Chapter 2

Analysis of Recombinants in Female Mouse Meiosis

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Abstract

During meiosis, homologous chromosomes (homologs) undergo recombinational interactions, resulting in the formation of crossovers (COs) or noncrossovers (NCOs). Both COs and NCOs are initiated by the same event: programmed double-strand DNA breaks (DSBs), which occur preferentially at hotspots throughout the genome. COs contribute to the genetic diversity of gametes and are needed to promote proper meiotic chromosome segregation. Accordingly, their formation is tightly controlled. In the mouse, the sites of preferred CO formation differ between male and female chromosomes, both on a regional level and on the level of individual hotspots. Sperm typing using (half-sided) allele-specific PCR has proven a powerful technique to characterize COs and all detectable NCOs at hotspots on male human and mouse chromosomes. In contrast, very little is known about the properties of hotspots in female meiosis. This chapter describes an adaptation of sperm typing to analyze recombinants in a hotspot, using DNA isolated from an ovary cell suspension enriched for oocytes.

Key words: Meiosis, Recombination, Crossover, Noncrossovers, Hotspot, Allele-specific PCR, Oocyte

1. Introduction

During meiosis, homologous chromosomes undergo recombinational interactions, which can yield crossovers (COs) or noncrossovers (NCOs). Studies in yeast have shown that both COs and NCOs initiate from the same event: the formation of programmed double-strand breaks (DSBs) (1). Subsequent repair of the DSBs through diverging pathways leads to the formation of the two different end products (2, 3). Whereas CO recombination leads to a reciprocal exchange of large chromosomal segments, NCO recombination results in a highly localized unidirectional transfer of genetic information.

Meiotic recombination events are not distributed evenly across the genome but tend to cluster in regions called hotspots (4). Analysis in sperm of human and mouse has shown that CO

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frequencies can vary considerably between hotspots, from 0.0004 to 2% (5, 6). Mammalian CO hotspots typically span 1–2 kb, often with the peak of COs and NCOs in the center (7, 8). The relative proportions of detectable COs and NCOs can vary widely from hotspot to hotspot, from a more than tenfold excess of COs to almost tenfold fewer COs than NCOs (9, 10).

The greater part of current knowledge of the relation between COs and NCOs in mammalian hotspots is derived from experiments with sperm from mice or men. However, large-scale CO analysis has shown that mouse chromosomes show sex-specific differences in CO recombination, both on a regional level and on the level of individual hotspots (11–13). What underlies these sex-specific differences remains elusive. Fully understanding the mechanisms and regulation of meiotic recombination thus requires parallel analysis of COs and NCOs in both male and female meiosis.

This chapter describes a strategy to analyze CO and NCO recombination in hotspots in female meiosis and assumes the hotspot of choice is already identified. For the identification of hotspots in the mouse see refs. 14, 15. The strategy we use to analyze COs and NCOs is based on allele-specific PCR, which has been very powerful in the analysis of mammalian CO hotspots in sperm (16, 17). We adapted this technique to allow parallel detection of COs and NCOs in mouse oocytes. Allele-specific PCR makes use of the presence of sequence polymorphisms (usually single nucleotide polymorphisms (SNPs), but also short insertion/deletions (indels)) between the parental haplotypes across the hotspot, both for the amplification of recombinants and for the mapping of COs and NCOs (Fig. 1). Inbred mouse strains with sufficient polymorphisms across the hotspot are crossed and recombination is analyzed in small pools of DNA purified from ovaries of the F1 hybrid mice. COs are selectively amplified by PCR with allele-specific forward and reverse primers (Fig. 1b), located outside the hotspot region. A half-sided allele-specific PCR with a pair of nested allele-specific primers on one side and nested non-allele-specific (universal) primers on the other allows parallel recovery of both COs and NCOs (Fig. 1c). The remaining internal polymorphisms are subsequently typed by hybridizing the PCR products with allele-specific oligos (ASOs) to map the CO and NCO breakpoints. The advantage of this technique is that recombination activity at all polymorphisms across a hotspot can be analyzed within the same experiment. In mouse, conversion tracts associated with NCOs are very short, typically 100 bp or less (10), so the detection of NCOs depends highly on the polymorphism density in the hotspot. This dependence may partially explain the above-mentioned variety in observed CO to NCO ratios between different hotspots in male meiosis. Additional information on allele-specific PCR and breakpoint mapping, but in sperm, is provided in refs. 18, 19. Additional information on the parallel

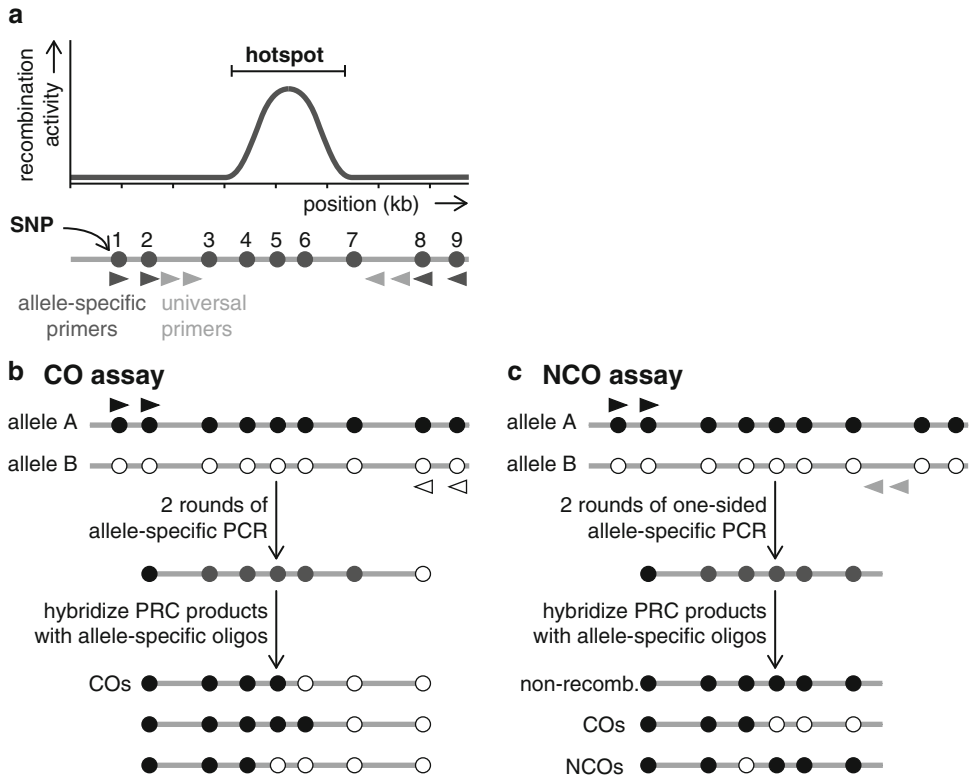

Fig. 1. Allele-specific PCR to amplify COs and NCOs across a hotspot. (**a**) Positions of PCR primers in relation to the hotspot. Depicted is a schematic representation of the sequence spanning a hotspot. Polymorphisms (e.g., SNPs) between the parental haplotypes are indicated as *closed circles* and *numbered*. In this example, allele-specific primers (*dark gray arrowheads*) are directed against SNPs 1, 2, 8, and 9. The remaining polymorphisms (SNPs 3–7) will later be used to map the COs and NCOs. Universal primers (*light gray arrowheads*) are located inside the positions of the allele-specific primers but still outside the hotspot region. (**b**) CO assay. Two rounds of nested allele-specific PCR are performed on pools of ovary DNA from the F1 of a cross between two mouse strains that have multiple SNPs across the hotspot. *Black and white circles* represent SNPs of the A and B parental haplotypes, respectively. Allele-specific primers are shown as *black or white arrowheads*. Only CO molecules will be amplified. To map the CO breakpoints, the remaining SNPs (*gray circles*) are typed by hybridizing the PCR products with allele-specific oligos (ASOs). (**c**) NCO assay. Two rounds of nested PCR are performed on small pools of F1 hybrid ovary DNA, with allele-specific primers on one side and universal primers on the other. This type of PCR will amplify non-recombinant molecules of one haplotype (the majority of PCR products) as well as both CO and NCO molecules. To map the CO and NCO breakpoints, the remaining SNPs (*gray circles*) are typed by hybridizing the PCR products with ASOs. Because the majority of the PCR products will be non-recombinants, only ASOs specific for the non-selected haplotype (in this example: allele B (*white*)) are used in SNP typing.

detection of recombinants in both male and female meiosis, focused on detection of NCOs involving a single polymorphism within a hotspot, is provided in ref. 20.

