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Isolation of Meiotic Recombinants from Mouse Sperm

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Abstract

Homologous recombination during meiosis is critical for the formation of gametes. Recombination is initiated by programmed DNA double-strand breaks which preferentially occur at hotspots dispersed throughout the genome. These double-strand breaks are repaired from the homolog, resulting in either a crossover or noncrossover product. Multiple noncrossover events are required for homolog pairing, and at least one crossover is critical for proper chromosome segregation at the first meiotic division. Consequently, homologous recombination in meiosis occurs at high frequencies. This chapter describes how to characterize crossovers and noncrossovers at a hotspot in mice using allele-specific PCR. Amplification of recombinant products directly from sperm DNA is a powerful approach to determine recombination frequencies and map recombination breakpoints, providing insight into homologous recombination mechanisms.

Keywords

Meiotic recombination; sperm; crossover; noncrossover; hotspot; allele-specific PCR; F1 hybrid mice; gene conversion; homolog

1. Introduction

Meiotic recombination does not occur evenly throughout the genome but instead clusters at “hotspots” which are estimated to occur every 25–100 kb in the human genome (1–4). Hotspots are presumed sites for programmed DNA double-strand breaks (DSBs) which initiate meiotic recombination between homologs (5, 6). Crossover (CO) recombination results in reciprocal exchange of flanking sequences (Fig. 15.1) and is essential for proper chromosome segregation during meiosis. Each chromosome requires at least one CO, termed the obligate CO, for proper alignment on the metaphase plate and to avoid meiotic non-disjunction which generates aneuploid gametes. However, COs are only one outcome of recombination. Estimates of DSBs indicate that COs are a fraction of recombinants (7), suggesting that noncrossovers (NCOs) account for a substantial proportion of inter-homolog DSB repair events. NCOs are the result of a patch-like repair of DSBs without exchange of flanking sequences (Fig. 15.1).

Experiments in yeast have shown that COs and NCOs derive from the same initiation events which diverge into distinct molecular pathways (8–11). The majority of COs are generated by the canonical DSB repair (DSBR) pathway via a double Holliday junction (dHJ) intermediate (12), while NCOs are thought to be primarily generated by the synthesis-dependent strand annealing (SDSA) pathway (Fig. 15.1). After DSB formation and 5′–3′ resection, a 3′ single-stranded tail invades the unbroken homologous template forming a D-

loop. In SDSA, the invading strand is extended by a DNA polymerase and is then displaced to re-anneal with the other 3' end. COs are thought to derive from polymerizing a more stable single-end invasion intermediate which then “captures” the other 3' end (second-end capture), generating a dHJ. Studies in yeast have shown that resolution of the dHJ primarily generates COs (13).

Meiotic recombination is an excellent system for gaining insight into DSB repair mechanisms, with a detailed understanding of these mechanisms requiring the isolation of both CO and NCO products. The powerful technique of sperm typing pioneered by the laboratories of Norman Arnheim and Alec Jeffreys enables characterization of recombination products (14–16). PCR with allele-specific forward and reverse primers is used to selectively amplify CO recombination products from isolated sperm DNA (CO assay, Fig. 15.1). PCR with one allele-specific primer and one universal primer (which can recognize both alleles equally well), followed by genotyping of products, can identify NCO and CO recombination products in the same analysis (NCO/CO assay, Fig. 15.1) (17). Analyses of human and mouse hotspots have shown that CO recombination activity can vary substantially between hotspots, from as low as 0.0004% to as high as 2% (18, 19). CO recombination breakpoints at mammalian hotspots typically span 1–2 kb with the peak of CO and NCO recombination breakpoints clustering in the center (17, 20), consistent with both COs and NCOs deriving from the same initiation events, presumably DSBs. The ratio of NCOs to COs at mammalian hotspots ranges between <1:12 and 9:1 (21, 22). Although it is likely that different hotspots have different propensities for CO or NCO formation, this broad range likely also reflects the ability to identify NCOs in the tested hotspots. NCO gene conversion tracts are very short (17), estimated to be ~100 bp in mouse (22). Thus, the ability to score NCOs at any hotspot is dependent upon the polymorphism density throughout the hotspot but especially close to the most frequent position(s) of recombination initiation.

Mouse is an ideal model in which to study meiotic recombination because of the evolutionary conservation of recombination mechanisms, the ability to generate and utilize mutants, and the availability of a large number of inbred strains, which provide genetic variation across meiotic hotspots. In this chapter, we describe methods to characterize COs and NCOs from mouse meiotic hotspots. For additional information with a focus on human hotspots, see (23).

2. Materials

To prevent contamination, keep all reagents and materials used for PCR in a separate area. Additionally, designated micropipettors are highly recommended.

2.1. PCR Buffers and Reagents

1. 10X Jeffreys' buffer (24): 450 mM Tris-HCl pH 8.8, 110 mM (NH₄)₂SO₄, 45 mM MgCl₂, 67 mM β-mercaptoethanol, 44 μM EDTA, 10 mM each dATP, dTTP, dGTP, and dCTP, and 1.13 mg/ml non-acetylated bovine serum albumin (BSA). Store in aliquots at –20°C (see Note 1).
2. 2 M Tris base (2-amino-2-(hydroxymethyl)-1,3-propanediol).

¹The original Jeffreys' buffer was formulated at 11.1X strength (24), but the 10X formulation described here is equally effective. Avoid repeated freezing and thawing. It is essential that the BSA be of high quality and non-acetylated (e.g., Ambion Ultrapure BSA, AM2616 <http://www.ambion.com/>) (33). Different preparations of 10X Jeffreys' buffer can affect the efficiency and specificity of PCRs. Make a large-scale batch (e.g., 10 ml) to allow progression through an entire experiment, but if a new preparation is needed, re-assess the primers, as in **Section 3.1.2**.

3. Taq DNA polymerase (Abgene AB-0192 <http://www.abgene.com/>).
4. Cloned Pfu DNA Polymerase.
5. Mouse inbred genomic DNA can be obtained from the Jackson Laboratory (<http://www.jax.org/dnares/index.html>).
6. S1 nuclease diluted to 10 U/μl in 20 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1 mM (CH₃CO₂)₂Zn (Zinc acetate), and 50% (v/v) glycerol. Can be stored at −20°C for up to 9 months.
7. 10X S1 buffer: 0.2 M CH₃COONa (Sodium acetate) pH 4.9, 10 mM Zn acetate, 1 M NaCl. Store in aliquots at −20°C.
8. S1 mix: 1X S1 buffer supplemented with 4.8 ng/μl sonicated salmon sperm DNA and 0.7 U/μl S1 nuclease. Freshly prepared.
9. Dilution mix: 10 mM Tris-HCl pH 7.5 and 5 μg/ml sonicated salmon sperm DNA (25). Freshly prepared.
10. 10X Tris-borate-EDTA (TBE) buffer.
11. Loading dye: 0.5X TBE, 30% (v/v) glycerol, supplemented with bromophenol blue.
12. 10 mg/ml ethidium bromide

2.2. Genomic DNA Isolation

1. F1 hybrid mice (e.g., C57BL/6 J × DBA/2 J).
2. 20X Sodium chloride–sodium citrate (SSC) buffer: 3 M NaCl and 0.3 M HOC(COONa)(CH₂COONa)₂·2H₂O (citric acid trisodium salt dihydrate), pH 7.0. Prepare 2X, adjust pH to 7.0 prior to use and dilute to 1X and 0.2X SSC from this stock.
3. 80 μm metal mesh (Sigma-Aldrich S3770).
4. β-Mercaptoethanol.
5. 10% (w/v) CH₃(CH₂)₁₁OSO₃Na (SDS).
6. 20 mg/ml proteinase K.
7. Phenol/chloroform/isoamyl alcohol 25:24:1 (v/v/v); saturated with 100 mM Tris pH 8.0.
8. Ethanol, 100% and 70%.
9. 3 M Na acetate, pH 5.2.
10. 5 mM Tris-HCl, pH 7.5.

