



## Detection of Homologous Recombination Intermediates *via* Proximity Ligation and Quantitative PCR in *Saccharomyces cerevisiae*

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### Abstract

DNA damage, including DNA double-stranded breaks and inter-strand cross-links, incurred during the S and G2 phases of the cell cycle can be repaired by homologous recombination (HR). In addition, HR represents an important mechanism of replication fork rescue following stalling or collapse. The regulation of the many reversible and irreversible steps of this complex pathway promotes its fidelity. The physical analysis of the recombination intermediates formed during HR enables the characterization of these controls by various nucleoprotein factors and their interactors. Though there are well-established methods to assay specific events and intermediates in the recombination pathway, the detection of D-loop formation and extension, the two critical steps in this pathway, has proved challenging until recently. Here, efficient methods for detecting key events in the HR pathway, namely DNA double-stranded break formation, D-loop formation, D-loop extension, and the formation of products *via* break-induced replication (BIR) in *Saccharomyces cerevisiae* are described. These assays detect their relevant recombination intermediates and products with high sensitivity and are independent of cellular viability. The detection of D-loops, D-loop extension, and the BIR product is based on proximity ligation. Together, these assays allow for the study of the kinetics of HR at the population level to finely address the functions of HR proteins and regulators at significant steps in the pathway.

### SUMMARY:

The D-loop capture (DLC) and D-loop extension (DLE) assays utilize the principle of proximity ligation together with quantitative PCR to quantify D-loop formation, D-loop extension, and product formation at the site of an inducible double-stranded break in *Saccharomyces cerevisiae*.

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DISCLOSURES:

The authors have nothing to disclose.

## Keywords

D-loop; genome stability; joint molecule; Rad51; break-induced replication

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## INTRODUCTION:

Homologous recombination (HR) is a high-fidelity mechanism of repair of DNA double-stranded breaks (DSBs), inter-strand cross-links, and ssDNA gaps, as well as a pathway for DNA damage tolerance. HR differs from error-prone pathways for DNA damage repair/tolerance, such as non-homologous end-joining (NHEJ) and translesion synthesis, in that it utilizes an intact, homologous duplex DNA as a donor to template the repair event. Moreover, many of the key intermediates in the HR pathway are reversible, allowing for exquisite regulation of the individual pathway steps. During the S, G2, and M phases of the cell cycle, HR competes with NHEJ for the repair of the two-ended DSBs<sup>1</sup>. In addition, HR is essential to DNA replication for the repair of replication-associated DNA damage, including ssDNA gaps and one-sided DSBs, and as a mechanism of DNA lesion bypass<sup>2</sup>.

A critical intermediate in the HR pathway is the displacement loop, or D-loop (Figure 1). Following end resection, the central recombinase in the reaction, Rad51, loads onto the newly resected ssDNA of the broken molecule, forming a helical filament<sup>2</sup>. Rad51 then carries out a homology search to identify a suitable homologous donor, typically the sister chromatid in somatic cells. The D-loop is formed when the Rad51-ssDNA filament invades a homologous duplex DNA, which leads to the Watson-Crick base pairing of the broken strand with the complementary strand of the donor, displacing the opposite donor strand. Extension of the 3' end of the broken strand by a DNA polymerase replaces the bases that were lost during the DNA damage event and promotes resolution of the extended D-loop intermediate into a dsDNA product through the synthesis-dependent strand annealing (SDSA), the double-Holliday junction (dHJ), or the break-induced replication (BIR) HR sub-pathways.

Assays that physically monitor the intermediates in the HR pathway permit the analysis of the genetic requirements for each step (i.e., pathway analysis). DSB formation, end resection, dHJs, BIR replication bubbles, and HR products are readily observed by Southern blotting<sup>3-7</sup>. Yet, Southern blotting fails to report on nascent and extended D-loops, and, thus, an alternative method to reliably measure these joint molecules is required<sup>4,8,9</sup>. One widely used strategy to analyze nascent D-loop formation is chromatin-immunoprecipitation (ChIP) of Rad51 coupled with quantitative PCR (qPCR)<sup>10,11</sup>. However, Rad51 association with dsDNA as measured by ChIP-qPCR is independent of sequence homology and the Rad51 accessory factor Rad54<sup>10,11</sup>. In contrast, an appreciable signal using the method of D-loop analysis presented here, called the D-loop capture (DLC) assay, depends on DSB formation, sequence homology, Rad51, and the Rad51 accessory proteins Rad52 and Rad54<sup>8</sup>. The finding that *Saccharomyces cerevisiae* Rad51-promoted D-loop formation depends on Rad54 *in vivo* is in agreement with numerous *in vitro* reconstitution experiments indicating that Rad54 is required for homology search and D-loop formation by budding yeast Rad51<sup>8,12-15</sup>.

Current approaches to measuring D-loop extension, primarily through semi-quantitative PCR, are similarly problematic. A typical PCR-based assay to detect D-loop extension amplifies a unique sequence, resulting from recombination between a break site and an ectopic donor and the subsequent recombination-associated DNA synthesis, *via* a primer upstream of the region of homology on the broken strand and another primer downstream of the region of homology on the donor strand. Using this method, the detection of recombination-associated DNA synthesis requires the non-essential Pol  $\delta$  processivity factor Pol32<sup>16</sup>. This finding conflicts with the observation that *POL32* deletion has only a mild effect on gene conversion *in vivo*<sup>17</sup>. Moreover, these PCR-based assays fail to temporally resolve D-loop extension and BIR product formation, suggesting that the signal results from dsDNA products rather than ssDNA intermediates<sup>17-19</sup>. The D-loop extension (DLE) assay was recently developed to address these discrepancies. The DLE assay quantifies recombination-associated DNA synthesis at a site ~400 base pairs (bp) downstream of the initial 3' invading end<sup>9</sup>. By this method, D-loop extension is independent of Pol32 and is detectable within 4 h post-DSB induction, whereas BIR products are first observed at 6 h. Indeed, a recent publication from the Haber and Malkova laboratories noted that using this method of preparation of genomic DNA singularly results in ssDNA preservation<sup>9,20</sup>.

Here, the DLC and DLE assays are described in detail. These assays rely on proximity ligation to detect nascent and extended D-loops in *S. cerevisiae* (Figure 2)<sup>8,9</sup>. BIR products can be quantified using this same assay system. For both assays, DSB formation at an HO endonuclease cut site located at the *URA3* locus on chromosome (Chr.) V is induced by the expression of the HO endonuclease under the control of a galactose-inducible promoter. Rad51-mediated DNA strand invasion leads to nascent D-loop formation at the site of an ectopic donor located at the *LYS2* locus on Chr. II. As the right side of the DSB lacks homology to the donor, repair *via* SDSA and dHJ formation is not feasible. Initial repair of the DSB by BIR is possible, but the formation of viable products is inhibited by the presence of the centromere<sup>21</sup>. This deliberate design prevents productive DSB repair, thereby avoiding the resumption of growth by cells with repaired DBSSs, which could otherwise overtake the culture during the time course analysis.

In the DLC assay, psoralen crosslinking of the two strands of the heteroduplex DNA within the D-loop preserves the recombination intermediate. Following restriction enzyme site restoration on the broken (resected) strand and digestion, the crosslinking allows for ligation of the unique sequences upstream of the homologous broken and donor DNAs. Using qPCR, the level of chimeric DNA molecule present in each sample is quantified. In the DLE assay, crosslinking is not required, and restriction enzyme site restoration and digestion followed by intramolecular ligation instead link the 5' end of the broken molecule to the newly extended 3' end. Again, qPCR is used to quantify the relative amounts of this chimeric product in each sample. In the absence of restriction enzyme site restoration, the DLE assay reports on the relative levels of the BIR (dsDNA) product that is formed following D-loop extension.

Representative results for each assay using a wild-type strain are shown, and readers are referred to Piazza et al.<sup>8</sup> and Piazza et al.<sup>9</sup> for the use of these assays for the analysis of

recombination mutants<sup>8,9</sup>. The intent of this contribution is to enable other laboratories to adopt the DLC and DLE assays, and support for them is available upon request.

## PROTOCOL:

### 1. Pre-growth, DSB induction, and sample collection

NOTE: Supplementation of all media with 0.01% adenine is recommended for Ade- strains.

1.1. Streak out the appropriate haploid strains (see Table 1) on yeast peptone dextrose adenine (YPDA) (1% yeast extract, 2% peptone, 2% glucose, 2% agar, 0.001% adenine) and grow for 2 days at 30 °C.

1.2. Use a single colony to inoculate 5 mL of YPD in a 15 mL glass culture tube. Grow cultures to saturation at 30 °C with shaking or rotation for aeration.

1.3. DLC assay: Prepare the 5x psoralen stock solution (0.5 mg/mL trioxsalen in 200-proof ethanol) in a fume hood by dissolving psoralen in a 50 mL conical tube wrapped in aluminum foil overnight at room temperature with continuous shaking or inversion. Seal screw top with a transparent film to prevent evaporation. Do not prepare more than 7 mL of 5x psoralen stock solution per 50 mL conical tube to ensure proper dissolution of the psoralen.

1.4. The next day, use 5 mL of the YPD grown overnight culture to inoculate 50–100 mL of YEP-lactate (1% yeast extract, 2% peptone, 2% w/w lactate, 0.001% adenine) in an appropriately sized flask (budding yeast grows optimally in a flask that is at least 5x the volume of the culture) to an OD<sub>600</sub> of ~0.03.

1.5. Grow the culture for ~16 h at 30 °C with shaking at 220 rpm. After ~16 h, measure the OD<sub>600</sub> of the culture and it should be ~0.5–0.8. Do not use under- or overgrown cultures.

1.6. For each time point, collect the appropriate volume of cells in a conical tube and place on ice. Typically, this is  $1.5 \times 10^8$  cells (approximately 7.5 mL of culture at OD<sub>600</sub> 1.0 for a haploid wild-type strain) for the DLC assay and  $1 \times 10^8$  cells (approximately 5 mL of culture at OD<sub>600</sub> 1.0) for the DLE assay.

1.7. To ensure the accuracy of the OD<sub>600</sub> values, prepare 1:5 dilutions for cultures with an OD<sub>600</sub> 1.0 to keep the OD reading at 0.2 or below. For wild-type strains, optimal time points for DLC analysis are between 2 h and 6 h, and optimal time points for DLE analysis are between 4 h and 8 h (see Figure 3 and Figure 4).

#### 1.8. DLC assay

1.8.1. Before each time point, prepare enough 1x psoralen solution (0.1 mg/mL trioxsalen, 50 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 20% ethanol) in a fume hood for all the samples in a 50 mL conical tube wrapped in foil. Leave at RT.

