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Gel Electrophoresis Assays for Analyzing DNA Double-Strand Breaks in *Saccharomyces cerevisiae* at Various Spatial Resolutions

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Summary

Meiotic recombination is triggered by programmed DNA double-strand breaks (DSBs), which are catalyzed by Spo11 protein in a type II topoisomerase-like manner. Meiotic DSBs can be detected directly using physical assays (gel electrophoresis, Southern blotting, and indirect end-labeling) applied to samples of genomic DNA from sporulating cultures of budding and fission yeast. Such assays are extremely useful for quantifying and characterizing many aspects of the initiation of meiotic recombination, including the timing of DSB formation relative to other events, the distribution of DSBs across the genome, and the influence on DSB formation of mutations in recombination factors and other gene products. By varying the type of gel electrophoresis and other parameters, the spatial resolution of DSB analysis can range from single nucleotides up to whole yeast chromosomes.

Keywords

yeast; meiotic recombination; Southern blotting; pulsed-field gel electrophoresis

1. Introduction

To detect meiotic DSBs in *S. cerevisiae*, genomic DNA is extracted from synchronously sporulating cultures, then digested with restriction enzyme(s) as necessary to yield an appropriate sized fragment. Subsequently, these DNA fragments are separated by gel electrophoresis, and both the parental length (unbroken) DNA and the DSB fragments are detected by Southern blotting and indirect end-labeling by hybridization to an appropriate probe (Fig. 1).

Meiotic DSBs do not occur randomly throughout the genome. Instead, DSBs (and the resulting recombination products) form preferentially within small (≤2 kbp) regions called hotspots (1). DSBs show specificfeaturesof distribution according to the resolution of the methods used to detect them. When observed at the resolution of a whole chromosome (100's to 1000's of kbp in *S. cerevisiae*), alternating hot and cold domains are observed that are typically on the order of 50–100 kbp wide (*see* Fig. 2 for an example). When examined at the resolution of individual genes (i.e., examining ~5–20 kbp at a time), individual hotspots can be observed that are separated by several kbp of DNA in which few, if any, DSBs are formed (Fig. 3A). Most hotspots in *S. cerevisiae* are located within transcriptional promoter regions (2). Mapping at yet higher resolution reveals that each hotspot consists of multiple DSB sites clustered within regions of ~75–250 bp (Fig. 3B). Finally, mapping at the resolution of individual nucleotides reveals that Spo11 cleaves the DNA to yield a two-

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nucleotide 5′ overhang and that some positions within hotspots are cut more frequently than others, although no obvious DNA sequence preference has yet emerged (3–6).

Because there is no single method for separating and detecting DNA fragments across all of the size ranges outlined above, it is necessary to choose DSB mapping strategies appropriate to the purpose of the experimental study. Here we describe procedures for DSB analysis at four levels of spatial resolution, which we define as chromosome level, medium resolution, high resolution, and nucleotide level mapping. These differ from one another primarily with respect to DNA sample preparation and the method of gel electrophoresis (pulsed-field, conventional agarose, polyacrylamide, or sequencing gel, respectively). These protocols are modified from established methods (5, 7, 8).

In many studies, the position and the frequency of meiotic DSBs are measured using *rad50S* or *sae2Δ* mutants, which are deficient in a process of endonucleolytic release of covalently attached Spo11 from DSB ends (5, 6, 9, 10). These mutants accumulate unrepaired DSBs, making observation of the normally transient meiotic DSB much easier. The frequency of DSBs varies from one hotspot to another and, in general, DSB frequencies measured in *rad50S* or *sae2* strains correlate well with overall recombination frequencies in corresponding *RAD50+* or *SAE2+*. However, it is important to note that DSB frequencies are substantially reduced in *rad50S* or *sae2* mutants in regions where replication is experimentally delayed (8), and recent genome-wide studies demonstrate that DSBs in certain regions are specifically underreported in *rad50S*-like mutants (11, 12).

To obtain well-synchronized meiotic samples, cells are precultured in presporulation medium (SPS), and then transferred to sporulation medium (SPM). In the SK1 strain background, the DSB frequency reaches maximum levels about 5–6 h after transfer to SPM. Premeiotic samples are harvested at 0 h (i.e., immediately upon transfer to SPM), and meiotic samples are harvested at appropriate time points thereafter (*see*Note 1).

The frequencies of meiotic DSBs are low, so it is important that genomic DNA is extracted gently to avoid mechanical shearing. Excessive shearing generates a high background of hybridizing signal in lanes from the gel electrophoresis, and can obscure weaker DSB signals and/or make DSBs difficult to quantify. DNA samples in solution should be mixed gently, and vigorous pipetting and vortexing should be avoided.

To observe DSBs at different levels of spatial resolution, the lengths of the parental and DSB fragments need to be considered, and an appropriate probe needs to be chosen for indirect end-labeling of Southern blots. Parental and DSB fragments should be well separated by gel electrophoresis in order to accurately estimate DSB frequencies. It is recommended that probes are designed within open reading frames so that the region to which the probe hybridizes is unlikely to itself be a site of DSB formation. Additional specifications are discussed in the individual methods sections below.

2. Materials

2.1. Media for yeast culture

1. YPD: 1% Bacto yeast extract, 2% Bacto peptone, 2% dextrose, 2% Bacto agar. Autoclave.

[1]These instructions assume the use of diploids of the SK1 strain background, with either *rad50S* or *sae2*Δ mutation. If other strain backgrounds are used, adjustments to the culture media and/or times for sample collection may be necessary.

2. YPG: 1% Bacto yeast extract, 2% Bacto peptone, 0.1% dextrose, 3% glycerol, 2% Bacto agar. Autoclave.
3. 0.1 *M* potassium biphthalate stock solution. Adjust to pH 5.5 with KOH.
4. Presporulation media (SPS): 1% Bacto yeast extract, 2% Bacto peptone, 0.17% Bacto yeast nitrogen base (without amino acids and without ammonium sulfate), 0.5% ammonium sulfate, 1% potassium acetate, 0.05 *M* potassium biphthalate. Autoclave.
5. Sporulation media (SPM): 1% potassium acetate. Autoclave.

2.2. Chromosome-level DSB mapping

2.2.1 Plug DNA preparation

1. 0.5 *M* EDTA pH 7.5 (adjust pH with NaOH). Autoclave. Store at room temperature.
2. SCE solution: 1 *M* sorbitol, 0.1 *M* sodium citrate, 0.06 *M* EDTA, pH 7.0. Filter-sterilize.
3. 1% LMP agarose mix: 1% low melting point agarose (Seaplaque GTG, Lonza), 0.125 *M* EDTA, pH 7.5. Microwave and equilibrate to 40°C. This solution should be made fresh the day of the experiment to prevent agarose hydrolysis.
4. Solution 1: SCE plus 5% β-mercaptoethanol plus 1 mg/mL zymolyase 100T. This solution must be made fresh and kept on ice until use.
5. Solution 2: 0.45 *M* EDTA pH 7.5, 0.01 *M* Tris-HCl pH 7.5, 7.5% β-mercaptoethanol, 10 μg/mL RNase A. This solution should be made fresh the day of the experiment from stock solutions (for preparation of 10 mg/mL RNase A, *see* **Subheading 2.3** below).
6. Solution 3: 0.25 *M* EDTA, pH 7.5, 0.01 *M* Tris-HCl pH 7.5, 1% sarkosyl (from a 10% sarkosyl solution, filter-sterilized), 1 mg/mL proteinase K (directly added as a powder). This solution may be prepared in advance without the proteinase K, filter-sterilized and kept at room temperature. Add Proteinase K just before use.
7. Plug storage solution: 0.05 *M* EDTA, pH 7.5, 50% (w/v) glycerol. Filter-sterilize and keep at room temperature.
8. Agarose plug molds (e.g., Biorad, catalog no. 170–3622).

2.2.2. Pulsed-field gel electrophoresis (PFGE)

1. TBE electrophoresis buffer, 10× stock solution: 890 m*M* Tris base, 890 m*M* boric acid, 20 m*M* EDTA pH 8.0. Store at room temperature.
2. Gel casting stand 14 cm by 21 cm (e.g., BioRad, catalog no.170–3704)
3. 14 cm wide, 15-well, 1.5 mm thick comb
4. PFGE system (BioRad CHEF-DRII or CHEF Mapper XA Chiller System, or equivalent).
5. Circulating variable-speed pump (BioRad catalog no. 170–3644 or equivalent)
6. Cooling module (BioRad catalog no. 170–3654 or equivalent)
7. 1.3% agarose gel: Weigh 1.95 g SeaKem agarose in 150 mL of 0.5× TBE. Microwave. Equilibrate at 55°C (*see*Note 2).