Compared to male meiosis, the analysis of COs and NCOs in female meiosis poses more of a challenge, mainly at the stage of sample collection and preparation. The number of oocytes that can be isolated from one female animal is about a 1,000-fold lower than the number of sperm cells that can be isolated from one male. A second problem is the high number of somatic cells in ovary

samples. Sperm samples easily reach a purity of >99%, whereas the fraction of oocytes in an ovary cell suspension is usually between 10 and 15%. We adapted a technique used in in vitro fertilization research to isolate oocytes (21) to enrich the ovary cell suspension for oocytes, up to 60%.

2. Materials

To prevent contamination, keep reagents and materials "before PCR" and "after PCR" separate. Ideally, a separate set of pipettes is dedicated to the "before PCR" steps. In addition, equipment (pipettes, tube-racks, etc.) that is used "before PCR" should be regularly exposed to UV light to inhibit amplification of possible contaminant DNA.

2.1. Preparation of an Ovary Cell Suspension and Enrichment for Oocytes

1. F1 hybrid female newborn mouse (see Note 1).
2. Scissors and forceps for dissection. Forceps #5 and #5/45 are recommended for dissecting the ovaries.
3. Dissecting needles.
4. Small petri dishes (∅ 35 mm).
5. Small glass embryo dish.
6. Clean slides. Keep slides for at least 20 min in 100% ethanol, transfer to a slide rack, and submerge in boiling filtered ddH_2O for 25 min. Air-dry and store in a dust-free environment (see Note 2).
7. Tabletop microcentrifuge.
8. Phosphate buffered saline (PBS): 140 mM NaCl, 1.9 mM NaH_2PO_4, 8.9 mM Na_2HPO_4, pH 7.3–7.4. Filter-sterilize and store at room temperature.
9. Fetal calf serum (FCS). Make 1 ml aliquots and store at –20°C.
10. Collagenase: 20 mg/ml in PBS. Make fresh and keep on ice until use.
11. DNase 1: 400 μg/ml in PBS. Store at –20°C. Thaw and keep on ice until use.
12. 20× Saline-sodium citrate buffer (SSC): 3 M NaCl, 300 mM sodium citrate-dihydrate. Adjust pH to 7.0, sterilize by autoclaving, and store at room temperature. Dilute with ddH_2O to make 1× SSC. Filter-sterilize and store at room temperature.

2.2. Preparation of Slides and Quantification of Oocytes

1. 12-well microscope slides, clean (see item 6 in Subheading 2.1), and coverslips.
2. Humidified box: use a box that can be closed off completely, line the bottom with water-saturated filter paper or tissues.

Rest the slides on supports to avoid direct contact of the slides with the paper.

3. Heating plate at 37°C.
4. Tabletop microcentrifuge.
5. Coplin jars.
6. Nail polish (clear) or rubber cement.
7. Fluorescence microscope.
8. Filtered ddH_2O.
9. 100 mM sucrose, filter-sterilize and store at room temperature.
10. 2% (w/v) paraformaldehyde (PFA), pH 9.2, 0.15% Triton X-100. Prepare fresh. Add 0.5 g of PFA to 20 ml of ddH_2O, add 1–2 drops 1 N NaOH, and dissolve by stirring at 60°C. Cool to room temperature and adjust the pH to 9.2. Add ddH_2O to a final volume of 25 ml and filter through a 0.2 μm filter. Add 37.5 μl Triton X-100 and mix well. Use on the same day.
11. 0.4% (v/v) Photoflo (Kodak) in filtered ddH_2O. Store at room temperature.
12. PBS (see Subheading 2.1).
13. Blocking buffer: 5% (w/v) nonfat dry milk, 5% (v/v) goat serum, 0.01% (w/v) sodium azide (NaN_3) in PBS. Store at −20°C. Prior to use, add phenyl methyl sulfonyl fluoride (PMSF, make a 1 M stock in DMSO, store at −20°C) to a final concentration of 1 mM (see Note 3). Centrifuge at maximum speed in a tabletop microcentrifuge for 30 min and use the supernatant within 2 h.
14. A primary antibody against a meiosis- or oocyte-specific antigen (e.g., SYCP3).
15. The appropriate secondary antibody conjugated to a fluorescent label.
16. Mounting medium with DAPI (4′,6-diamidino-2-phenylindole): 10 μg/ml DAPI in Vectashield (Vector Laboratories Inc.). Store in the dark at 4°C.

2.3. DNA Extraction

1. Sterile petri dish.
2. Sterile razor blade or scalpel.
3. Size 80 μm mesh (Screens for CD-1, size 80 mesh; Sigma-Aldrich).
4. Tabletop microcentrifuge.
5. Heating block or water bath at 55°C.
6. Spectrophotometer.
7. 1× SSC (see Subheading 2.1).
8. β-Mercaptoethanol.

9. 10% (w/v) sodium dodecyl sulfate (SDS).
10. Proteinase K: 20 mg/ml in ddH_2O. Make fresh and keep on ice until use.
11. Phenol:Chloroform:Isoamyl alcohol 25:24:1 (v/v/v), saturated with 100 mM Tris–HCl pH 8.0.
12. Ethanol, 100% and 80%.
13. 3 M sodium acetate, pH 5.2.
14. 5 mM Tris–HCl, pH 7.5
15. Optional (see Note 4): Long polyacrylamide (LPA; e.g., GenElute™-LPA, Sigma-Aldrich).
16. Loading dye: 30% (v/v) glycerol in electrophoresis buffer of choice, add bromophenol blue to give blue color. Store at room temperature.

2.4. Allele-Specific PCR and Half-Sided Allele-Specific PCR

1. Thermal cycler.
2. 11.1× PCR buffer: 495 mM Tris–HCl pH 8, 121 mM ammonium sulfate, 49.5 mM $MgCl_2$, 73.7 mM β-mercaptoethanol, 48.4 μM EDTA pH 8, 11 mM dATP, 11 mM dTTP, 11 mM dGTP, 11 mM dCTP, 1.24 mg/ml bovine serum albumin (non-acetylated ultrapure BSA, Ambion). Store in 100–500 μl aliquots at −20°C (see Note 5).
3. 2 M Tris base (Tris(hydroxymethyl)aminomethane, Ultra grade for molecular biology, Fluka Chemie).
4. Taq polymerase.
5. Turbo *pfu* polymerase (Stratagene).
6. Genomic DNA of both parental genotypes. This can be prepared as described (see Subheading 3.3) or ordered from the Jackson Laboratory (http://www.jax.org/dnares/index).
7. S1 nuclease, diluted to 10 U/μl in S1 storage buffer (20 mM Tris–HCl pH 7.5, 50 mM NaCl, 0.1 mM zinc acetate, 50% (v/v) glycerol). Store at −20°C for up to 6 months.
8. S1 digestion mix: 20 mM sodium acetate pH 4.9, 1 mM zinc acetate, 100 mM NaCl. Aliquot and store at −20°C. Add 0.7 U/μl of S1 nuclease before use.
9. Dilution buffer: 10 mM Tris–HCl pH 7.5, 5 μg/ml sonicated salmon sperm DNA.
10. Loading dye (see Subheading 2.3).

2.5. Mapping CO and NCO Breakpoints by Hybridization with ASOs

1. 96-well dot blot manifold.
2. Hybridization oven with rotator.
3. Hybridization bottles.
4. Screw-cap microcentrifuge tubes.
5. Phosphorimager screen.