2.3. Quantification and Quality Assessment of Genomic DNA

1. SYBR green included in a qPCR master mix (e.g., Brilliant® II SYBR® Green QPCR Master Mix, Stratagene).
2. ROX reference dye (1:500).
3. Stratagene Mx3005 real-time PCR instrument or equivalent.

2.4. Allele-Specific Oligonucleotide (ASO) Hybridization

1. 96-Well dot-blot manifold.

2. Denaturation buffer: 0.5 M NaOH, 2 M NaCl, 25 mM EDTA.
3. Whatman filter paper, grade 3.
4. Nylon hybridization membranes (e.g., HybondTM-XL).
5. Multichannel 30–300 μ l pipettor.
6. Stratalinker or equivalent.
7. 10 U/ μ l T4 polynucleotide kinase.
8. 10X kinase mix: 700 mM Tris-HCl pH 7.5, 100 mM MgCl₂, 50 mM spermidine trichloride (Sigma S2501), and 20 mM dithiothreitol. Aliquot and store at -20°C .
9. Kinase Stop Solution: 25 mM EDTA, 0.1% SDS, 10 μ M ATP. Aliquot and store at -20°C .
10. 10 mCi/ml (γ -³²P)ATP.
11. 50X Denhardt's solution: 1% (w/v) Ficoll 400, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) BSA (Fraction V). Filter sterilize and store at 4°C .
12. Tetramethylammonium chloride (TMAC) hybridization solution: 3 M TMAC, 0.6% SDS, 10 mM NaPO₄ pH 6.8, 1 mM EDTA, 4 μ g/ml yeast RNA in 5X Denhardt's solution. Store at 4°C .
13. TMAC wash solution: 3 M TMAC, 0.6% SDS, 10 mM NaPO₄ pH 6.8, 1 mM EDTA. Store at 4°C .
14. Hybridization mesh.
15. 3 mg/ml sonicated salmon sperm DNA.
16. Rotisserie hybridization oven and bottles.
17. Phosphorimager and screen.
18. 0.1% SDS for stripping membranes.
19. 2X and 3X SSC.

2.5. Cloning and Confirmation of NCOs

1. TOPO[®] TA cloning kit (Invitrogen).
2. Competent cells (e.g., TOP10[®] chemically competent, Invitrogen).
3. LB agar plates supplemented with 50 μ g/ml of ampicillin.
4. 40 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal).
5. 82 mm nylon hybridization membranes (e.g., HybondTM-XL).
6. India ink.
7. Cloning denaturation buffer: 0.5 M NaCl, 0.5 M NaOH.
8. Cloning neutralization buffer: 1.5 M NaCl, 0.5 M Tris-HCl pH 7.5.
9. 2X and 3X SSC.

3. Methods

Several mouse recombination hotspots have been characterized, some of which have been shown to be differentially active in different strain combinations (e.g., (26–30)). These

characterized hotspots represent a tiny fraction of hotspots within the mouse genome, however, such that the approximate position of many additional hotspots can be inferred from crossover maps from a number of sources, for example, recombinant inbred strains (27) or recombinants directly from F1 hybrids (30). Once a known hotspot has been chosen for further analysis or a candidate hotspot region has been narrowed down for investigation, polymorphisms between the two mouse strains used in the crosses must be identified. A goal of the Mouse Genome Project is a haplotype map at 20 kb resolution of 48 mouse strains, with in-depth sequencing of 17 mouse strains (<http://www.sanger.ac.uk/resources/mouse/genomes/>). However, as yet, DNA sequencing may be necessary to identify/confirm polymorphisms between strains in the region of interest. Once polymorphisms are identified, the methods below detail how to design primers, extract DNA, amplify recombinants from sperm from F1 hybrids, and map recombination breakpoints.

The following nomenclature will be used throughout (see Fig. 15.1), with the example of C57BL/6J (the “B” strain) and DBA/2J (the “D” strain) as the strain combination to generate F1 hybrids (B × D). Reciprocal recombinants are referred to as B to D and D to B, which can be amplified using forward (Bf) and reverse (Dr) primers for the B to D orientation, with 1° (Bf1 and Dr1) or 2° (Bf2 and Dr2) PCR primer sets. Universal primers that recognize both strains equally well are designated as “U.”

3.1. Designing and Assessing Allele-Specific and Universal Primers

Allele-specific PCR primers must be designed and tested to be both efficient in amplifying a single recombinant from a complex genomic milieu and highly specific for the recombinant. In both CO and NCO assays, two serial rounds of amplification are used to ensure efficiency and specificity. Nested sets of allele-specific PCR primers that encompass the hotspot are required. Most mammalian hotspots span 1–2 kb, but the allele-specific primer binding sites may not immediately flank the hotspot. Long-range PCR is reliably efficient and accurate up to 10 kb, although with high-quality DNA and highly efficient allele-specific PCR primers up to 12 kb products can be isolated. The first external round of PCR (1° PCR) requires the primers be both specific and highly efficient. Primers for the second, nested PCR (2° PCR) can be less efficient as the input DNA is not as complex and recombinant molecules should represent a substantial proportion; however, these primers should still have high specificity.

3.1.1. General Guidelines for Primer Design

1. Analyze the sequence of interest for repeat regions and areas of low complexity. Programs for this purpose can be found in sequence analysis software and on the web, for example, <http://www.repeatmasker.org/>. Avoid repetitive regions for design of universal primers and, if possible, for allele-specific primers.
2. Universal primers are targeted to regions with no polymorphisms. Design universal primers with 50% G/C content and a length of 20–22 nucleotides. Universal primers should also be tested as detailed below to ensure that they efficiently and equivalently amplify both strains and do not compromise the efficiency of the allele-specific reactions.
3. For allele-specific primers, the locations of polymorphisms dictate the sequence. Some sites readily allow for efficient and specific amplification, while others do not; the characteristics that define “good” sites vs. “bad” are not simple and so sites must be tested empirically. Allele-specific primers can range between 16 (high G/C content) and 24 (low G/C content) nucleotides, but most are in the 18–20 nucleotide range. Importantly, the polymorphic nucleotide (or nucleotides in the case of an insertion/deletion polymorphism) is located at the 3′ end of the primer. Four allele-specific primer sets are shown in Table 15.1.

4. To increase specificity of a mildly non-specific primer, the primer can be shortened by one or two nucleotides. To increase efficiency, the primer length can be increased (e.g., compare primers to POLY4020 and POLY5465 in Table 15.1) or, especially for primers with low G/C content, additional non-templated G or C nucleotides (e.g., GGGG) can be added to the 5' end of the primer (19). The non-templated nucleotides serve to increase the efficiency of the PCR only after the initial target has been amplified. If amplification of a particular polymorphic site is critical and allele specificity cannot be achieved with these modifications, an intentional mismatch can be included one to two nucleotides 5' to the polymorphic site (*see* Note 2). In this case, optimization of MgCl₂ concentration and increasing the length of the primer may be required to get efficient amplification.

