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A Proximity Ligation-Based Method for Quantitative Measurement of D-Loop Extension in *S. cerevisiae*

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Abstract

Homologous recombination faithfully restores the sequence information interrupted by a DNA double-strand break by referencing an intact DNA molecule as a template for repair DNA synthesis. DNA synthesis is primed from 3'-OH end of the invading DNA strand in the displacement loop (D-loop). Here, we describe a simple and quantitative proximity ligation-based assay to study the initiation of homologous recombination-associated DNA synthesis initiated at the D-loop and final product formation. The D-loop extension assay overcomes the semiquantitative nature and some limitations of the current PCR-based technique and facilitates the study of the recombination-associated DNA synthesis.

1. INTRODUCTION

1.1 DNA Synthesis in Homologous Recombination

Homologous recombination (HR) faithfully repairs complex DNA damage including DNA double-stranded breaks (DSBs) by referencing an intact donor template in the form of the sister chromatid, a homolog, or an ectopic sequence. DNA strand invasion posits the 3'-OH of the invading DNA strand on the donor template (Fig. 1A). HR-associated DNA synthesis recovers the sequence information disrupted by the DSB. The extended D-loop is the last intermediate common to all HR subpathways (double Holliday Junction, Synthesis-Dependent Strand Annealing, Break-Induced Replication (BIR)) and their associated repair outcome (Heyer, 2015).

1.2 Current Method for Detection of Initiation of Recombination-Associated DNA Synthesis and Its Limitations

The kinetics and regulation of multiple steps of HR up to the repair product can be studied in detail following the controlled and synchronous induction of a site-specific DSB in *Saccharomyces cerevisiae* (White & Haber, 1990). The initiation of DNA synthesis has so far been studied semiquantitatively using PCR-based assays. These assays score the amplification of the unique sequence produced upon DNA synthesis templated from an ectopic sequence, with one primer upstream of the region of homology on the broken molecule and the other downstream of the region of homology on the donor (Aylon,

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Liefshitz, Bitan-Banin, & Kupiec, 2003; Hicks, Yamaguchi, & Haber, 2011; White & Haber, 1990). This approach has some inherent limitations. First, semiquantitative PCR methods are labor intensive and imprecise. Second, the length of the fragment generated by the PCR imposes a limit on the length of homology that can be provided between the broken molecule and the donor, as long ectopic homologies cause PCR artifacts. Since the length of homology dictates the efficiency of the upstream HR steps such as homology search and D-loop stability (Forget & Kowalczykowski, 2012) with consequences for the crossover/noncrossover HR outcome (Inbar, Liefshitz, Bitan, & Kupiec, 2000; Jinks-Robertson, Michelitch, & Ramcharan, 1993; Mehta, Beach, & Haber, 2017), this experimental approach constrains the study of recombinational DNA synthesis to particular substrates. Third, in the case of BIR, the kinetics of DNA synthesis initiation and final product formation, which entails several hundred kilobases of DNA synthesis, is not temporally resolved by these assays (Jain et al., 2009; Lydeard, Jain, Yamaguchi, & Haber, 2007; Malkova, Naylor, Yamauchi, Ira, & Haber, 2005). This is unexpected given that the conservative DNA synthesis of BIR produces long-lived newly synthesized ssDNA that can be detected by physical means (Saini et al., 2013) and that one study showed a correlation between length of DNA synthesis required for BIR completion and the time of appearance of the dsDNA BIR product (Donnianni & Symington, 2013). These results indicate a delay between DNA synthesis initiation and final BIR product formation. Finally, detection of DNA synthesis initiation by PCR-based assays was found to be dependent on Pol32 (a nonessential Pol δ subunit), while DSB repair by gene conversion, which requires DNA synthesis, is not (Jain et al., 2009; Lydeard et al., 2010). It suggests that these methodologies either fail to detect the ssDNA produced upon D-loop extension (DLE) or disproportionately detect the final dsDNA product.

These considerations prompted the development of an alternative and independent methodology for the study of HR-associated DNA synthesis. We developed a quantitative assay based on the proximity ligation principle common to chromosome conformation techniques that overcomes these limitations (Fig. 1A) (Dekker, Rippe, Dekker, & Kleckner, 2002). We also discuss possible refinements of this methodology.

2. METHODS

2.1 Materials

2.1.1 Equipment

- Standard yeast culture equipment (120rpm shaker and rotator at 30°C, 100- and 250-mL culture flasks, 15- and 50-mL conical tubes).
- Dry bath at 30°C, 37°C, 55°C, and 65°C.
- Water bath at 16°C.
- A quantitative PCR instrument (in our case a Roche LightCycler 96).
- Benchtop centrifuge with 50-mL conical tubes adaptors.
- Microcentrifuge.

- Vortex.

