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Purification of *Saccharomyces cerevisiae* Homologous Recombination Proteins Dmc1 and Rdh54/Tid1 and a Fluorescent D-loop Assay

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Abstract

Budding yeast Dmc1 is a member of the RecA family of strand exchange proteins essential for homologous recombination (HR) during meiosis. Dmc1 mediates the steps of homology search and DNA strand exchange reactions that are central to HR. To achieve optimum activity, Dmc1 requires a number of accessory factors. Although methods for purification of Dmc1 and many of its associated factors have been described (Binz *et al.* 2006; Van Komen *et al.* 2006; Chi *et al.* 2006; Nimonkar *et al.* 2007; Cloud *et al.* 2012; Busygina *et al.* 2013; Chan *et al.* 2014), Dmc1 has been particularly difficult to purify because of its tendency to aggregate. Here, we provide an alternative and simple high-yield purification method for recombinant Dmc1 that is active and responsive to stimulation by accessory factors. The same method may be used for purification of recombinant Rdh54 (a.k.a. Tid1) and other HR proteins with minor adjustments. We also describe an economical and sensitive D-loop assay for strand exchange proteins that uses fluorescent dye-tagged, rather than radioactive, ssDNA substrates.

Keywords

Saccharomyces cerevisiae; strand exchange; Dmc1; Tid1; Rdh54; overexpression; purification; fluorescent D-loop assay

1. Introduction

In eukaryotic cells, Dmc1 is essential for, and functions specifically during, meiosis. During meiosis, chromosomes are replicated and undergo homologous recombination (HR) (Brown & Bishop 2014). Meiotic recombination is then initiated by programmed double-strand DNA breaks which are processed by a group of specific proteins and nucleases to generate 5' duplex junctions with 3' single-stranded DNA (ssDNA) overhangs. In *Saccharomyces cerevisiae*, Dmc1 with the help of associated factors, binds ssDNA to form nucleoprotein filaments. These filaments in turn search the genome for homologous DNA sequences. Once

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a homologous sequence is found, Dmc1 then catalyzes strand invasion and strand exchange, resulting in a heteroduplex with a displaced strand called the displacement loop (D-loop). The *in vitro* D-loop assay models the *in vivo* reaction using ssDNA oligo as substrate for the assembly of nucleoprotein filaments by Dmc1 (or other recombinases) together with a negatively supercoiled plasmid that carries a sequence identical to that of the oligo. After strand exchange, the ssDNA forms a heteroduplex inside the plasmid resulting in a D-loop (Fig. 1A). The D-loop assay is one of the most commonly used biochemical functional assays for recombinases.

Genetic and biochemical studies have provided many insights into the mechanism of HR. Purified HR proteins in native or recombinant forms have facilitated many of these biochemical studies. However, some recombination proteins, including budding yeast Dmc1, are difficult to purify. The key challenge for purification of Dmc1 is its tendency to aggregate. The Rad54 family translocase Rdh54/Tid1 (Brown & Bishop 2014) is also difficult to purify because of its instability; proteolysis tends to occur during purification. This chapter describes an alternative method for purification of (His)₆-tagged versions of Dmc1 and Rdh54/Tid1 proteins (Fig. 2A). We have also used modest modification of the same approach to purify other HR proteins and believe the method is broadly applicable to other (His)₆-tagged DNA binding proteins. We also describe a fluorescent dye based D-loop assay for functional studies of HR proteins.

2. Overexpression of Dmc1

2.1 General Comments

Dmc1 is overexpressed from the plasmid pNRB150 (Hong *et al.* 2001) that has pET11a as its backbone. Dmc1 is (His)₆-tagged at its N-terminal, and the (His)₆-tagged protein has been demonstrated to be expressed at normal levels during meiosis following targeting into yeast cells, where it functions normally (Hong *et al.* 2001). The recombinant protein is expressed from a T7 promoter. Overexpression of Dmc1 is toxic to *E. coli* cells, thus the following protocol is optimized for induction of high levels of expression with minimum impact of toxicity on protein yield. The expression procedure below has been applied to Rdh54/Tid1 and other proteins with adjustment of the concentration of IPTG, temperature, and the duration of induction.