6. Geiger counter.
7. Whatmann filter paper.
8. Hybond-XL nylon membrane (Amersham/GE Healthcare).
9. Hybridization mesh (optional).
10. Denaturation buffer: 0.5 M NaOH, 2 M NaCl, 25 mM EDTA. Store at room temperature.
11. 2× SSC and 3× SSC: make 20× SSC (see Subheading 2.1), dilute in ddH_2O to make 2× and 3× SSC.
12. 10 mCi/ml (γ^{32}-P) ATP.
13. T4 polynucleotide kinase.
14. 10× kinase mix: 700 mM Tris–HCl pH 7.5, 100 mM $MgCl_2$, 50 mM spermidine trihydrochloride, 20 mM dithiothreitol (DTT). Aliquot and store at −20°C.
15. Sonicated salmon sperm DNA.
16. Tetramethylammonium chloride (TMAC) hybridization solution: 3 M TMAC, 0.6% (w/v) SDS, 10 mM sodium phosphate pH 6.8, 1 mM EDTA, 4 μg/ml yeast RNA, in 5× Denhardt's solution (see Note 6). Store at 4°C, prewarm before use.
17. TMAC wash solution: 3 M TMAC, 0.6% (w/v) SDS, 10 mM sodium phosphate pH 6.8, 1 mM EDTA (see Note 6). Store at 4°C, prewarm before use.
18. 0.1% (w/v) SDS.

3. Methods

3.1. Preparation of an Ovary Cell Suspension and Enrichment for Oocytes

1. Kill the newborn pups by decapitation.
2. Dissect the ovaries from the female newborns and store them in fresh PBS on ice until further use (see Note 7). Also collect a somatic tissue (e.g., liver or spleen) and store at −20°C. This will be used as a negative control for meiotic recombination.
3. Decapsulate each ovary on a clean slide with a droplet (25–50 μl) of PBS under the dissection microscope. Repeat for all collected ovaries.
4. Collect the ovaries in a small embryo dish containing 200 μl fresh PBS + 5% FCS at room temperature until all ovaries have been decapsulated.
5. Place the embryo dish under the dissection microscope and add 20 μl collagenase and 5 μl DNase 1 (see Note 8).
6. Using the dissecting needles, puncture and gently shake the ovaries at regular intervals for 1 h.
7. Remove as much debris as possible.

8. Collect the cell suspension in an eppendorf tube. Add PBS + 5% FCS to a final volume of 1.5 ml.
9. Centrifuge in a tabletop microcentrifuge for 2.5 min at 800 rpm ($60 \times g$). Discard the supernatant.
10. Gently resuspend the cell pellet in 1.5 ml PBS.
11. Centrifuge in a tabletop microcentrifuge for 2.5 min at 800 rpm ($60 \times g$). Discard the supernatant.
12. Resuspend the cells in 100–200 µl 1× SSC. Take a small aliquot (5–15 µl) of the cell suspension for slide preparation and quantification, store the remainder at –20°C until further use (see Note 9).

3.2. Preparation of Slides and Quantification of Oocytes

1. Place a clean 12-well microscope slide in a humidified box. Put a 5 µl drop of 100 mM sucrose in one or more wells.
2. Add 5 µl of the ovary cell suspension to the drop of 100 mM sucrose, put the lid on the humidified box and wait for 5 min.
3. Add 10 µl of 2% PFA, 0.15% Triton X-100 to the drop, and close the humidified box for ~1 h.
4. Remove the lid and leave for ~30 min.
5. Transfer the slide to a 37°C heating plate and allow the drop to dry to the consistency of toffee. This takes about 10~15 min.
6. Rinse the slide three times in filtered ddH_2O, dip once in 0.4% Photoflo, and finish with a rinse with 0.4% Photoflo, using a pipette.
7. Air-dry the slides at room temperature.
8. Either wrap the slides in aluminum foil and store at –80 ° C until further use (see Note 10) or continue with immunocytological labeling (steps 9–25).
9. Wash the slide three times for 5 min in PBS.
10. Cover the slide with 500 µl blocking buffer.
11. Incubate for 30 min in a closed humidified box at room temperature.
12. Prepare 100 µl per slide of a meiosis- or oocyte-specific primary antibody, diluted in blocking solution (see Note 11).
13. Centrifuge the dilution for 30 min at maximum speed in a tabletop microcentrifuge at 4°C.
14. Pour the blocking buffer off of the slides and add 100 µl of the supernatant of centrifuged primary antibody dilution to the slide (see Note 12).
15. Cover the slide with a coverslip and incubate in a closed humidified box for ~2 h at 37°C (see Note 11).
16. Wash the slides three times for 5 min in PBS.

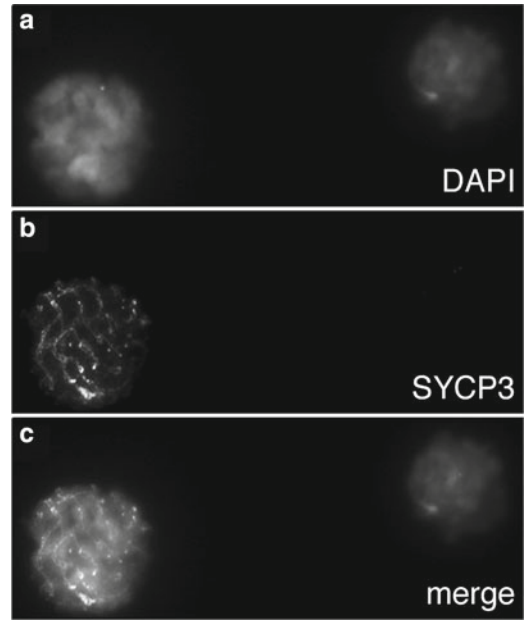

Fig. 2. Immunofluorescent labeling of SYCP3 and DAPI staining of ovary cells to identify oocytes. The nucleus on the left is from an oocyte and that on the right from a somatic cell. Both nuclei are stained with DAPI; however, only oocytes will show a positive SYCP3 signal.

17. Cover the slide with 500 µl blocking buffer and incubate for 30 min in a closed humidified box at room temperature.
18. Prepare 100 µl per slide of the secondary antibody, diluted in blocking solution, and centrifuge for 30 min at maximum speed in a tabletop microcentrifuge at 4°C.
19. Pour the blocking buffer off of the slides, add 100 µl of the supernatant of the centrifuged secondary antibody dilution to the slide, and cover with a coverslip.
20. Incubate the slide in a closed humidified box for 1 h at 37°C.
21. Wash the slide three times for 5 min in PBS.
22. Dip the slide three times in filtered ddH_2O (see Note 13).
23. Mount the slides in 10 µl Vectashield + DAPI and seal the coverslip with nail polish.
24. Using a fluorescence microscope, count the total number of nuclei (DAPI-positive) and the number of oocytes (positive for the meiosis- or oocyte-specific antibody) per well (see Fig. 2). The percentage of oocytes usually ranges between 40 and 60%.
25. Calculate the number of oocytes and total number of cells in the stored cell suspension.

3.3. DNA Extraction

Steps 1–10 describe the first steps for DNA extraction from somatic tissues. For DNA extraction from an ovary cell suspension, start from step 9. To avoid cross-contamination or mixing up the samples,

it is preferable that DNA extractions from somatic tissue and ovaries are not performed side by side.