8. *S. cerevisiae* pulsed-field size marker (Mid-range I or II PFGE size markers from New England Biolabs)
9. 10 mg/mL ethidium bromide solution

2.2.3. Southern blotting

1. UV crosslinker (Stratagene Stratalinker or equivalent); vacuum blotter; nylon membrane (positively charged, Hybond N+, GE Healthcare, or equivalent)
2. PFGE transfer solution: 0.6 *M* NaCl, 0.4 N NaOH. Store at room temperature.
3. 20× SSC: 3 *M* NaCl, 0.3 *M* citrate. Adjust to pH 7.0 with 1 *N* HCl. Store at room temperature.

2.2.4 Southern blot hybridization and washing

1. ^{32}P-labeled double-stranded DNA probe, prepared by random priming using an appropriate commercial kit according to manufacturer's instructions (e.g., Amersham Rediprime™ II DNA Labeling System, GE Healthcare). The DNA template for the labeling reaction should be generated by PCR from genomic DNA and should be gel-purified by agarose gel electrophoresis and extracted using an appropriate commercial gel extraction kit. It is sometimes convenient to use the gel purified material from this initial PCR to seed a secondary PCR with the same primers as a way of generating larger quantities of highly purified template. The amplified template DNA can be stored indefinitely at −20°C.
2. 1 *M* phosphate buffer pH 7.2: 38.6 g $NaH_2PO_4 \cdot H_2O$, 193 g $Na_2HPO_4 \cdot 7H_2O$, distilled water to 1 L. Autoclave. Store at room temperature.
3. Hybridization solution: 1% bovine serum albumin (BSA), 0.5 *M* phosphate buffer pH 7.2, 7% SDS, 1 m*M* EDTA pH 8.0. Dissolve BSA in the phosphate buffer, then add SDS (high SDS concentration prevents BSA from dissolving). Filter sterilize. Store at −20°C.
4. Wash solution: 40 m*M* phosphate buffer pH 7.2, 1% SDS, 1 m*M* EDTA pH 8.0.
5. Hybridization oven and bottles (35 × 300 mm).

2.3 Medium resolution mapping

2.3.1 Genomic DNA extraction

1. 1.2 *M* sorbitol. Autoclave. Store at room temperature.
2. Zymolyase solution: Dissolve 100 mg of zymolyase 20T in 1 mL of 1 *M* sorbitol. Store at 4°C up to 2–3 months.
3. β-mercaptoethanol, isopropanol, 100% ethanol, all stored at room temperature. 70% ethanol, stored at −20°C.
4. Extraction solution: 50 m*M* EDTA pH 8.0, 50 m*M* Tris-HCl pH 8.0, 0.5% SDS. Autoclave. Store at room temperature.
5. Proteinase K: Prepare 10 mg/mL solution in water. Store at −20°C.
6. 5 *M* potassium acetate. Autoclave. Store at room temperature.

[2]Be careful that the agarose solution is not too hot when you pour it because this may partially melt the DNA agarose plugs and detach them from the comb.

7. TE buffer: 10 m*M* Tris-HCl pH 7.5, 1 m*M* EDTA pH 8.0. Autoclave. Store at room temperature.
8. RNase A: Prepare 10 mg/mL solution in 10 m*M* Tris-HCl pH 7.5, 15 m*M* NaCl, 1 m*M* EDTA pH 8.0. Incubate in boiling water bath for 15 min, then at room temperature for 1 h. Store at −20°C.
9. 3 *M* sodium acetate pH 5.2: adjust pH with acetic acid. Autoclave. Store at room temperature.

2.3.2. Agarose gel electrophoresis—Restriction enzymes; electrophoresis power supply; agarose gel electrophoresis apparatus suitable for running 30 cm-long gels; agarose; TBE buffer (*see* **Subheading 2.2.2**), 6× loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll PM400); 10 mg/mL ethidium bromide solution.

2.3.3. Southern blotting

1. Vacuum blotter; nylon membrane (positively charged); 20× SSC (*see* **Subheading 2.2.3**)
2. Depurination solution: 0.25 *N* HCl. Store at room temperature.
3. Denaturing solution: 1.5 *M* NaCl, 0.5 *N* NaOH. Store at room temperature.
4. Transfer solution: 1.5 *M* NaCl, 0.25 *N* NaOH. Store at room temperature.
5. ^{32}P-labeled double-stranded DNA probe (*see* **Subheading 2.2.4**).

2.4. High resolution mapping

2.4.1. Polyacrylamide gel electrophoresis

1. Polyacrylamide gel electrophoresis apparatus and power supply; gel casting plates and combs (8 × 8 cm, 1 mm thick); 40% acrylamide solution (19:1 acrylamide:bis-acrylamide); urea; TEMED; 10× TBE (*see* **Subheading 2.2.2**).
2. 3× loading buffer: 95% formamide, 10 m*M* EDTA pH 8.0, 0.1% bromophenol blue, 0.1% xylene cyanol FF. Store at −20°C.
3. APS: 10% ammonium persulfate. Store at −20°C.

2.4.2. Southern blotting—Whatman 3MM filter paper; transfer apparatus (BioRad Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell, or equivalent); Nylon membrane (uncharged, GeneScreen™ Hybridization Transfer Membrane (PerkinElmer) or equivalent); UV crosslinker; 20× SSC (*see* **Subheading 2.2.3**).

2.5. Nucleotide resolution mapping

2.5.1. Sample preparation

1. Phusion™ High-Fidelity DNA Polymerase (Finnzymes).
2. SequiTherm EXEL™ II DNA Sequencing Kit (Epicentre).
3. NucleoSpin Extract II (Macherey-Nagel).
4. Primers a, b, c, d in Fig. 4.

2.5.2. Electrophoresis—High voltage electrophoresis power supply; sequencing gel electrophoresis apparatus; sequencing gel plates (30 × 40 cm); plate coating reagent

(Sigmacote); shark tooth combs and spacers (0.4 mm thick). *See* **Subheading 2.4.1** for acrylamide gel solutions and loading buffer.

2.5.3. Southern blotting—UV crosslinker; 20× SSC (*see* **Subheading 2.2.3**); 35 × 45 cm blotting paper (BioRad); Whatman 3MM filter paper; electro transfer apparatus (TE 90 - GeneSweep™ Sequencing Gel Transfer Unit (Hoefer Scientific Instruments)).

2.5.4 Probe preparation and hybridization—Taq polymerase, 333 μ*M* dNTP mix without dCTP, [α-^{32}P] dCTP (6000 Ci/mmol), Taq amplification buffer, 50 m*M* $MgCl_2$, probe template DNA (*see* **Subheading 2.2.4**), 20 μ*M* PCR primer b (Fig. 4).

3. Methods

3.1. Synchronous meiotic cultures

This section is based on (13) with minor modifications.

1. Day −4: Patch *rad50S* (or *sae2Δ*) diploid on YPG plate and incubate at 30°C for at least 6 h for the selection of functional mitochondria. Streak diploid on YPD plate to generate single colonies and incubate at 30°C for 2 days.
2. Day −2: Pick a single colony and inoculate in 1 mL of SPS. Shake overnight at 250 rpm, 30°C.
3. Day −1: Calculate the cell density either by measuring OD_{600} or directly counting cells in a hemacytometer. Inoculate cells in 5 mL of SPS at cell density of 0.5×10^7 cells/mL and shake at 250 rpm, 30°C for 6 h. Inoculate cells in SPS at cell density of $2–4 \times 10^5$ cells/mL and shake at 250 rpm, 30°C overnight (*see*Note 3).
4. Day 0: 16 h after the inoculation, cell density should be $2–4 \times 10^7$ cells/mL. Harvest cells by centrifugation at 2600*g* for 5 min and wash with one vol of pre-warmed SPM. Then resuspend cells in two vol of pre-warmed SPM (*see*Note 4).
5. Collect 0 h (premeiotic) sample and return the flask to the shaker. Shake at 250 rpm, 30°C. Collect meiotic samples at appropriate times after transfer to SPM (*see*Note 5). Refer to individual subsections below for appropriate volumes and methods for harvesting cells.

3.2. Chromosome-level DSB mapping

This method is suitable for observing whole chromosomes and DSB fragments of 0.2–2.0 Mbp after separation by pulsed-field gel electrophoresis (PFGE). It is necessary to prepare high molecular weight DNA under conditions of gentle cell lysis to prevent any mechanical DNA breakage. This is achieved by embedding cells in low-melting point agarose as soon as they are collected and performing all enzymatic reactions (preparation of spheroplasts, lysis and DNA purification) in the resulting DNA “plugs”. Enzymatic degradation of chromosomal DNA is prevented by maintaining high concentrations of EDTA, which inhibits nucleases, at all steps of DNA plug preparation. For indirect end-labeling on Southern blots, a good choice for probe is often the open reading frame closest to one extremity of the chromosome of interest. A unique, unrepeated gene should be chosen.