2.2 Equipment, Buffers, and Reagents

- Standard shaker that holds baffled flask and allows temperature control
- Six 2.8 liter (lit) baffled flasks
- Luria broth (LB) (10g Bacto-tryptone, 5g yeast extract, and 10g NaCl per lit, adjust to pH 7 – 7.5 with NaOH, sterilized)
- 40% glucose, 100 mg/ml ampicillin, 34 mg/ml chloramphenicol, 5 M NaCl
- Centrifuge with rotor for 0.5 lit (FiberLite F10) or 1 lit bottles (Sorvall H6000A)
- Standard SDS-PAGE gel apparatus, 12% SDS-PAGE gels

2.3 Expression Procedure

Dmc1 is overexpressed in the *E. coli* strain Rosetta(DE3)pLysS (Novagen, chloramphenicol⁺)

1. Transform Rosetta(DE3)pLysS competent cells with pNRB150 (Ampicillin⁺). Grow cells on agar plates containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol at room temperature till the colonies are ~ 2 mm in diameter (in about 2 – 3 days). Or, include 1% glucose in agar plates if cells are to be grown at 37°C. [NB: glucose is necessary for suppressing expression from the T7 promoter carried by pET11.]
2. **Starting culture:** In a 2 lit flask, inoculate 600 ml LB (supplemented with 1% glucose, 100 µg/ml ampicillin, 17 µg/ml chloramphenicol) with about 20 colonies. Let the culture stand at room temperature overnight.
3. Next morning, grow cells in a shaker at 37 °C (230 – 250 rpm) for 1.5 h or until A₆₀₀ reaches 0.5 – 0.7.
4. Inoculate each lit LB (supplemented with 1% glucose, 100 µg/ml ampicillin, 17 µg/ml chloramphenicol) in a 2.8 lit baffled flask. Add a sufficient volume of starting culture to make the starting A₆₀₀ of the medium around 0.05. This is typically between 50 – 90 ml of starting culture per flask. Grow cells in a shaker at 37 °C (230 – 250 rpm) for about 2 – 2.5 h or until A₆₀₀ reach 0.7 – 1.0. Save 1 ml culture for SDS-PAGE gel analysis.
5. Induce expression of Dmc1 by adding 0.5 ml of 1 M IPTG per lit culture (final concentration of 0.5 mM IPTG). After 1 h induction at 37°C, adjust the shaker temperature to 30 °C; and allow temperature to taper down slowly from 37°C to 30°C while still shaking and continue cell growth for another 1 h; i.e., a total of 2 h induction. Save 1 ml induced culture for SDS-PAGE gel analysis.
6. Harvest cells by centrifugation at 5,000 rpm (Sorvall H6000A rotor) for 20 min at 4°C. Wash cell pellets by suspending cells in Cell lysis buffer (25 mM sodium phosphate buffer, pH 7.5 + 1 mM dithiothreitol (DTT) + 1 M NaCl + 50 mM imidazole; see details below) and combine cells from all 6 lit cultures (yield about 12 g cells) into one bottle. Centrifuge cells again and store the cell pellet at – 20°C. Proceed to purification within one week. Store cells at –80°C if longer storage is required.
7. Analyze protein extracts from cultures above to determine the expression level of Dmc1 using a 12% SDS-PAGE gel. In general, 150 µl aliquots of the culture, taken before and after induction by IPTG, should be adequate for gel analysis.

3. Purification of Dmc1

3.1 General Comments

Some practical suggestions on the purification of Dmc1: (a) try to achieve a high level of protein expression while avoiding the formation of inclusion bodies (i.e. avoiding conditions that result in an insoluble form of the protein that goes to the pellet when the extract is

cleared by centrifugation); (b) over-load Ni^{2+} resin to encourage specific his-tagged protein binding and minimize non-specific protein binding; 1 ml Ni^{2+} resin binds about 30 mg of (His)₆-tagged Dmc1; (c) reduce protein concentration after elution from Ni^{2+} column to no more than 4 – 5 mg/ml to avoid aggregation of Dmc1; (d) use high salt in the lysis buffer to release Dmc1 from *E. coli* genomic DNA; (e) wash the protein-bound Ni^{2+} resin excessively before elution, to remove DNA and protein contaminants, in particular DNA nucleases and helicases; (f) avoid concentrating Dmc1 by Centricon because the protein tends to bind to the membrane; (g) avoid dialysis of Dmc1 because it is likely to either precipitate or stick to dialysis membrane; (f) avoid detergents in the buffer as these may affect biochemical assays.

The protein overexpression and purification protocols detailed here have been adopted for Rdh54/Tid1 (details are provided below).