2.1.2 Reagents

- YPD medium: 1% (w/v) of Bacto yeast extract (BD Biosciences), 2% (v/w) Bacto peptone (BD Biosciences), 2% (v/w) D-glucose. Autoclave and store at room temperature (RT).
- YEP-lactate medium: 1% (w/v) of Bacto yeast extract (BD Biosciences), 2% (v/w) Bacto peptone (BD Biosciences), 2% Sodium DL-lactate (60%, Sigma-Aldrich). Autoclave and store at RT.
- Galactose 20% (w/v). Dissolve by stirring upon moderate heating. Filter sterilize and store at RT.
- Spheroplasting buffer: 0.4M sorbitol, 0.4MKCl, 40mM Sodium Phosphate Buffer pH 7.2, 0.5mM MgCl₂. Filter sterilize and store at 4°C.
- Zymolyase 100T solution: 2% D-glucose, 50mM Tris-HCl pH 7.5, 5mg/mL Zymolyase 100T (US Biological). Vortex well and store up to 3 months at 4°C.
- Cutsmart buffer 10 : 500mM Potassium Acetate, 200mM Tris-Acetate pH 8.0, 100mM Magnesium Acetate, 1mg/mL BSA. Store at 4°C or 20°C.
- *Hind*III-HF 20,000U/mL (NEB, R3104L).
- Ligation buffer 10 : 500mM Tris-HCl pH 8.0, 100mM MgCl₂, 100mM DTT, 10mM ATP pH 8.0, 25µg/mL BSA. Store at -20°C.
- Ligation mix (660µL per sample): Ligation buffer 1.2×, 82.5 µg/mL BSA, 7µg/mL (Bayou Biolabs), or 240U/mL (NEB M0202L) DNA T4 ligase. Make fresh right before use.
- 10% Triton X-100.
- 1% and 10% SDS solution.
- 25:24:1 Phenol:chloroform:isoamyl alcohol.
- 3M Sodium Acetate pH 5.2.
- 100% Isopropanol.
- 70% Ethanol.
- TE pH 8.0.
- RNase A 10mg/mL (Promega, cat. EN0531).
- Proteinase K 10mg/mL.
- Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, cat. 4367659).
- LightCycler Multiwell plate 96 white (Roche, cat. 04729692001).
 - Hybridization oligonucleotides

The “hybridization oligonucleotides” (ol2007 and ol2046; Table 1) are used to restore the restriction sites on the ssDNA produced by resection and DNA synthesis (in the 5′ and 3′ unique sequences flanking the region of homology “A,” respectively). They must be properly oriented and encompass the restriction site with at least ~80nt internal to the restriction fragment, in order to remain bound during the enzyme inactivation steps (55°C) and up to the ligation step of the procedure.

○ Quantitative PCR primers

The qPCR primers must have similar T_m and be positioned so as to generate amplicons of similar size (100–180bp). The primers used in this study are listed in Table 1 and their purpose described below. Amplification efficiency must be determined for each pair of primers.

2.1.3 S. cerevisiae Strains—The genotype of the wild-type (WDHY5511), *rad51* (WDHY5573), and *pol32* (WDHY5574) haploid *S. cerevisiae* strains (W303 *RAD5* background) used in this study is listed in Table 2. The *HO* gene (which encodes the site-specific HO endonuclease) under the control of the *GAL1* promoter has been inserted at the *trp1-1* locus alongside the *hphMX* marker to allow for controlled HO endonuclease expression upon galactose addition in the culture media. The HO-cut site (HOcs) at *MAT* on chromosome (Chr.) III has been mutated (*MAT-inc*) to preclude cutting at *MAT*. The DSB-inducible construct and the donor locus are depicted in Fig. 1B, and their annotated sequences are provided in Dataset S1 in the online version at <https://doi.org/10.1016/bs.mie.2017.11.024>. The HOcs together with a 2-kb-long sequence “A” (2065bp of the *LYS2* gene, coordinates 4-2068) and a 327-bp fragment of the PhiX genome flanked by multiple restriction sites replaces the *URA3* gene on Chr. V. The donor consists of the A sequence replacing the *LYS2* gene on Chr. II. These two loci have been extensively used by others to study DSB repair by HR (Inbar & Kupiec, 1999; Mine-Hattab & Rothstein, 2012). They are located in the middle of chromosome arms and represent unconstrained and untethered chromosomal regions (Agmon, Liefshitz, Zimmer, Fabre, & Kupiec, 2013).

2.2 The D-Loop Extension Assay

2.2.1 Rationale—The rationale of the DLE assay is depicted in Fig. 1A. It relies on the proximity ligation principle (Dekker et al., 2002) that the collision (and ligation) probability of two physically tethered DNA extremities will remain unaffected by the overall strand concentration. Hence, ligation performed in dilute conditions, which disfavor intermolecular ligation, allows detecting physically linked DNA extremities. In the DLE assay, the copying of a restriction site uniquely present in the 3′ flanking region of the donor upon DLE will cause its physical tethering with the restriction site present on the 5′ flanking region of the broken molecule (Fig. 1B). The two restriction sites ablated in ssDNA can be restored upon hybridization of long oligonucleotides (see below). Detection of the final dsDNA repair product does not require hybridization oligonucleotides. This criterion is used to discriminate the initiation of DNA synthesis from the final product formation. The chimeric molecule formed between the 5′ and the 3′ flanking regions of the donor upon

intramolecular ligation can be detected by a short quantitative PCR that does not encompass the region of homology (Fig. 1A).