[3]The volume of SPS should be less than 20% of the flask volume to ensure good aeration. Use a flask of ≥250 mL for 50 mL of SPS culture.
[4]The volume of SPM should be less than 10% of the flask volume to ensure good aeration, e.g., use a 1 L flask for 100 mL of SPM culture.
[5]Appropriate time points to collect cells are dependent on the purpose of the experiment. Usually, the DSB frequency reaches a maximum by 5–6 h after transfer to SPM in the SK1 background when using *rad50S* or *sae2Δ* mutants.

3.2.1. Preparation of agarose-embedded DNA ("plugs")—This method has been described in (14).

1. Collect 15 mL of meiotic culture (30 mL if at times before replication, i.e., 0–2 h after meiotic induction). (This is equivalent to 3×10^8 cells (6×10^8 before replication), and represents [$(13 \times 10^6$ bp) × (650×10^6) × 2 {diploid} × 2 {after S phase} × (3×10^8 cells)/(6.022×10^{23})] = 17 μg of DNA. This is sufficient for six loadings and PFGE runs.
2. Centrifuge in 50 mL conical tubes 4 min in a table-top centrifuge at 3200*g*.
3. Resuspend in 5 mL of 50 m*M* EDTA, pH 7.5. Vortex until completely resuspended.
4. Repeat steps 2 and 3.
5. Repeat step 2.
6. While washing, make the following mix: for five samples, mix 0.83 mL of 1% LMP agarose mix with 0.17 mL of Solution 1 (adjust quantities according to the number of samples you are processing). Vortex and keep at 40°C (*see*Note 6).
7. After the last wash, eliminate supernatant and resuspend the cell pellet in 100 μL of 50 m*M* EDTA pH 7.5. Transfer to an microcentrifuge tube. Equilibrate 30 s at 40°C.
8. Add 200 μL of pre-warmed LMP agarose/Solution 1 mix. Vortex briefly. Pipette into plug mold. 90 μL fills each block. Work quickly to fill the plugs before the solution begins to harden.
9. Chill blocks for 10 min at 4°C.
10. Using a thin spatula, express blocks into 3 mL of Solution 2. Invert gently several times to mix. Incubate 1 h at 37°C.
11. Carefully pour off Solution 2 and replace with 3 mL of Solution 3. Mix by gentle inversion. Incubate overnight at 50°C.
12. Carefully pour off Solution 3. Wash twice with 3 mL of 50 m*M* EDTA for 15 min on a rotating wheel. Equilibrate with 3 mL of storage buffer for 15 min. Pour off and replace with 3 mL of fresh storage buffer. Store plugs in storage buffer at −20°C.

3.2.2. Pulsed-field gel electrophoresis

1. Prepare 2.2 L of 0.5× TBE.
2. Use 150 mL of 0.5× TBE to make the 1.3% agarose gel. Microwave and keep at 55°C.
3. Put the remaining buffer in the CHEF apparatus tank and switch on the circulating pump.
4. Switch on the cooler and set the thermostat to 14°C.
5. Using a clean razor blade, cut one third of a plug (this represents about 1.8 μg DNA) and immerse in 1.5 mL of filter-sterilized 0.5× TBE in a 2 mL Eppendorf tube (buffer mixing and exchange is more efficient in a 2 mL rather than 1.5 mL tube). Rotate 15 min on a rotating wheel.

[6]The mixture of Solution 1 and the LMP agarose mix should be kept at 40°C as briefly as possible (maximum 3–4 min) in order not to inactivate the zymolyase enzymatic activity. Therefore, it is better not to make an agarose mix for more than 6 samples at a time.

6. Prepare the gel casting stand and comb.
7. Using a spatula, take the plug out of solution and lay it on a tooth of the comb. Reserve one tooth for size markers. Seal each sample on the comb with one drop of the reserved 1.3% agarose gel. Let the comb stand horizontally for ~5 min to allow the sealing agarose to set.
8. Install the comb vertically in the gel casting stand and slowly pour the agarose into the stand. Let the gel set for 30 min at room temperature.
9. When set, using a razor blade, cut the drop of set agarose on each tooth of the comb that goes above the gel surface. Then slowly remove the comb, so that the DNA sample plugs remain in the gel.
10. Dismantle the gel from the casting stand and install it in the PFGE electrophoresis tank filled with pre-cooled running buffer. Take care when placing the gel on the bottom of the tank so that it will not move during the electrophoresis.
11. Let the gel equilibrate to the buffer temperature for 15 min.
12. Begin electrophoresis: for separating fragments from 425 kb to 15 kb, use the following conditions: 15.1 s initial switch time; 25.1 s final switch time; 120° switch angle; 6 V/cm (120 V in the CHEF and DRII Biorad apparatuses); 46 h run time (*see*note 7). An example of separation of DSB fragments after such pulsed-field gel electrophoresis conditions is shown in Fig. 2A.

3.2.3. Southern blotting

1. After electrophoresis, stain the gel for 20 min in 0.5 μg/mL ethidium bromide. Rinse in water for 5 min and photograph the gel.
2. Place the gel on a tray in a UV crosslinker and UV irradiate with 180 mJ/cm^2. This step is necessary to nick the DNA to allow efficient transfer.
3. Equilibrate the gel in 250 mL of PFGE transfer buffer for 15 min.
4. Cut the nylon membrane and wet first in water, then in PFGE transfer buffer for 1 min. Assemble the membrane, gasket and gel on the blotter.
5. Set up the vacuum blotter according to manufacturer's instructions. Place the gel on the blotter.
6. Pour PFGE transfer solution directly onto the gel.
7. Vacuum transfer at 55 cm Hg for 2 h, watching that the gel does not become dry. If it does, add more transfer solution.
8. Rinse the membrane with 2× SSC. Dry the membrane on Whatman 3MM paper for 1 h. Dry the membrane completely if storage is required.

3.2.4. Hybridization and wash

1. Prepare 50 mL of hybridization solution.
2. Place the blot into a hybridization bottle and rinse blot with 50 mL of 0.5 *M* phosphate buffer.

[7]If you have more than 15 samples to run on the same gel, you may run a 14 cm long by 21 cm wide gel using the same gel casting stand, but with a 21 cm wide comb. The running conditions must be adjusted such that total run time is 30.5 h but all the other conditions are unchanged. An example of such a run is shown in Fig. 2B.

3. Prehybridization: Pour 25 mL of hybridization solution into the bottle and rotate at 65°C for 1 h in a hybridization oven.
4. Place remaining 25 mL of hybridization solution at 65°C.
5. During prehybridization, prepare radioactively labeled probe.
6. Purify the radiolabeled probe using a gel filtration spin column. Denature the probe at 100°C for 5 min. Place on ice.
7. Discard the hybridization solution in the bottle. Add denatured probe to the fresh hybridization solution that has been equilibrated to 65°C (**step 4**). Pour hybridization solution containing the probe into the hybridization bottle.
8. Rotate hybridization bottle at 65°C overnight (16–48 h).
9. Warm the wash buffer to 65°C.
10. Discard the hybridization solution.
11. Pour approximately 50 mL of wash buffer into the bottle. Wash at 65°C for 15 min. Discard wash buffer. Repeat three times.
12. Remove blot from the hybridization bottle. (Further washing in a tray in a 65°C water bath may be conducted if necessary to remove background.)
13. Wrap the blot with plastic wrap (*see*Note 8).
14. Expose to phosphor screen or film.

3.3. Medium resolution DSB mapping

DSB mapping using separation by conventional agarose gel electrophoresis is suitable for observing DSBs across ~2–15 kb regions, with a resolution of ~50–100 bp. For accurate quantification, parental fragments of less than 10 kb are recommended so that transfer during Southern blotting is efficient. Restriction enzymes can be chosen as appropriate to give good spatial separation between the DSB fragments and the parental fragment (*see* Fig. 3A). We generally use 0.5–1 kb DNA fragments as probes, which can be efficiently labeled by random priming. Ideally, the probe should hybridize to sequence close to one end of the parental size restriction fragment (Fig. 3A).