3.2 Equipment

- Bio-Rad glass Econo-column
- Dounce glass homogenizer (40 ml, Kontes Glass Co.)
- French press (or other equipment for the efficient lysis of bacterial cells).
- Standard SDS-PAGE gel apparatus, 12% SDS-PAGE gels
- Ultracentrifuge
- Type 60 or Type 70 titanium rotor (Beckmann) or equivalent
- Rotisserie apparatus
- Ni^{2+} Sepharose High Performance resin (GE Healthcare) or equivalent resin.
- FPLC system (Bio-Rad or equivalent system)
- Mono Q column (1 ml resin, GE HealthCare, HR 5/50 GL)
- PD-10 desalting column containing Sephadex G-25 resin (GE Healthcare).

3.3 Buffers and Reagents

- 2x Sodium phosphate buffer (2x Buffer P), pH 7.5: 50 mM buffer containing 2 mM DTT and 20% glycerol.
- 2x Tris-HCl buffer, pH 7.5 (2x Buffer T): 50 mM buffer containing 2 mM DTT and 20% glycerol.
- 5 M NaCl.
- 2 M imidazole (adjust to pH 7.4 with HCl).
- Cell lysis buffer P: (1x Buffer P) + 1 M NaCl + 50 mM imidazole [NB: low concentration imidazole is added to minimize binding of host cell proteins].
- Protease inhibitor cocktail tablet (EDTA-free, Roche protease inhibitor cocktail tablet. Add 1 tablet per 50 – 100 ml Cell lysis buffer immediately before use).

- Wash buffer T: (1x Buffer T) + 0.5 M NaCl + 30 mM or 50 mM imidazole (detail later).
- Elution buffer T: (1x Buffer T) + 0.5 M NaCl + 80 mM, or 350 mM, or 500 mM imidazole (detail later).
- Storage buffer T: (1x Buffer T) + 0.5 M NaCl + 1 mM EDTA + 10% glycerol.

3.4 Procedure

3.4.1 Cell lysate preparation at 4°C: To frozen cell pellet from 6 lit culture, add 120 ml ice-cold Cell lysis buffer P plus protease inhibitors and allow cells to thaw on ice. Resuspend cells using a Dounce homogenizer until no cell clumps remain. Then lyse cells using a French Press; repeat 3 passes at 18,000 – 20,000 psi. Centrifuge the lysate at $120,000 \times g$ (use Beckman rotor Type60 or Type70) for 30 min at 4°C. Collect the clear supernatant and avoid cell debris. If debris is observed, filter the lysate through a 0.45 μm low protein binding membrane (e.g. Millipore Stericup).

3.4.2 Batch/gravity flow Ni^{2+} column purification: Perform all steps at 4°C or in the cold room. Save 50 μl sample from each step for SDS-PAGE gel analysis.

1. **Ni^{2+} resin preparation:** Wash Ni^{2+} resin with double distilled water and equilibrate resin with Cell lysis buffer P (without protease inhibitors) as suggested by the manufacturer. Use about 0.7 ml packed Ni^{2+} resin for lysate from 1 lit cell culture if the expression is estimated to be ~ 20 mg. Equilibrate resin with 15 ml of Cell lysis buffer P in a 50 ml centrifuge tube for 10 min on a rotisserie apparatus at low speed. Pellet resin in a clinical centrifuge for 1 min.
2. **Protein binding to resin:** Add the cleared lysate to the buffer equilibrated Ni^{2+} resin. Gently rotate the sample on a rotisserie at low speed for 1 h at 4 °C. After binding, pellet resin in a clinical centrifuge for 1 min, remove almost all of the supernatant without disturbing the resin pellet. The resin will change color from light blue to grey when bound with protein. Proceed to wash the resin.
3. **Batch-wash the resin** with 45 ml Cell lysis buffer P + 50 mM imidazole for 1 min with gentle shaking. Centrifuge as in step 2 above. Repeat batch-wash once more.
4. **Gravity Ni^{2+} column wash:** Resuspend resin in ~5 ml Wash buffer T + 30 mM imidazole. Transfer the resin mixture into a glass Econo-column (~1.5 cm diameter) with an end cap in place. Allow resin to settle in the column for about 5 min. Remove end cap and allow buffer to drain till it reaches the top of the resin bed. Avoid air bubbles, gently layer 5 ml Wash buffer T + 30 mM imidazole onto the top of the resin. Set up a continuous buffer siphon system for auto-wash by gravity and change buffer sequentially as follows:
 - a. 40 – 50 column volume (CV) of Wash buffer T + 30 mM imidazole.
 - b. 10 – 20 CV Wash buffer T + 50 mM imidazole.