1.8.2. Centrifuge the samples at  $2,500 \times g$  for 5 min at 4 °C. Resuspend the pellet in 2.5 mL of 1x psoralen solution in a fume hood and transfer to a 60 mm × 15 mm Petri dish.

Alternatively, resuspend the pellet in 2.5 mL of TE1 solution (50 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0) for a no-crosslinking control.

1.8.3. Crosslink the samples. For a UV crosslinker fit with long-wave (365 nm) bulbs, position the Petri dishes 1–2 cm below the UV light source with the lid removed atop a plastic or plexiglass plate that has been pre-chilled at  $-20^{\circ}\text{C}$ . For a UV light box, place the Petri dishes directly atop the UV light source. Expose the samples for 10 min with gentle shaking.

NOTE: It is recommended to set the UV light source atop an orbital shaker set at  $\sim 50$  rpm.

1.8.4. In a fume hood, transfer the sample into a new 15 mL tube. Rinse the Petri dish with 2.5 mL of TE1 solution and add this to the tube. Centrifuge the samples at  $2,500 \times g$  for 5 min at  $4^{\circ}\text{C}$ , properly dispose of the supernatant, and store the pellet at  $-20^{\circ}\text{C}$ . Samples can be stored for up to 1 week before moving to the next step.

## 1.9. DLE assay

1.9.1. Centrifuge the samples at  $2,500 \times g$  for 5 min at  $4^{\circ}\text{C}$ . Wash the cell pellet in 2.5 mL of cold TE1 solution before repeating the spin and storing the pellets at  $-20^{\circ}\text{C}$ . Samples can be stored for up to 1 week before moving to the next step.

1.10. For sample collection at 0 h, collect the samples prior to the addition of 20% galactose. For subsequent timepoints, induce DSB formation by adding 20% galactose to the cultures to a final concentration of 2%. Collect the remaining samples as described above, pellet, and freeze relative to the time post-DSB induction (i.e., the 4 h sample is collected 4 h after the addition of 20% galactose).

## 2. Cell spheroplasting, lysis, and restriction site restoration

2.1. Thaw the samples on ice. Preheat a dry bath to  $30^{\circ}\text{C}$ .

2.2. Resuspend the samples in 1 mL of spheroplasting buffer (0.4 M sorbitol, 0.4 M KCl, 40 mM sodium phosphate buffer pH 7.2, 0.5 mM  $\text{MgCl}_2$ ) and transfer to a 1.5 mL microcentrifuge tube.

2.3. Add 3.5  $\mu\text{L}$  of zymolyase solution (2% glucose, 50 mM Tris-HCl pH 7.5, 5 mg/mL zymolyase 100T; 17.5  $\mu\text{g}/\text{mL}$  zymolyase final concentration). Mix gently by tapping or inversion. Incubate at  $30^{\circ}\text{C}$  for 15 min, and then place on ice. During the 15 min incubation, obtain liquid nitrogen or dry ice.

2.4. Centrifuge for 3 min at  $2,500 \times g$  at  $4^{\circ}\text{C}$  and place the samples on ice. Wash the samples 3x in 1 mL of spheroplasting buffer. Centrifuge the samples for 3 min at  $2,500 \times g$  at  $4^{\circ}\text{C}$ .

2.5. Resuspend the samples in 1 mL of cold 1x restriction enzyme buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 100  $\mu\text{g}/\text{mL}$  BSA pH  $\sim 8.0$  at RT) and centrifuge for 3 min at  $16,000 \times g$  at  $4^{\circ}\text{C}$ . Place the samples on ice. Repeat the wash 1x.

2.6. Resuspend the samples in 1 mL of cold 1x restriction enzyme buffer. Split the sample (0.5 mL each) into two 1.5 mL microcentrifuge tubes. Centrifuge the samples for 3 min at  $16,000 \times g$  at 4 °C.

2.7. Resuspend one tube from each sample in 180  $\mu$ L of 1.4x restriction enzyme buffer with hybridizing oligos (see Table 2) and one tube in 180  $\mu$ L of 1.4X restriction enzyme buffer without hybridizing oligos. Each hybridizing oligo is resuspended in 1x TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and used at a final concentration of 7 nM. The 1x TE replaces the hybridizing oligos in the 1.4x restriction enzyme buffer without hybridizing oligos.

NOTE: The hybridizing oligos must be stored at  $-20$  °C in small aliquots at the working dilution. The concentration of the hybridizing oligos may require optimization; see section 7, DLC and DLE assay troubleshooting.

2.8. Snap freeze the samples in liquid nitrogen or dry ice/ethanol and store at  $-80$  °C. Samples can be stored at this stage for several months.

### 3. Restriction enzyme digest and intramolecular ligation

3.1. Thaw the samples on ice. Preheat one dry bath to 65 °C and another to 37 °C.

3.2. Pipet 36  $\mu$ L of the sample into a new 1.5 mL microcentrifuge tube on ice. Promptly return the remaining sample to  $-80$  °C for storage.

3.3. Add 4  $\mu$ L of 1% SDS (0.1% final concentration) and mix by gently tapping the side of the tube. Incubate at 65 °C for 15 min with gentle tapping every 5 min. Place samples on ice immediately following the incubation.

NOTE: This SDS treatment promotes the denaturation of DNA-associated proteins, solubilization of the nuclear envelope, and chromatin accessibility in advance of the restriction enzyme digest and intramolecular ligation steps.

3.4. Add 4.5  $\mu$ L of 10% Triton X-100 (1% final concentration) and mix by pipetting. Add 20–50 U of restriction enzyme (*Eco*RI-HF or *Hind*III-HF) to each sample and incubate at 37 °C for 1 h with gentle agitation every 20–30 min. During this time, preheat a dry bath to 55 °C and preset a water bath to 16 °C.

3.5. Add 8.6  $\mu$ L of 10% SDS (1.5% final concentration) to each sample and mix by pipetting and tapping. Incubate at 55 °C for 10 min. Add 80  $\mu$ L of 10% Triton X-100 (6% final concentration) to each sample and mix by pipetting.

3.6. Add 660  $\mu$ L of 1x ligation buffer without ATP (50 mM Tris-HCl pH 8.0, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 2.5  $\mu$ g/mL BSA) + 1 mM ATP pH 8.0 + T4 DNA ligase (8 U/sample) to each sample and mix by gentle inversion. Incubate at 16 °C for 1.5 h with inversion every 30 min. Place the samples on ice immediately following the incubation.

#### 4. DNA purification

4.1. Preheat one dry bath to 65 °C and another to 37 °C. Add 1 µL of 10 mg/mL proteinase K (prepared in 1x TE pH 8.0) to each sample (12.5 µg/mL final concentration). Incubate at 65 °C for 30 min and place the samples on ice immediately following the incubation until they have cooled.

4.2. Transfer the samples to 2 mL tubes. Working in a fume hood, add an equal volume (~800 µL) of phenol/chloroform/isoamyl alcohol (P/C/IA; pH 8.0) to each sample. Vortex the samples for ~30 s and centrifuge the samples for 5–10 min at 16,000 × *g* in a microcentrifuge.

4.3. Carefully remove 600 µL of the upper phase of each sample into a new 1.5 mL tube. Properly dispose of the lower phase and 2 mL tubes.

4.4. Precipitate the DNA by adding a 1/10 volume of 3 M sodium acetate pH 5.2 (~60 µL) to each sample, followed by 1 volume of isopropanol (~660 µL). Invert the samples 5x–10x and incubate at RT for 30 min.

4.5. Place the samples on ice for 2 min, and then centrifuge the samples at 16,500 × *g* for 15 min at 4 °C in a microcentrifuge. Return the samples to ice, pour off the supernatant, and drain the tube on a paper towel.

4.6. Wash the DNA pellet with 200 µL of 70% ethanol. Centrifuge at 16,500 × *g* for 3 min at 4 °C, place the samples back on ice, pour off the supernatant, and remove the residual alcohol with a pipet. Dry the samples with the caps of the tubes open at 37 °C for 15–20 min.

4.7. Resuspend the DNA pellets in 50 µL of 1x TE by vortexing. Incubate at RT for 30 min, vortex, and then incubate at 37 °C in a dry bath for 30 min. Vortex the samples again, and then place them on ice. Samples can be stored at this stage at –20 °C for several months, but it is advisable to proceed immediately for the decrosslinking (DLC only) and qPCR steps.

#### 5. Psoralen crosslink reversal (for DLC assay only)

5.1. Pipet 9 µL of purified DNA into a PCR tube on ice. Add 1 µL of 1 M KOH (0.1 M final concentration). Incubate the samples at 90 °C for 30 min in a thermocycler.

5.2. Add 19.73 µL of sodium acetate solution (0.1 M sodium acetate, 9.6 mM Tris-HCl pH 8.0, 1.0 mM EDTA pH 8.0). Samples can be stored at this stage at –20 °C for several months, but it is advisable to proceed immediately to the qPCR step.

#### 6. Quantitative PCR, controls, and analysis

6.1. Using 2 µL of purified DNA, with or without crosslinking, set up a 20 µL qPCR reaction according to the manufacturer's instructions. Set up each reaction in duplicate. For both the DLC and DLE assays, there are five control reactions and one DLC/DLE quantification reaction, or a total of six reactions per sample, run in duplicate. Supplementary Table S1 and

Supplementary Table S2 provide a template for setting up these reactions and analysis, and the sequences of the qPCR primers are listed in Table 3.

6.2. qPCR cycling conditions need to be optimized for each qPCR kit.

6.2.1. Use the following DLC qPCR conditions, depending on the qPCR kits used: initial denaturation (95 °C for 3 min); 50 rounds of amplification (95 °C for 15 s, 61 °C for 25 s, 72 °C for 15 s with a single acquisition); melting curve analysis (95 °C for 5 s, 65 °C for 1 min, 97 °C with continuous acquisition); and cooling (37 °C for 30 s).

6.2.2. Use the following qPCR conditions for the DLE assay: initial denaturation (95 °C for 5 min); 50 rounds of amplification (95 °C for 15 s, 60 °C for 30 s, 72 °C for 15 s with a single acquisition); melting curve analysis (95 °C for 5 s, 65 °C for 1 min, 97 °C with continuous acquisition); and cooling (37 °C for 30 s). Note that optimization for different qPCR machines/kits may be required.

6.3. DLC assay

6.3.1. Controls: See the list of qPCR primers in Table 3. A map of the primer binding sites is shown in Figure S1. For supplementary sequence files for the relevant genomic features and amplicons, check the A plasmid Editor (ApE) files; Supplementary Sequence Files 1–5.