3.3.1. Genomic DNA extraction—

This section is based on (7). (*See*Note 9)

1. Collect samples from synchronous meiotic culture (*see* **Subheading 3.1**). The amount of DNA per mL of culture increases according to the progression of meiosis as the yeast cells undergo premeiotic DNA replication. To adjust for this increase, we collect cells from the following volumes of SPM culture: 0 h, 34 mL; 2 h, 31 mL; 3 h, 27 mL; 4 h, 24 mL; after 5 h, 20 mL. Harvest cells by centrifugation 5 min at 3200*g* in a tabletop centrifuge. Resuspend cell pellet in 1 mL sterile distilled water, transfer to a 1.5 mL Eppendorf tube, and pellet cells with a brief (30 s) spin in a microcentrifuge. Store pellets at −20°C.
2. Add 200 μL of 0.5 *M* EDTA pH 8.0, 800 μL of 1.2 *M* sorbitol, 10 μL of β-mercaptoethanol and 10μL of zymolyase solution to frozen yeast cells. Suspend

[8]Try to avoid leaks to protect the phosphor screen and to prevent the blot from drying. If the blot is kept moist, the probe can be stripped by washing twice with 1% SDS for 15 min. However, once the blot is dried with the hybridized probe, it becomes difficult to strip the probe.

[9]If the region to be analyzed is larger than 10 kb, it is recommended to extract and digest genomic DNA in low melting point agarose as described in **Subheading 3.2.1**.

yeast cells by vortexing, making sure there are no clumps. Incubate at 37°C for 45 min.

3. Centrifuge 4500*g* in a microcentrifuge for 5 min. Gently remove supernatant by pipetting and discard.
4. Add 500 μL of extraction solution and 10 μL of proteinase K. Gently suspend the spheroplasts by pipeting. Incubate at 65 °C for 1 h.
5. Add 200 μL of 5 *M* potassium acetate, and mix immediately. Chill tubes on ice for 10 min.
6. Centrifuge 16,000*g* in a microcentrifuge for 30 min at 4°C. Transfer supernatant to new tube. Add 500 μL of isopropanol and invert tube 4–5 times to mix.
7. Centrifuge 16,000*g*, 5 min. Discard supernatant.
8. Add 1 mL of 70%ethanol. Centrifuge 16,000*g* for 5 min at 4°C. Discard supernatant. Centrifuge for 1 min at 4°C. Remove residual ethanol by pipeting.
9. Add 300 μL of TE and 3 μL of RNase A. Do not try to suspend the pellet at this stage, because it causes random DNA shearing. Incubate at 37°C for 1 h.
10. Add 30 μL of 3 *M* sodium acetate pH 5.2 and 750 μL of 100% ethanol. Mix by inversion. Chill sample at −20°C for 1 h. Centrifuge 16,000*g* for 20 min at 4°C. Discard supernatant.
11. Add 1 mL of 70% ethanol. Centrifuge for 5 min at 4°C. Discard supernatant. Centrifuge for 1 min at 4°C. Remove residual ethanol by pipeting.
12. *Briefly* dry the pellet at 37°C for 20 min. Add 100 μL of TE. Store overnight at 4°C: the DNA will gently dissolve during this step (*see*Note 10).
13. Store dissolved DNA at −20°C.

3.3.2. Restriction enzyme digestion and electrophoresis

1. Mix approximately 1 μg of genomic DNA with restriction enzyme buffer, water, and restriction enzyme (10–20 units), in 20 μl total volume (*see*Note 11).
2. Digest DNA for 3 h. Heat inactivate the restriction enzyme.
3. Prepare an appropriate concentration agarose gel with 1× TBE.
4. Add 4 μl of 6× loading buffer to samples. Mix.
5. Load samples on gel along with appropriate size standards (*see*Note 12).
6. Run samples in 1× TBE at >70 V overnight until dyes migrate to appropriate position (*see*Note 13)
7. Place the gel in 1× TBE containing 0.5 μg/mL of ethidium bromide. Agitate for 20 min. Take a photograph of the gel (*see*Note 14).

[10]If a residual pellet exists after overnight incubation at 4°C, mix the DNA by gentle tapping. Do not vortex. Often, meiotic samples are cloudy, presumably because of polysaccharide or other components from the ascus or spore walls. However, this does not affect later steps (restriction enzyme digestion, etc.).

[11]Usually 10 μl of sample corresponds to approximately 1 μg of DNA. For more precision to ensure that the quantity of DNA is similar from sample to sample, the DNA concentration can be quantified by Hoechst dye fluorescence using a fluorometer.

3.3.3. Southern blotting (see Note 15)

1. Cut the nylon membrane to the appropriate size for the gel. Set up the vacuum blotter according to manufacturer's instructions. Place the gel on the blotter.
2. Pour depurination solution directly onto the gel. Apply vacuum at 55 cm Hg for approximately 15 min, taking care that the gel does not dry out. The bromophenol blue dye will turn yellow (*see*Note 16).
3. Drain residual depurination buffer and pour denaturation solution directly onto the gel. Denature for approximately 45 min. During this step, the bromophenol blue dye will become blue again.
4. Pour transfer solution directly onto the gel. Transfer for 2 h.
5. Take the membrane out. Fix DNA to the membrane by soaking in freshly prepared 0.4 *N* NaOH for 5 min.
6. Rinse the membrane with 2× SSC. Dry the membrane on Whatman 3MM paper for 1 h. The membrane is ready to use for hybridization as described in **Subheading 3.2.4**, or it can be dried completely at this stage if storage is required.

3.3.4 Quantification of DSB frequency—Parental and DSB bands can be visualized using a Phosphoimager and quantified using ImageQuant (Molecular Dynamics) as described in (7). The frequency of DSBs is calculated as the percent of radioactivity in DSB fragments relative to the total radioactivity in the lane (i.e., parental and DSB fragments). Appropriate exposure times can be selected to allow accurate determination of the intensity of the parental band (i.e., within the linear response range of the phosphor screen). Background signals are often present in the lane because of random shearing of genomic DNA during preparation of samples, etc; these background signals should be subtracted.

3.4. High resolution DSB mapping

DSBs can be detected at higher resolution (±10~20 bp) using polyacrylamide instead of agarose gel electrophoresis (*see* Fig. 3B). We generally obtain better resolution and signal strength using denaturing (6% polyacrylamide containing 8 *M* urea) rather than nondenaturing gels. Good resolution is achieved when DSB fragments are 150–300 bp long. To provide sufficient spatial separation from DSB fragments of this size, parental fragments should be 500–1000 bp. Restriction sites should be chosen with these size ranges in mind. The size of the DNA probe fragment should be 100–200 bp, which is often difficult to label

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[12]For accurate DSB mapping, it is important to include appropriate DNA size standards on the same gel, and it is also important that they appear in the autoradiograph of the Southern blot after probing. There are two convenient ways to achieve this goal. The first is to use a commercially available molecular weight marker, which is run in an adjacent lane in the gel (e.g., lambda DNA digested with BstE II; *see* Fig. 3A, **lane M**). To visualize the marker on the autoradiograph, marker DNA is also added to the random primed labeling reaction at a probe to marker ratio of 1000:1. Alternatively, molecular weight markers can be prepared from yeast genomic DNA. To do so, approximately 1 μg of 0 h sample (i.e., DNA from a premeiotic culture) is digested with the same restriction enzyme used to digest the meiotic samples. Then, an aliquot of the digested DNA is digested with an appropriate second enzyme which cuts within the region of interest. A set of such double digests is then pooled with the undigested DNA after heat inactivation of the restriction enzymes (*see* Fig. 3B, **lane M**). The amount of DNA that is subjected to double digestion should be adjusted dependent on the expected DSB frequency, such that the marker fragments are not stronger than the DSB signal. A typical starting point would be to perform the secondary digestions on aliquots of 1% of the first digest (i.e., ~0.01 μg of DNA). Either method works well for providing size standards, but the second method provides somewhat more accurate size information because it allows one to control for DNA sequence composition and for the amount of DNA loaded in the lane.

[13]1 × TAE may be used instead of TBE. If so, it is essential to circulate the buffer during electrophoresis.

[14]If the restriction digest worked well, each sample will show the same pattern on ethidium stained gel.

[15]We obtain good results using a vacuum blotter to transfer DNA from the gel to a hybridization membrane. Alternative methods using capillary transfer under neutral or denaturing conditions may also be satisfactory.

[16]Depurination is not necessary for DNA fragments less than 5 kb, but we observe that transfer is partial for DNA fragments larger than this. Thus, depurination (and subsequent nicking of apurinic sites) is critical to obtain accurate estimation of DSB frequencies. The depurination step can be replaced by UV treatment at 120 mJ/cm^2, which produces alkali-labile photoproducts in the DNA.

to sufficient specific activity by random priming. Therefore, we use PCR to amplify the probe fragment in the presence of [α-^{32}P] dCTP.