2.2.2 Procedure

2.2.2.1 Pregrowth, Site-Specific DSB Induction, and Sample Collection

- Inoculate 5mL of YPD with a fresh colony and grow to saturation overnight at 30°C with shaking.
- Dilute in 80mL YEP-lactate in a 250-mL flask so that the cells are in exponential phase at an $OD_{600} \sim 0.5$ the next morning.
- Harvest a “no DSB” sample prior to galactose addition. Add 2% galactose. At each time point, harvest 5×10^7 cells by centrifugation at 4°C. Wash once in cold TE and store the pellet at -20°C.

2.2.2.2 Yeast Cells Spheroplasting and Restriction Sites Restoration

- Resuspend cells in 1mL of spheroplasting buffer.
- Add 4 μ L of Zymolyase 100T solution (20 μ g/mL final concentration Zymolyase 100T) and spheroplast cells 20min at 30°C. This time may need to be adjusted with each Zymolyase 100T solution.
- Wash cells two times in 1mL of spheroplasting buffer at 2500 $\times g$. Prepare 3mL of Cutsmart 1 \times buffer per sample.
- Wash cells two times in 1mL of Cutsmart 1 \times buffer at 16,000 $\times g$. These washing steps eliminate phosphatase contaminants of the Zymolyase solution and gently lyse cells. Prepare 360 μ L of Cutsmart 1.4 \times with and without hybridization oligonucleotides (olWDH2007 and olWDH2046) at a 3.5nM final concentration for each sample.
- Split the last Cutsmart 1 \times wash in two tubes. Resuspend one pellet in Cutsmart 1.4 \times with, and the other in Cutsmart 1.4 \times without, hybridization oligonucleotides. The final cell concentration is ~ 7000 cells/ μ L. When present, hybridization oligonucleotides are in a 3000-fold excess over cells and will restore restriction sites present on resected and newly synthesized ssDNA. In the absence of hybridization oligonucleotides, dsDNA products (i.e., final BIR products) can be detected.
- Store the lysed cells at -80°C or proceed immediately.

2.2.2.3 Restriction Digestion and Ligation

- Thaw cells on ice. Prewarm dry baths at 65°C and 37°C. Prewarm a water bath at 16°C.
- Take 36 μ L of resuspended spheroplasts (2.5×10^5 cells) in a new microfuge tube. Solubilize chromatin by adding 4 μ L of SDS 1% (0.1% final concentration). Mix by vortex.

- Incubate at 65°C for 15min. Vortex every 5min.
- Put on ice. Quench SDS by adding 4.5µL of Triton X-100 10% (1% final concentration). Mix with pipette.
- Digest DNA with 20–50U of *Hind*III-HF for 1h at 37°C. Finger-vortex every 20min. Prewarm a dry bath at 55°C.
- Denature *Hind*III with 9µL of SDS 10% (2% final concentration) at 55°C for 10min. Vortex.
- Put on ice. Quench SDS with 80µL of Triton X-100 10%. Mix by pipetting up and down.
- Add 660µL of ligation mix on ice. The cell concentration is $\sim 3 \times 10^3$ cells/µL in 800µL. Mix by inversion and incubate at 16°C for 90 min. Mix by inversion every 30 min. Thaw Proteinase K on ice.

2.2.2.4 DNA Purification

- Add 20µg of Proteinase K and vortex. Digest 30 min at 65°C.
- Put on ice. Transfer in a 2-mL microfuge tube.
- Add an equal volume (i.e., 800µL) of phenol:chloroform:isoamyl alcohol (25:24:1) and vortex thoroughly for at least 30s.
- Centrifuge 5 min at $16,000 \times g$ and carefully recover ~ 600 µL of the upper phase in a new 1.5-mL microfuge tube.
- Add 1:10 volume of 3M Sodium Acetate pH 5.2 and 1 volume of isopropanol. Mix by inverting several times.
- Incubate at RT for 30min.
- Pellet DNA at $16,000 \times g$ for 15min at RT. Discard supernatant and drain well. A small pellet may be visible.
- Wash the pellet in 200µL ethanol 70%. Carefully remove the supernatant with a pipette. Drain well.
- Dry the pellet at 37°C for 15min.
- Rehydrate the pellet in 50µL TE pH 8.0 at RT for 30min. Vortex.
- Add 20µg of RNase A. Incubate at 37°C for 30min. Put on ice.

2.2.2.5 Quantitative PCR

- Set up the 20µL qPCRs according to the Power SYBR Green PCR Master Mix manufacturer instructions. Use 2µL of DNA ($\sim 10^5$ genomes) per reaction. Each reaction is run in duplicate. The different primer pairs to be used and their purpose are shown in Table 1.
- The qPCR cycles are as follow: one initial denaturation step (5min at 95°C), 50 amplification steps (15 s at 95°C and 30 s at 60°C followed by a fluorescence

recording), and a final melting curve (65–97°C with 0.11°C/s increment and continuous fluorescence recording).