6.3.1.1. Genomic DNA at *ARG4*: Use oIWDH1760/oIWDH1761 to amplify dsDNA located at *ARG4*. Use this reaction as a loading control and normalize all other reactions except the DLC signal reaction to this control.

6.3.1.2. Intramolecular ligation efficiency at *DAP2*: Use the 1,904 bp fragment created by *EcoRI* digestion for intramolecular ligation in parallel with the DLC ligation. Amplification across this ligation junction reports on the intramolecular ligation efficiency and serves as a control to which the DLC signal is normalized.

6.3.1.3. DSB induction: Use oIWDH1766/oIWDH1767 to amplify a region that spans the induced DSB.

6.3.1.4. Psoralen crosslinking (if not decrosslinked) and resection: Use oIWDH2019/oIWDH2020 to amplify the unique PhiX region downstream of the *EcoRI* recognition site. Without crosslink reversal, use the ratio of the ssDNA (no crosslinking) over *ARG4* (crosslinked dsDNA) to determine the crosslinking efficiency. With crosslink reversal, resection will lead to a progressive decrease from 1 to 0.5 of the signal relative to *ARG4*.

6.3.1.5. *EcoRI* recognition site restoration and cutting: Use oIWDH1768/oIWDH1764 to amplify a region that spans the restored *EcoRI* recognition site upstream of the DSB on the resected strand. oIWDH1769/oIWDH1763 amplify a region that spans the *EcoRI* restriction enzyme site at *DAP2*. Perform *EcoRI* cleavage at this site to use as intramolecular ligation control.

6.3.2. DLC signal: Use oIWDH1764/oIWDH1765 to amplify the chimeric DNA molecule created by intramolecular ligation of the resected (invading) strand and the donor.



6.3.3. Analysis: Calculate the average and standard deviation of the Cp values for each of the duplicate reactions. Use the *ARG4* genomic DNA qPCR Cp values as a reference to normalize all the other control qPCRs. Normalize the DLC signal to the intramolecular ligation control at *DAP2*. See Figure 3 for typical DLC signal values at 2 h.

#### 6.4. DLE assay

6.4.1. Controls: See the list of qPCR primers in Table 3. A map of the primer binding sites is shown in Figure S1. For supplementary sequence files for the relevant genomic features and amplicons, check the A plasmid Editor (ApE) files.

6.4.1.1. Genomic DNA at *ARG4*: See section 6.3.1.1.

6.4.1.2. Intramolecular ligation efficiency at *YLR050C*: Use the *HindIII* digestion to create a 765 bp fragment that will undergo intramolecular ligation in parallel with the DLE ligation. Amplification across this ligation junction reports on the intramolecular ligation efficiency and serves as a control to which the DLE signal is normalized.

6.4.1.3. DSB induction: See section 6.3.1.2.

6.4.1.4. *HindIII* recognition site restoration and cutting: Use oIWDH2010/oIWDH2012 and oIWDH2009/2011 to amplify a region that spans the *HindIII* restriction enzyme sites on the broken strand where it has been resected and extended, respectively.

6.4.2. DLE signal: Use oIWDH2009/oIWDH2010 to amplify the chimeric DNA molecule created by intramolecular ligation of the resected end of the invading strand upstream of the DSB to the newly extended end downstream of the DSB.

6.4.3. Analysis: Calculate the average and standard deviation of the Cp values for each of the duplicate reactions. Use the *ARG4* genomic DNA qPCR Cp values as a reference to normalize all the other control qPCRs. Normalize the DLE signal to the intramolecular ligation control at *YLR050C*. Typical DLE signal values at 6 h are reported in Figure 4 and previous publications<sup>9</sup>.

## REPRESENTATIVE RESULTS:

### DLC assay

The DLC assay detects both nascent and extended D-loops formed by the invasion of a site-specific DSB into a single donor (Figure 2). Psoralen crosslinking physically links the broken strand and the donor *via* the heteroduplex DNA within the D-loop. Restriction enzyme site restoration with a long, hybridizing oligo on the resected strand of the break allows for restriction enzyme cleavage, followed by ligation of the broken strand to the proximal donor to form a chimeric product that is quantified by qPCR. Notably, the DLC signal depends on the psoralen crosslinking, the hybridizing oligo, the central recombinase, Rad51, and the Rad51 accessory factors Rad52 and Rad54<sup>8</sup>. Deletion of the DNA helicases/topoisomerases Sgs1-Top3-Rmi1, Mph1, and Srs2 leads to an increased DLC signal.

Figure 3 shows the representative results for the standard wild-type strain at 2 h post-DSB induction in triplicate with and without hybridizing oligo. A sample lacking in a key step, psoralen crosslinking, is also shown in duplicate.

As shown in Figure 3, psoralen crosslinking is a critical step. There is practically no detectable signal without it<sup>8</sup>. Crosslinking efficiency is measured based on the ratio of ssDNA to dsDNA amplification. Unlike dsDNA, ssDNA experiences minimal psoralen crosslinking, and, thus, a high signal indicates successful crosslinking. Crosslinking efficiency varies depending on the time between sample collection and preparation for qPCR (Figure 3, bottom left panel). The more time between sample collection and preparation, the less signal will be observed for the crosslinking efficiency qPCR control. Significant intersample variation in the signal observed for the crosslinking efficiency qPCR control is a cause for concern, and the time course should be discarded.

*ARG4* Cp values are similar between the with- and without-hybridizing oligo samples (Figure 3, top-left panel). A low Cp value indicates that more amplifiable DNA is present. This explains why the *ARG4* Cp values for the without-crosslinking samples are significantly lower: crosslinking interferes with qPCR amplification. This difference between the with- and without-crosslinking samples applies to all the qPCRs except the *EcoRI* cleavage qPCR control, which will amplify ssDNA/non-crosslinked dsDNA. All the qPCR controls, but not the DLC signal, are normalized to the *ARG4* qPCR signal.

For all the samples, the intramolecular ligation qPCR control is within the appropriate range (Figure 3, top middle panel), and there is robust DSB induction, as evidenced by the low signal for the qPCR control that amplifies across the HO endonuclease recognition site (Figure 3, top-right panel). In the with-hybridizing oligo samples, efficient *EcoRI* cutting is observed, and this qPCR control gives a low signal (Figure 3, bottom middle panel). Conversely, the without-hybridizing oligo with-crosslinking samples give a high signal, similar to what is shown for the crosslinking efficiency qPCR control, since, in this case, uncut ssDNA is being amplified and normalized to the *ARG4* qPCR signal (dsDNA).

In contrast to the other qPCRs, the qPCR signal for the DLC assay is normalized to the intramolecular ligation qPCR control, since the chimeric molecule quantified by the DLC qPCR depends on ligation. The median DLC signal at 2 h with hybridizing oligo is  $0.030 \pm 0.0055$  (Figure 3, bottom right panel), in keeping with previously published results for this assay<sup>8</sup>. As expected, this signal depends on both the hybridizing oligo and psoralen crosslinking.

## DLE assay

The DLE assay allows for the accurate monitoring of D-loop extension in response to a site-specific DSB (Figure 2). It was demonstrated previously that the DLE signal depends on Rad51, the central recombinase in the reaction, which mediates strand invasion and is, thus, required for recombination-associated DNA synthesis<sup>9</sup>. In addition, the DLE signal depends on the catalytic subunit of Pol  $\delta$ , Pol3 (DR, AP, WDH, unpublished data) but not the non-essential processivity factor Pol32. In contrast to the DLC signal, which first becomes detectable at 2 h post-DSB induction, the DLE signal first noticeably increases

at 4 h post-DSB induction, rises dramatically between 4 h and 6 h, and begins to plateau thereafter, with much of the increase in signal between 6 h and 8 h attributable to BIR product formation<sup>8,9</sup>.

As the chimeric ligation product quantified in the DLE assay is single-stranded, the cell spheroplasting and lysis step is critical. Decreased DLE signal can result from issues with this step, which may release nucleases and lead to degradation of the target ssDNA.

Figure 4 shows representative results for the standard wild-type strain at 6 h post-DSB induction in triplicate with and without hybridizing oligos. The wild-type sample without hybridizing oligos represents the dsDNA BIR product alone, whereas the with-oligo signal is derived from both the ssDNA of the extended D-loop and the dsDNA BIR product. A third sample is included as an example of a failed experiment.

*ARG4* Cp values were similar between the with- and without-hybridizing oligos samples (Figure 4, top-left panel). *ARG4* Cp values were noticeably lower for the failed sample, indicating that this sample has more genomic DNA than the successful samples. The qPCR signals for the qPCR controls, but not the DLE signal, were normalized to the *ARG4* qPCR signal. The intramolecular ligation qPCR control revealed an acceptable signal for the with- and without-hybridizing oligos samples (between ~0.15–0.35) but a substantially lower signal for the failed sample (Figure 4, top-middle panel). In this failed sample, the high amount of genomic DNA indicated by the *ARG4* qPCR control likely caused the intramolecular ligation to fail, since a high concentration of genomic DNA will lead to intermolecular ligation.

In all three samples, there was robust DSB induction (Figure 4, top-right panel). *HindIII* cleavage on both the resected and extended strands depends on the presence of the hybridizing oligos. On the extended strand, it additionally depends on D-loop extension. Thus, there was a significant difference in amplification across the *HindIII* cleavage site on the resected strand between the with- and without-oligo samples (Figure 4, bottom-left panel) and a smaller difference in amplification across the *HindIII* recognition site on the extended strand between these samples (Figure 4, bottom-middle panel).

As the DLE signal depends on intramolecular ligation, it is normalized to the intramolecular ligation qPCR control. The median DLE signal at 6 h with hybridizing oligos was  $0.53 \pm 0.17$  (Figure 4, bottom right panel), consistent with previously published results for this assay<sup>9</sup>. DLE signal for the wild-type sample without hybridizing oligos was similarly compatible with this prior publication. The DLE signal was lower than expected for the failed sample, likely reflecting the issues with that sample mentioned above.

### Crosslink reversal

Psoralen intercalated between ApT/TpA base pairs in dsDNA can become covalently linked through its furan and pyrone rings to one or both opposing thymine bases upon UV irradiation, resulting in (predominantly furan) mono-adducts or inter-strand di-adducts (i.e., crosslinks), respectively<sup>22</sup>. These modifications are expected to block DNA polymerase's progression, thus inhibiting the DNA synthesis reaction integral to quantitative PCR.