1. Prepare a chunk (2–3 mm^3) of somatic tissue (use fresh or stored at −20°C); put the tissue in a sterile petri dish.
2. Finely chop up the sample in both directions until mushy, add 1 ml 1× SSC.
3. Use a sterile transfer pipette to wash off the petri dish; collect the macerated tissue in 1× SSC at a corner of the dish.
4. Make a cone out of the mesh, place it on top of an eppendorf tube and pipette the sample up and down through the mesh.
5. Spin for 2 min in a tabletop centrifuge at 3,000 rpm (830 × *g*).
6. Remove the supernatant; spin again briefly and remove residual 1× SSC.
7. Resuspend the pellet in 1 ml 1× SSC (make sure there are no clumps, vortex if needed).
8. Repeat the 1× SSC washes at least two more times.
9. Resuspend the somatic cell pellet in 960 µl 1× SSC. For DNA extraction from ovaries, pool the ovary cell suspensions (see Note 14) and add 1× SSC to a final volume of 960 µl.
10. Add 120 µl β-mercaptoethanol, 100 µl 10% SDS, and 20 µl freshly made proteinase K (20 mg/ml).
11. Invert to mix; incubate 2–3 h at 55°C with occasional gentle mixing.
12. Split the contents of the tube into two tubes (600 µl each).
13. Add an equal volume of phenol:chloroform:isoamyl alcohol, mix well, and spin for 5 min in a tabletop microcentrifuge at maximum speed.
14. Use a 1 ml pipet tip with the tip cut off to transfer the aqueous layer (the top layer) to a clean eppendorf tube.
15. Re-extract residual DNA from the organic layer by adding 200 µl 1× SSC, 0.15% SDS, mix well, and spin for 5 min in a tabletop centrifuge at maximum speed. Pool the aqueous layer with aqueous solution from step 14.
16. Repeat steps 13–15.
17. Add 2 volumes of ice-cold absolute ethanol to aqueous solution, tap and invert tube until the DNA precipitates (see Note 4).
18. Spin for 5 min in a tabletop centrifuge at maximum speed, remove the supernatant, and wash the pellet with 1 ml 80% ethanol.
19. Spin for 5 min in a tabletop centrifuge at maximum speed and remove the supernatant.

20. Dissolve each pellet in 100 µl ddH_2O; pool the appropriate DNA solutions and reprecipitate by adding 1/10 volume of 3 M sodium acetate, pH 5.2, and 3 volumes of 100% ice-cold ethanol.
21. Repeat steps 18 and 19.
22. Air-dry the pellet and dissolve in 50–100 µl 5 mM Tris–HCl pH 7.5 (see Note 15).
23. Determine the concentration of the DNA by measuring the absorbance at 260 nm in a spectrophotometer. Add 5 mM Tris–HCl pH 7.5 to a working stock concentration of ~100 ng/µl. Store at −20°C.
24. Run a sample on a 0.8% agarose gel in the presence of a marker in various known amounts to verify the DNA concentration and to check the quality of the DNA. Good quality DNA should be high molecular weight and give a discrete band at the resolution limit of the gel.

3.4. Designing Allele-Specific and Universal PCR Primers

CO and NCO assays require two to three pairs of nested PCR primers that flank the region of the hotspot (see Fig. 1). Although most mammalian hotspots span a region of 1–2 kb, most PCR primer pairs need to be further apart because of the dependence on the presence of SNPs for the allele-specific PCR primers. Using the long-range PCR protocol described in this chapter, amplification of sequences up to 13 kb is feasible (see Note 16).

The behavior of PCR primers is difficult to predict so all primers have to be tested and optimized (described in Subheading 3.5). Below are some general guidelines for designing allele-specific and universal PCR primers:

1. Avoid repetitive regions and make sure the primer sequence is unique. On the Web, http://www.repeatmasker.org will show repeat regions in the sequence of interest and http://www.ncbi.nlm.nih.gov/blast allows determining whether the primer sequence is unique.
2. If possible, design primers with a GC content of at least 50% and a length between 18 and 22 nucleotides. For allele-specific primers, the polymorphic nucleotide that determines the allele specificity is always at the 3′ end of the primer. This means that the GC content of an allele-specific primer is dictated by the sequence context of the selected polymorphic nucleotide. If needed, the length of the allele-specific can be reduced down to 14 nucleotides (for high GC content) or increased up to 25 nucleotides (for low GC content) (see Notes 17 and 18).
3. The allele-specific primers should be located externally of and relatively close to the universal primers (see Fig. 1). Designing the allele-specific primers externally of the universal primers allows for the use of the same allele-specific primers in both the CO and NCO assay.

3.5. Testing and Optimizing Allele-Specific and Universal Primers

1. Set up PCRs in a total volume of 8 μl with 0.72 μl 11.1× PCR buffer, supplemented with 12 mM Tris base, 0.03 U/μl Taq polymerase, 0.003 U/μl turbo Pfu polymerase, 0.2 μM of each primer, and 10 ng of genomic DNA per reaction.
2. Perform each PCR on genomic DNA from both parental strains that were used to generate the F1 hybrid that is going to be analyzed, and at various annealing temperatures (usually at 56, 59, 62, and 65°C).
3. PCRs with both forward and reverse universal primers serve as positive control and should amplify both parental strains equally efficiently and specifically at the optimal annealing temperature.
4. To test allele-specific primers and to determine their optimal annealing temperature, set up PCRs with an allele-specific primer in combination with a universal primer (e.g., allele-specific forward with universal reverse). The size of the resulting fragment should be as close as possible to the expected fragment size for the CO or NCO assay.
5. Cycle with the following conditions: 2 min 96°C (denaturation), followed by 35 cycles of amplification (20 s 96°C, 30 s at the annealing temperature and extension at 65°C for ~90 s/kb).
6. Analyze by adding 3 μl loading dye to each reaction and running 7.5 μl on a 0.8% agarose gel with 0.5 μg/ml ethidium bromide. Include longer exposure times when photographing the gel to enable visualization of weak bands.
7. The optimal annealing temperature for each allele-specific primer is the temperature at which the primer is both specific and efficient. Also note that the allele-specific primers that will be used together in a CO assay have to amplify both specifically and efficiently at the same annealing temperature (see Note 18).

3.6. CO Assay: Amplification of CO Molecules by Allele-Specific PCR

In most cases it is advisable to set up a pilot experiment with varying amounts of input DNA to assess the activity of the hotspot before embarking on any large-scale experiments. If the activity of the hotspot is already known, proceed from step 9.

For both the pilot experiment and the subsequent large-scale CO assay, the DNA input has to be calculated. Since one haploid mouse genome contains approximately 3 pg of DNA, 6 pg of DNA will on average contain one molecule of each parental allele. From our experience, only about half of the molecules will actually generate PCR products, due to primer inefficiency and DNA damage. This means that on average one amplifiable molecule of each parental allele will be found in 12 pg of DNA. However, for both the CO and NCO, only the amplifiable molecules that are derived from oocytes should be considered. The DNA extracted from ovaries is a mix of DNA from somatic cells and oocytes and this mix varies

between different preparations of an ovary cell suspension. Using the result from Subheading 3.2, step 25, calculate the final percentage of oocytes in the pooled ovary cell suspension that was used for the DNA extraction (Subheading 3.3, step 9). Given that oocytes have double the genomic content (4C vs. 2C) of most somatic cells, which are in G1/G0 phase, and assuming equal loss during the extraction for oocytes and somatic DNA, calculate the fraction of oocyte-derived DNA in the ovary DNA sample. If 12 pg of DNA contains on average one amplifiable molecule of each parental allele, then (12 pg/the fraction of oocyte DNA) will contain on average one oocyte-derived amplifiable molecule (see Note 19).

