low magnesium" (1 m*M* Mg-acetate with 10% PEG).

1. Aliquot 10 µL of freshly prepared stop solution into twelve 1.5-mL microcentrifuge tubes. Label sets of four tubes with "high," "intermediate," and "low." In each set, label a tube "0 min," "5 min," "15 min," and "30 min."
2. Prepare three reaction mixtures by adding 10 µL of 10X coupled reaction buffer and 2 µL of pyruvate kinase to 40 µL of water in 1.5-mL microcentrifuge tubes. Label one tube "high," the second "intermediate," and the last "low."
3. To the tube labeled "high,add 12 µL of 0.1 *M* Mg-acetate, to the tube labeled "intermediate" add 6 µL of 0.1 *M* Mg-acetate, and to the tube labeled "low," add 1 µL 0.1 *M* Mg-acetate. Also, add to the tube labeled "low," 25 µL of 40% PEG (*see* **Note 13**). Mix the solution in each tube well with a vortex mixer.
4. To each tube, add RecA protein and SSB protein to a final concentration of 5 µ*M* and 1 µ*M*, respectively. Also add pUC19 scDNA and 5'-end-labeled linear pUC19 duplex DNA to a final concentration of 20 µ*M* and 10 µ*M*, respectively (*see* **Note 14**). Bring the volume of each reaction mixture up to 100 µL with water. Mix gently using a vortex mixer.
5. Incubate the reaction mixtures for 5 min at 37°C. Remove a 20 µL aliquot from each reaction, and add to the corresponding tube containing stop solution (for example "high, 0 min"). Continue incubation of the remainder of the reaction mixture at 37°C. Mix the stopped aliquots thoroughly with a vortex mixer and place on ice (*see* **Note 15**).
6. Start the reaction by adding 20 helicase U of the DNA helicase (*see* **Note 17**), mix gently, and continue incubation at 37°C. Take additional aliquots at 5, 15, and 30 min and stop the reaction by adding each aliquot to the appropriate tube containing the stop solution. Mix each aliquots thoroughly using a vortex mixer and place on ice.
7. Deproteinize the DNA in each of the samples by incubating the aliquots at 37°C for 5 min.
8. Load 20 µL of each sample onto a 1% (w/v) agarose gel (13 × 26 × 0.5 cm) with 12 (1 × 0.15 cm) wells and run the gel at 1.5 V/cm for 12 h with 1X TAE as the running buffer. To visualize the DNA, dry the gel onto Whatman DE81 paper (*see* **Note 10**) and expose to either X-ray film or a phosphorimager screen. **Fig. 1C** is a representation of an autoradiogram for a typical time-course of D-loop formation mediated by RecQ helicase and *E. coli* RecA protein.

4. Notes

1. SSB protein is present in these reactions to trap the ssDNA produced by a DNA helicase so that this ssDNA does not reanneal after passage of the helicase ***(11,25–27)***. Because of differences in helicases from species to species, it is desirable, where possible, to use a DNA helicase and an SSB protein from the same or related species.
2. The molar nucleotide concentration of duplex DNA can be determined spectrophotometrically using the extinction coefficient of 6500 $M^{-1}cm^{-1}$ at a wavelength of 260 nm ***(16)***.
3. SSB protein is present in these reactions to remove secondary structure in ssDNA, which inhibits RecA protein function ***(28)***. Because of differences in recombination proteins from species to species, it is desirable, where possible, to use RecA-like and SSB proteins from the same or related species.
4. Exchange of unincorporated label and buffer can also be achieved by precipitating the DNA with ethanol twice ***(21)***. In this case, determine the DNA concentration spectrophotometrically using the extinction coefficient of 6500 $M^{-1}cm^{-1}$ at a wavelength of 260 nm. To confirm that all the ATP has been removed, calculate the ratio of the absorbance at 260 nm to the absorbance at 280 nm. The value for this ratio should be 1.8–1.9; a higher value than this standard is indicative of contamination by ATP.
5. In our hands, DNA loss after this step is <10%, which is acceptable for these experiments. It is not unusual to gain between 2–10 µL of volume from the spin column; therefore, it is important to determine the final volume of the DNA solution. Please note that this treatment of the DNA imparts ultraviolet light-absorbent material to the DNA solution, so the DNA concentration cannot be accurately determined spectrophotometrically. In most cases, the absorbent material can be washed out of the column by five successive washes with water (totaling approx 5 column volumes). To wash the column once, centrifuge column as per the suppliers' instructions to remove the original buffer, resuspend the matrix in 400 µL of water, then centrifuge as before. After washing in this manner five times, follow the suppliers' instructions for exchange of unincorporated label and buffer in the DNA sample. The concentration of the recovered DNA can then be determined spectrophotometrically as described in **Note 3**.
6. If the DNA helicase of interest is incapable of initiating unwinding from flush dsDNA ends, then the DNA substrate must be modified to contain the appropriate initiation site for the DNA helicase. In most cases, the addition of short (>100 nt) ssDNA tails are sufficient for most DNA helicases, but the polarity of the tail must be determined for each helicase. Linear dsDNA can be resected to contain ssDNA tails by use of a dsDNA-dependent exonuclease ***(29,30)***; for example, *E. coli* Exonuclease III will generate 5'-tails, and λ Exonuclease will produce 3'-tails. Exonuclease III and λ Exonuclease are available from United States Biochemical. Keep in mind that if tailed DNA substrates are used in the coupled reaction, the ssDNA regions must be heterologous to the scDNA recipient. Otherwise, RecA protein, alone, will pair the tailed dsDNA with the scDNA recipient without the need for a DNA helicase.