Consequently, most dsDNA templates cannot be amplified (Figure 5A,B). In contrast, the absence of base pairs in ssDNA makes it less prone to psoralen crosslinking. It is, thus, amplified more readily than dsDNA, which distorts the relative quantification of ssDNA versus dsDNA and of dsDNA amplicons of different lengths and ApT/TpA content (Figure 5A,B). To overcome these limitations, a base- and heat-catalyzed reversal of the psoralen crosslink reversal step<sup>23</sup> was applied prior to the quantitative PCR. This method only leaves the minor species of pyrone-side mono-adducts<sup>23,24</sup>. It led to an 80-fold recovery of dsDNA loading and circularization control amplicons, indicating that the great majority of template molecules had at least one furan-side monoadduct or inter-strand crosslink (Figure 5B,C). The comparison of the Cp values of the dsDNA loading control before and after crosslink reversal provides an estimate of the crosslinking efficiency, which should be in the range shown here. Beyond short amplicons, this procedure can restore templates up to 3 kb long (Figure S2). No change was observed for the ssDNA amplicon, consistent with a lack of psoralen crosslinking to ssDNA (Figure 5B–D). It also shows that the crosslink reversal procedure does not detectably damage DNA<sup>23</sup>. The recovery of the DLC chimera amplicon, which contains a crosslinked dsDNA segment ligated to a non-crosslinked ds-ssDNA segment (50 bp and 118 bp/nt; Figure 5A) was intermediate to that of dsDNA and ssDNA amplicons, with an 8-fold improvement in recovery (Figure 5B,C). Crosslink reversal did not affect the relative levels of the two dsDNA amplicons, with the circularization control remaining in the 20%–25% range relative to the loading control (Figure 5E). However, it changed the relative amount of the ssDNA amplicon relative to the dsDNA loading control from a 40-fold excess to the 0.5-fold expected for an ssDNA relative to a dsDNA template (Figure 5D). Likewise, the partly ssDNA DLC signal decreased from  $6.6 \times 10^{-2}$  to  $6.6 \times 10^{-3}$  relative to the dsDNA circularization controls (Figure 5F). This leads us to estimate the number of D-loop joint molecules at an inter-chromosomal donor detected by this approach 4 h post-DSB induction to be an average of 1.3% of the total broken molecules in the cell population. Such absolute estimates could not be made with psoralen-based distortion of dsDNA and ssDNA amplification, which highlights the value of this additional crosslink reversal step.

## DISCUSSION:

The assays presented allow for the detection of nascent and extended D-loops (DLC assay), D-loop extension (DLE assay), and BIR product formation (DLE assay with no hybridizing oligonucleotides) using proximity ligation and qPCR. ChIP-qPCR of Rad51 to sites distant from the DSB has previously been used as a proxy for Rad51-mediated homology search and D-loop formation. However, this ChIP-qPCR signal is independent of the sequence homology between the break site and a potential donor, as well as the Rad51-associated factor Rad54, and is, thus, more likely to represent a transient association between the Rad51-ssDNA filament and dsDNA rather than a D-loop intermediate<sup>10,11</sup>. In contrast, the DLC signal depends on DSB formation, Rad51, Rad52, Rad54, and shared sequence homology between the DSB and the donor site assayed<sup>8</sup>. Moreover, increased DLC signals are observed in the absence of the Mph1 and Srs2 helicases, and the Sgs1-Top3-Rmi1 helicase-topoisomerase complex, consistent with previous reports that these three factors can disassemble Rad51/Rad54-made nascent D-loops *in vitro*<sup>8,25–27</sup>. The DLE assay similarly

represents an improvement over previous methods to follow recombination-associated DNA synthesis, as it can distinguish between D-loop extension and BIR product formation<sup>19</sup>.

As discussed above, the qPCR controls, including those for the genomic DNA, DSB induction, psoralen cross-linking, intramolecular ligation, and oligonucleotide hybridization, are critical to the success and reproducibility of these assays. Raw genomic DNA qPCR values should be approximately equivalent across samples. Low Cp values for the *ARG4* genomic DNA control indicate excess DNA, and the number of cells collected should be adjusted. High Cp values for this control indicate insufficient DNA recovery or contamination with reagents that interfere with qPCR. Following spheroplasting, cell lysis can be observed using a standard light microscope and equal volumes of sample and sterile water. If insufficient lysis is observed upon the addition of water, the zymolyase solution must be remade, or the incubation at 30 °C should be prolonged. Samples can also be lost or contaminants introduced during DNA purification by P/C/IA extraction. For the efficient recovery of DNA, one should ensure that the pH of the P/C/IA has been adjusted to ~8.0 and that the bottom phase is not disturbed while removing the upper phase. Lastly, inefficient resuspension of the DNA pellet in 1x TE can result in low Cp values. A longer incubation at 37 °C and vortexing will improve the resuspension of the DNA pellet.

In addition to the genomic DNA loading control, the DSB induction and restriction enzyme cleavage control reactions should also be similar across samples. HO endonuclease or restriction enzyme cutting at the site of the DSB or restriction enzyme recognition site prevents amplification across this region; therefore, typical normalized qPCR values for these controls are near zero, and a high qPCR value indicates insufficient cleavage. If a high signal at the site of the DSB is observed, the galactose solution should be remade. For mutants with a known cell cycle defect, DSB induction should be quantified by plating equal amounts of culture grown according to the protocol (see section 1) on YPDA and YPA media supplemented with galactose. Colonies that grow on media containing galactose represent yeast in which end-joining created an uncleavable HOcs. If there are significantly more end-joining events in a mutant of interest relative to the wild type, a correction must be applied to compensate for this difference in DSB induction, which will affect the DLC/DLE signal.

Three primer pairs (olWDH1764/olWDH1768, olWDH2010/olWDH2012, and olWDH2009/olWDH2011) assess restriction enzyme site restoration by the hybridizing oligos and cutting by the *EcoRI*-HF and *HindIII*-HF restriction enzymes. Moreover, the intramolecular ligation controls also depend on adequate restriction enzyme digestion. Thus, a sample with low intramolecular ligation efficiency and a high signal for one of these three primer pairs has insufficient restriction enzyme cutting. Additional restriction enzymes should be provided in subsequent preparations, and the efficacy of the restriction enzyme should be assessed on genomic DNA. The olWDH1769/olWDH1763 primer pair represents an additional control for the DLC assay, which measures *EcoRI* cleavage at *DAP2*, where intramolecular ligation efficiency is also measured. A sample with an adequate intramolecular ligation signal but a high signal for one of these three primer pairs has inadequate restriction enzyme site restoration by the hybridizing oligos. To address this problem, duplicate samples should be collected and the concentration of the affected

hybridizing oligo(s) should be varied. Typical qPCR values obtained for these reactions with and without hybridizing oligos can be found in Figure 3 and Figure 4 and in Piazza et al.<sup>8</sup> and Piazza et al.<sup>9</sup>.

For both the DLC and the DLE assay, an intramolecular ligation efficiency of 15%–35% as normalized to the genomic DNA control is considered normal. As the detection of nascent and extended D-loops and the BIR product is dependent on efficient ligation, samples with low ligation signals must be discarded. The 10x ligation buffer lacking ATP should be stored at 4 °C for no more than 6 months. Collecting too many cells can lead to intermolecular ligation, which will result in low intramolecular ligation efficiency and DLC/DLE signal.

Though these controls for the DLC and DLE assays report on nearly all the sensitive steps, it is still possible to obtain non-physiological values for the DLC or DLE signal when these controls are within the appropriate range. A low DLC or DLE signal may result from errors in the cell spheroplasting step, which is extremely sensitive. One should process only a few samples in parallel and keep them at 4 °C at all times. A high/low DLC/DLE signal can also result from collecting too many/few cells at each time point. This problem can be addressed by collecting multiple OD<sub>600</sub>s of cells at each time point for each sample.

There are several technical and conceptual limitations to the DLC and DLE assays in their present form. First, the psoralen-mediated inter-strand crosslink density is ~1 in 500 bp<sup>8</sup>. Therefore, an increased DLC signal can either indicate that there are more D-loops in the population, that the average length of the D-loops in the population has increased (assuming that D-loops can be smaller than 500 bp), or both. Furthermore, the likelihood that a D-loop will be captured by the DLC assay decreases with decreasing D-loop length. Given that very short D-loops may account for a significant fraction of the total D-loop population in certain mutant backgrounds, this limitation of the assay must be considered when interpreting results. Second, the DLC assay requires DNA crosslinking, whereas the DLE assay does not. Previously, for a given experiment, this meant that DLC and DLE samples had to be collected and analyzed separately. The method shown in Figure 5 achieves robust crosslink reversal, alleviating the need to collect multiple samples from the same culture. The introduction of a second *EcoRI* restriction enzyme site on the broken strand, downstream of the *HindIII* recognition site, will enable sequential DLC and DLE analysis.

In addition to these technical limitations, the DLC and DLE assay system currently does not permit the recovery of viable HR products because the right side of the inducible DSB lacks homology to the donor. To better understand the kinetics and mechanism of second end engagement and synthesis, the system could be modified such that repair using a proximal or distal region of homology shared between the second end of the break and the donor is feasible (Figure 6). Looking forward, it may prove insightful to combine the DLC and DLE assays with other technologies, such as ChIP-qPCR, high-throughput chromosome conformation capture (Hi-C), and *in vivo* D-loop mapping, to achieve a comprehensive analysis of the kinetics and regulation of the steps in the HR pathway, including break formation, end resection, Rad51 filament formation, nascent D-loop formation, D-loop extension, D-loop reversal, second end engagement, second end synthesis, and resolution<sup>28</sup>.