3.1.2. Testing Specificity and Efficiency of Primers

1. Set up PCR optimization reactions in a total volume of 10 μ l with 1X Jeffreys' PCR buffer, supplemented with an additional 12.5 mM Tris base, 0.03 U/ μ l Taq polymerase, 0.006 U/ μ l Pfu polymerase, 0.2 μ M of each primer, and 12.5 ng of genomic DNA. *See* Table 15.2 for an example optimization experiment that tests two forward, two reverse, and one universal primer sets at four different annealing temperatures.
2. Each reaction should be carried out with genomic DNA from each inbred strain (in this example, B and D) used to generate the F1 hybrid to be examined (B $\times$ D). Test several annealing temperatures ranging from 56 to 67° C.
3. Use universal primers such that the amplified products approach the size predicted for your CO or NCO assay. Avoid amplifying regions larger than your intended CO or NCO products to avoid potential contamination issues. Conditions for the optimization of PCR are denaturation (1 min at 96° C) followed by 30 cycles of amplification (20 s at 96° C, 30 s at the annealing temperature, and 60 s/kb at 65° C for extension) (*see* Note 3).
4. Analyze PCR products by agarose gel electrophoresis. To aid visualization of weak bands, include additional ethidium bromide in the loading buffer (50 ng/ml) and/or transfer the gel for Southern blotting and take a long exposure of the membrane. Examples of good and bad allele-specific primers are shown in Fig. 15.2, including a bad primer that can be modified.

3.2. Genomic DNA Isolation

To prevent contamination, all samples are preferably prepared in a PCR hood with laminar flow or alternatively in a separate area from what will be used for analysis of PCR products. Preparation of somatic DNA should always be performed separately and with cleaned (*see* Note 4) or designated instruments. All steps are performed at room temperature except where noted.

²The addition of an intentional mismatch to an allele-specific primer (Amplification-Refractory Mutation System, or ARMS) has been used successfully in multiple contexts (34). The mismatch can be placed between 1 and 5 nucleotides 5' to the polymorphic site.

³The 65°C extension temperature for long-range PCR is lower than the commonly utilized 72°C, but it increases efficiency of the amplification and may reduce PCR artifacts caused by annealing of incompletely synthesized products (23).

⁴We routinely expose our dissecting instruments, pipettors, and tube racks to ultraviolet light from a Stratalinker or equivalent instrument to prevent DNA crosscontamination (follow the instructions provided for your instrument). Also, aerosol-resistant tips are used for all pre-PCR steps.

3.2.1. Extracting DNA from Sperm and Somatic Tissue

1. Somatic and sperm DNA should always be prepared from the same mice. Dissect the somatic tissue of choice (e.g., spleen, brain, or liver) first and then dissect the cauda epididymides (Fig. 15.3) from 6- to 8-week-old male mice (*see* Notes 5 and 6). Place the tissue to be extracted in a clean Petri dish. With a fresh razor blade, finely chop the sample until homogenized. Add 1 ml of 1X SSC and continue to homogenize with the razor blade.
2. Repeatedly pipet up and down with a transfer pipet to resuspend and retrieve the sample to filter through an 80 μ m mesh into a 1.5 ml screw cap tube. Rinse the plate with an additional 0.5 ml of 1X SSC and add through the filter into the same tube.
3. Pellet the cells in a microcentrifuge at 2,700 rcf (~5,000 rpm) for 1 min (*see* Note 7). Carefully remove the supernatant with a 1 ml pipet tip and resuspend the pellet in 1 ml of 1X SSC by vortexing. Repeat one time.
4. For epididymides: Remove 5 μ l and assess the cells under a microscope. Sperm should represent >95% of the cells in the mix. If so, go on to Step 5. If somatic cells represent a more significant portion, *see* Note 8.
5. Briefly spin the cells, and remove the residual 1X SSC and resuspend in 960 μ l of 0.2X SSC. Pellet the cells as in Step 3. Repeat one time and resuspend in 960 μ l of 0.2X SSC.
6. Add 120 μ l of β -mercaptoethanol (*see* Note 9), 20 μ l of freshly prepared 20 mg/ml proteinase K, and 100 μ l of 10% SDS. Invert to mix and incubate at 55°C for 2 h, inverting occasionally.
7. Split the resulting slurry into four tubes of ~320 μ l each. Add an equivalent volume of phenol:chloroform:isoamyl alcohol (25:24:1) to extract the protein. Mix well and spin at 15,000 rcf for 5 min.
8. For the subsequent phenol extractions: Use 1 ml pipet tips with the ends removed at an angle with a clean razor blade. Transfer the top aqueous layer into a clean screw cap 1.5 ml tube. Leave a significant portion of the aqueous phase at the interphase to prevent protein contamination.
9. Re-extract the phenol layer from Step 8 by adding 200 μ l of 1X SSC and 20 μ l of 10% SDS. Mix well by inverting vigorously and spin as in Step 7. Combine the aqueous phase from the re-extraction to the previous sample.
10. Add 500 μ l of phenol:chloroform:isoamyl alcohol (25:24:1) to the combined aqueous phases and spin as in Step 7. Remove the aqueous phase to a fresh tube. Precipitate the DNA by adding 1 ml of -20°C 100% ethanol. Mix well and spin at 15,000 rcf for 5 min. Decant supernatant and wash the pellet with 70% ethanol. Repeat the centrifugation step and decant the supernatant. Briefly spin and remove the last of the supernatant with a pipet tip.

⁵Some mice have delayed development of the epididymides and should be dissected at 8 weeks of age (e.g., F1 hybrid A/J x DBA/2 J mice).

⁶Dissected epididymides and control tissues can be stored at -80°C until use.

⁸If somatic cells are a significant proportion of the sperm preparation, specifically lyse somatic cells by adding SDS to a final concentration of 0.15%. Somatic cells will lyse, but sperm are resistant to this lower concentration of SDS (35). Mix briefly by inverting and spin at 10,000 rpm for 3 min. Remove the supernatant with a 1 ml pipet tip and discard.

⁹ β -Mercaptoethanol reduces disulfide bonds that inter- and intramolecularly connect protamines which highly compact sperm DNA. Protamines are cysteine- and arginine-rich proteins that replace histones in sperm.

11. Immediately resuspend the pellet in 75 μ l of ddH₂O and pool the four aliquots together. Add 1/10 volume of 3 M Na acetate (pH 5.2) and three volumes of -20°C 100% ethanol. Mix well and spin as in Step 10. Decant supernatant and wash the pellet with 70% ethanol. Air dry the pellet (*see* Note 10). Resuspend in 100 μ l of 5 mM Tris pH 7.5. Incubate at 55°C for 1 h with occasional mixing, transfer to 4°C overnight. Store indefinitely at -20°C .

3.2.2. Quantification and Quality Assessment of Genomic DNA

1. Test several amounts of your genomic DNA (e.g., 1, 2, and 3 μ l into 1 ml each) and quantify the concentration by measuring the absorbance at 260 nm with a spectrophotometer (*see* Note 11). Make a working stock concentration of DNA of 20 ng/ μ l in 5 mM Tris pH 7.5 and retest the absorbance at 260 nm. Adjust the concentration as necessary.
2. Run 1, 5, and 10 μ l samples of the working stock concentration of genomic DNA preparations on an agarose gel to verify that the DNA is of high molecular weight and to confirm the relative concentrations. Make sure to include sperm and somatic DNA on the same gel for comparison.
3. If comparison of absolute recombination frequency between samples is essential to your study, the concentration of different DNA samples can be equalized after assessment by quantitative PCR (qPCR). Design universal primers that generate a 100–150 bp amplified product of the same sequence regardless of the parental strain (i.e., containing no polymorphisms) and completely located within your experimental primary (1°) PCR product for CO and NCO assays (*see* below). Ensure that these primers equivalently amplify both inbred strains used to generate the F1 hybrid of interest, by testing them with serial dilutions of known quantities of pure inbred genomic DNA. Compare samples with at least three replicates containing 0.2 ng of genomic DNA with SYBR green for qPCR in a final volume of 20 μ l. qPCR conditions in a Stratagene Mx3005 or similar are denaturation (10 min at 95°C) followed by 40 cycles of amplification (30 s at 95°C , 1 min at 60°C , and 30 s at 72°C) and finishing with 1 min at 95°C , 30 s at 55°C , and 30 s at 95°C .