3.4.1. Denaturing PAGE gel electrophoresis and semi-dry transfer

1. Prepare restriction enzyme digested premeiotic and meiotic samples (**Subheading 3.3.2**).
2. Ethanol precipitate all samples. Rinse with 70% ethanol. Dry pellets. Dissolve each pellet in 6μL of TE and 3 μL of 3× loading buffer.
3. For each gel, prepare 10 mL of 6% acrylamide gel solution: 4.2 g urea, 1.5 mL 40% acrylamide (19:1), 1 mL 10× TBE, 4 mL H_2O.
4. Add 10 μL of TEMED and 100 μL of 10% APS. Mix.
5. Pour the gel immediately into gel casting plates. Insert the comb (12 well) into the gel solution. Normally, the gel will be completely polymerized within 30 min.
6. Assemble the gel into the electrophoresis apparatus with 1× TBE as the running buffer. Flush urea from the wells using a pipet. Load each well with 9 μl of 1× loading buffer (diluted from 3× stock with TE). Pre-run at 240 V for 30 min (*see*Note 17).
7. Denature samples at 100°C for 5 min. Place samples on ice.
8. Flush urea from the wells using a pipet. Load samples. Start electrophoresis at 240 V.
9. During electrophoresis, cut the uncharged nylon membrane and five pieces of Whatman 3MM paper to the size of the gel.
10. Stop electrophoresis when the xylene cyanol dye has migrated close to the end of the gel. Under these conditions, xylene cyanol comigrates with single-stranded DNA of approximately 100 bases.
11. Remove the gel from the gel plates. Equilibrate the gel, membrane, and Whatman 3MM in 0.5× TBE for 15 min.
12. Assemble the sandwich for the semi-dry transfer apparatus (+ anodic side: two Whatman 3MM sheets, membrane, gel, three Whatman 3MM sheets: − cathodic side). Remove all air bubbles by rolling a pipet over the surface of each layer.
13. Run the transfer at 400 mA for 1 h.
14. Remove the membrane and fix the DNA to the membrane by UV crosslinking at 120 mJ/cm^2. Rinse the membrane once with 2× SSC. Dry the membrane completely if storage is required.

3.4.2 PCR-mediated probe labeling

1. Prepare labeling mix on ice as following:

333 μ*M* dNTP mix, without dCTP	0.5 μL

[17]This step is to check the quality of the gel. If there is a problem (e.g., leaks or air bubbles that cannot be removed, etc.), discard the gel and prepare it again.

[α-^{32}P] dCTP (400 Ci/mmol)	5 μL
20 μ*M* Forward Primer*	0.625 μL
20 μ*M* Backward Primer*	0.625 μL
10× Buffer	1.25 μL
50 m*M* $MgCl_2$	0.625 μL
Taq polymerase (5 U/μL)	0.25 μL
Template DNA fragment	5 ng
Water	to 12.5 μL

*These are the same primers used originally to amplify the template fragment from genomic DNA.

2. PCR conditions: 25 cycles of 94°C for 1 min, X°C for 30 s, 72°C for 1 min (X = primer-specific Tm).
3. Purify and denature the labeled probe as in **Subheading 3.2.4, step 6**.
4. Perform hybridization and wash as described in **Subheading 3.2.4**, except at 57°C instead of 65°C.
5. Expose the blot to phosphorimager screen.

3.5. Nucleotide resolution DSB mapping

In order to detect DSB sites at single nucleotide resolution, DSB fragments must be separated on a sequencing gel. Before carrying out this type of analysis, it is recommended to first determine the position and frequency of DSBs at the locus of interest by performing high resolution mapping (**Subheading 3.4**). Based on the result of this mapping, a restriction enzyme that cuts 150–200 bp from DSB site can be chosen, along with primer sets to amplify probe and template for sequence standards (*see* Fig. 4).

Meiotic DSBs made by Spo11 have 2-nucleotide 5′ overhangs (*see*Note 18). In *rad50S* or *sae2Δ* mutants, Spo11 remains covalently attached to the 5′ ends. Even with extensive proteinase K treatment, oligo-peptides of Spo11 still remain covalently attached to 5′ DSB ends, which causes the 5′-terminal DNA strand to migrate slower on sequencing gels and thus makes it difficult to map the 5′ ends accurately (3–6). To circumvent this problem, 5′ ends are mapped indirectly by filling in the 3′ ends with a DNA polymerase that does not add extra nucleotides. After the fill-in reaction, the ends of the DSB fragments will be blunt and the filled-in 3′ ends will match the original 5′ ends (*see* Fig. 5). The filled-in 3′ ends and untreated (original) 3′ ends can then be detected side by side on the same gel by probing a Southern blot with a strand-specific probe (Fig. 5). Sequence standards are prepared by linear amplification in the presence of dideoxy nucleotide triphosphates (ddNTPs) with a primer that corresponds to the end of the sequence cleaved by the restriction enzyme (Fig. 4, primer a). This method is based on (5, 15).

After defining DSB sites at single-nucleotide resolution by this method, it is advisable to confirm the accuracy by mapping the DSB ends on the other side of the hotspot. This is accomplished by repeating the procedure using an appropriate restriction digestion and probe, as diagrammed in Fig. 4 (probe B, primer c, primer d, and RE2).

[18]We have mapped breaks at single nucleotide resolution at several DSB hotspots (Murakami et al., manuscript in preparation). When the 5′ ends were mapped independently from both sides of DSB hotspots using separate restriction digests and probes, we confirmed that DSB ends always have 2-nucleotide 5′ overhangs, as reported previously (4, 5). However, it is important to note that some 3′ DSB ends were not identical to what was expected based on the mapping of 5′ ends from the other side of the DSB site. Specifically, 3′ ends were often 1–2 nucleotides longer than expected. Therefore, it appears that 3′ ends may sometimes be filled in by DNA polymerase, either in vivo or during the preparation of genomic DNA (B. de Massy, personal communication).

3.5.1. Preparation of probes, sequence standards, and samples

1. Based on the high resolution DSB mapping performed as described in **Subheading 3.4**, choose two restriction enzymes, which cleave 150–200 bp on either side of the DSB site (RE1 and RE2 in Fig. 4).
2. Design DNA primers a, b, c, d as described in Fig. 4. Amplify Probe A, Probe B, and template DNA for sequencing standard by PCR. Gel-purify and extract all fragments. Quantify the DNA concentrations.
3. Using the template DNA fragment with primer a or primer d, prepare two sets of G, A, T, and C sequence standards using a thermocycle sequencing kit (e.g., SequiTherm EXEL™ II DNA Sequencing Kit (Epicentre), or similar). These sequence standards need to be appropriately diluted with TE to approximately match the intensity of the DSB bands. For a DSB hotspot where ~20% of DNA molecules are broken, an 80-fold dilution is appropriate. For a DSB hotspot where ~8% of DNA molecules are broken, a 200-fold dilution is appropriate.
4. Prepare two separate restriction enzyme digests (RE1 in Fig. 4), each of which contains approximately 1 μg of genomic DNA from a meiotic sample (*see*Note 19). One sample will be for 5′ end mapping and the other for 3′ end mapping. Also prepare four digests of sample from a 0 h culture (one each for the G, A, T, and C sequence standards) (*see*Note 20). Digest for 3 h, then heat inactivate the restriction enzyme.
5. Purify all of the samples using a commercial extraction kit (e.g., NucleoSpin Extract II kit (Macherey-Nagel), or similar). Elute DNA with 39.1 μL (5′ end sample) or 20 μL (the rest of the samples) of 1 m*M* Tris-HCl, pH 8.5 (this is a five-fold dilution of the elution buffer supplied with the extraction kit).
6. For the 5′ end sample, prepare 50 μL of a fill-in reaction mixture using Phusion™ High-Fidelity DNA Polymerase (FINNZYMES) as follows:

DNA	39.1 μL
5× buffer	10 μL
25 m*M* dNTP mix	0.4 μL
Polymerase	0.5 μL
Total volume	50 μL

7. Incubate at 72°C for 5 min. Purify DNA as in step 2, eluting DNA in 20 μL.
8. Dry all samples by speed-vac (takes about 1–2 h).
9. Dissolve the 5′ and 3′ samples in 4 μL of TE.
10. Dissolve the 0 h DNA samples with 4 μL of either G, A, T or C sequence standard, which has been appropriately diluted (see step 3 of **Subheading 3.5.1**).
11. Add 2 μL of 3× loading buffer to each sample. Mix. Samples may be stored at −20°C.