In summary, the DLC and DLE assays permit the quantification of nascent and extended D-loops, D-loop extension, and BIR product formation using the principle of proximity ligation. These assays represent major advancements in the field, as they are the first to permit the semi-quantitative measurement of D-loop formation and extension independent of cellular viability.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGMENTS:

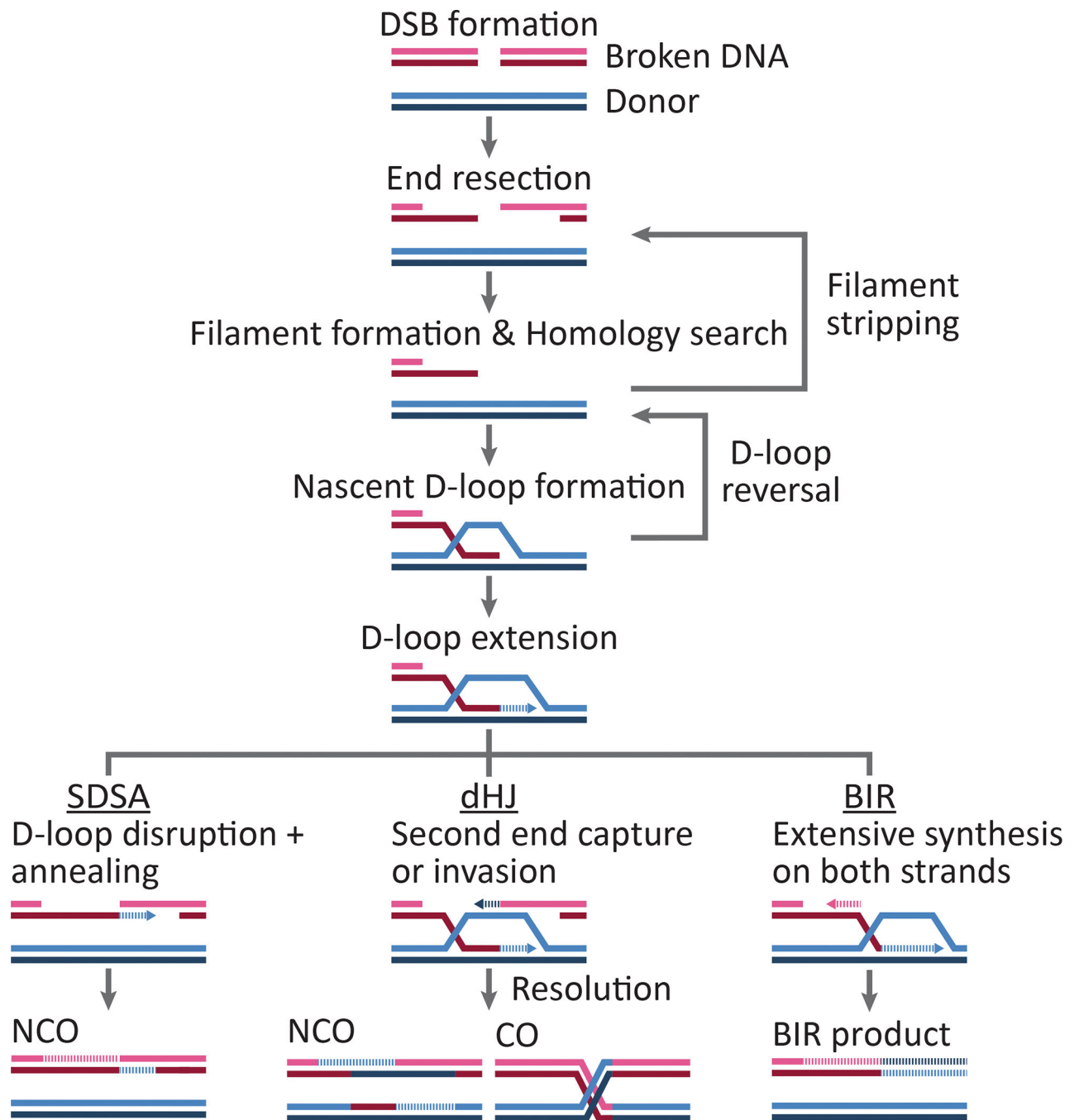
The work in the Heyer laboratory is supported by grants GM58015 and GM137751 to W.-D.H. Research in the Piazza laboratory is supported by the European Research Council (ERC-SiG 3D-loop, grand agreement 851006). D.R. is supported by T32CA108459 and the A.P. Giannini Foundation. We thank Shih-Hsun Hung (Heyer Lab) for sharing his DLC/DLE assay results and for additionally validating the changes to the assays that are detailed in this protocol.

## REFERENCES:

1. Symington LS, Gautier J Double-strand break end resection and repair pathway choice. *Annual Review of Genetics*. 45 (1), 247–271 (2011).
2. Heyer W-D. Regulation of recombination and genomic maintenance. *Cold Spring Harbor Perspectives in Biology*. 7 (8), a016501 (2015). [PubMed: 26238353]
3. Mimitou EP, Symington LS Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. *Nature*. 455 (7214), 770–774 (2008). [PubMed: 18806779]
4. Bzymek M, Thayer NH, Oh SD, Kleckner N, Hunter N Double Holliday junctions are intermediates of DNA break repair. *Nature*. 464 (7290), 937–941 (2010). [PubMed: 20348905]
5. Chen H, Lisby M, Symington LS RPA coordinates DNA end resection and prevents formation of DNA hairpins. *Molecular Cell*. 50 (4), 589–600 (2013). [PubMed: 23706822]
6. Mazón G, Symington LS Mph1 and Mus81-Mms4 prevent aberrant processing of mitotic recombination intermediates. *Molecular Cell*. 52 (1), 63–74 (2013). [PubMed: 24119400]
7. Saini N et al. Migrating bubble during break-induced replication drives conservative DNA synthesis. *Nature*. 502 (7471), 389–392 (2013). [PubMed: 24025772]
8. Piazza A et al. Dynamic processing of displacement loops during recombinational DNA repair. *Molecular Cell*. 73 (6), 1255–1266.e4 (2019). [PubMed: 30737186]
9. Piazza A, Koszul R, Heyer W-D. A proximity ligation-based method for quantitative measurement of D-loop extension in *S. cerevisiae*. *Methods in Enzymology*. 601, 27–44 (2018). [PubMed: 29523235]
10. Sugawara N, Wang X, Haber JE In vivo roles of Rad52, Rad54, and Rad55 proteins in Rad51-mediated recombination. *Molecular Cell*. 12 (1), 209–219 (2003). [PubMed: 12887906]
11. Renkawitz J, Lademann CA, Kalocsay M, Jentsch S Monitoring homology search during DNA double-strand break repair in vivo. *Molecular Cell*. 50 (2), 261–272 (2013). [PubMed: 23523370]
12. Petukhova G, Stratton S, Sung P Catalysis of homologous DNA pairing by yeast Rad51 and Rad54 proteins. *Nature*. 393 (6680), 91–94 (1998). [PubMed: 9590697]
13. Wright WD, Heyer W-D. Rad54 functions as a heteroduplex DNA pump modulated by its DNA substrates and Rad51 during D-loop formation. *Molecular Cell*. 53 (3), 420–432 (2014). [PubMed: 24486020]
14. Tavares EM, Wright WD, Heyer W-D, Cam EL, Dupaigne P. In vitro role of Rad54 in Rad51-ssDNA filament-dependent homology search and synaptic complexes formation. *Nature Communications*. 10 (1), 4058 (2019).

15. Crickard JB, Moevus CJ, Kwon Y, Sung P, Greene EC Rad54 drives ATP hydrolysis-dependent DNA sequence alignment during homologous recombination. *Cell*. 181 (6), 1380–1394.e18 (2020). [PubMed: 32502392]
16. Lydeard JR, Jain S, Yamaguchi M, Haber JE Break-induced replication and telomerase-independent telomere maintenance require Pol32. *Nature*. 448 (7155), 820–823 (2007). [PubMed: 17671506]
17. Jain S et al. A recombination execution checkpoint regulates the choice of homologous recombination pathway during DNA double-strand break repair. *Genes & Development*. 23 (3), 291–303 (2009). [PubMed: 19204116]
18. Donnianni RA, Symington LS Break-induced replication occurs by conservative DNA synthesis. *Proceedings of the National Academy of Sciences of the United States of America*. 110 (33), 13475–13480 (2013). [PubMed: 23898170]
19. Liu L et al. Tracking break-induced replication shows that it stalls at roadblocks. *Nature*. 590 (7847), 655–659 (2021). [PubMed: 33473214]
20. Liu L, Sugawara N, Malkova A, Haber JE Determining the kinetics of break-induced replication (BIR) by the assay for monitoring BIR elongation rate (AMBER). *Methods in Enzymology*. 661, 139–154 (2021). [PubMed: 34776210]
21. Pham N et al. Mechanisms restraining break-induced replication at two-ended DNA double-strand breaks. *The EMBO Journal*. 40 (10), e104847 (2021). [PubMed: 33844333]
22. Cimino GD, Gamper HB, Isaacs ST, Hearst JE Psoralens as photoactive probes of nucleic acid structure and function: Organic chemistry, photochemistry, and biochemistry. *Annual Review of Biochemistry*. 54 (1), 1151–1193 (1985).
23. Yeung AT, Dinehart WJ, Jones BK Alkali reversal of psoralen cross-link for the targeted delivery of psoralen monoadduct lesion. *Biochemistry*. 27 (17), 6332–6338 (1988). [PubMed: 3219339]
24. Shi YB, Spielmann HP, Hearst JE Base-catalyzed reversal of a psoralen-DNA cross-link. *Biochemistry*. 27 (14), 5174–5178 (1988). [PubMed: 3167039]
25. Prakash R et al. Yeast Mph1 helicase dissociates Rad51-made D-loops: Implications for crossover control in mitotic recombination. *Genes & Development*. 23 (1), 67–79 (2009). [PubMed: 19136626]
26. Liu J et al. Srs2 promotes synthesis-dependent strand annealing by disrupting DNA polymerase  $\delta$ -extending D-loops. *eLife*. 6, e22195 (2017). [PubMed: 28535142]
27. Fasching CL, Cejka P, Kowalczykowski SC, Heyer W -D. Top3-Rmi1 dissolve Rad51-mediated D loops by a topoisomerase-based mechanism. *Molecular Cell*. 57 (4), 595–606 (2015). [PubMed: 25699708]
28. Shah SS, Hartono SR, Chédin F, Heyer W-D. Bisulfite treatment and single-molecule real-time sequencing reveal D-loop length, position, and distribution. *eLife*. 9, e59111 (2020). [PubMed: 33185185]





**Figure 1: Homologous recombination and resolution sub-pathways.**

Following DNA damage that results in a one- or two-ended DSB (shown) or an ssDNA gap, 5' to 3' resection of the DNA ends reveals 3' ssDNA overhangs on which the Rad51 filament forms, aided by its accessory factors. Rad51 then searches the genome for an intact duplex DNA (i.e., the donor) on which to template the repair event. This process culminates in DNA strand invasion, in which the broken strand Watson-Crick base pairs with the complementary strand of the double-stranded DNA donor, displacing the opposite strand and forming the nascent D-loop. This D-loop can either be reversed to allow a Rad51