3.2.3. Assessing the Amplification Efficiency of Genomic DNA—Even with careful genomic DNA extraction, shearing will occur and proteinase K digestion can be incomplete, both of which can result in non-amplifiable genomic DNA. In order to accurately quantify recombination activity at your locus, the amplification efficiency across your hotspot for each genomic DNA sample used in your study must be calculated.

1. For each genomic sample to be assayed, perform a series of PCRs using allele-specific primers directed against one side of the hotspot and universal primers on the other (Fig. 15.4). Because each strain and allele-specific primer must be checked separately, four separate sets of reactions are generated for all samples.
2. Each PCR is seeded with 12 pg of genomic DNA from a dilution generated from your working stock. As the haploid mouse genome is ~ 3 pg, this corresponds to two copies of the region of interest from each parental strain. Multiple sets of reactions are required to get an accurate assessment of amplification efficiency, so

¹⁰Do not overdry the pellet as this will hamper resuspension. It is best to air dry in a PCR laminar flow hood to prevent potential contamination.

¹¹If the genomic DNA is viscous, abrogating accurate pipetting, a portion can be digested with a restriction enzyme(s) to reduce viscosity. Choose an enzyme that does not cleave within any regions being amplified. After digesting the DNA overnight, the sample can be phenol:chloroform extracted and ethanol precipitated. Proceed with quantification and assessment of DNA quality.

we routinely perform 24–48 PCRs per set, such that each DNA sample is tested in 1–2 96-well plates.

3. Perform the primary (1°) PCRs in a total volume of 8 μ l, as described in **Section 3.1.2**. Conditions for the PCR are denaturation (1 min at 96°C) followed by 26 cycles of amplification (20 s at 96°C, 30 s at the optimized annealing temp, and 60 s/kb at 65°C for extension).
4. Immediately upon completion of the 1° PCR, S1 nuclease digest the reactions to eliminate single-stranded DNA by seeding 0.5 μ l of each 1° PCR into 5 μ l of S1 Mix per reaction and incubating for 20 min at room temperature. Add 45 μ l of dilution buffer to each well.
5. Use 1.6 μ l of the S1-digested samples to seed a secondary (2°) 8 μ l PCR with nested allele-specific and universal primers. There is no need to inactivate the S1 nuclease prior to addition to the 2° reaction. Conditions for the 2° PCR are denaturation (1 min at 96°C) followed by 27 cycles of amplification (20 s at 96°C, 30 s at the optimized annealing temp, and 60 s/kb at 65°C for extension).
6. The samples are then analyzed by agarose gel electrophoresis and the number of positive wells (N_{pos}), negative wells (N_{neg}), and total wells (N_{tot}) are determined. To calculate the amplification efficiency that corresponds to a particular combination of allele-specific primers in an experiment (e.g., a CO assay using Bf1 to Dr1 followed by Bf2 to Dr2), use the well-count numbers from the allele combinations that had the smaller N_{pos} . In the example shown in Fig. 15.4, the smaller N_{pos} corresponds to the Bf test ($N_{\text{pos}} = 22$ for Bf test vs. $N_{\text{pos}} = 26$ for the Dr test).
7. Use the Poisson approximation $p(n) = \frac{\mu^n e^{-\mu}}{n!}$ to estimate the amplification efficiency. Determine μ_{amp} , the average number of molecules amplified/well. By setting $n = 0$, μ_{amp} can be calculated because $p(0) = \frac{N_{\text{neg}}}{N_{\text{tot}}}$ and therefore, $\mu_{\text{amp}} = -\ln\left(\frac{N_{\text{neg}}}{N_{\text{tot}}}\right)$. The amplification adjustment factor is the average number of amplifiable molecules per seeded molecule and is calculated as $\frac{\mu_{\text{amp}}}{m}$, where m is the number of molecules seeded in each reaction ($m = 2$). In later calculations, the variance for μ_{amp} ($\sigma_{\mu_{\text{amp}}}^2$) will need to be estimated, which is $\sigma_{\mu}^2 = \frac{1}{N_{\text{neg}}} - \frac{1}{N_{\text{tot}}}$. For the example depicted in Fig. 15.4, $\mu_{\text{amp}} = 0.6131$, the amplification adjustment factor is 0.3065, and $\sigma_{\mu_{\text{amp}}}^2 = 0.0177$. The amplification adjustment factor is typically 0.3–0.8.

3.3. Amplification of Recombinants from Sperm DNA

For amplification of COs, two rounds of nested allele-specific PCR are performed (e.g., Bf1 to Dr1 and Bf2 to Dr2, Fig. 15.1). For amplification of NCOs, two rounds of nested PCR are performed, using allele-specific primers to only one side of the hotspot with universal primers to the other side (e.g., Bf1 to Ur1 and Bf2 to Ur2, Fig. 15.1). Note that the latter primer sets amplify parental, non-recombinant DNA (the major amplification product), while also amplifying NCOs and COs. An advantage of this method is that the relationship between NCOs and COs at the hotspot can be discerned; moreover, NCOs are amplified non-selectively. Other approaches have been developed that selectively isolate NCOs based on either hybridization or PCR (23, 31).

3.3.1. Amplification of COs—As the recombination activity of mammalian hotspots is quite variable, a series of input DNA concentrations (hereafter referred to as “pools”) should be tested to determine the ideal range before performing large-scale experiments. As the haploid sperm DNA content is ~3 pg, 6 pg of mouse sperm DNA contains one copy of the

allele being amplified. Calculate the amplification efficiency as outlined in **Section 3.2.3** to determine the number of amplifiable molecules in the input pools by dividing the desired number of molecules by the amplification adjustment factor. In the example shown in **Section 3.2.3**, one amplifiable molecule would be found in 19.58 pg of DNA (6 pg/0.3065). To control for contamination and any PCR-derived artifacts, include negative controls with no DNA (mock) and somatic DNA.

1. Set up a series of PCR amplifications (*see* Fig. 15.1, CO assay) with multiple small pools containing varying concentrations of sperm DNA. For example, in a 96-well plate, set up 12 reactions of 100, 200, 500, 1,000, and 3,000 amplifiable molecules per well. Include 12 wells with no DNA for contamination controls. Also, set up 24 wells with 2,400 amplifiable molecules of somatic DNA, such that the total amount of amplifiable molecules tested for somatic and sperm DNA is equivalent (57,600 molecules, in this example).
2. Set up the PCRs in a total volume of 8 μ l with 1X Jeffreys' PCR buffer, supplemented with an additional 12.5 mM Tris base, 0.03 U/ μ l Taq polymerase, 0.006 U/ μ l Pfu polymerase, 0.2 μ M of each primer, and the desired amount of genomic DNA. Conditions for the 1° PCR are denaturation (1 min at 96°C) followed by 26 cycles of amplification (20 s at 96°C, 30 s at the optimized annealing temp, and 60 s/kb at 65°C for extension). *See* Note 12.
3. Immediately upon completion of the 1° PCR, S1 nuclease digest the reactions as outlined in **Section 3.2.3**, Step 4. Add 45 μ l of dilution buffer and use 1.6 μ l to seed the 2° PCRs.
4. Set up and perform the 2° PCRs in a total volume of 8 μ l, as outlined above with nested allele-specific primers. Thirty cycles should be sufficient to see positive reactions by 2° PCR.
5. Add 2.5 μ l of loading dye and run 3 μ l of each sample on an agarose gel. The somatic and mock controls should show no positive reactions (*see* Note 12). It should be apparent that increasing concentrations of input DNA result in a larger fraction of positive wells.
6. Use the calculations outlined in **Section 3.2.3**, Step 7 to estimate μ , the average number of recombinants per well in each pool size. Select a range of input DNA that corresponds to 0.4–0.8 recombinants per pool and perform a number of CO amplifications. A low (0.4) and high (0.8) input in 1–2 96-well plates per orientation (Bf to Dr vs. Df to Br) is ideal to map CO breakpoints. This range gives an ample number of CO events but not so many as to make accurately estimating the frequency by Poisson correction problematic. Make sure to include mock and somatic DNA controls in each experiment. For accurate assessment of the distribution of CO breakpoints, aim to isolate ~100 COs per orientation. *See* Table 15.3 for an example of the PCR set-up for a CO assay.
7. To later estimate the recombination frequency in total and per interval, note the number of positive, negative, and total wells/experiment. Keep a record of these data for each DNA sample and each input amount.