2.2.3 Analysis, Normalization, and Controls—Quantitative PCR results were analyzed on the LightCycler 96 Software 1.1 (Roche). With the exception of the DLE PCR (see below), all the amplification results are normalized to the reference *ARG4* locus on Chr. VIII (primers oWDH1760 and oWDH1761), with correction of the amplification efficiency (determined experimentally for each couple of primers). Several control reactions are performed (Fig. 2):

- The efficiency of DSB formation at HOcs is measured with the oWDH1766 and oWDH1767 primers. It is normalized to the uncut levels (i.e., prior to galactose addition). The results obtained by this method are not different from Southern blot quantification (Fig. 3A).
- The efficiency of *Hind*III digestion 5′ of the region of homology on the broken molecule with primers oWDH2010 and oWDH2012. It is normalized to an “uncut” control.
- The efficiency of *Hind*III digestion 3′ of the region of homology on the donor molecule with primers oWDH2009 and oWDH2011. It also measures primer extension as the copy number increases over the time course (Fig. S2B in the online version at <https://doi.org/10.1016/bs.mie.2017.11.024>).
- The intramolecular ligation efficiency of a 765-bp linear dsDNA fragment from Chr. XII (overlapping *YLR050C* and *YLR051C*) with primers oWDH2052 and oWDH2053. In a typical experiment, ~20% of the molecules have been circularized (Fig. S1A in the online version at <https://doi.org/10.1016/bs.mie.2017.11.024>). The DLE signal is normalized to this signal.

The DLE signal produced by the intramolecular ligation of the extended molecule is obtained with the primers oWDH2009 and oWDH2010. This amplification is normalized to the intramolecular ligation efficiency determined with primers oWDH2052 and oWDH2053.

3. RESULTS AND DISCUSSION

3.1 DLE Kinetics in Wild-Type Cells and Genetics Requirements

The formation of the site-specific DSB at the HOcs is achieved rapidly upon HO expression, with >90% of the molecules cut and resection initiated within an hour after induction (Fig. 3A). The chimeric ligation product expected upon D-loop extension (hereafter referred to as the DLE signal) appears with the expected slower kinetics than the previous steps of DSB formation and resection (Fig. 3B). The first unambiguous amplification is detected 2h post-HO induction, the amount of which increases sharply (13-fold) between 2 and 6h (Fig. 3B). At that time the DLE signal reaches approximately half that of the dsDNA control, consistent with the fact that the initial substrate is ssDNA, not dsDNA. The additional 1.4-fold increase observed at 8h can be attributed to dsDNA repair product formation (see below).

As expected, the DLE signal depends on a functional HR pathway, as it is reduced to background levels in a *rad51* mutant (Fig. 3C). Furthermore, we find that the initiation of DNA synthesis and extension of at least 405nt (Fig. 1B) is achieved independently of Pol32 (Fig. 3C). Pol32 is the Pol δ subunit dispensable for gene conversion but required for the extensive synthesis underlying BIR (Lydeard et al., 2007, 2010). This result suggests that Pol32 promotes BIR not by enabling the initiation of DNA synthesis, but rather by promoting extensive DNA synthesis required for BIR, consistent with its characterized biochemical role as a processivity factor for Pol δ (Burgers & Gerik, 1998; Johansson, Garg, & Burgers, 2004). The PCR-based assay showed a dependence on Pol32 for extension of 45nt during BIR (Jain et al., 2009; Lydeard et al., 2007). We thus suspect that the amplification resulted not from the ssDNA extension intermediate but from the final BIR product (see also below).

3.2 Restriction Sites Restoration Allows to Distinguish DNA Synthesis Initiation and BIR Product Formation

An anticipated requirement for the DLE assay is the restoration of the restriction sites in the ssDNA produced by the extension of the D-loop (Fig. 4A). This is achieved upon hybridization of long oligonucleotides at these two loci prior to the digestion step (Fig. 4A). Oligonucleotides are designed (i) to encompass the restriction site and (ii) to hybridize a sufficient amount of ssDNA (we used 80nt) internal to the restriction fragment to withstand the restriction enzyme denaturation step, so as to enable ligation at the subsequent step. Following induction of DSB formation at HOcs, we monitored the *Hind*III digestion of both the upstream (5') region flanking the A homology on the DSB-inducible Chr. V and the downstream (3') region flanking the A homology on the donor Chr. II in the presence or absence of their respective hybridization primers ol2007 and ol2046 (Fig. S2 in the online version at <https://doi.org/10.1016/bs.mie.2017.11.024A> and B, respectively). DNA upstream of the DSB-inducible construct is efficiently digested in its dsDNA form prior and up to 1h after DSB induction at HOcs, independently of the presence of ol2007 (Fig. S2A in the online version at <https://doi.org/10.1016/bs.mie.2017.11.024>). After that, restriction digestion depends on ol2007 (Fig. S2A in the online version at <https://doi.org/10.1016/bs.mie.2017.11.024>), consistent with the resection kinetics observed by Southern blot (Fig. 3A).

The digestion of the *Hind*III site downstream of the donor is achieved independently of ol2046 up to 2h post-DSB induction (Fig. S2B in the online version at <https://doi.org/10.1016/bs.mie.2017.11.024>). After that, the digestion requires ol2046, consistent with the rise of the DLE signal (Fig. 3B). Although less sensitive than the DLE assay, this decrease in *Hind*III digestion in the absence of ol2046 is a secondary readout of DLE, limited to the molecule in the ssDNA form as final dsDNA BIR products are digested. Consequently, the undigested signal plateaus at 50% of the dsDNA control at 6 and 8h (Fig. S2B in the online version at <https://doi.org/10.1016/bs.mie.2017.11.024>).