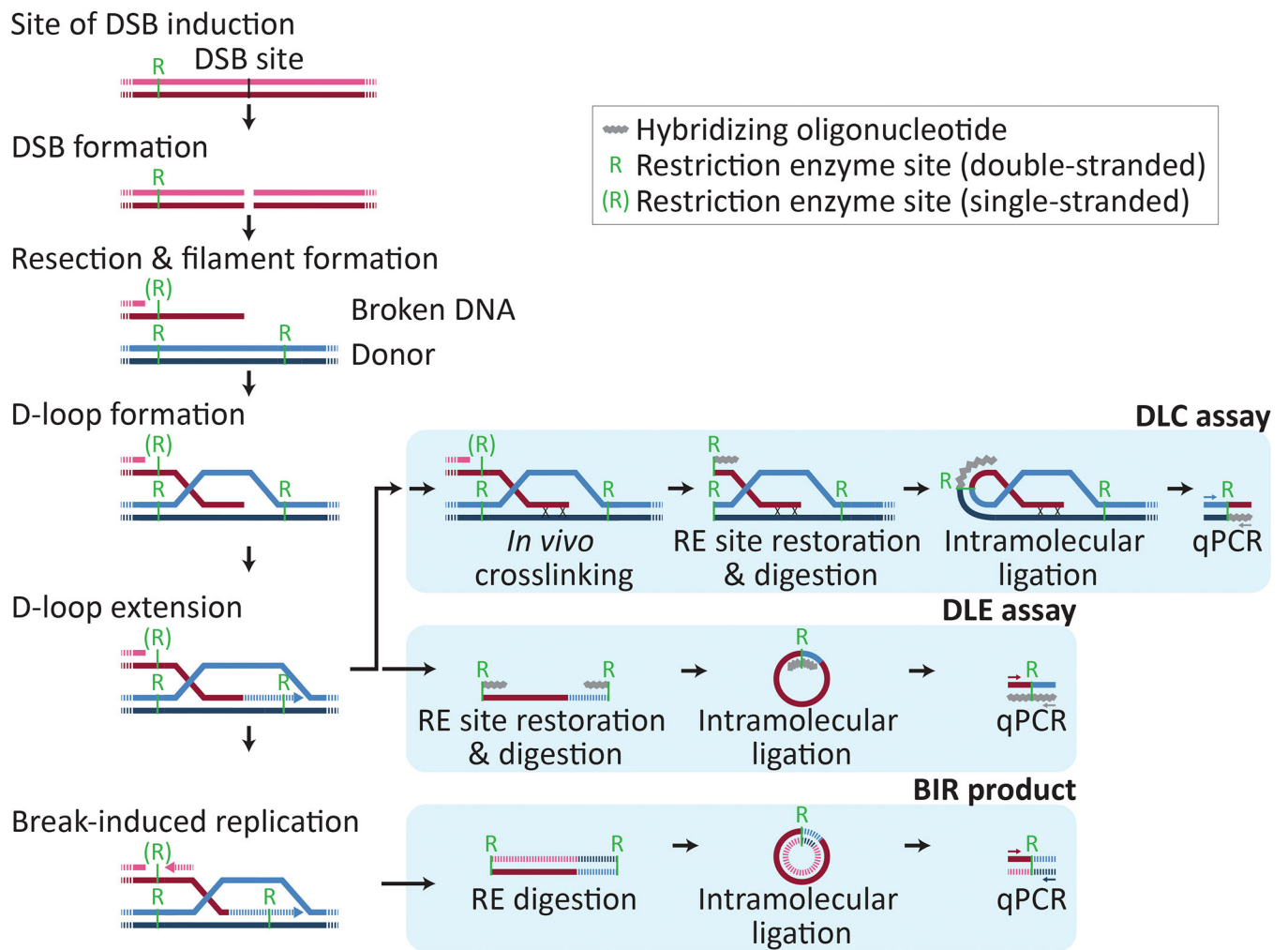
homology search to select a different donor or extended by a DNA polymerase to replace the bases lost during the DNA damage event. Three HR sub-pathways are available to resolve this extended D-loop intermediate into a product. First, the extended D-loop can be disrupted by a helicase, permitting the newly extended end of the break to anneal to the second end in a process termed synthesis-dependent strand annealing (SDSA). Fill-in DNA synthesis and ligation then lead to product formation. Alternatively, the second end of the break can anneal to the displaced donor strand, forming a double-Holliday junction (dHJ). Nucleolytic resolution of the dHJ results in either a crossover (CO) or non-crossover (NCO), whereas dHJ dissolution (not shown) results in only NCO products. Lastly, failure to engage the second end of the DSB results in break-induced replication (BIR), a mutagenic process in which thousands of base pairs are copied from the donor onto the broken strand. This process can extend as far as the converging replication fork or the end of the chromosome.

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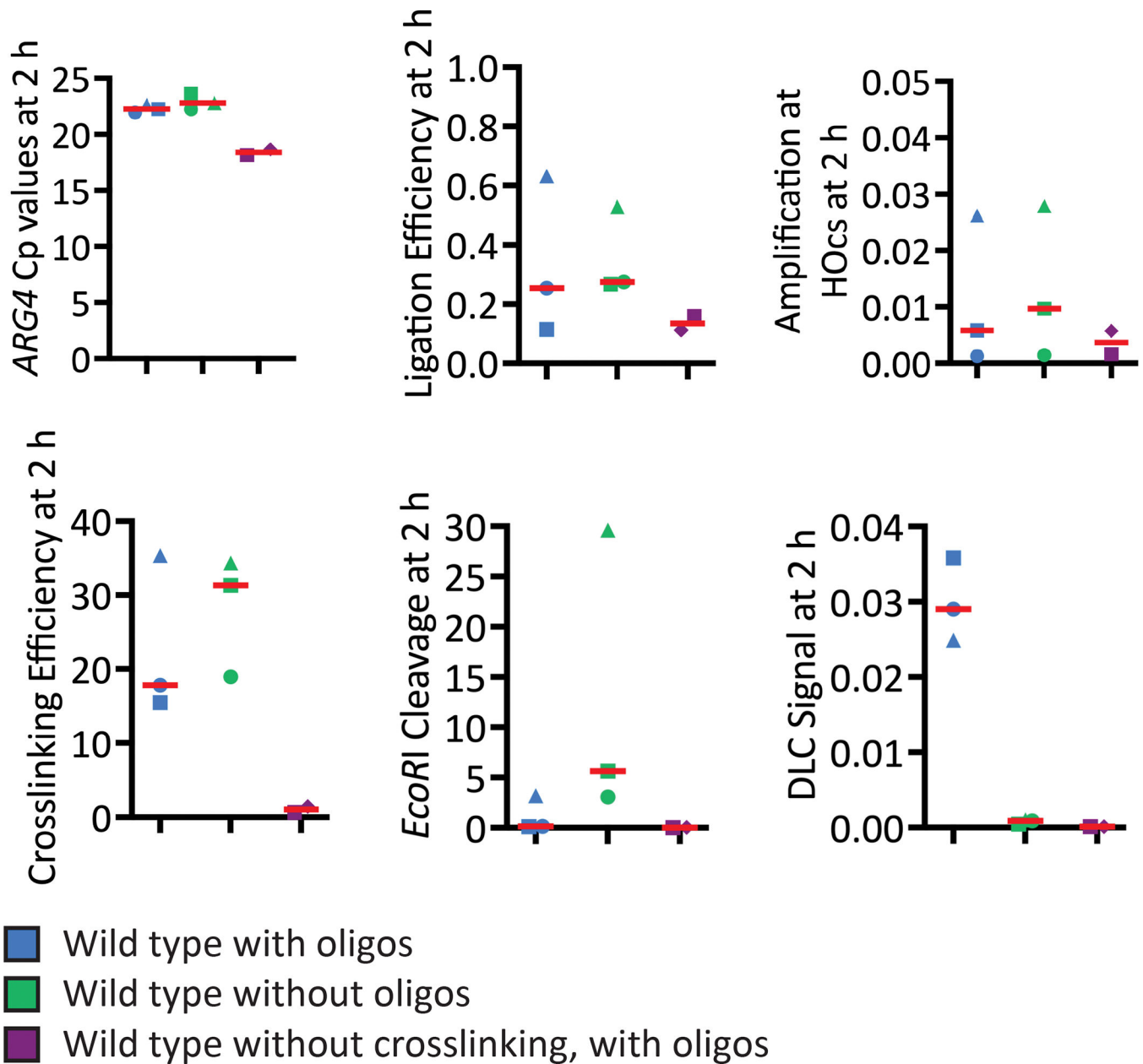
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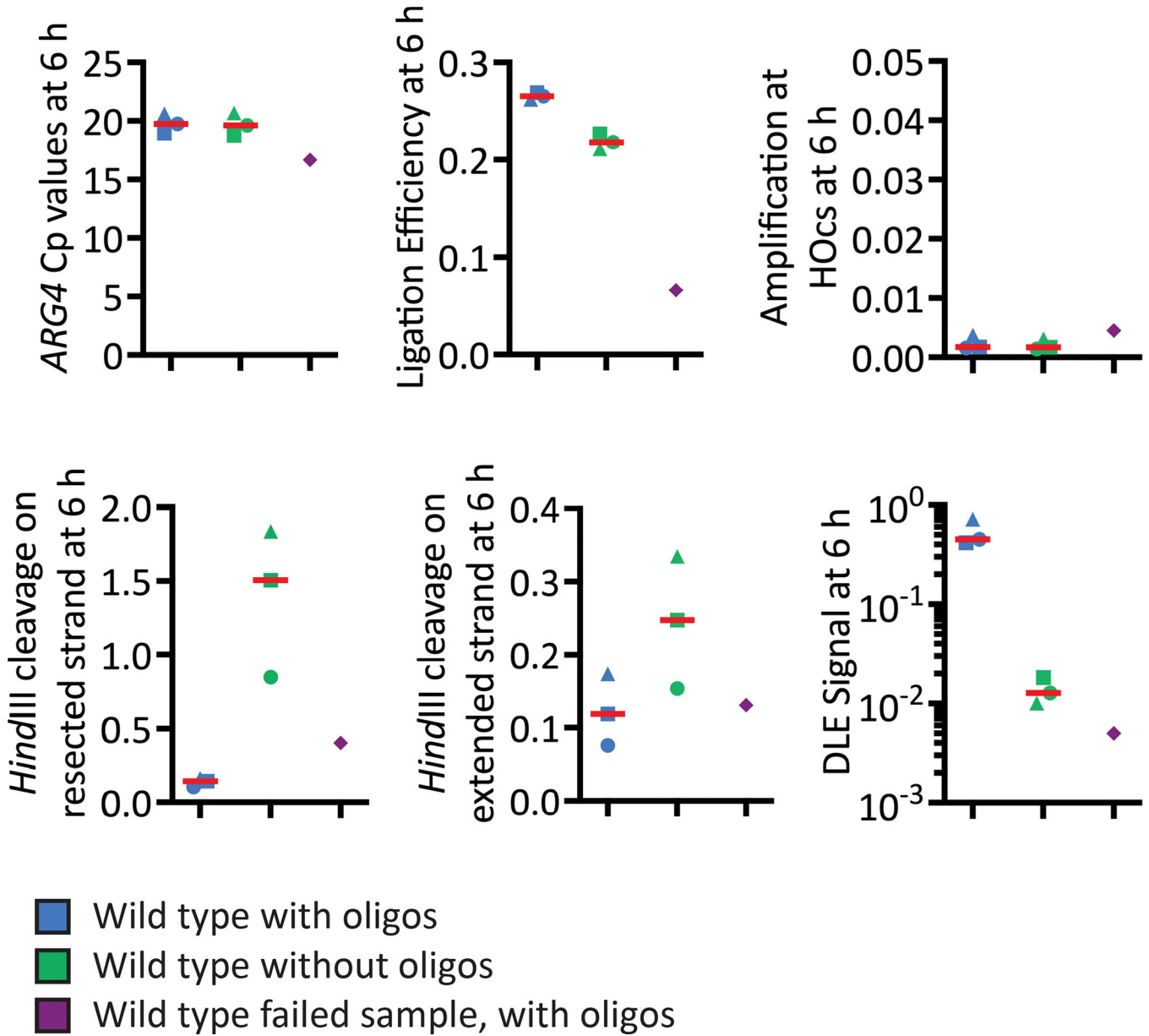