1. Set up PCRs with various amounts of ovary DNA, with somatic control DNA, and with no DNA (the latter two are negative controls). For instance, in a 96-well plate, set up 16 reactions each with 2,000, 1,000, 500, and 250 oocyte-derived amplifiable molecules per well. This pilot setup allows screening of 60,000 oocyte-derived amplifiable molecules at once, so even in very weak hotspots (recombination frequency $<1 \times 10^{-5}$), COs will be detected. As a negative control for contamination, set up eight reactions with no DNA. As a negative control for PCR artifacts, set up 24 reactions with 2,500 amplifiable molecules of somatic DNA. Both the total input and the maximum input per well of amplifiable molecules for somatic and ovary DNA should be in the same range (see Note 20).
2. Set up the first round of PCR with an outside forward allele-specific primer and the outside reverse primer that is specific for the other haplotype (Fig. 1b).
3. The total volume for each first PCR should be 8 μl with 0.72 μl 11.1× PCR buffer, supplemented with 12 mM Tris base, 0.03 U/μl Taq polymerase, 0.003 U/μl turbo Pfu polymerase, 0.2 μM of each allele-specific primer, and the determined amount of DNA. Start the PCR with 1 min denaturation at 96°C, followed by 25 cycles of amplification (20 s 96°C, 30 s at the optimized annealing temperature and extension at 65°C for ~90 s/kb).
4. As the first PCR is almost finished, prepare for S1 digestion by setting up a new 96-well plate with 5 μl of S1 digestion mix per well (see Note 21). Immediately upon completion of the first PCR, add 0.5 μl of each reaction to the corresponding well with the S1 digestion mix, and incubate at room temperature for 20 min. Dilute by adding 45 μl of S1 dilution buffer.
5. Set up the second round of PCR with the inside forward allele-specific primer and the inside reverse primer that is specific for the other haplotype. The direction and allele specificity of the nested primers has to be the same as in the first PCR (see Fig. 1b). The total volume for each second PCR should be 8 μl (0.72 μl 11.1× PCR buffer, supplemented with 12 mM

Tris base, 0.03 U/μl Taq polymerase, 0.003 U/μl turbo Pfu polymerase, and 0.2 μM of each allele-specific primer).

6. Seed each reaction with 1.6 μl of the S1 digested first PCR. Conditions for the second PCR are as described for the first PCR (see step 3), with 30–35 amplification cycles.
7. Add 3 μl loading dye to each reaction and run 5 μl of each on a 0.8% agarose gel with 0.5 μg/ml ethidium bromide. Increasing oocyte DNA input should correspond with an increase in the number of positive reactions and the negative controls should not show any PCR products.
8. Use the Poisson approximation to estimate the average number of COs per well (m) for each pool size: $m = -\ln$ (number of negative reactions/total number of reactions).
9. For large-scale CO assays, select an input DNA amount that yields an average of 0.4–0.6 COs per well. Using larger pools will make estimating the CO frequency less accurate because many of the positive PCRs will contain more than one CO molecule. On the other hand, smaller pools will decrease the number of positive reactions, increasing the number of PCRs required. About 100 COs are needed to accurately characterize the CO distribution in a hotspot.
10. Perform large-scale allele-specific PCRs with the selected amount of input oocyte DNA per well, as described above for the pilot experiment. Include negative controls with somatic DNA and no DNA. Do the same for the reciprocal CO orientation by switching the haplotypes targeted by the allele-specific primers (see Note 22). Adjust the annealing temperature in the reciprocal PCRs according to the optimization described in Subheading 3.5.

3.7. Mapping CO Breakpoints by Hybridization with ASOs

1. In a new 96-well plate, set up a third round of PCR with nested universal forward and reverse primers in a final volume of 22 μl for each reaction (1.98 μl 11.1× PCR buffer, supplemented with 12 mM Tris base, 0.03 U/μl Taq polymerase, 0.003 U/μl turbo Pfu polymerase, and 0.2 μM of each universal primer). Reserve the first column of wells for positive and negative controls and transfer 0.6 μl of each positive second PCR to one of the other wells. Keep a record of the origin (plate and well number) of each reaction. In the first column, seed two wells with 5 ng of genomic DNA from each parental strain that was used to generate the F1 hybrid. Seed the remaining six wells with 0.6 μl of randomly picked somatic controls from the second PCR.
2. The conditions for the third PCR are as described above (Subheading 3.6, step 3), with 35 amplification cycles.
3. Add 10 μl of loading dye to each reaction and run 2 μl of each on a 0.8% agarose gel with 0.5 μg/ml ethidium bromide. PCR amplification should be uniformly high.

4. Cut three Whatmann filter papers and ten pieces of nylon membrane to the appropriate size for the 96-well dot blot manifold, according to the manufacturer's instructions. Wet filter paper and a membrane with ddH_2O, assemble the dot blot manifold, and apply vacuum.
5. Add 300 µl of denaturation buffer to each well of the third PCR plate and pipet gently up and down to mix (see Note 23). Transfer 30 µl from each well to the corresponding well of the dot blot manifold. Make sure all the liquid is pulled through the membrane (see Note 24) and rinse each well with 150 µl 2× SSC. Remove the membrane, air-dry, and repeat for all replicate membranes. Ensure all membranes are completely dry before proceeding with the hybridization steps (see Note 25).
6. Prepare a working stock of 8 ng/µl of the ASO for each polymorphic site to be tested. ASOs are typically 18 nucleotides long with the SNP site located at the eighth base from the 5′ end (see Note 26). For each polymorphism, ASOs specific to both parental genotypes are used twice: once with one ASO to be labeled for hybridization and the ASO for the alternative allele as a competitor, followed by a hybridization in which the labeled and competitor ASO are switched. Store ASOs at a concentration of 800 ng/µl in ddH_2O at –20°C and dilute 1:100 to make the working stock.
7. For each dot blot, set up a 10 µl ASO labeling reaction in a screw-cap microcentrifuge tube for each polymorphism with 1 µl 10× kinase mix, 0.35 µl T4 polynucleotide kinase, 7.8 µl ddH_2O, 0.2 µl (γ-^{32}P)-ATP, and 1 µl of 8 ng/µl ASO. Incubate at 37°C for 45 min. Spin down briefly and inactivate the T4 polynucleotide kinase by incubating at 65°C for 20 min. Spin down briefly again and add 20 µl of 8 ng/µl unlabeled ASO of the other allele as competitor (see Note 27).
8. Soak the dot blots in 3× SSC and place in a small hybridization bottle with the DNA side facing inward. If blots from more than one experiment are to be hybridized with the same probe, they can be stacked with alternating layers of hybridization mesh. Stacks of up to eight blots have been successfully hybridized with the same probe. Keep in mind that the amount of probe and volumes of solutions have to be increased accordingly when hybridizing multiple blots. In addition, the length of time for all subsequent (pre-)hybridization and wash steps has to be increased to allow the solutions to fully penetrate the stack of blots.
9. For each blot, pre-hybridize with 3 ml of TMAC hybridization buffer at 58°C for 10–20 min in a rotator hybridization oven (see Note 28).
10. Discard the buffer and replace with 2.5 ml fresh TMAC hybridization buffer supplemented with 21 µg freshly denatured

sonicated salmon sperm DNA (boil for 5 min prior to use). Incubate in the rotator oven at 53°C for 5–10 min.

11. Add the probe solution containing the labeled ASO and the unlabeled competitor ASO and hybridize in the rotator oven for 1 h at 53°C.
12. Discard the hybridization solution. Wash three times with 3 ml pre-warmed TMAC wash buffer per blot at 56°C with rotation for 5–10 min per wash, followed by a 15 min wash with 4 ml pre-warmed TMAC wash buffer per blot.
13. Rinse the blots three times in the bottles with 2× SSC at room temperature, then transfer them from the bottle to a tray with 2× SSC and wash an additional two to three times. Blot off excess liquid, seal the dot blots in Saran wrap, and expose on a phosphorimager screen (see Note 29).
14. Strip the probes from the blots by repeated washes in boiling 0.1% SDS. Monitor probe removal using a Geiger counter and continue the washes until the signal is sufficiently reduced. For this step, all blots can be combined in a single tray with hybridization mesh between the layers of blots to increase the stripping efficiency.
15. Rinse the stripped blots several times in water and reprobe immediately or allow to air-dry and store free of dust at room temperature until further use (see Note 30). Continue probing and stripping the dot blots until all polymorphisms of interest have been hybridized with labeled ASOs from both parental genotypes. Ideally, include one or two SNPs on each side outside the hotspot region. To account for variation between the replicate dot blots, the same blot should be hybridized with labeled ASOs specific for both parental alleles for a given polymorphism. Blots can be stripped and reprobed up to ten times without significant loss of signal.
16. Score the positive signals on the dot blots (see Fig. 3), combine the data for both alleles in a table and order the COs according to their location (see example in Table 1).
17. Most PCR products will show a single CO breakpoint (e.g., Table 1, well 2A). However, some wells will contain mixed molecules, with a positive hybridization signal for both alleles at one or more polymorphisms (e.g., Table 1, wells 2C and 3C). This happens when two or more CO molecules are amplified in the same PCR. Only the two CO breakpoints that can be identified (both ends of the stretch of mixed SNPs) are scored. (In the example in Table 1, well 2C shows a CO breakpoint between SNP 3 and 4 and between SNP 6 and 7.) Note that it is possible that the PCR contained three or more CO molecules, but this assay does not allow for the identification of more than two. The occurrence of mixed molecules increases