As expected, the DLE signal up to 6h depends on both ol2007 and ol2046 (Fig. 4B). At the latest time point 15% of the DLE signal is independent of the hybridization oligonucleotides (Fig. 4C), indicating that the circularized molecule was dsDNA. The conversion of a fraction

of the extended ssDNA into dsDNA satisfyingly explains why the DLE signal keeps increasing from 6 to 8h past the 50% expected from a normalization on dsDNA, while the undigested donor plateaus (Fig. S3 in the online version at <https://doi.org/10.1016/bs.mie.2017.11.024>). Hence, the DLE signal obtained in the presence and in the absence of hybridization oligonucleotides enables kinetic analysis of both the initiation of DLE (ssDNA) and the formation of the BIR product (dsDNA). Subtraction of the dsDNA component of the DLE signal (i.e., half of the dsDNA signal) enables a quantitative determination of the molecules that have primed and extended DNA synthesis up to the restriction site in a cell population (Fig. 4D).

These results show that the initiation of DLE and the completion of BIR, which involves asynchronous lagging strand synthesis (Donnianni & Symington, 2013; Saini et al., 2013), are temporally distinct: initiation of DLE occurs mainly between 4 and 6 h post-DSB induction, while conversion to dsDNA occurs in a delayed fashion, after 6 h. These observations and the Pol32-independent nature of DNA synthesis up to 405nt differ from results obtained with PCR-based methods, which might preferentially detect final dsDNA products (Jain et al., 2009; Jain, Sugawara, Mehta, Ryu, & Haber, 2016; Lydeard et al., 2007; Malkova et al., 2005).

3.3 Possible Refinements of the DLE Assay

The assay is dependent on the availability of compatible restriction sites in the vicinity of the region of homology between the DSB-inducible and the ectopic donor. To overcome this constraint, which dictates the minimum extension length detected at any given locus, genetic engineering may be required. By placing periodically spaced restriction sites, and by using site-specific hybridization oligonucleotides and qPCR primers in independent reactions, such engineering could allow mapping the kinetics of DLE tracts of various length (Fig. 5A). The resolution would only be limited by the hybridization oligonucleotide length (~80nt), to avoid restriction sites overlap in case a single restriction enzyme is being used.

A second possible development aimed at weeding out dsDNA repair products is to include a restriction site within the region of homology (Fig. 5B). A benefit of such site would be to more straightforwardly allow distinguishing the extending D-loops (ssDNA) from the repair product (dsDNA) than to compare DLE levels in the presence or absence of the ol2046 oligonucleotide. Indeed, in such a case restriction digestion of dsDNA would prevent the ligation of the two unique flanking regions by physically untethering them while leaving the ssDNA extension product intact (Fig. 5B).

Finally, our knowledge of mitotic DNA synthesis initiation gained with PCR-based assays mostly comes from ectopic DSB repair systems (Lydeard et al., 2007, 2010; Tsaponina & Haber, 2014; White & Haber, 1990). The assay presented here is amenable to the study of DNA synthesis in the context of allelic DNA repair by using restriction site polymorphisms between the DSB-containing homolog and the donor homolog.

4. CONCLUSION

We developed a quantitative proximity ligation-based assay for the study of DNA synthesis during recombinational DSB repair. It provides an alternative and independent approach to semiquantitative PCR-based methods and overcomes certain technical limitations. The versatility of the assay grants further developments to study in greater details the initiation and extension steps of DNA synthesis on different templates and various physiological and mutant contexts.

Supplementary data to this article can be found online at <https://doi.org/10.1016/bs.mie.2017.11.024>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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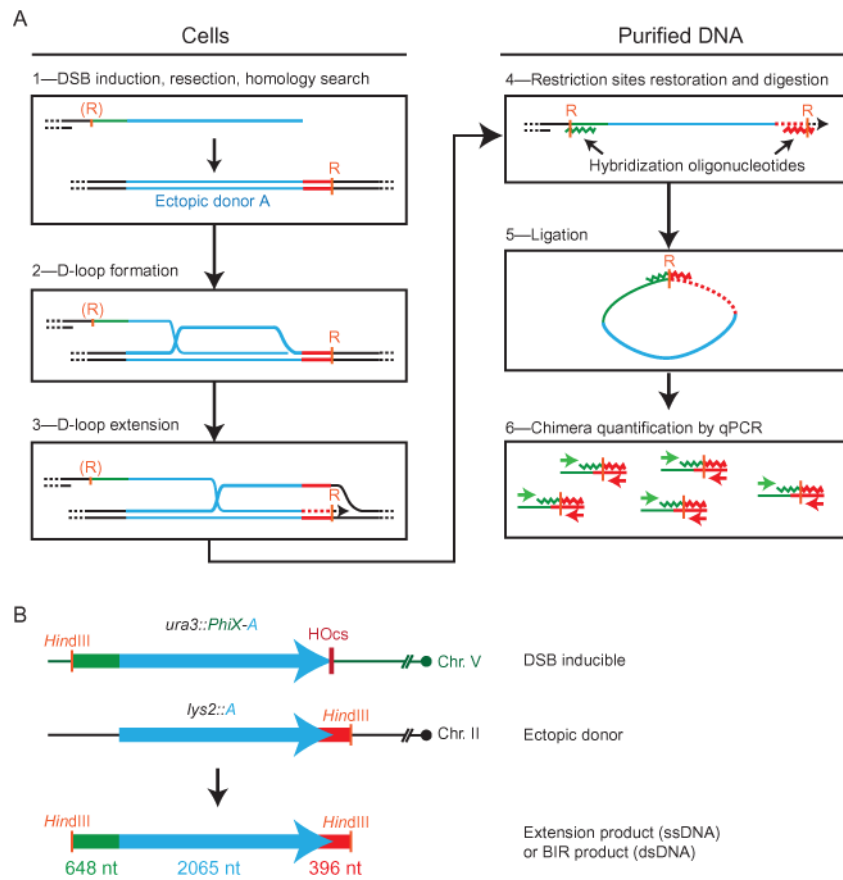
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**Fig. 1.**