**Figure 2: The premise of the D-loop capture (DLC), D-loop extension (DLE), and break-induced replication (BIR) product formation assays.**

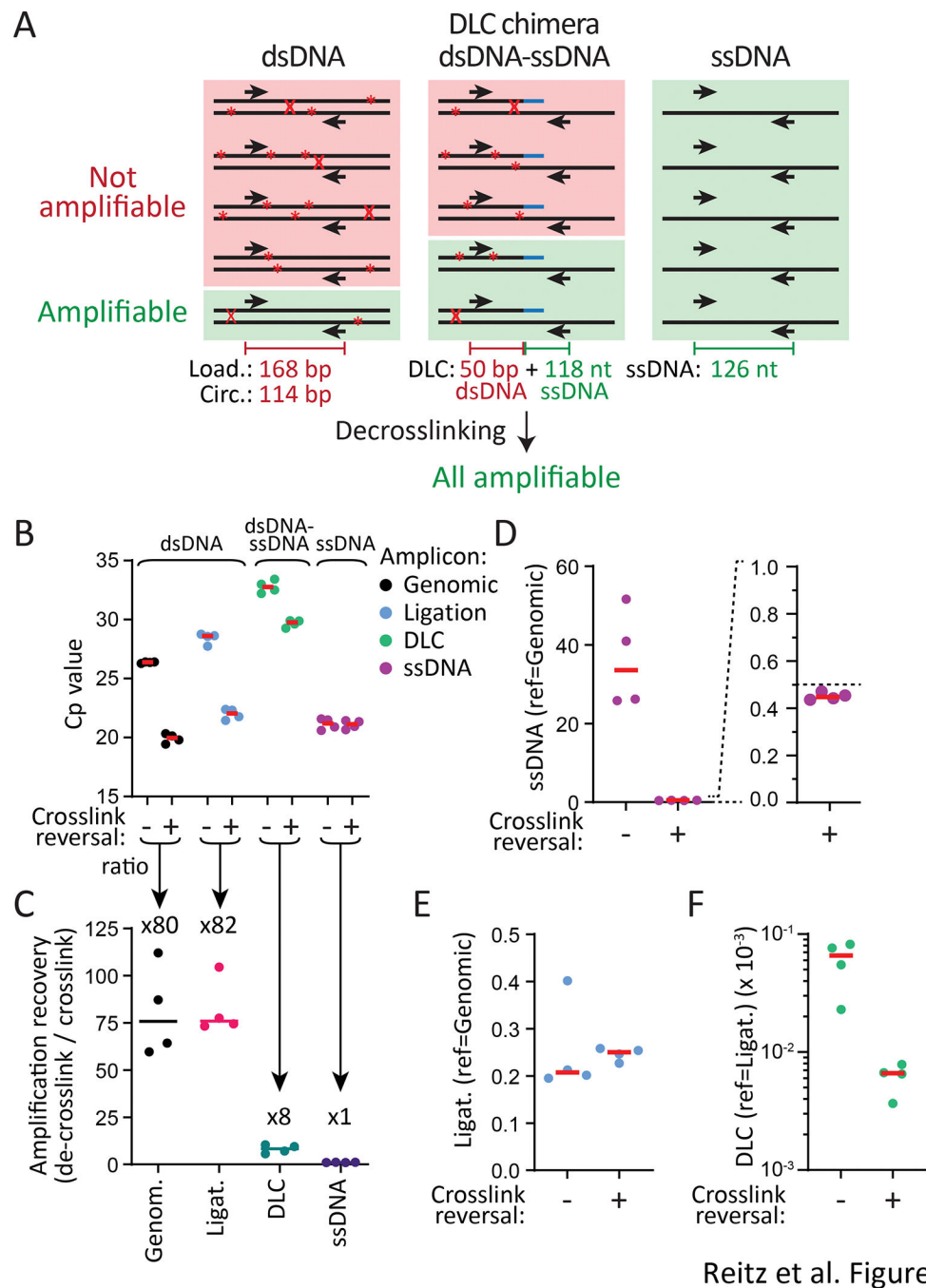
DSB formation is driven by a site-specific endonuclease under the control of the *GAL1* promoter. DSB induction leads to the formation of a nascent D-loop. In the DLC assay, inter-strand crosslinking of the DNA preserves this structure, which is then extracted. Restriction enzyme site restoration is achieved *via* hybridization with a long oligonucleotide, and then the DNA is digested and ligated to form a product that can be quantified by quantitative PCR (qPCR). The DLE assay differs in that the DNA is not cross-linked, and instead, the intramolecular ligation product forms between the two ends of the ssDNA on one side of the break, the 3' end having been extended by a DNA polymerase. qPCR is again used to quantify the formation of the chimeric ligation product. The detection of D-loop extension *via* the DLE assay likewise requires restriction enzyme site restoration. In contrast, the double-stranded BIR product is detected using the DLE assay primers without the hybridizing oligonucleotides. R indicates that a restriction enzyme site is competent for enzyme cleavage; (R) indicates a restriction enzyme site that cannot be cut.



**Figure 3: Representative results from DLC assay analysis of D-loops at 2 h post-DSB induction.** Samples were collected, prepared, and analyzed by qPCR as described in this protocol. Blue symbols represent results for the standard wild-type strain with hybridizing oligos for  $n = 3$ . Green symbols represent results for the wild-type strain without hybridizing oligos for  $n = 3$ . The thick red line shows the median. The purple symbols represent samples without psoralen crosslinking but with hybridizing oligos for  $n = 2$ . Symbols indicate that the samples are derived from the same culture. Inter-experimental differences in crosslinking efficiency can introduce variability into certain qPCR controls but are not problematic as long as there is no inter-sample variability in these qPCR controls within an experiment.



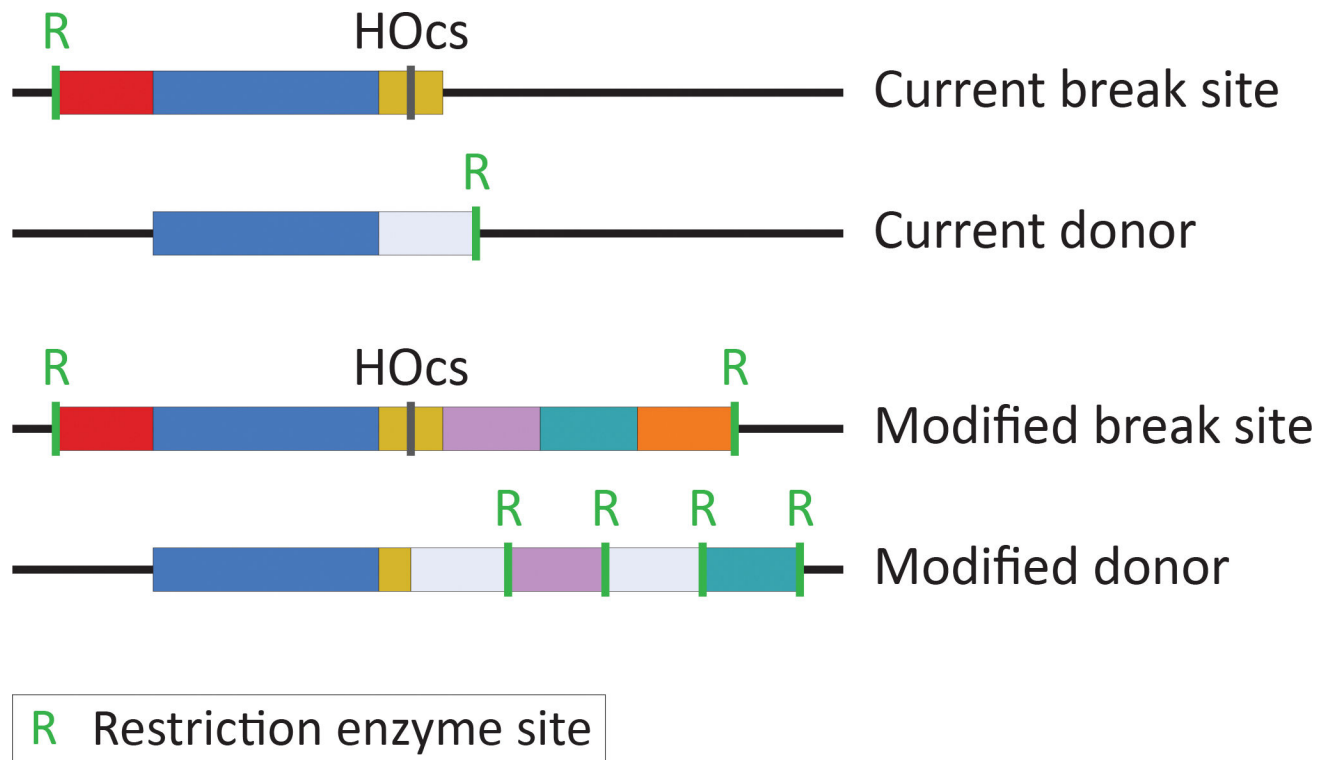
**Figure 4: Representative results from DLE assay analysis 6 h post-DSB induction.** Samples were collected, prepared, and analyzed by qPCR as described in this protocol. Blue symbols represent results for the standard wild-type strain with hybridizing oligos for n = 3. Green symbols represent results for the wild-type strain without hybridizing oligos for n = 3. The thick red line shows the median. Note that the with- and without-hybridizing oligos samples are derived from the same cultures. The purple diamond represents a failed sample without hybridizing oligos for n = 1. Symbols indicate the samples are derived from the same culture.