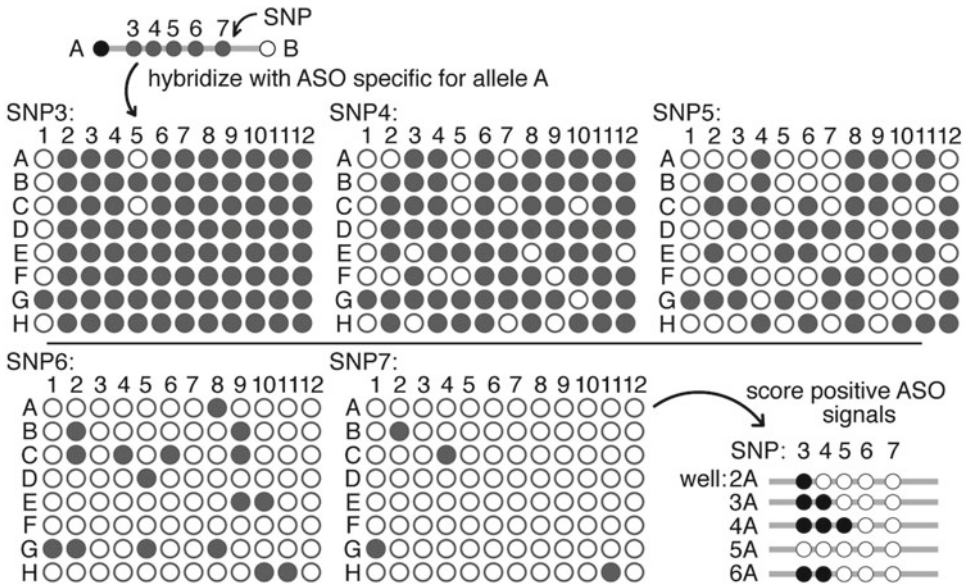

Fig. 3. CO breakpoint mapping. Allele-specific PCR will only amplify CO molecules, in this example molecules that are of the A genotype on the left side and of the B genotype on the right side (indicated by *black and white circles*, respectively). The remaining SNPs (*gray circles*) will be typed by ASO hybridization to determine the CO breakpoint for each CO molecule. The PCR products are transferred to *dot blots*, where every dot represents one PCR and ideally one (but possibly more) CO molecule. In this example, the first column in the plate contained 6 negative controls (wells 1A–1F) and the positive controls for alleles A and B (wells 1G and 1H, respectively). The *dot blots* are hybridized with ASOs that are specific for one of the parental haplotypes (in this example, the A alleles), for each subsequent polymorphism across the hotspot (SNPs 3–7 in this example, shown from *left to right*; positive hybridization signals are shown as *gray circles*, negative signals as *white*). The ASO hybridization signals are scored (in this example, results for wells 2A–6A, *black circles* represent positive hybridization signals, i.e., presence of the A alleles. *White circles* represent negative hybridization signals). For a CO assay the data for the A alleles are combined with the data for the B alleles (not shown).

with increasing amount of input DNA, which is why the initial pool size should not be too big (see Subheading 3.6, step 9) (see Note 31).

18. Sometimes one observes no switch from one haplotype to the other for any of the analyzed SNPs (see Table 1, well 2B). This either means that the CO breakpoint is located between one of the terminal SNPs and the inside allele-specific PCR primer or that the allele-specific PCR is not specific enough and is amplifying non-recombinant DNA. In either case, these wells are discarded from the analysis. In the case of mixed molecules only the single CO breakpoint that can be identified will be scored (see Table 1, well 3F, the CO breakpoint between SNP 5 and 6). Typing more SNPs outside the hotspot region may help avoid the problem of the apparent absence of a CO breakpoint. On the other hand, if the problem is due to nonspecific amplification, indicated by a high frequency of positives in the PCRs on somatic DNA, re-optimizing the allele-specific primers may help (see Subheading 3.5).

Table 1
Calculations for the CO assay

Well		Pool size	SNP				
Column	Row		3	4	5	6	7
2	A	300	A	B	B	B	B
2	F	300	A	B	B	B	B
3	E	300	A	B	B	B	B
3	H	300	A	B	B	B	B
2	C	300	A	M	M	M	B
2	D	300	A	A	B	B	B
2	H	300	A	A	B	B	B
3	A	300	A	A	B	B	B
3	B	300	A	A	B	B	B
3	C	300	A	A	M	B	B
2	E	300	A	A	A	B	B
3	D	300	A	A	A	B	B
3	F	300	M	M	M	B	B
3	G	300	A	A	A	B	B
2	G	300	A	A	A	A	B
2	B	300	A	A	A	A	A

SNP interval	3–4	4–5	5–6	6–7
Number of COs	5	5	5	2
Analyzable pools	14	13	14	15
Negative pools	9	8	9	13
P(0)[a]	0.643	0.615	0.643	0.867
m[b]	0.442	0.486	0.442	0.143
Poisson-corrected number of COs[c]	6.186	6.312	6.186	2.147
Number of analyzed molecules[d]	4,200	3,900	4,200	4,500
Length SNP interval (bp)	500	200	300	600
Recombination fraction (cM)[e]	0.147	0.162	0.147	0.048
Recombination activity (cM/Mb)[f]	295	809	491	80

CO molecules that contained only the A allele are light gray ("A," data taken from columns 2 and 3 from the dot blots in Fig. 3), those containing only the B allele are dark gray ("B"), and those amplification products containing both parental alleles are black ("M"). The CO breakpoints are sorted according to their position. The number of COs per inter-polymorphism interval is corrected using Poisson approximation and the CO activity in cM/Mb is calculated for each interval

[a] $P(0) = (\text{number of negative pools}/\text{number of analyzable pools})$

[b] $m = -\ln(P(0))$

[c] The Poisson-corrected number of COs = $m \times$ number of analyzable pools

[d] Number of analyzed molecules = (number of analyzable pools × pool size)

[e] Recombination fraction = $100 \times$ (Poisson-corrected number of COs/number of analyzed molecules) in cM

[f] Recombination activity = (Recombination fraction/length of SNP interval in bp) $\times 10^6$

19. For each interval, count the number of COs. To account for the presence of possible hidden COs (i.e., two CO or more molecules with the same breakpoint), apply a Poisson correction. For this, count the number of analyzable pools per SNP interval. This number includes the pools that were included in the first and second PCR of the CO assay but were negative. For the example in Table 1, we assume 31 pools were initially analyzed for the retrieval of the 16 COs in columns 2 and 3 of the dotblot in Fig. 3. There are five COs in the SNP 4–5 interval (wells 2D, 2H and 3A–3C) and the number of analyzable pools is 28 (a total of 31 pools were initially analyzed, with 16 positive in the second PCR. Of the 16 COs, well 2B is discarded for all SNP intervals because it shows no switch between alleles across the analyzed SNPs. Wells 2C and 3F are considered not analyzable for the SNP 4–5 interval because the interval falls in a stretch of mixed molecules, which does not allow identification of any breakpoint in this interval). Because the fraction of negative pools is known: $P(0)$ = (number of negative pools/number of analyzable pools), the Poisson approximation can be used to calculate the average number of COs (m) per well: $m = -\ln P(0)$.
20. With m known, the expected (Poisson-corrected) total number of COs for each SNP interval can be calculated: Poisson-corrected number of COs = $m \times$ number of analyzable pools (see Table 1).
21. Calculate the recombination fraction in centimorgans (cM) for each SNP interval: recombination fraction = (Poisson-corrected number of COs/total number of analyzed molecules) × 100.
22. Calculate the recombination activity in cM/Mb for each interval: recombination activity = (recombination fraction/length of the interval in bp) × 10^6 (see Table 1).
23. An example graph of the recombination activity across a hotspot is depicted in Fig. 4.
24. Calculate the overall Poisson-corrected CO frequency across the hotspot by calculating m for the second PCRs as described in Subheading 3.6, step 8. The overall CO frequency = (m/the number of amplifiable molecules per well).