3.3.2. Amplification of NCOs and COs: the NCO/CO Assay—As the large majority of DNA amplified in this assay will be of the parental genotype, the input pool sizes are much smaller than in the CO assay. With smaller pools, NCOs and COs can be detected

¹²The number of amplification cycles in 1°, 2°, and 3° PCRs can be modified as needed to reduce potential low-grade, non-specific amplification of non-recombinant DNA or to enhance less efficient reactions.

within the context of a large excess of non-recombinant, parental DNAs. Because NCO gene conversion tracts are short and frequently encompass only a single polymorphism, the ability to score NCOs at any hotspot is highly dependent upon the polymorphism density.

1. Set up 8 μ l PCRs as detailed in **Section 3.3.1** using allele-specific forward primers against universal reverse primers (e.g., Bf1 to Ur1) or vice versa. Perform the assay in bulk in 96-well plates with 30 amplifiable molecules per well (*see* Note 13). In a separate PCR machine, set up eight positive hybridization control reactions (*see* Note 14) with the alternate allele-specific primer (e.g., Df1 to Ur1).
2. Immediately upon completion of the 1° PCRs, add 35 μ l of dilution buffer to each well (*see* Note 15).
3. Use 0.6 μ l of the diluted 1° PCRs to seed 15 μ l 2° PCRs containing the nested allele-specific and universal primers for both the experimental plate(s) and the positive hybridization controls.
4. Conditions for the 2° PCR are denaturation (1 min at 96°C) followed by 36 cycles of amplification (20 s at 96°C, 30 s at the optimized annealing temperature, and 60 s/kb at 65°C for extension).
5. To later estimate the recombination frequency in total and per interval, note the number of positive, negative, and total wells/experiment. Frequencies will be determined after allele-specific oligonucleotide (ASO) mapping detailed in the next section. Keep a record of these data for each DNA sample and each input amount.
6. Once the NCO frequency has been determined (**Section 3.4.3**), a suitable number of PCRs should be performed with somatic DNA, as a negative control, to assess the frequency of PCR mis-incorporations at particular alleles and to confirm that the NCOs observed are meiosis-specific.

3.4. Genotyping Recombinants by Allele-Specific Oligonucleotide (ASO) Hybridization

For CO breakpoint mapping, most of the reactions should contain a single recombinant. Amplified DNA from positive 2° PCRs can be purified using standard techniques and cloned or sequenced. If higher yields are needed from the 2° PCRs, a tertiary (3°) round of PCR can be performed using either nested allele-specific or universal primers. Alternatively, positive 2° reactions (and some negative reactions for controls) can be subjected to 3° PCR to generate enough DNA for dot-blotting and allele-specific oligonucleotide (ASO) hybridization. For breakpoint mapping in the NCO/CO assay, the ASO approach is the preferred method and is outlined here.

3.4.1. Generation of Replica Dot-Blots for ASO Hybridization

1. For the CO assay, perform 3° PCRs with either nested allele-specific or universal primers in a total volume of 30 μ l seeded with 0.75 μ l of the 2° PCRs as outlined in **Section 3.3.1**, Step 2. Use all positive 2° PCRs and include some somatic controls. Also, for a positive hybridization control, PCR amplify genomic DNA from each parental strain that generated the F1 hybrid. Conditions for the 3° PCR are

¹³If NCOs are hard to distinguish by ASO hybridization (described in **Section 3.4**), reduce the concentration of amplifiable molecules in the 1° PCRs. We routinely use between 10 and 40 molecules per reaction.

¹⁴Generate a master mix without the allele-specific primer and remove an aliquot for the positive control PCRs. Then add the allele-specific primers to each reaction to a final concentration of 0.2 μ M. Generate the positive control reactions in a separate PCR machine and handle with extreme care throughout the experiment. Routinely perform all steps with experimental samples prior to removing the positive hybridization controls from the PCR machine.

¹⁵S1 nuclease digestion is not routinely used for these assays but can be added for increased stringency if necessary as outlined in **Section 3.2.3**, Step 4.

denaturation (1 min at 96°C) followed by 21 cycles of amplification (20 s at 96°C, 30 s at the optimized annealing temperature, and 60 s/kb at 65°C for extension). Add 7.5 µl of loading dye to each sample.

2. For the NCO/CO assay, directly use the 2° PCR. We routinely perform a duplicate 2° PCR to generate a larger amount of amplified DNA for dot-blotting (*see Note 16*). Combine the two 2° PCRs and add 7.5 µl of loading dye to each sample. The eight positive control tubes from the first round of the 2° PCR should be combined and 60 µl of loading dye added.
3. Prior to generating the dot-blots, load 1.0 µl of randomly chosen samples (always include the positive hybridization controls) on an agarose gel to check the quality and quantity of the PCRs.
4. For the NCO/CO assay, replace one well of the 96-well plate (*see Note 17*) with 30 µl of positive hybridization control (Fig. 15.5). Additionally add positive hybridization control DNA to 5 wells of the 96-well plate at ratios of 1:10, 1:30, 1:100, 1:300, and 1:1,000 (Fig. 15.5). In the ideal scenario, the positive signal from an NCO or CO product should be close to or exceed the signal observed in the 1:30 dilution.
5. Following manufacturer's instructions for the 96-well dot-blot manifold, cut Whatman filter paper (two to three pieces) and nylon membranes (10 replicates) to the appropriate sizes. Wet the filter paper and a membrane with ddH₂O and assemble the manifold, applying a vacuum.
6. Add 280 µl of denaturation buffer to each PCR and pipet up and down to mix (*see Note 18*). Generate replica dot-blots by loading 30 µl/well onto the assembled dot-blot manifold. Rinse each well with 150 µl of 2X SSC. Remove the membrane and repeat until all replicas are generated. UV crosslink the membranes with a Stratalinker or similar apparatus and proceed to hybridization.

3.4.2. Probe Preparation and ASO Hybridization Conditions—For CO assays, for each polymorphism prepare probes to each parental genotype. Perform one round of hybridization with one parental genotype, followed by stripping the ASO probe and reprobing with the other genotype. For NCO/CO assays, hybridize only with the probes for the parental genotype that was not amplified. For example, if the B genotype was amplified (Fig. 15.1), probe only with D genotype ASOs (Fig. 15.5).

1. ASOs are 18-mers that contain the polymorphism in the middle of the oligonucleotide, typically the 8th or 12th nucleotide from the 5' end in the case of a single nucleotide polymorphism. Frequently short insertion/deletion polymorphisms are found at hotspots and can be used to generate excellent ASOs (and allele-specific primers); design the ASO with the insertion/deletion polymorphism in the center as well. ASOs are stored in ddH₂O at 0.8 mg/ml. Prior to use, dilute in ddH₂O to 8 µg/ml (1:100). For each ASO hybridization, ASOs specific to both parental DNAs are required – one to hybridize and the other as a

¹⁶A single 15 µl 2° PCR is sufficient to generate five replica blots for ASO hybridization. If your assays require more replicas, a duplicate 2° PCR can be performed and combined with the previous to generate 10 replica blots. Alternatively, a 20 µl reaction can be seeded with 0.8 µl of the 1° PCR to generate seven to eight replica blots. Note: the 2° PCR for the eight positive hybridization controls provides DNA for up to 20 replica blots.