**Figure 5: Representative results from psoralen crosslink reversal.**

(A) Psoralen-DNA mono-adducts (\*) and inter-strand crosslinks (X) specifically occur on dsDNA and prevent its amplification by DNA polymerases, unlike ssDNA templates. This difference introduces a bias in the quantification of dsDNA- and ssDNA-containing templates by qPCR. This bias can be overcome upon reversal of the psoralen crosslink. (B) Representative Cp values of dsDNA (loading, circular), ssDNA, and mixed ds-ssDNA (DLC) amplicons obtained 4 h post-DSB induction. Data represent individual values and the median of four biological replicates. (C) Amplification recovery upon crosslink reversal,

calculated from the  $C_p$  values in **(B)**. **(D)** The ssDNA amplification relative to the dsDNA loading control with and without psoralen crosslink reversal. Upon reversal, the ssDNA amplicon amplifies at the expected 0.5 of the dsDNA loading control. **(E)** The dsDNA circularization control relative to the dsDNA loading control with and without psoralen crosslink reversal. **(F)** The DLC signal relative to the dsDNA circularization control.



**Figure 6: Current DLC/DLE assay system and the proposed modifications.**

Above: Current DLC/DLE assay break site and donor. Below: Planned modifications to the DLC/DLE assay break site and donor. (I) The 117 bp HO endonuclease cut site is indicated in yellow. To prevent confounding effects while monitoring D-loop disruption, the left side of the HOcs (74 bp) will be introduced into the donor, such that recombination between the two creates a perfectly matched D-loop lacking a 3' flap. (II) To make the system repairable and, thus, more physiological, DNA homologous to the donor (indicated in teal and lilac) will be inserted into the right side of the HOcs. (III) Invasion and extension by the strand to the right of the HOcs will be monitored using sequences unique to that side of the break (indicated in orange). (IV) Additional evenly spaced restriction enzyme sites and sequences unique to the donor will allow D-loop extension (*via* invasion from the left side of the HOcs) to be monitored at more distant sites. In this modified system, synthesis-dependent strand annealing (SDSA) or double-Holliday junction (dHJ) formation can occur at the sites shown in teal or lilac.



**Table 1:**  
***S. cerevisiae* strain used for DLC and DLE assay analysis.**

Genotype of the haploid budding yeast strain used in this study. The strain is available upon request.

Additional strains available for DLC/DLE assay analysis can be found in Piazza et al.<sup>8</sup> and Piazza et al.<sup>9</sup>.

Strain Name	Background	Genotype
WDHY5877/APY266	W303 <i>RAD5+</i>	<i>MATa-inc, ura3::LY-HOcs; lys2::LY; trp1::GAL-HO-hphMX; can1-100; his3-11,15; ade2-1; leu2-3,112; RAD5</i>

**Table 2:**  
**Hybridizing oligonucleotides used for DLC and DLE assay analysis.**

The sequences of the long, hybridizing oligonucleotides used in the DLC and DLE assays. Additional SDS-PAGE purification of the hybridizing oligonucleotides by the custom oligonucleotide provider is recommended.

Name	Sequence (5'–3')
olWDH1770	CGAAATCATCTTCGGTTAAATCCAAAACGGCAGAAGCCTGAATGAAACATATGAACCAATTGGAGGACGTCAATGAATTCTGGGGATCCATTGCAT
olWDH2007	TCTGCTCGGAGATTACCGAATCAAAAAAATTTCAAAGAAACCGGAATCAAAAAAAGAACAAAAAAGATGAATTGAAAAGCTTTATGG
olWDH2046	AATCTTTGTGAAGCTTCGCAAGTATTCATTTAGACCCATGGTGGAACCCTAGTGTGAATGGCAAAGTGGTGATAGAGTTCATAGAATTGGTCAGT

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**Table 3:**  
**qPCR primers used for DLC and DLE assay analysis.**

The qPCR primer pairs for the DLC and the DLE assays and descriptions of their purposes. Note that oIWDH1764, oIWDH2009, and oIWDH2010 are used in two qPCRs.

Name	Sequence (5'-3')	Amplicon Size (bp)	Purpose
oIWDH1760	CAGCGGGCTTGCAGAAGTTG	168	Quantify amount of genomic DNA at <i>ARG4</i> . Used as a dsDNA loading control for all qPCRs.
oIWDH1761	GGCCAATTAGTTCACCAAGACG		
oIWDH2125	AGACAGAATTGGCAAAGATCC	135	Alternative forward primer at <i>ARG4</i> that can be used with oIWDH1761 as a dsDNA loading control.
oIWDH1766	GTTTCAGCTTCCGCAACAG	168	Quantify DSB induction. DSB formation results in low signal.
oIWDH1767			
oIWDH1762	ACTTCGAATTCGGCACTTC	114	Quantify intramolecular ligation efficiency of <i>EcoRI</i> -derived fragments. Intramolecular ligation of a 1904-bp fragment at <i>DAP2</i> is performed in parallel with the DLC assay ligation.
oIWDH1763	CGATGAAACGTTAAGTGACCAC		
oIWDH2019	CTTTAACCGGACGCTCGA	126	Quantify psoralen crosslinking in samples with extensive resection.
oIWDH2020	TTGAGTTTATTGCTGCCGTC		
oIWDH1768	AGGAGCACAGACTTAGATTGG	248	Used with oIWDH1764, this qPCR measures <i>EcoRI</i> recognition site restoration and cutting upstream of the break on the resected strand. Restriction enzyme site restoration and cleavage results in a low signal.
oIWDH1764	AGAGCGGTCAGTAGCAATCC		See oIWDH1768, oIWDH1765.
oIWDH1769	GAAGCCAGTACCACGACC	160	Used with oIWDH1763, this qPCR measures <i>EcoRI</i> cutting at <i>DAP2</i> . Restriction enzyme cleavage results in a low signal.
oIWDH1765	CACACGCGAAAAACCGCC	167	Used with oIWDH1764, this qPCR quantifies the chimeric DLC ligation product.
oIWDH2052	ATGTGCCTTCTACCGCTC	111	Quantify intramolecular ligation efficiency of <i>HindIII</i> -derived fragments. Intramolecular ligation of a 765-bp fragment at <i>YLR050C</i> is performed in parallel with the DLE assay ligation.
oIWDH2053	TCAAGCGTGGTTACATTCCTTAC		
oIWDH2010	TGCTCGGAGATTACCGAATC	144	Used with oIWDH2009, this qPCR quantifies the chimeric DLE ligation product.
oIWDH2012	CGAGGCATATTTATGGTGAAGG	162	Used with oIWDH2010, this qPCR measures <i>HindIII</i> recognition site restoration and cutting upstream of the break on the resected strand. Restriction enzyme site restoration and cleavage results in a low signal.
oIWDH2009	CACCACTTTGCCATTCAACAC	118	See oIWDH2010, oIWDH2011.
oIWDH2011	TGCGAGGTTTCTTGTCAG		Used with oIWDH2009, this qPCR measures <i>HindIII</i> recognition site restoration and cutting downstream of the break on the newly extended strand. Restriction enzyme site restoration and cleavage results in a low signal.

Name of Material/ Supplies	Company	Catalog Number	Comments/Description
<b>1. Pre-growth, DSB induction, and sample collection</b>			
<b>Equipment</b>			
Rotator set to 30° C			
15 mL glass culture tubes			
250 mL, 500 mL, or 1 L flasks			
15 and 50 mL conical tubes			
Benchtop centrifuge at 4° C with 15 and 50 mL conical tube adapters			
Benchtop orbital shaker or tube rotator/revolver			
60 mm × 15 mm optically clear petri dishes with flat bottom			Suggested: Corning, Catalog Number 430166; or Genesee Scientific, Catalog Number 32-150G
UV crosslinker or light box with 365 nm UV bulbs set atop an orbital shaker			Suggested: Spectrolinker XL-1500 UV Crosslinker (Spectronics Corporation) or Vilbert Lourmat™ BLX-365 BIO-LINK, Catalog Number 611110831
<b>Materials</b>			
Bacto yeast extract	BD Difco	212750	For media preparation
Bacto peptone	BD Difco	211840	For media preparation
D-(+)-glucose	BD Difco	0155-17-4	For media preparation
Agar	Fisher	BP1423500	For media preparation
60% w/w sodium DL-lactate syrup	Sigma-Aldrich	L1375	For media preparation
Trioxsalen	Sigma-Aldrich	T6137	For psoralen cross-linking
<b>2. Spheroplasting, lysis, and restriction enzyme site restoration</b>			
<b>Supplies</b>			
1.5 mL microcentrifuge tubes			
Dry bath for incubation at 37° C			
Refrigerated microcentrifuge or microcentrifuge placed at 4° C			
Liquid nitrogen or dry ice/ethanol			
<b>Materials</b>			
10X restriction enzyme (CutSmart) buffer (500 mM potassium acetate, 200 mM Tris-acetate, 100 mM magnesium acetate, 1 mg/mL BSA, pH 7.8-8.0)			
Zymolyase 100T	US Biological	Z1004	For spheroplasting
<b>3. Restriction enzyme digest and intramolecular ligation</b>			

Name of Material/ Supplies	Company	Catalog Number	Comments/Description
<b>Supplies</b>			
1.5 mL microcentrifuge tubes			
Dry bath(s) for incubation at 37° C, 55° C, 65° C			
Water bath for incubation at 16° C			
<b>Materials</b>			
EcoRI-HF	New England Biolabs	R3101	Restriction enzyme digest for DL.C assay
HindIII-HF	New England Biolabs	R3104	Restriction enzyme digest for DLE assay
T4 DNA ligase	New England Biolabs	M0202	Intramolecular ligation
<b>4. DNA purification</b>			
<b>Supplies</b>			
1.5 and 2 mL microcentrifuge tubes			
Dry bath(s) for incubation at 37° C and 65° C			
<b>Materials</b>			
Phenol/chloroform/isoamyl alcohol (P/C/IA) at 25:24:1	Sigma-Aldrich	P2069	DNA purification
<b>5. Psoralen cross-link reversal</b>			
<b>Supplies</b>			
Thermocycler/PCR machine			
<b>6. qPCR</b>			
<b>Supplies</b>			
Lightcycler 480	Roche	5015278001	qPCR machine used by the authors
Lightcycler 96	Roche	5815916001	qPCR machine used by the authors
<b>Materials</b>			
SYBR Green I Master Mix	Roche	4707516001	qPCR kit used by the authors
SsoAdvanced Universal SYBR Green Super Mix	BioRad	1725271	qPCR kit used by the authors
LightCycler 480 96-Well Plate, white	Roche	4729692001	96-well plates for qPCR