3.8. NCO Assay: Amplification of CO and NCO Molecules by Half-Sided Allele-Specific PCR

The setup for the NCO assay (see Fig. 1c) is the same as for the CO assay (see Subheading 3.6) with the following exceptions:

1. Instead of the combination of forward allele-specific primers with reverse allele-specific primers that are specific to the opposite parental haplotype, the NCO assay is performed with allele-specific primers on only one side. Primers in the opposite direction will be universal primers. This "half-sided" PCR will

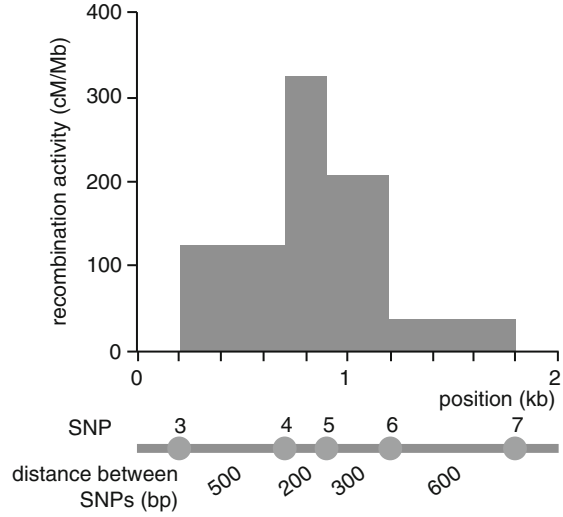

Fig. 4. CO activity across a hotspot. The position of the SNPs is indicated by *gray circles*. Data are from the example in Table 1.

amplify molecules that are CO, NCO, and non-recombinant, with the majority of products being of the latter type (see Fig. 1c).

2. Because most PCR products will be non-recombinant molecules, the pool size of input DNA needs to be much smaller than for the CO assay, usually about 15 amplifiable molecules per well. Having too many non-recombinant molecules in the PCR interferes with the detection of recombinants in the later ASO hybridization steps. As the input pool size is fixed, there is no need for a pilot experiment with different input pool sizes.
3. S1-nuclease digestion is usually not used in NCO assays (see Note 32). Immediately upon completion of the first PCR, add 35 µl dilution buffer to each well. Seed the second PCR with 0.6 µl of the diluted first PCR.
4. The second PCR for the NCO assay is set up in a larger reaction volume, usually 22 µl. The bigger reaction volume is needed because there is no re-amplification (or third PCR) prior to transferring the PCR products to dot blots.

3.9. Mapping NCOs and CO Breakpoints by Hybridization with ASOs

The procedure for mapping NCO and CO breakpoints by ASO hybridization is similar to the procedure for COs (see Subheading 3.7), with the following exceptions:

1. The second PCR is not re-amplified, but used directly. Add 10 µl of loading dye to each well and check the quantity and

quality of the PCR products by running 2 μl on a 0.8% agarose gel with 0.5 μg/ml ethidium bromide.

2. Prepare positive and negative hybridization controls by separately setting up and performing first and second half-sided PCRs on 5 μg genomic DNA from each parental strain that was used to generate the F1 hybrid. The negative hybridization control will be of the same haplotype as was selected for in the half-sided allele-specific PCR. The positive hybridization control is of the same haplotype as the ASOs that will be hybridized. For each parental haplotype, set up about 16 reactions of 22 μl and pool the products for each haplotype separately after completion of the second PCR. Add loading dye and check the quantity and quality of the PCR products by running 2 μl on a 0.8% agarose gel with 0.5 μg/ml ethidium bromide.
3. Replace one well on the 96-well plate of the completed second half-sided allele-specific PCR with 30 μl of the positive hybridization control. Add positive hybridization control to 6 wells in the following dilutions: 1:10, 1:30, 1:100, 1:300, 1:1,000, and 1:3,000. Empty one well, wash three times with water, and add 30 μl of negative hybridization control. Add 300 μl of denaturing buffer to each well and continue as described in Subheading 3.7.
4. Hybridize the dot blots with only the ASOs specific for the non-selected haplotype from the half-sided allele-specific PCR.
5. Use the dilution series of the positive hybridization control as a tool in scoring the hybridization signals. In general, hybridization signals that are stronger than the signals of 1:100 diluted positive controls are considered positive, i.e., represent bona fide CO or NCO molecules against the background of amplified non-recombinant molecules.
6. Score the positive hybridization signals and transfer the data to a table (see Fig. 5 and Table 2). Order the COs and NCOs separately according to their positions. Calculate the CO activity for each SNP interval and across the hotspot as described in Subheading 3.7. The overall CO frequency across the hotspot found in this assay should be comparable to the frequency found in the CO-specific assay.
7. Count the number of NCOs at each SNP. Calculate the Poisson-corrected average NCO number per well (m) for each SNP: $m = -\ln(\text{number of negative pools}/\text{number of analyzable pools})$. Note that pools that contain a CO are not analyzable for NCOs for the SNPs that give a positive signal in the ASO hybridization.

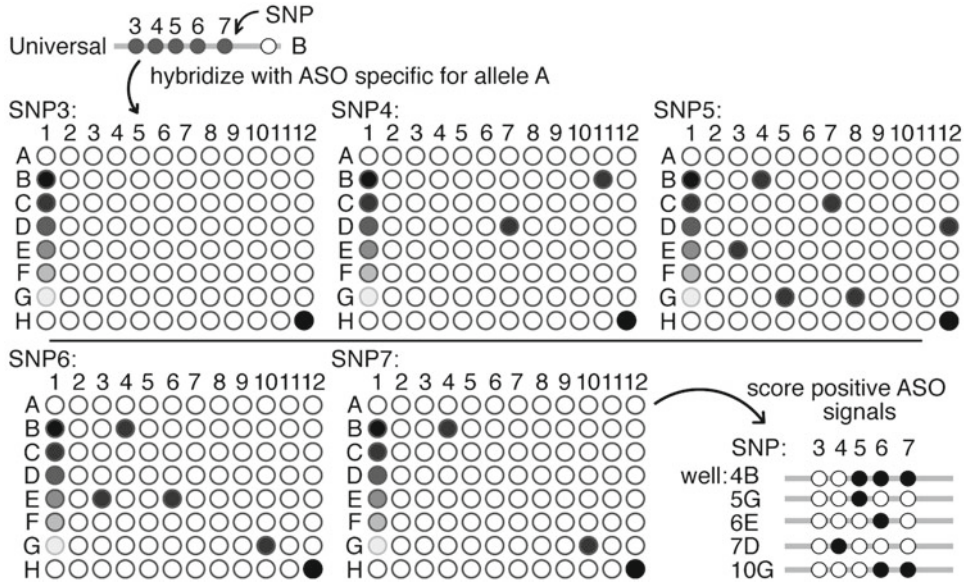

Fig. 5. CO and NCO breakpoint mapping. Half-sided allele-specific PCR will amplify CO, NCO, and non-recombinant molecules. In this example, amplified molecules are of the B genotype (indicated by *white circle*) on the right side and either genotype on the left side. The remaining SNPs (*gray circles*) will be typed to determine the CO or NCO breakpoint for each recombinant molecule. The PCR products are transferred to *dot blots*, where *every dot* represents one amplification product. In this example, the first column in each plate contained the controls with the negative hybridization sample (amplified from parental haplotype B) in well 1A and the dilution series of the positive hybridization control (amplified from parental haplotype A) in wells 1B–1G. Well 12H contains the undiluted positive hybridization control. The *dot blots* are hybridized with ASOs specific for the parental haplotype that was not selected for in the PCR (in this example, the A haplotype), for each SNP across the hotspot (SNPs 3–7 in this example, shown from left to right; positive hybridization signals are shown as *gray circles*, negative signals as *white*). The positive ASO hybridization signals are then scored. The example (*bottom right*) shows two COs (wells 4B and 10G) and three NCOs (wells 5G, 6E, and 7D), with *black circles* representing the presence of A alleles on the recombinant molecule (i.e., positive hybridization signals on the *dot blots*) and *white circles* representing inferred B alleles on the recombinants (i.e., negative hybridization signals).