¹⁷Remove the amplification reaction from one well and rinse the well several times with water. For example, use the lower right-hand well, 12H, to avoid potential crosscontamination of wells during the 2X SSC rinses of the dot-blots.

¹⁸The loading dye marks the membrane, assisting in dot-blot preparation, but fades in the denaturation buffer, so prepare dot-blots immediately.

competitor. For example in Fig. 15.5, ASOs to D were labeled and ASOs to B served as the competitor.

2. In screw cap microcentrifuge tubes, assemble the kinase reaction in a final volume of 10 μ l containing 1X kinase mix, 0.35 μ l T4 polynucleotide kinase (10 U/ μ l), 0.2 μ l (γ -³²P)ATP (10 mCi/ml), and 1 μ l ASO (8 μ g/ml). Incubate at 37°C for 45 min. Add 20 μ l of Kinase Stop Solution, mix and centrifuge the sample. Add 20 μ l unlabeled competitor ASO (8 μ g/ml) (*see* Note 19).
3. Wet the dot-blot membranes with 3X SSC and place in a small hybridization bottle (DNA facing inside). Multiple dot-blots from separate experiments can be hybridized with the same probe by separating the membranes with hybridization mesh and increasing the volume of solutions accordingly.
4. Pre-hybridize a single membrane in a rotisserie hybridization oven with 3 ml of pre-warmed TMAC hybridization buffer (*see* Note 20) at 56°C for 10–15 min. Pour off this buffer and add 2.5 ml of fresh TMAC hybridization buffer supplemented with 7 μ l of 3 mg/ml sonicated salmon sperm DNA (freshly boiled for 5 min and stored on ice until use). Reduce and Jasin the hybridization oven temperature to 53°C and rotate for an additional 10 min.
5. Add the ASO probe directly into the hybridization buffer at the bottom of the bottle, swirl the bottle to distribute, and continue to incubate at 53°C with rotation for 45 min.
6. Wash the membranes three times with 2.5 ml of pre-warmed TMAC wash buffer over 20 min with rotation at 56°C. Change solution to 4 ml of pre-warmed TMAC wash buffer for a final wash of 15 min.
7. Rinse the membrane in the bottle two times with 3X SSC and transfer to a tray containing 3X SSC. Blot off excess liquid, wrap in plastic, and expose for 45 min to 1 h on a phosphorimager screen (*see* Note 21).
8. For CO breakpoint mapping, strip the membranes and reprobe the same membrane with the labeled ASO of the opposite genotype. It is important to use the same membrane to account for dot-blot to dot-blot variation. Strip probes from membranes by repeatedly washing with boiled 0.1% SDS until the signal is sufficiently reduced when scanning with a Geiger counter.

3.4.3. Scoring COs and NCOs—Once the ASO hybridization has been performed, the information can be assembled to generate CO and NCO maps. It is important that polymorphisms that flank the hotspot are included in the ASO hybridization to assess the genotype of recombinants and to avoid including parental DNA that was non-specifically amplified in the quantification.

3.4.4. Scoring COs from the CO Assay

1. In the CO assays, the majority of amplification-positive reactions should contain only a single recombinant (*see* Fig. 15.6). The genotype of these recombinants and

¹⁹A single ASO kinase reaction can be used for hybridizing two dot-blots. A master kinase mix can be prepared and used to label multiple ASOs. Labeled ASOs are stable at 4°C or –20°C for several days.

²⁰TMAC binds to A/T-rich sequences and eliminates the hybridization bias for G/C base pairing and allows different ASO hybridizations to be performed at the same temperature regardless of sequence content (36). TMAC is highly toxic and must be handled and disposed of following institutional guidelines.

²¹Do not allow the membranes to dry at any point as they will be repeatedly stripped and reprobed. If a phosphorimager is not available, expose the membranes to autoradiography film with an intensifying screen at –80°C for ~3 h to overnight. If the ASO probe is somewhat non-specific, rewash the membranes with TMAC wash buffer at increasing temperatures up to ~62°C.

the location of the CO breakpoints should be readily apparent, as a positive signal will be seen for only one ASO at every site. However, particularly in larger pool sizes, two or more recombinants can be amplified in the same well, and if they do not share the same breakpoints, polymorphisms will score positively for both ASOs at some sites (in Fig. 15.6c, wells 3 and 9). In these cases, the two exchanges from the unique to mixed genotypes are scored as two CO breakpoints. A small number of pools may contain two independent recombinants with identical breakpoints (i.e., invisible doubles); the problem of multiples is kept to a minimum by keeping the fraction of positive pools within the suggested range (0.4–0.8).

2. Positive signals from somatic controls will likely reflect non-specific amplification of parental DNA and can help identify problematic allele-specific primers. If such non-specific amplification is observed at a significant frequency, any breakpoints that appear to occur between the outermost genotyped polymorphisms and the internal allele-specific primers are also likely to be from non-specific amplification of parental DNA and should be discounted.
3. Occasionally a CO will flip between genotypes multiple times across the hotspot (see Note 22). These could be bona fide discontinuous gene conversion tracts; however, as they complicate the mapping of CO breakpoints, they may be counted in the overall CO frequency but eliminated from the breakpoint map. Such events are rare in our experience, so omitting them has little impact on the shape of the breakpoint map.

3.4.5. Scoring NCOs and COs from the NCO/CO Assay

1. Examples of NCO/CO assay dot-blot are shown in Fig. 15.5. COs show no hybridization with the ASO probe(s) for polymorphisms on one side of the hotspot and then flip to showing hybridization with the remaining polymorphisms, whereas the NCO conversion tracts often encompass only a single polymorphism. As the pool size is 30 amplifiable molecules, the intensity of the positive hybridization signals for both COs and NCOs should fall between those observed from the 1:100 and the 1:10 dilutions of the positive control. The CO frequency should concur with what was observed in the CO assay and can be used as a gauge for the success of the NCO/CO assay.
2. Very faint hybridization signals should be evaluated carefully, for example, by comparing with somatic control plates. A signal fainter than the 1:100 dilution control should be discounted as potential PCR mis-incorporation or a hybridization artifact.
3. Due to the fact that the pool size is small, multiple recombinants per well are rare, but they do occur. See Section 3.5 for statistical analysis which takes this into account and see Section 3.6 for a method for cloning recombinants derived from the NCO/CO assay.
4. If a hotspot shows preferential initiation of meiotic recombination on one parental chromosome over the other (e.g., $B \gg D$, Fig. 15.1), the frequency of NCOs can be quite low for the non-initiating parent (D). Nonetheless, both orientations of COs (e.g., B to D and D to B; Fig. 15.1) are equivalent in frequency and serve as a critical control in this instance.

²²The frequency of discontinuous gene conversion tracts and other more complex events has been reported to be ~5% in some mouse hotspots (27, 28).

3.5. Calculation of CO and NCO Frequencies

CO and NCO frequencies are estimated using similar approaches. To account for multiple events per well (some of which may have identical breakpoints), overall numbers of COs and NCOs are Poisson-corrected to estimate the true frequency. Figure 15.6 shows a model CO experiment with 16 polymorphisms typed across the hotspot. In this experiment, 300 amplifiable molecules per well were assayed in 72 wells, with 56 negative wells and 16 positive wells, 2 of which contained at least two COs. Total activity across the hotspot and activity for each interval on Fig. 15.6 were calculated as described below. For further information on recombination statistics, see (31, 32).