[19]If the DSB signals are too weak, more genomic DNA can be used. We have confirmed that up to 5 μg of DNA can be loaded in a single lane.

[20]Mixing the sequence standard with genomic DNA from the 0 hr sample allows for equal amounts of total DNA to be loaded in each lane. This controls for the effects of genomic DNA on the migration pattern on the sequencing gel, and is essential for accurate DSB mapping.

3.5.2. Denaturing polyacrylamide gel electrophoresis

1. Wash a pair of 30 × 40 cm front and back gel plates with soap and water. Wipe well with 100% ethanol and dry.
2. Siliconize one side of each plate by wetting a Kimwipe with Sigmacote solution and wiping the whole plate. After the solution dries, wipe with water and then with 100% ethanol.
3. Assemble gel plates with 0.4 mm spacers. Carefully seal the side and bottom of plates with sticky tape.
4. Prepare 60 mL of 6% acrylamide gel solution as follows: 25.2 g urea, 9 mL of 40% acrylamide (19:1); 6 mL of 10× TBE; 24 mL of H_2O.
5. Add 60 μL of TEMED and 600 μL of 10% APS. Mix.
6. Pour the gel immediately using a large syringe. With the short plate on top, raise the top of the gel sandwich to about a 30° angle from the bench top and carefully pour the acrylamide between the plates along one side. Insert the flat side of a 0.4 mm shark tooth comb at the top of the gel. Avoid introducing bubbles in the gel sandwich.
7. The acrylamide should polymerize within 2 h. The polymerized gel may be used immediately, or may be wrapped in plastic film and stored at room temperature over night.
8. Remove sticky tape from the gel sandwich. Place the gel sandwich in a sequencing electrophoresis apparatus.
9. Pour 1× TBE into the apparatus. Remove the shark tooth comb. Wash the top and bottom of the gel using a syringe with needle to remove acrylamide fragments, urea, and air bubbles.
10. Insert the teeth side of the shark tooth comb into the gel sandwich. Flush wells using a syringe with needle. Load 1× loading buffer (diluted from stock with TE) to all wells. Pre-run at 1700 V, 70 W for 30 min (*see*Note 17).
11. Prior to loading, denature all samples at 100°C for 5 min and chill on ice. Flush wells using a syringe with needle. Load samples gently to avoid spillover between lanes.
12. Begin running the gel at 1700 V, 70 W.
13. Observe the migration of the xylene cyanol dye, which comigrates with DNA strands of approximately 100 nucleotides. Stop electrophoresis at an appropriate time after the xylene cyanol dye has run out of the gel (*see*Note 21).
14. Remove the gel sandwich from the electrophoresis apparatus. Place the sandwich in a cold room (4°C) until the plates are cool. This step makes it easier to handle the gel during the transfer step.

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[21]The electrophoresis time depends on the length of the DSB fragment to be resolved. The following are optimal total times using the conditions described in this protocol:

150 nucleotides	190 min
180 nucleotides	250 min
220 nucleotides	290 min
240 nucleotides	320 min

3.5.3. Southern blotting by electro-transfer—This step assumes the use of TE 90 GeneSweep™ Sequencing Gel Transfer Unit (Hoefer Scientific Instruments).

1. Prepare blotting paper and membrane: Cut the GeneScreen™ uncharged nylon membranes to the size of the gel plus 2 cm. Prepare two sheets of 35 × 45 cm blotting paper. Prepare a tray containing 1× TBE to soak the membrane and blotting paper.
2. Place the gel sandwich on the bench with the short plate on top. Very slowly disassemble the gel sandwich so that the gel remains on the bottom plate. Take out the side spacers. If wrinkles or air bubbles developed between the gel and the plate, pour a small amount of 1× TBE onto the gel and gently roll the area smooth with a pipette.
3. Wet a sheet of blotting paper with 1× TBE. Drain off excess buffer with Whatman 3MM paper. Lay the blotting paper on the gel, starting from the top of the gel. Carefully roll out wrinkles and air bubbles with a pipette.
4. To lift the gel from the glass plate onto the blotting paper, rapidly peel off the blotting paper, taking care not to damage the gel.
5. Place the blotting paper with gel side up onto the GeneSweep platform.
6. Wet the nylon membrane with 1× TBE. Blot off excess buffer with Whatman 3MM paper. Lay the membrane onto the gel. Do not move the membrane after it makes contact with the gel. Gently roll out wrinkles and air bubbles with a pipette.
7. Wet another sheet of blotting paper with 1× TBE. Drain excess buffer with Whatman 3MM paper. Lay the paper on top of the nylon membrane. Gently roll out wrinkles and air bubbles with a pipette.
8. Lower the GeneSweep arm onto the left edge of the sandwich. Start the transfer. As the arm slides across the sandwich, the display should read between 1.8 and 2 A. If the current is less than 1 A, the sandwich may be too dry. If so, stop the run, lift the arm, and pour 1× TBE onto the sandwich. Gently roll out excess buffer with a pipette and start the run again.
9. When the arm reaches the right end of the sandwich, stop the run and lift the arm.
10. Carefully take off the upper blotting paper and the nylon membrane. Leave the membrane on theblotting paper (to prevent the membrane from drying out) and immediately fix the DNA to the membrane by UV crosslinking at 120 mJ/cm^2. The membrane can then be removed from the blotting paper.
11. Rinse the membrane once with 2× SSC. The membrane is now ready for hybridization (next section), or can be dried if storage is required.

3.5.4. Strand-specific probe preparation and hybridization

1. Prepare labeling mix on ice as following (*see*Note 22):

333 μ*M* dNTP mix without dCTP	0.5 μL
[α-^{32}P] dCTP (6000 Ci/mmol.)	5 μL (50 μCi)

[22]If the DSB hybridization signals are too weak, increase the total volume with the same concentrations of all reagents. We have used up to 50 μL of total reaction volume successfully.

20 μ*M* Primer*	0.625 μL
10× Buffer	1.25 μL
50 m*M* $MgCl_2$	0.625 μL
Taq polymerase (5 U/μL)	0.25 μL
Template DNA fragment**	5 ng
Water	to 12.5 μL

*Primer b,

**Probe A in Fig. 4

2. PCR conditions: 25 cycles of 94°C for 1 min, X°C for 30 s, 72°C for 1 min (X = primer specific Tm).

3. Purify and denature labeled probe as described in step 6 of **Subheading 3.2.4**.

4. Perform hybridization and wash as described in **Subheading 3.2.4**, except at 57°C instead of 65°C.

5. Expose the blot to the phosphorimager screen. Compare migration of the DSB bands to the sequencing standards to determine the positions of 5′ and 3′ DSB ends (*see* Fig. 6 for an example).

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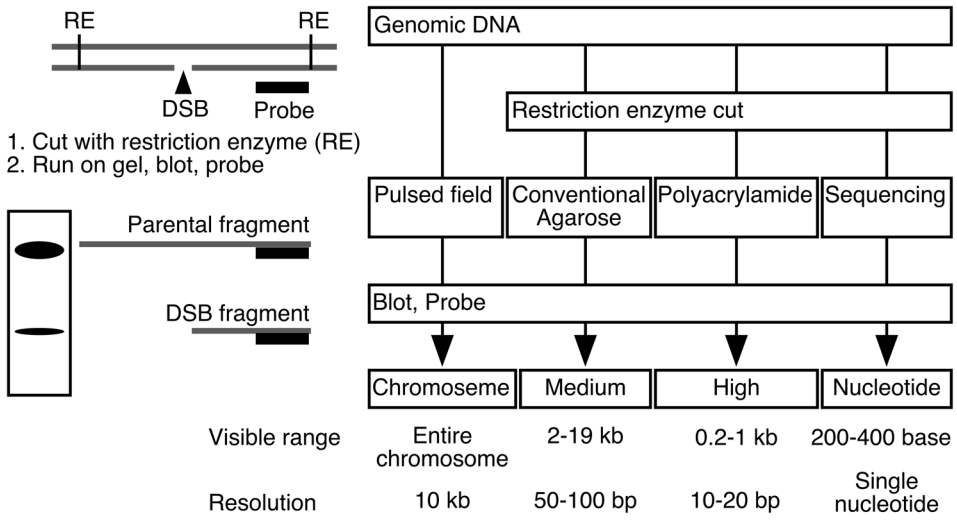

Fig. 1.

The procedure of DSB mapping at four different resolutions.

Left: General procedure of DSB mapping. Genomic DNA is digested with restriction enzyme(s) to yield appropriately sized DNA fragments (for chromosome-level mapping, this step is skipped). The DNA fragments are separated by electrophoresis on an appropriate gel according to the size of the fragment, then detected by Southern blot hybridization. Right: Comparison of methods and resolution of chromosome, medium, high, and nucleotide level resolution mapping.