Rationale of the D-loop extension assay. (A) (1) Site-specific DSB induction and resection enables homology search and (2) DNA strand invasion of an ectopically positioned donor. (3) Priming of DNA synthesis from the 3'-OH end of the invading molecule will lead to the copy of the restriction site located downstream of the donor. The two compatible restriction sites are now present on the same ssDNA molecule. (4) Following DNA extraction and restoration of the restriction sites upon annealing of long oligonucleotides, the extended ssDNA can be digested. The product of this digestion is a ssDNA with short dsDNA extremities. (5) Proximity ligation of these extremities, achieved in dilute conditions, produces a unique chimeric molecule. (6) The amount of this chimera is determined by quantitative PCR, and is the readout of the amount of D-loops extended past this restriction site on the donor in the cell population. R denotes a restriction site in its dsDNA form and (R) a restriction site in its ssDNA form (uncuttable). (B) Experimental system in haploid *S. cerevisiae* used to study initiation of DNA synthesis of an ectopic donor located at *LYS2* on chromosome (Chr.) II upon site-specific DSB induction at *URA3* on Chr. V. The DSB is repairable only by BIR, which in this context is inviable so as to prevent dilution of repair intermediates by fast repair events that would proliferate. The extent of DNA synthesis measured in this assay is at least 405nt (396nt up to the *HindIII* site, 6nt of restriction site, and 3nt of overhang to enable cutting by the enzyme).

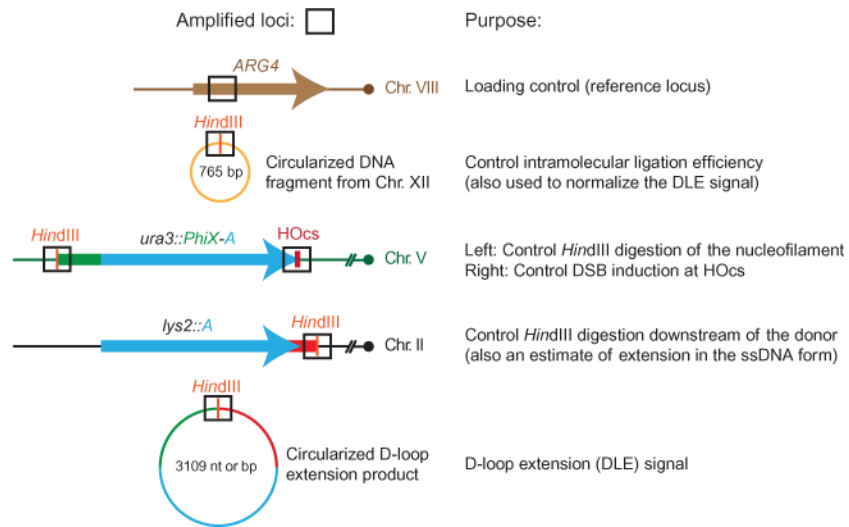


Fig. 2. Quantitative PCRs performed in the DLE assay. Loci for PCR amplification and their purpose are indicated.