1. For total CO activity across the hotspot (Fig. 15.6b), calculate the mean number of recombinants per well (μ_{rec}) and the variance of μ_{rec} ($\sigma_{\mu_{\text{rec}}}^2$) as shown above in **Section 3.2.3**, Step 7 for different DNAs (i.e., biological replicates) and pool sizes separately. The frequency of recombination (F) equals μ_{rec} divided by the number of amplifiable molecules per well (m). The variance of the frequency (σ_{freq}^2) takes into account the variance from both the estimation of μ_{rec} and the μ_{amp} and equals $F^2 \left(\frac{\sigma_{\mu_{\text{rec}}}^2}{\mu_{\text{rec}}^2} + \frac{\sigma_{\mu_{\text{amp}}}^2}{\mu_{\text{amp}}^2} \right)$. The standard deviation (σ_{freq}) is the square root of the variance.
2. For CO activity for each interval, determine the number of Poisson-corrected COs per interval. Quantify different DNAs and input pool sizes separately. Order the COs in a tabular format and count the number of breakpoints within each interval (Fig. 15.6c). Include the intervals between the nested allele-specific primers and the outermost tested polymorphisms. In the event of wells with more than one CO (e.g., wells 3 and 9), the first and last breakpoints are considered unambiguous, whereas the ones in between are considered ambiguous (as they may contain hidden CO breakpoints). On a per interval basis, calculate the number of wells with ambiguous breakpoints and subtract that from the total number (72 in this example), to obtain the total number of unambiguous intervals (I_{tot}). Calculate the negative intervals (I_{neg}) by subtracting the number of breakpoint intervals from I_{tot} . Note that the number of positive wells in the experiment shown in Fig. 15.6c is 16, but there are 18 breakpoint intervals because of two wells with multiple events. Similar to **Section 3.2.3**, Step 7, calculate the average number of breakpoints per interval, μ_{int} . The Poisson-corrected number of COs per interval is $\mu_{\text{int}} \times I_{\text{tot}}$. To calculate centiMorgans (cM), divide the Poisson-corrected number of COs per interval by the total number of corrected input molecules per interval (Total DNA = $m \times I_{\text{tot}}$), and multiply by 100. For CO activity in cM/Mb, divide cM by the number of bp in the interval and multiply by 10^6 . See the intervals in the example in Fig. 15.6c for calculations.
3. For NCO frequency across the entire hotspot or at a particular polymorphism, calculate as in Steps 1 and 2, respectively.

3.6. Cloning and Confirmation of NCOs

ASO hybridization is a powerful method to detect NCOs, but further information can be gleaned by cloning. For example, while most wells containing NCOs will display conversion of only a single polymorphism, a significant fraction may encompass two or more polymorphisms. These latter wells may arise from a single NCO by co-conversion of adjacent polymorphisms or from two separate NCOs found in the same well by chance, which can be distinguished by cloning. NCOs derived from the NCO/CO assay represent a small fraction of the amplified DNA, with the non-recombinant parental genotype in large excess (i.e., ~1 in 30). Here is a straightforward method to clone and confirm the identity of NCOs from a sea of parental DNA.

1. After identifying wells with NCOs of interest, use 0.6 μ l from the 1° PCR to seed a 2° PCR in a total volume of 15 μ l. These PCRs are identical to the 2° PCRs performed for the NCO/CO assay in **Section 3.3.2**, Steps 3 and 4, with two exceptions: they do not include Pfu polymerase and the concentration of Taq polymerase is increased to 0.0037 U/ μ l. Also, after 36 PCR cycles, an extended extension step at 65° C for 7 min is added.
2. Run 1–2 μ l of these 2° PCRs on an agarose gel to confirm the quality and quantity of the amplified DNA.
3. Perform a TOPO® TA (Invitrogen) ligation reaction with 1 μ l of the 2° PCR (*see* Note 23), following the manufacturer's instructions.
4. Transform 1–2 μ l of the ligation reaction into competent cells (e.g., TOP10® chemically competent) by standard protocols. Prepare LB agar plates supplemented with 50 μ g/ml ampicillin (or other antibiotics depending upon the vector used) and spread with 40 μ l of 40 mg/ml X-gal. Plate the transformants at several concentrations to obtain an adequate number of colonies to ensure the presence of colonies with the NCO, but not too dense to impair picking these colonies later. A good range to plate is 5, 20, and 75% of the transformation mix. Incubate overnight at 37°C.
5. Let plates come to room temperature. Place dry 82 mm disc nylon membranes (e.g., Hybond™-XL) on the plates and leave for 30 s. At this time, puncture the membrane at three spots around the perimeter of the disc with an 18-gauge needle soaked in India ink to orient the membranes (*see* Note 24).
6. Place the membrane colony side up on Whatman filter paper soaked with cloning denaturation buffer for 2–5 min, followed by two subsequent incubations on Whatman filter paper soaked with cloning neutralization buffer for 3 min.
7. Wash in 2X SSC, dry the membranes, and crosslink in a Stratalinker or equivalent.
8. Store the agar plates wrapped at 4°C.
9. Perform ASO hybridization as outlined in **Section 3.4.2** to identify NCOs. For example, if these were amplifications with Bf and Ur primers, probe with an ASO to detect the D polymorphism in bacterial colonies containing NCOs (e.g., black polymorphism 4 in Fig. 15.7a). Next, strip and reprobe (*see* Note 25) with the ASO to detect the B polymorphism at this position (e.g., gray polymorphism 4). The B probe will hybridize to the vast majority of the bacterial colonies, but will not hybridize to a bona fide NCO at this polymorphism. For potential co-conversions, strip and re-probe for the other converted D polymorphism (e.g., black polymorphism 5 in Fig. 15.7a). As the same colonies hybridize to both D-specific ASOs, this NCO is surmised to be a co-conversion of both polymorphisms. Confirmation of this interpretation can be obtained by probing with additional ASOs, for example, to rule out D parental DNA contamination. Figure 15.7b shows the expected hybridization patterns in the case of two separate NCOs.
10. Print the phosphorimager scans onto transparencies and align with the agar plates. Pick colonies of interest to isolate plasmid DNA to confirm the identities of NCOs

²³TOPO® TA cloning is highly efficient, but blunt-end cloning can also be used. Blunt-end PCR products with T4 DNA polymerase and ligate into a blue-white selection plasmid. In this case, Pfu polymerase can be included in the 2° PCRs.

²⁴Cutting one or two notches in the membrane also helps to orient the membrane to the film.

²⁵It is important to determine the potential genotypes of NCOs by ASO hybridizations of dot-blots first, and based on that decide which ASOs to probe membranes from colony lifts. Colony lifts are more sensitive to stripping than dot-blots, so some colonies can be lost after multiple reprobings (for example, *see* asterisk in Fig. 15.7a).

surmised from ASO hybridization, using restriction mapping and/or DNA sequencing.

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Fig. 15.1.

Homologous recombination pathways and assays to amplify COs and NCOs. *Top:* A double-strand break (DSB) is generated on the *gray* chromosome (e.g., B, C57BL/6 J) and the *black* chromosome (e.g., D, DBA/2 J) serves as the homologous donor for repair. After the DSB is generated, 5' ends are resected and one 3' single-stranded tail invades the homolog to generate a displacement loop (D-loop). The invading strand serves as a primer to initiate DNA repair synthesis (*dotted lines*). At this point the two major pathways diverge. Most crossovers (CO) are generated by the canonical DSB repair (DSBR) pathway and most noncrossovers (NCO) are generated by synthesis-dependent strand annealing (SDSA). In DSBR, the second 3' end of the DSB is "captured" to generate a double Holliday junction

(dHJ) intermediate which can be resolved to form COs. In SDSA, the invading strand is displaced and anneals to the other 3' end of the DSB and subsequently repaired to form NCOs. Only one chromatid from each homolog is shown for simplicity, but importantly, sister chromatids are present throughout. *Middle*: After meiosis, recombining chromatids segregate, and after spermiogenesis sperm are formed. *Circles* represent polymorphisms between the B and D genotypes. *Bottom*: Assays to isolate COs and NCOs by PCR in microtiter plates. In the CO assay, nested, allele-specific PCR is performed on small pools of sperm DNA, using primers that flank the hotspot. Only COs are amplified in this assay. In the NCO/CO assay, nested PCR is also performed but only one set of primers is allele-specific, whereas the other set is "universal" (recognizing both parents). In this assay, the majority of products are from the parental genotype, but NCOs and COs are also amplified. In both assays, *white circles* represent polymorphisms derived from one or the other parent.