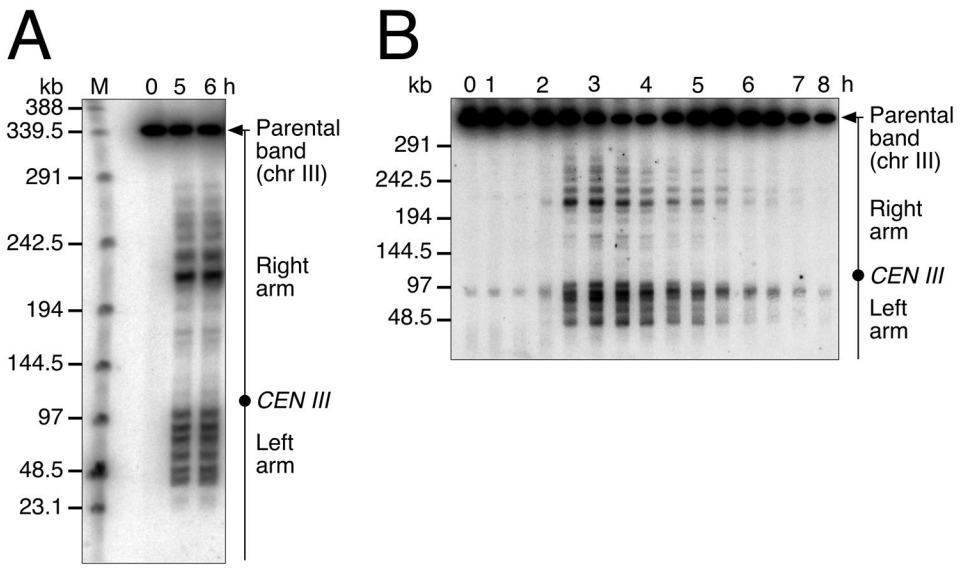

Fig. 2.

Chromosome level mapping.

Cells from a s*ae2*Δ strain (MJL 2305) **(A)** or a wild-type strain (MJL 1071) **(B)** were harvested at the indicated times in meiosis and processed for genomic DNA plug preparation and pulsed-field gel electrophoresis as described in the text. After Southern blotting, the membrane was probed with a chromosome III left end fragment (*CHA1*), revealing, in addition to the unbroken chromosome III (340 kb), the DSB fragments of chromosome III produced during meiosis. In **(A)**, a 14 cm wide by 21 cm long gel was used, and electrophoresis time was for 46 h. In **(B)**, a 21 cm wide by 14 cm long gel was used and electrophoresis time was 30.5 h. The size standard (M) is a *Hind* III digest of bacteriophage λ DNA and λ DNA concatamers. **(B)** is adapted with permission from (8).

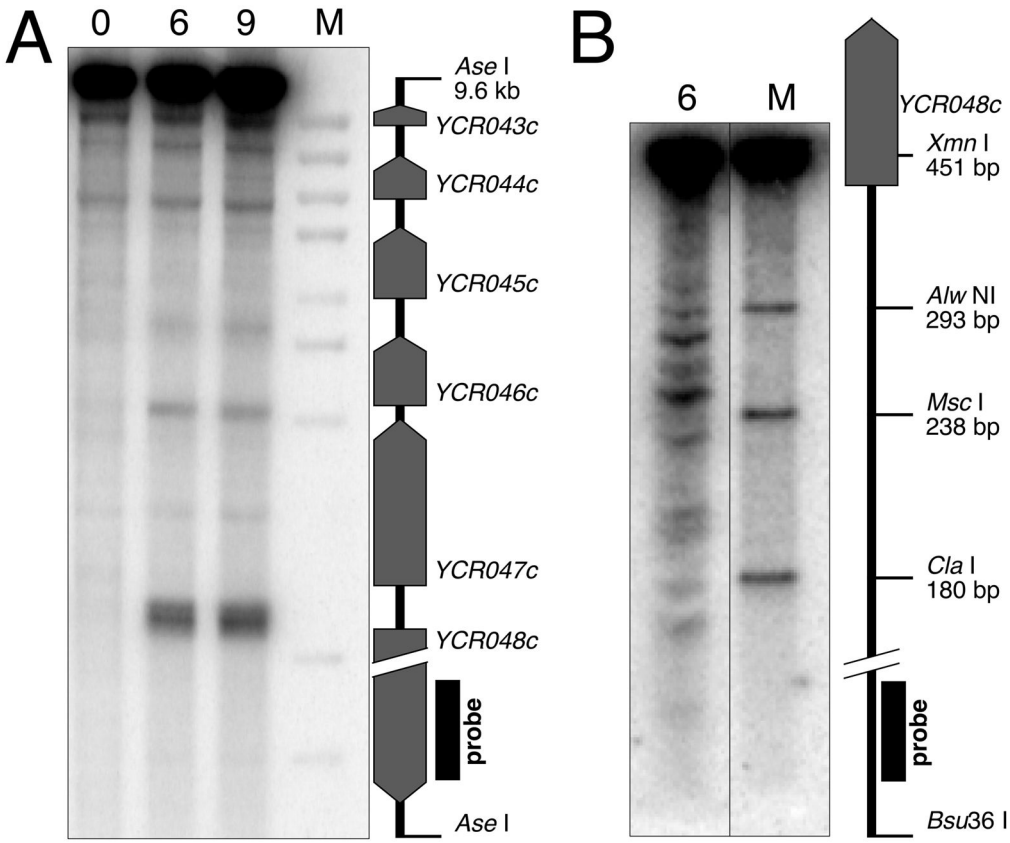

Fig. 3.

Medium and high resolution mapping of DSB in a *rad50S* strain.

(**A**) Medium resolution mapping of DSBs around *YCR048w*. Numbers above the panel indicate the time (in h) after transfer to SPM. The lane marked M includes 200 ng of λ *Bst*E II digest. Genomic DNA was digested with *Ase* I. The parental fragment is 9.6 kb. Both the genomic DNA and the marker were separated on a 0.8 % agarose gel and detected using a radiolabeled probe containing 100 ng of the probe DNA fragment and 0.1 ng of λ DNA.

(**B**) High resolution mapping of DSBs in the *YCR048w* promoter region. DSBs, which appeared as a single band in normal resolution mapping, are observed as multiple break sites when analyzed at this resolution. Genomic DNA extracted from meiotic cells (6 h) was digested with *Xmn* I and *Bsu*36 I. For the molecular weight marker, genomic DNA purified from 0 h cells was digested with *Xmn* I and *Bsu*36 I. Aliquots (1% of the digested DNA each) were further digested with either *Alw*N I, *Msc* I or *Cla* I. After heat inactivation of the restriction enzymes, the differently digested DNA fragments were mixed together and loaded in the lane marked M. These DNA fragments were separated on a 6% polyacrylamide gel containing 8 *M* urea and detected by Southern blotting and indirect end labeling.

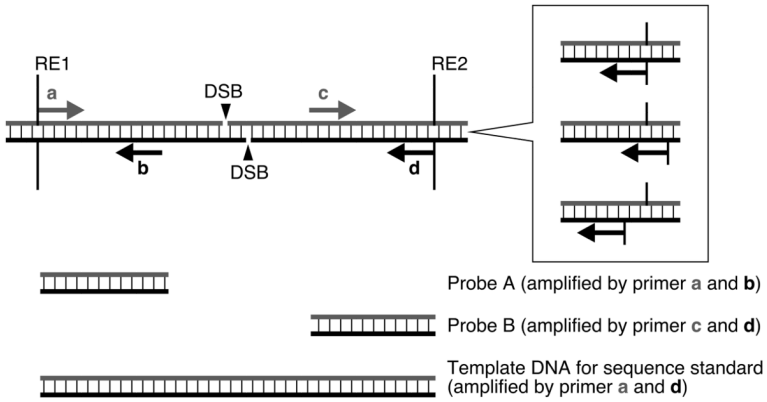

Fig. 4.

Primers required for nucleotide level mapping.

For nucleotide resolution mapping, genomic DNA is digested with an appropriate restriction enzyme (RE1 or RE2), which cleaves 150–200 bp from DSB sites. Four primers (a, b, c, and d) are used for following procedures: to amplify probes A and B and a template DNA for sequence standards; to prepare strand-specific probe (primers b and c); to prepare sequence standards (primers a and d). Primers a and d are designed to be suitable to the shape of restriction enzyme cut as illustrated in the box on the right.