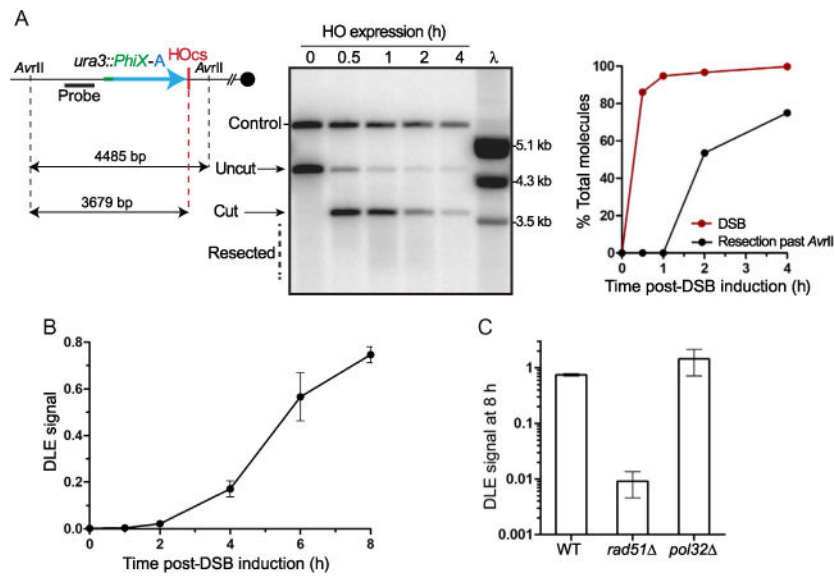


Fig. 3. Kinetics of D-loop extension and genetic requirements. (A) Southern blot analysis of the kinetics of DSB formation at HOcs following HO expression induction and resection at the *AvrII* site located 1.5kb upstream of the region of homology “A.” The loading control corresponds to an additional probe hybridizing the *RAD54* locus. The Southern blot procedure is as described (Piazza, Wright, & Heyer, 2017). (B) Kinetics of D-loop extension in wild-type cells. The DLE signal is normalized to the intramolecular ligation efficiency (Fig. S1A in the online version at <https://doi.org/10.1016/bs.mie.2017.11.024>). Data represent mean \pm SEM of biological triplicates. (C) The DLE signal is Rad51 dependent and Pol32 independent. Intramolecular ligation efficiency is similar in all strains (Fig. S1C in the online version at <https://doi.org/10.1016/bs.mie.2017.11.024>), and DSB formation at HOcs was verified in the *rad51* mutant (Fig. S1B in the online version at <https://doi.org/10.1016/bs.mie.2017.11.024>). Data represent mean \pm SEM of parallel biological replicates.

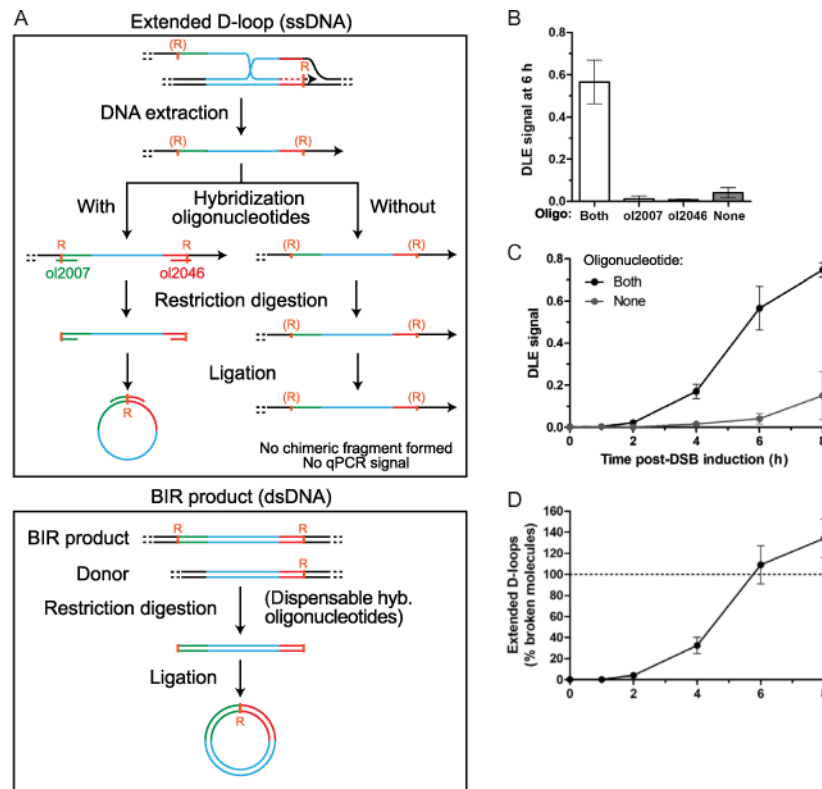


Fig. 4. Restoration of the restriction sites in ssDNA is required for DLE signal. (A) The extended D-loop prior to BIR product formation is in ssDNA form. The restriction digestion step of the DLE assay thus requires restoration of these sites. This is achieved upon annealing of the “hybridization oligonucleotides” ol2007 and ol2046 to the sites located upstream and downstream of the region of homology, respectively. This digestion step enables the subsequent intramolecular ligation reaction which provides the readout of the DLE assay. Digestion of the BIR product (dsDNA) does not require restriction site restoration. Consequently, the intramolecular ligation product is generated independently of the presence of the hybridization oligonucleotides. (B) The DLE signal at 6h post-DSB induction depends on both ol2007 and ol2046. (C) DLE kinetics in the absence of hybridization oligonucleotides reveal the fraction of DLE signal originating from dsDNA products at 6 and 8h. (D) Proportion of extended molecules in the cell population deduced from the DLE signal in the presence and absence of hybridization oligonucleotides (C). Data represent mean SEM of biological replicates.