Fig. 15.2.

Assessing allele-specific primers. Southern blots of six allele-specific primer optimizations are shown, three of which are assessed to be non-specific and three of which are specific. Each PCR comprises one allele-specific primer, as indicated, and one universal primer. The input DNA (B, D, or no DNA) and annealing temperatures of the PCRs are also indicated. Arrows indicate the expected size of the amplified DNA. Note that the Southern blots are overexposed to detect non-specific amplification. *Top*: Three non-specific primers. The primer on the far *right* shows highly efficient amplification of B DNA, but also amplifies D DNA at a significantly lower level; this primer may have improved performance if shortened by one or two nucleotides. *Bottom*: Three allele-specific primers. The primer on the far *right* is highly specific, but is not as efficient as the other two. This primer can be used successfully for 2° PCRs.

Fig. 15.3.
Diagram of the *left* male mouse testis. Mature sperm are isolated from the cauda epididymis.

Fig. 15.4. Strategy for determining amplification efficiency. For each DNA preparation, four separate PCRs are required to determine amplification efficiency. Each PCR contains 12 pg of DNA per well, and 48 wells are typically assayed. At the *bottom* of each experimental design, sample results are shown.

Fig. 15.5.

Mapping NCOs and COs using ASO hybridization. **(a)** PCR strategy for the NCO/CO assay. In this example, B parental DNA and recombinants are amplified, such that dot-blots are probed with ASOs that recognize D polymorphisms. **(b)** DNA is amplified in a 96-well plate and then replica dot-blots are generated using a 96-well manifold for ASO hybridization. Six separate polymorphisms (POLY1-6) are tested in this example. All recombinants identified in this example are indicated with *circles* in the *left* diagram, with two recombinants highlighted on each blot (*, CO in well 6B; #, NCO in well 9D). Positive controls (*boxes*) are amplified D DNA located at the 12H location and a dilution series at the indicated ratios of D into B DNA located at 1A through 1E. **(c)** Maps of all NCOs and COs identified in B.

Fig. 15.6.

Calculating recombination frequency. **(a)** PCR strategy for the CO assay in which COs are isolated in the B to D orientation. **(b)** Calculations to determine the total CO recombination frequency across the hotspot. **(c)** Breakpoint maps for COs amplified from the 16 positive wells. Polymorphisms that hybridized to both parental genotypes in wells 3 and 9 are indicated with *half gray/half black circles*. CO frequency calculations for each interval are indicated below.

Fig. 15.7.

Cloning NCOs to determine whether NCOs have undergone co-conversion. NCOs are amplified and cloned with a large excess of parental DNA and identified by ASO hybridization. The phosphorimager scans of a membrane hybridized with three ASOs that recognize the indicated polymorphisms are shown for each case. **(a)** A co-converted NCO with the indicated genotype. Hybridization patterns for four colonies containing NCOs are depicted below each scan. Note that the hybridization signal from one of the colonies was reduced after multiple re-probings (*asterisk*). **(b)** Two distinct NCOs with the indicated genotypes. Hybridization patterns for eight colonies containing NCOs are depicted below each scan. The colonies hybridize to different probes, indicating that they are derived from

distinct NCOs (four colonies are type 1 and four colonies are type 2). *Open circles*, hybridization negative; *filled circles*, hybridization positive.

Table 15.1

Allele-specific primers

Polymorphism	PCR	Primer	Sequence	Length	%GC
POLY665	1°	Bf3	ATAAGCACGTATTGAGGCC	20	45
		Df3-1	AAGCACGTGTTGAGGCC	18	56
POLY697	2°	Bf4-1	CAGCAGCTGAGTTAAAACT	19	42
		Df4-1	CAGCAGCTGAGTTAAAAACA	19	42
POLY4020	2°	Bf4020	TCTCCAACAGTGGGGGAT	18	56
		Df4020	TCTCCAACAGTGGGGGAC	18	61
POLY5465	1°	Bf5465	GTGTCACATTTCAGTTGATGT	21	38
		Df5465	GTGTCACATTTCAGTTGATGC	21	43

Table 15.2

Primer optimization example

Master mix	1X	132X	Remaining components per reaction (see below)			
10X Jeffreys' PCR buffer	1.0	132				
2 M Tris base	0.0625	8.25	10 μM f primer	0.2		
Taq polymerase (5 U/μl)	0.06	7.92	10 μM r primer	0.2		
Pfu polymerase (2.5 U/μl)	0.024	3.17	ddH ₂ O	7.49		
Total	1.109	151.34	12.5 μg/ml DNA	1.0		
Forward mix	55X (μl)	Reverse mix	55X (μl)	Positive mix	10X	
Master mix, 132X	61	Master mix, 132X	61	Master mix, 132X	11.09	
10 μM universal r primer	11	10 μM universal f primer	11	10 μM universal f primer	2 μl	
ddH ₂ O	412	ddH ₂ O	412	10 μM universal r primer	2 μl	
				ddH ₂ O	74.9 μl	
88 μl each into 5 tubes		88 μl each into 5 tubes		9 μl each into 9 tubes (see below)		
Forward primer mixes			Reverse primer mixes			
Rx	Primer	Amt (μl)	Rx	Primer	Amt (μl)	
A	10 μM Bf1	2	F	10 μM Br1	2	
B	10 μM Df1	2	G	10 μM Dr1	2	
C	10 μM Bf2	2	H	10 μM Br2	2	
D	10 μM Df2	2	I	10 μM Dr2	2	
E	10 μM Uf1	2	J	10 μM Ur1	2	
9 μl each into 9 tubes/wells			9 μl each into 9 tubes/wells			
PCRs (in tube or well)			Annealing temperature			
Rx	DNA	Amt (μl)	56°C	59°C	62°C	65°C
1	12.5 μg/ml C57BL/6 J	1.0	Pos1	Pos1	Pos1	Pos1
2	12.5 μg/ml DBA/2 J	1.0	Pos2	Pos2	Pos2	Pos2
–	No DNA		Pos-			
1	12.5 μg/ml C57BL/6 J	1.0	A1	A1	A1	A1
2	12.5 μg/ml DBA/2 J	1.0	A2	A2	A2	A2
–	No DNA		A-			
1	12.5 μg/ml C57BL/6 J	1.0	B1	B1	B1	B1
2	12.5 μg/ml DBA/2 J	1.0	B2	B2	B2	B2
–	No DNA		B-			
Continue for all samples...						

Table 15.3

CO assay example

Master mix	1X	232X		
10X Jeffreys' PCR buffer	0.8	185.6		
2 M Tris base	0.05	11.6		
BfI	0.16	37.1		
DrI	0.16	37.1		
Taq polymerase (5 U/ μ l)	0.048	11.1		
Pfu polymerase (2.5 U/ μ l)	0.0216	5.0		
Total	1.24	287.5		
Wells	18 mock (no DNA) (μ l)	36 somatic DNA (2,025 mol/well) (μ l)	80 sperm DNA (300 mol/well) (μ l)	80 sperm DNA (600 mol/well) (μ l)
Master mix, 232X	22.3	44.6	99.2	99.2
DNA (20 ng/ μ l) ^a	0	71.4	23.5	47
ddH ₂ O	121.7	171.96	517.3	493.8

Plate 8 μ l per well in 2X 96-well plates with 8 mock, 16 somatic controls, 36 sperm DNA at 300 amplifiable molecules/well and 36 sperm DNA at 600 amplifiable molecules/well.

^aNote that the amplification adjustment factor was previously calculated to be 0.3065 for the sperm DNA preparation. We are simplifying here by using the same number for somatic DNA.