[NB: this extensive washing procedure is critical for purity; we discourage the temptation to reduce wash volumes].

5. **Protein elution:** Manually elute his-tagged Dmc1 by the following steps, and collect 1 – 1.5 ml fractions (depending on the size of Ni²⁺ column), elute till A₂₈₀ reaches baseline:
 - a. 5 CV, Elution buffer T + 80 mM imidazole
 - b. 10 CV, Elution buffer T + 300 mM imidazole
 - c. 5 CV, Elution buffer T + 500 mM imidazole
6. Read A₂₈₀ of the fractions. A Nanodrop apparatus is particularly convenient for this purpose. Dilute peak fractions containing high Dmc1 concentration to about 4 – 5 mg/ml using Storage buffer T as soon as possible after elution to avoid Dmc1 precipitating out of solution.
7. Analyze fractions (10 – 20 µl per fraction) on a 12% SDS-PAGE gel. Pool peak fractions according to protein purity as revealed by the SDS gel.
8. Typically, Dmc1 is estimated to be near 95% homogeneity after just the Ni²⁺ resin column (Fig. 2B). If no further purification step needs to be taken, proceed to remove imidazole and exchange Dmc1 into Storage buffer T using a PD-10 or equivalent column according to manufacturer's instructions. Store the purified protein at –80°C.

3.4.3 Cleaning and storage of Ni²⁺ resin column: (Ni²⁺ Sepharose high performance resin can be reused 7 – 9 times without regeneration)

After the last elution step, wash the column sequentially with 5 CV of Wash buffer T containing 500 mM imidazole; 5 CV of distilled water; 5 CV of 20% ethanol containing 0.05% sodium azide. Cap the top and bottom of column and store it in the cold room.

3.5 FPLC Ion Exchange Chromatography:

If Dmc1 is still not pure enough after the Ni²⁺ resin step, proceed to Mono Q anion exchange column for additional purification at 4°C.

3.5.1 Buffers and reagents

- Buffer A1 = 1x Buffer T
- Buffer B1 = 1x Buffer T + 1 M KCl

3.5.2 Procedure

1. Exchange about 20 mg Dmc1 eluted from the Ni²⁺ column into (Buffer A1 + 200 mM KCl) using PD-10 column which also removes the imidazole.
2. Equilibrate a Mono Q column with 5 – 10 CV of (Buffer A1 + 200 mM KCl).
3. Load the sample onto Mono Q.

4. Using FPLC software, set automated program as follows: Wash the column with 10 CV (Buffer A1 + 200 mM KCl). Elute Dmc1 with a 30 ml linear gradient of (200 – 800 mM KCl or 20% – 80% buffer B1 at 0.8 – 1 ml/min, collect 1 ml fractions.
5. Analyze proteins in peak fractions on a 12% SDS-PAGE gel. The prominent peak should contain intact Dmc1 which is eluted at ~ 350 mM KCl. Pool fractions containing Dmc1, exchange into storage buffer by the PD10 column, keep Dmc1 concentration below 4 – 5 mg/ml, and store aliquots at –80°C.

3.6 Notes

The overall yield of near-homogenous Dmc1 is around 7 mg/lit culture. Dmc1 is a stable protein that sustains full D-loop activity for 3 – 5 freeze/thaw cycles when stored at concentrations higher than 30 μ M at – 80°C. Nonetheless, the purified protein should be stored in aliquots and minimize the number of freeze/thaw cycles.

The concentration of Dmc1 may be determined spectrophotometrically using a molar extinction coefficient of 11,520 $M^{-1}cm^{-1}$ at 280 nm; molecular weight of (His)₆-tagged Dmc1 is 37.4 kDa; use the ratio of molecular weight to molar extinction coefficient to convert Dmc1 A_{280} to concentration in mg/ml. For Dmc1, 1 A_{280} = 3.25 mg/ml.