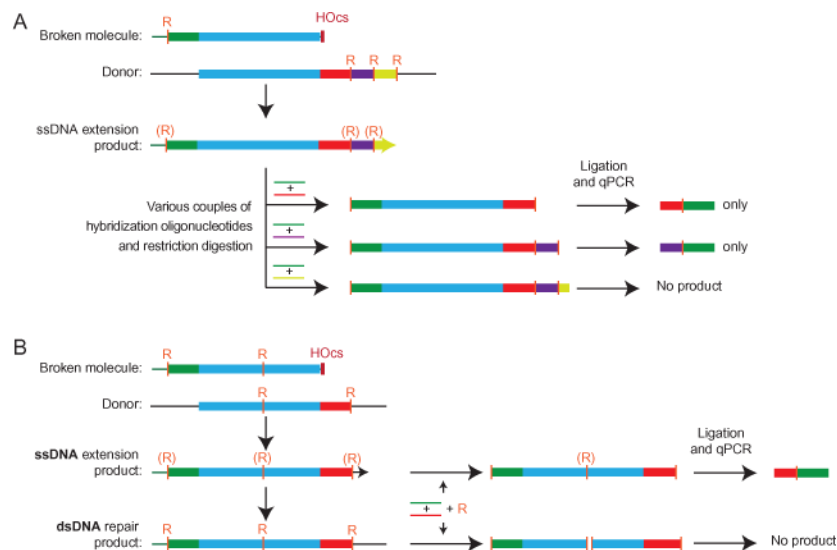


Fig. 5. Possible refinements of the DLE assay. (A) Multiple restriction sites downstream of the donor provided with the appropriate hybridization oligonucleotides would enable determining the kinetics of initiation as well as the extent of DNA synthesis primed from a D-loop. Either the same or compatible restriction enzymes could be used. (B) Restriction digestion of a site present in the homologous region would restrict the analysis to ssDNA extension product by eliminating the dsDNA repair product from the procedure. (A, B) R denotes a restriction site in its dsDNA form and (R) a restriction site in its ssDNA form (uncuttable).

Table 1

Primers Used in This Study

| Name | Sequence (5'-3') | Purpose |
|---|--|---|
| Quantitative PCR | | |
| o1WDH1760 | AGACAGAAATGGCAAAGATCC | Reference locus (<i>ARG4</i> , Chr. VIII); used for copy number reference and dsDNA loading control in all quantitative PCR experiments |
| o1WDH1761 | GGCCAATTAGTTCACCAAGACG | |
| o1WDH2062 | ATGTGCCCTTCTACCCTC | Quantify the intramolecular ligation (i.e., circularization) efficiency of a control 765- bp <i>HindIII</i> fragment (<i>YLR050C</i> ; Chr. XII) |
| o1WDH2063 | TCAAAGCGTGGTTACATTCCTTAC | |
| o1WDH1766 | GTTTCAGCTTTCCGCAACAG | Measure DSB formation at the HOcs. DSB induction causes a loss of signal |
| o1WDH1767 | GGCGAGGTATTGGATAGTTCC | |
| o1WDH2010 | TGCTCGGAGATTACCGAATC | Quantify the <i>HindIII</i> cutting efficiency on the broken molecule (resected) |
| o1WDH2012 | CGAGGCATATTATGGTGAAGG | |
| o1WDH2009 | CACCACTTTGCCAATCAACAC | Quantify the <i>HindIII</i> cutting efficiency downstream of the donor |
| o1WDH2011 | TGCCGAGGTTTTCTTGGTCAG | |
| o1WDH2009 | CACCACTTTGCCAATCAACAC | Quantify the chimera formed upon circularization of the extended invading molecule (DLE-signal). To be normalized on the circularization efficiency |
| o1WDH2010 | TGCTCGGAGATTACCGAATC | |
| <i>HindIII</i> restriction sites restoration | | |
| o1WDH2007 | TCTGCTCGGAGATTACCGAATCAAAAAAATTTCAAAGAACCCGGAATCAAAAAAAGAACAAAAAAGATGAATTTGAAAAGCTTTTATG GACCgac | Hybridize at the <i>HindIII</i> site in the <i>URA3</i> promoter on the resected broken molecule |
| o1WDH2046 | AACTCTTTGTGAAGCTTCGCAAGTATTCATTTAGACCCATGGTGAACCTTAGTGTGATGGCAAAGTGTGATAGAGATTCATAGAGATTCATAGATTGGTCAAGTAT | Hybridize at the <i>HindIII</i> site downstream of the A donor on the extended invading molecule |

Table 2Relevant Genotype of Haploid *Saccharomyces cerevisiae* Strains Used In This Study

| Strain | Relevant Genotype |
|----------|---|
| WDHY5511 | <i>ura3::A-HOcs, lys2::A, trp1::GAL-HO-hphMX, his3D200, can1-100, leu2-3, 112, ade2-1, RAD5</i> |
| WDHY5573 | <i>ura3::A-HOcs, lys2::A, trp1::GAL-HO-hphMX, his3D200, can1-100, leu2-3,112, ade2-1, RAD5 rad51::LEU2</i> |
| WDHY5574 | <i>ura3::A-HOcs, lys2::A, trp1::GAL-HO-hphMX, his3D200, can1-100, leu2-3,112, ade2-1, RAD5 pol32::KanMX</i> |

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