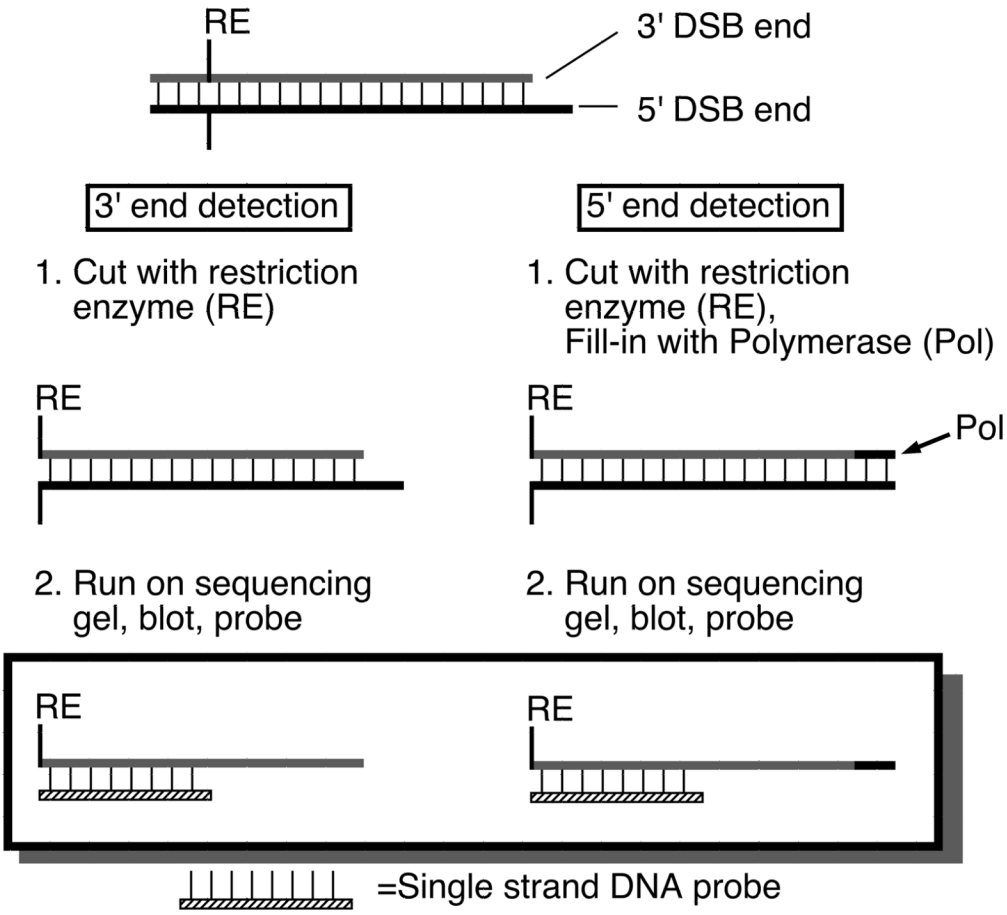

Fig. 5.

Overview of procedure for nucleotide level resolution mapping of 5′ and 3′ DSB ends. Genomic DNA is purified from meiotic cell and digested with an appropriate restriction enzyme (RE). After the separation on a sequencing gel and Southern blotting, 3′ DSB ends are detected with a strand-specific DNA probe. For the indirect detection and mapping of 5′ ends, 3′ ends are filled-in with DNA polymerase to match the size of the 5′ ends.

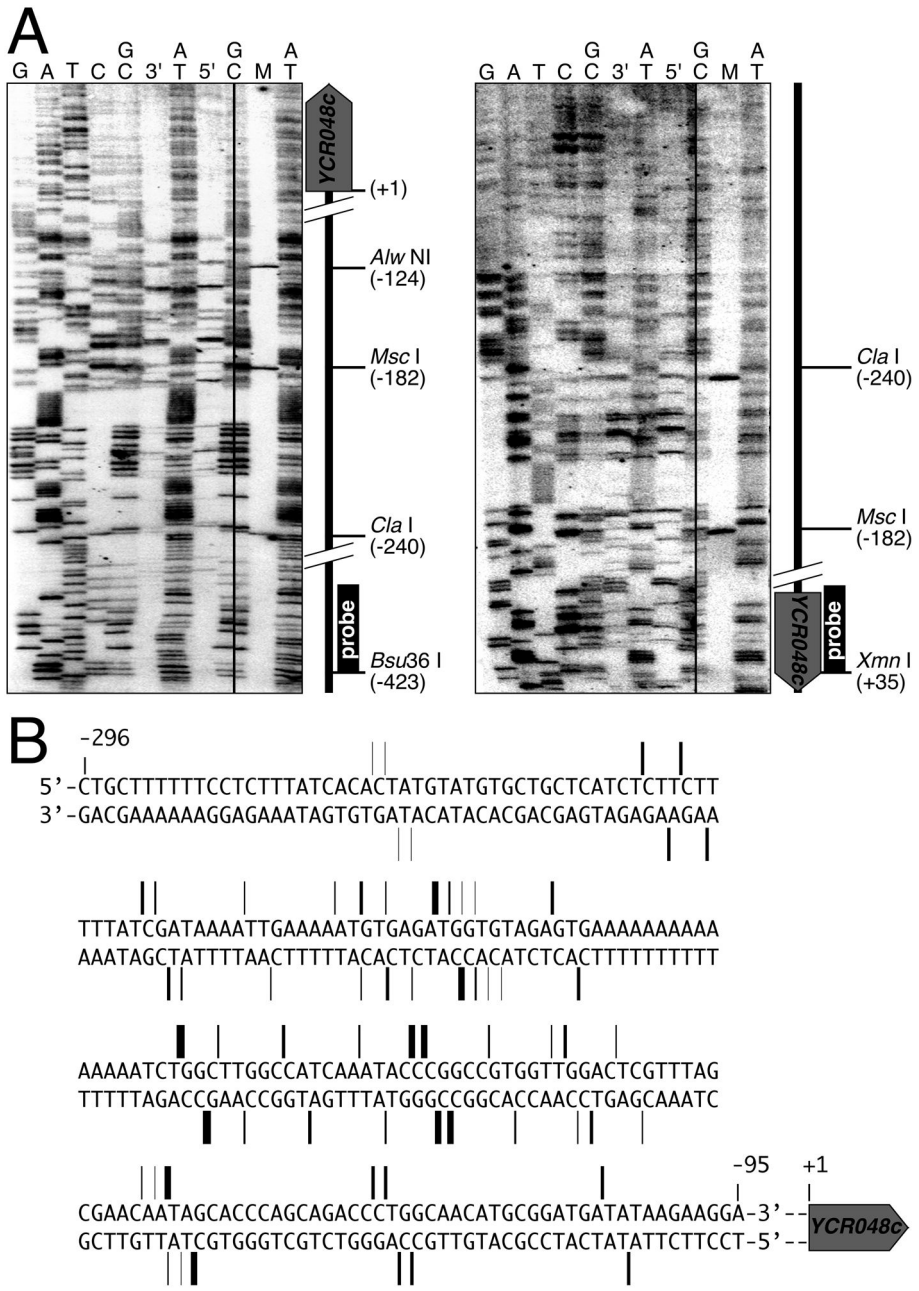

Fig. 6.

Nucleotide level resolution mapping of DSB at *YCR048w* promoter region.

(**A**) Examples of sequencing gel mapping of DSBs. Images of Southern blots probed with two different probes are shown. Samples were digested with *Bsu*36 I (left) or *Xmn* I (right) and probed with single-stranded DNA in the vicinity of each restriction enzyme site (illustrated on the right of each panel; *see also* Fig. 4). Lanes marked 5′ and 3′ contain separate DSB end-mapping samples, prepared using genomic DNA purified from cells 9 h after transfer to SPM. Lanes marked G, A, T, and C are nucleotide sequence standards. Lanes marked GC and AT are sequencing standard containing pooled G+C or A+T standards, respectively. All of the sequence standards contain additional restriction-digested genomic DNA (from a 0 h culture) so that the amount of total genomic DNA is the same as

in the DSB sample lanes. Lanes marked M contain DNA from 0 h cells, digested with either *Bsu*36 I (left) or *Xmn* I (right), plus 1% of DNA subjected to secondary digest with either *Alw*N I, *Msc* I, or *Cla* I (*see* Note 12). Numbers correspond to positions relative to the translation start of *YCR048w*.

(**B**) Location of DSBs in the *YCR048w* promoter region. The vertical bars represent DSB locations, defined by mapping of DSB 5′ ends using probes on both sides of the DSB hotspot. Thickness of the bars provides a semi-quantitative representation of the signal strength of each DSB band on the Southern blots.