Budding yeast Rdh54/Tid1 can be overexpressed from pET21a-Tid1 (Chi *et al.* 2006); the protein is (His)₆-tagged at the C-terminal and purified by the two-column method described here with modification of the FPLC step. After the first step of Ni²⁺ resin, the partially purified Rdh54/Tid1 should be further purified by FPLC on Mono S column instead of Mono Q because Rdh54/Tid1 is a basic protein. The protocol for Mono S will be the same as for Mono Q described above except a different buffer is required. Buffer A2 = 25 mM potassium phosphate buffer, pH 7.5 + 1 mM DTT + 10% glycerol; Buffer B2 = Buffer A2 + 1 M KCl. Rdh54/Tid1 is eluted at ~ 360 mM KCl. Full-length Rdh54/Tid1 is relatively stable in our hands and also could sustain about 3 cycles of freeze/thaw without compromising its activity. The yield of intact Rdh54/Tid1 (Fig. 2B) was 1.3 mg from 6 lit culture (~ 12 g cells). As mentioned previously, Rdh54/Tid1 is prone to proteolysis and has a tendency to be truncated into smaller size proteins. A 75 kDa protein fragment that is missing its N-terminal is often recovered in substantial quantity after the Ni²⁺ affinity step. This fragment is separated from the full-length protein by the Mono S column (Fig. 2B).

4. D-loop Assay Using Fluorescent Dye-tagged ssDNA

The displacement loop (D-loop) assay is one of the most common biochemical assays for characterizing the activity of RecA-type of recombinases. Perhaps the most commonly used version of the D-loop assay uses radioactively labeled (³²P) ssDNA oligos. Here, we introduce a method that replaces the ³²P-ssDNA substrate with a fluorescent dye Quasar670-labeled ssDNA. Fluorescent dye Quasar670 (LGC BioSearch Technologies) is a Cy5 (Cyanine 5) analog that renders bright signal suitable for detection using the 633 or 647 nm laser lines.

We have compared the feasibility of Quasar670-ssDNA (alias Cy5-ssDNA) to ^{32}P -ssDNA in D-loop assays (Fig. 1). In this assay, equal amounts of Cy5-ssDNA and ^{32}P -ssDNA were mixed and used as substrate for Dmc1. The assay results showed that Cy5-ssDNA assay is comparable in sensitivity to the ^{32}P -ssDNA assay. D-loop band signals remain linear when reaction products were diluted by half, 5 fold, and 10 fold (Fig. 1C). The D-loop activity (the D-loop yield expressed as a percentage of input plasmid DNA) is similar using either ^{32}P -ssDNA or Cy5-ssDNA (Fig. 1B & 1D). The advantages of Cy5-ssDNA over ^{32}P -ssDNA are: (a) Cy5-ssDNA is very stable when stored at -20°C in contrast to ^{32}P -ssDNA which rapidly loses radioactivity (half-life is 14.3 days); typically a preparation of ^{32}P labeled ssDNA is only suitable for use in the D-loop assay for 2 weeks. A 200 nmole synthesis of Quasar670-ssDNA is sufficient for $\sim 75,000$ D-loop reactions (10 μl reaction with Cy5-ssDNA at 30 nM). The cost of Cy5-ssDNA is about 100 times cheaper than ^{32}P -ssDNA. (b) Comparing gel images, the Cy5 method yields bands that are sharper than those produced by ^{32}P -ssDNA. (c) After electrophoresis, the agarose gel can be imaged directly for Cy5 fluorescence signal without any further gel manipulation. That is, there is no loss of D-loop assay product signal. When ^{32}P -ssDNA is used, the agarose gel must be dried onto a positively charged membrane (to capture and retain small DNA oligos), the dried gel is then exposed to image plate for a period of time before imaging. Some loss of D-loop or ssDNA signal can occur during transfer of DNAs to the filter, reducing reproducibility. It should also be noted that positively charged membranes, such as from Roche Diagnostics, or DE81 from Whatman, are quite expensive (\$6 – 10 per membrane of 10 cm $\times$ 15 cm per gel) and add significantly to the cost of the ^{32}P method. Overall the fluorescence version of the D-loop assay gives better resolution, is far more convenient, and is much less costly than the ^{32}P method.

A typical D-loop assay that assesses the Dmc1 activity stimulated by its accessory factor Hop2-Mnd1 using Cy5-ssDNA will be described below.