7. The concentration of SSB protein used here produces optimal unwinding for both RecQ helicase and RecBCD enzyme. For an uncharacterized helicase, the concentration of SSB protein should be varied to achieve maximal unwinding activity. Keep in mind that the ssDNA binding stoichiometry of *E. coli* SSB protein is dependent on the ionic conditions of the solution: at magnesium ion conditions greater than 1–2 m*M* (at 37ºC), the site size is approx 15 nucleotides per SSB monomer ***(31)***.
8. The aliquots are kept on ice to prevent the re-annealing of the individual ssDNA strands.
9. For the best results, the concentration of DNA helicase in the reaction should be limiting relative to the duplex DNA substrate so that the initial rate of unwinding can be accurately measured. For an uncharacterized DNA helicase, vary the DNA helicase concentration over a 10- to 100-fold range to achieve a hyperbolic unwinding curve with an obvious linear initial slope (*see* **Fig. 2B**).
10. The agarose gel is dried onto Whatman DE81 paper to minimize DNA loss. Gel drying time can be reduced by flattening the gel prior to drying. To flatten the gel, place the gel on Whatman DE81 paper cut to the size of the gel, which is atop several sheets of filter paper and lab matting. Cover the gel with a single layer of plastic wrap. Place a rigid sheet of Lucite or a wooden board over the gel, followed by a 15–20-lb. weight. Dry the gel onto the Whatman DE81 paper once the gel is between 1–2 mm in thickness.
11. An alternative method to visualize the DNA is to stain the wet gel with 0.5 µg/mL ethidium bromide in water for 1 h, destain with water for 1 h, and illuminate the gel using an ultraviolet light box. In this case, the amount of radioactivity in each band can be determined by excising the individual bands and analyzing each with a scintillation counter.
12. Each buffer condition provided here supports homologous pairing and DNA strand exchange by *E. coli* RecA protein. The magnesium requirements for *E. coli* RecA protein are ≈4 m*M* magnesium ion in dilute solution ***(32)***, or as low as 1 m*M* magnesium ion in a buffer supplemented with a volume exclusion agent, such as the PEG used here ***(33)***.
13. The 40% PEG solution is extremely viscous, so the solution must be drawn up into the micropipet slowly to measure the proper volume of PEG. Pipet the PEG solution into the buffer slowly, taking care to expel all of the solution into the microcentrifuge tube. Mix well by pipeting the buffer and PEG mixture up and down several times until a homogenous mixture is obtained.
14. The concentrations of RecA protein and SSB protein used here produce optimal results when using *E. coli* RecA protein. For an uncharacterized RecA-like protein, vary the concentration of each protein to achieve maximal pairing activity. Keep in mind that a saturating amount of RecA protein is achieved at a stoichiometry of 1 RecA monomer to 3 nucleotides of ssDNA ***(34)***. In addition, the ssDNA binding stoichiometry of *E. coli* SSB protein is dependent on the ionic conditions of the solution: at magnesium ion conditions greater than 1–2 m*M* (at 37°C), the site size is approx 15 nucleotides per SSB monomer ***(31)***. It is

also important to note excessive amounts of SSB protein or addition of SSB protein to ssDNA before RecA protein will inhibit RecA protein, because these two proteins compete for binding to ssDNA ***(28)***. Also, though an excess of *E. coli* RecA protein does not inhibit DNA strand exchange, excessive amounts of *Saccharomyces cerevisiae* Rad51 protein inhibit DNA strand exchange by binding to the dsDNA ***(35)***.

15. Each time-point is placed on ice to minimize thermal dissociation of the D loops formed by RecA protein.
16. RecA protein and the DNA helicase may not display optimal activity at the same concentration of magnesium, so it is important to test a range of magnesium concentrations ***(16,17)***. As stated above (*see* **Note 12**), *E. coli* RecA protein is capable of pairing under all the conditions presented here.
17. Helicase units are defined and determined in **Subheading 3.2.** In our hands, 20 U of helicase activity produce sufficient ssDNA for use as a substrate for *E. coli* RecA protein. This amount of helicase corresponds to approx 2.5 n*M* RecBCD enzyme ***(17)*** or 100 n*M E. coli* RecQ helicase ***(16)***. However, the amount of DNA helicase needed for optimal coupled pairing must be determined empirically for each DNA helicase. To optimize the reaction, DNA helicase concentration should be varied over a 10- to 100-fold range. Keep in mind that the optimum may be very sharp, because the DNA helicase being tested may also be capable of disrupting the joint molecules formed by RecA protein, as is the case for the *E. coli* RecQ helicase ***(16)***.

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