4.1 Buffers and reagents

- D-loop assay reaction buffer: 25 mM Tris-HCl, pH 7.5; 1 mM dithiothreitol, 5 mM MgCl_2 , 3 mM ATP, 250 nM CaCl_2 , 100 $\mu\text{g/ml}$ BSA.
- Quasar670-ssDNA (or Cy5-ssDNA): 5' Quasar670-modified 90 mer ssDNA oligo, stored in 10 mM Tris-HCl, pH 7.5; final concentration in assay is 30 nM (i.e., 2.7 μM nucleotides), ordered from LGC BioSearch Technologies. 90 mer ssDNA sequence:
5' AAAAGGCCTCTAGGTTCTTTGTTACTTCTTCTGCCGCCTGCTTCAAA
C CGCTAACAAATACCTGGGCCCCACCACACCGTGTGCATTTCGTA.
- Final concentrations of yeast proteins: Dmc1, 3 μM ; Hop2-Mnd1, 0.5 μM .
- Plasmid pRS306 with homologous sequence to Cy5-ssDNA: negatively supercoiled, purified by standard protocol using cesium chloride equilibrium gradient and without dsDNA denaturation with NaOH during cell lysis; final concentration of plasmid molecules is 5 nM in assay.
- Deproteinization solution: equal volume of 10% SDS and 10 mg/ml protease K.

- Gel, gel buffer, and electrophoresis buffer: 0.9 % agarose gel (10 cm × 14.5 cm) in 1x TAE (40 mM Tris-HCl, pH 7.5, 5 mM sodium acetate, 1 mM EDTA).
- Gel loading buffer: 25 mM Tris-HCl, pH 7.5; 50% glycerol, no dye.

4.2 Procedure

1. Mixture A: in an 8 µl reaction volume, mix 2 µl D-loop reaction buffer (5X stock), 4 µl double distilled water, 1 µl of Dmc1 (30 µM stock) and 1 µl of Cy5-ssDNA (300 nM stock). Incubate the mixture in a water bath at 37°C for 5 min.
2. Mixture B: mix 1 µl Hop2-Mnd1 (5 µM stock) with 1 µl plasmid pRS306 (50 nM stock), incubate at room temperature for 3 min.
3. Add Mixture A to Mixture B; incubate at 37°C for 20 min to form D-loops.
4. Add 2 µl of Deproteinization solution, incubate at 37°C for 5 min.
5. Add 2 µl Gel loading buffer. Make sure there is no dye in the buffer because many dyes fluoresce and interfere with the Cy5 signal.
6. Load the mixture onto agarose gel and resolve the DNA species at room temperature by electrophoresis at 8 volts/cm for 1 hr 45 min.
7. Image the gel directly for Cy5 fluorescence signal using the 647 – 670 nm laser line at 570 PMT in a Typhoon 9200 Imager or the equivalent. At this PMT value, both the D-loop band and the unreacted ssDNA band are at the linear range, i.e., the signals are under saturation.

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Fig. 1. Comparison of D-loop assays using Cy5-ssDNA vs ^{32}P -ssDNA.

(A) Cartoon of the D-loop assay. (B) Hop2-Mnd1 (H) stimulation of Dmc1 (D) D-loop formation using both fluorescent Cy5-ssDNA and radioactive ^{32}P -ssDNA in the same reaction mixture. Equal amount (15 nM each) of the two ssDNAs were added to the reaction. The “0” lane has no protein. After the D-loop formation step, the reaction mixture was diluted by half, 5 fold, and 10 fold using reaction buffer. The dilution step was followed by deproteinization and other steps. After gel electrophoresis, the agarose gel was first imaged directly for Cy5 signal in Typhoon 9200 Imager. Subsequently, the same gel was dried onto

a positively charged membrane, and exposed to an image plate overnight. The radioactive signal from the image plate was acquired using the same Imager. (C) The signal of D-loop bands from diluted reactions in (B) were quantified and plotted. (D) The D-loop activity of the reactions are plotted.

Fig. 2.

(A) **A flow chart** showing steps of the two-column method in the purification of (His)₆-tagged Dmc1 and Rdh54/Tid1. (B) Overexpression and purification of Dmc1 and Rdh54/Tid1 proteins. Lysates from Rosetta(DE3)pLysS *E. coli* cells before and after induction of expression by IPTG were analyzed on 12% SDS-PAGE for Dmc1 and 8% for Rdh/Tid1. Gel staining was with Coomassie Brilliant Blue. The purified proteins (2 µg Dmc1 [37 kDa], 2.5 µg Rdh54/Tid1 [105 KDa]) were analyzed on separate gels. Arrows indicate the position of Dmc1 and Rdh54/Tid1; an asterisk indicates the position of the 75 kDa C-terminal Rdh54/

Tid1 fragment (4 μ g fragment). The intact Rdh54/Tid1 was separated from the truncated protein by Mono S column.

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