8. Divide the Poisson-corrected NCO frequency per well by the pool size to give the NCO frequency per molecule for each SNP: NCO frequency = (m/pool size).
9. Calculate the overall NCO frequency across the hotspot as described for COs (see Subheading 3.6, step 8). Keep in mind that for the overall NCO frequency, each co-conversion (see Table 2, well 3E) should be counted as one event.
10. NCO activity across a hotspot can also be visualized by plotting either the relative or absolute NCO numbers for each SNP (see Fig. 6). Again, co-conversions are counted as one event, so each SNP involved will contribute an equal part of the event. For the example in Table 2, well 3A, this means that 0.5 of the recombinant involves SNP 5 and 0.5 of the recombinant involves SNP 6.

Table 2
Calculations for the NCO assay

Well		Pool size	SNP				
Column	Row		3	4	5	6	7
4	B	15			A	A	A
10	G	15				A	A
7	D	15		A			
11	B	15		A			
5	G	15			A		
7	C	15			A		
8	G	15			A		
12	D	15			A		
3	E	15			A	A	
6	E	15				A	
SNP			**3**	**4**	**5**	**6**	**7**
Observed number of NCOs			0	2	5	2	0
Analyzable pools[a]			88	88	87	86	86
Negative pools			88	86	82	84	86
Poisson-corrected number of NCOs[b]			0.000	2.023	5.149	2.024	0.000
Poisson-corrected NCO frequency[c]			0.000	0.023	0.059	0.024	0.000

SNPs that showed a positive hybridization signal in a well are shaded gray ("A"), SNPs that showed no positive hybridization signal are left blank. The CO and NCO breakpoints are sorted separately, according to their position. Shown is the calculation of the Poisson-corrected NCO frequency per SNP; for CO calculations see Table 1

[a]In the 96-well plate in Fig. 5, 8 wells were occupied by controls, leaving 88 wells available for analysis

[b]See Table 1 for the calculation of the Poisson-corrected number of recombinants

[c]The Poisson-corrected NCO frequency = (−ln(number of negative pools/number of analyzable pools))/pool size

4. Notes

1. Most of the oocytes in newborns are in the dictyate stage and have completed recombination. Although the number of oocytes in an ovary is the highest shortly before birth and rapidly decreases following birth (22), it is more convenient to use newborns rather than sacrificing dams to recover embryos.

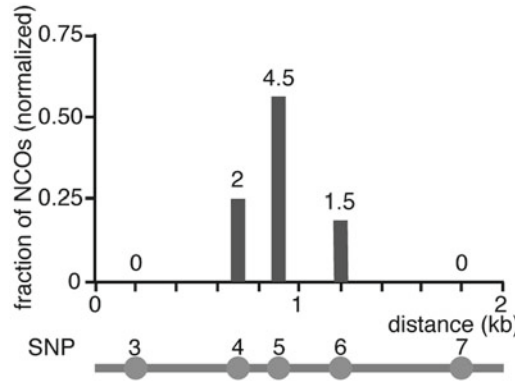

Fig. 6. NCOs across a hotspot. The fraction of Poisson-corrected NCOs per SNP is plotted against the distance in kb. The position of the SNPs is indicated by *gray circles*. The co-conversions were normalized (see Subheading 3.9 step 7), and the observed normalized number of NCOs is shown above each bar. Data are from the example in Table 2. Note that the polymorphism density in the hotspot as depicted is rather low, meaning that likely a great part of the NCOs across this hotspot remains undetected.

2. The objective is to remove all traces of grease and dust from the slides to reduce background in the immunofluorescence. Boiling the slides in a slide rack allows for free circulation between the slides and improves the removal of dust and grease.
3. Sodium azide and PMSF are added to inhibit bacterial growth and proteolytic breakdown, respectively, during the immune-incubation steps. PMSF is not stable in aqueous solutions and has to be added shortly before use. Note that both sodium azide and PMSF are highly toxic.
4. LPA can be added as a carrier to aid DNA precipitation.
5. Because the behavior of the primers can differ between different batches of 11.1× PCR buffer, it is advisable to make enough for at least one complete experiment (including optimization of the primers). Primers have to be re-optimized for every new batch of 11.1× PCR buffer.
6. TMAC allows the hybridization of oligos of different G/C content at the same temperature (23). Note that TMAC is highly toxic.
7. The ovaries can be kept on ice for some time but try to process them as quickly as possible. The most efficient way is to dissect out the ovaries of all the females in the litter, then continue with preparation of the cell suspension.

8. The treatment with collagenase and DNase I is required to remove part of the somatic cells during the subsequent wash steps and helps in making a clump-free cell suspension.
9. Additionally, 5–15 μl aliquots can be taken from the different preceding wash steps, included in the preparation of slides and quantified. This will give an indication of the change in the cellular composition of the cell suspension at each step.
10. Wrapped airtight, the slides can be kept at −80°C for several years.
11. The optimal dilution and incubation time depends on the antibody and should be tested beforehand.
12. The blocking buffer with the antibody is centrifuged to remove aggregates of denatured protein/antibody to help reduce background signals.
13. If the secondary antibody is conjugated to fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC), adding a last dip in FITC buffer (150 mM NaCl, 50 mM sodium carbonate, pH 9.5, filter-sterilized) helps reduce fading of the fluorophore.
14. Avoid extracting DNA from too small amounts of ovary cell suspension; aim for a pooled suspension from at least 45 animals.
15. Do not over dry the pellet, as this will make the pellet harder to dissolve. If needed, dissolution can be aided by incubation at 50°C for up to 1 h.
16. Even though amplicon sizes up to 13 kb are feasible, the PCR efficiency is usually better for shorter ones.
17. Because the behavior of the allele-specific primers is unpredictable, it is advisable to test different lengths for the allele-specific primers simultaneously, e.g., an allele-specific primer of 20, 21, and 22 nucleotides long with the SNP at the 3′ end.
18. Only two nested allele-specific primers on each side of the hotspot are needed for the CO and NCO assays, however, the allele-specific primers that will be used together in a CO assay have to amplify both specifically and efficiently at the same annealing temperature. If possible, design and test allele-specific primers directed against more than two SNPs on either side of the hotspot.
19. Alternatively, set up the PCR assuming 100% oocyte DNA in the DNA sample and recalculate the number of oocyte-derived amplifiable molecules upon completion of the CO assay.
20. The somatic control is included to assess the level of nonspecific amplification, which tends to increase with higher input of negative control DNA. To aid the interpretation of the CO

assay, the amount of input DNA for the somatic control should not exceed the maximum input of ovary DNA.

21. S1 nuclease digestion improves the specificity of allele-specific primers and removes PCR artifacts by removing single-stranded DNA from the PCR products. PCR artifacts such as panhandle loop structures can interfere with the detection of COs (16). The plate with S1 digestion mix can be prepared several hours in advance and kept at 4°C until use.
22. Reciprocal allele-specific PCRs serve two purposes. First, they provide an internal control because the CO frequency should be comparable for both orientations. Second, differences in the initiation of recombination on the parental alleles will be revealed (10, 24).
23. Ensure the 96-well plate used for PCR is capable of holding this volume (~330 µl) beforehand.
24. Remove bubbles by poking a pipette tip in the well. Make sure to use a clean tip for every bubble to avoid cross-contamination. After the wash step with 2× SSC a single needle can be used for all bubbles.
25. Cross-linking the DNA to the membrane is not routinely performed for the Hybond-XL membranes but can be included. See the manufacturer's instructions.
26. If a particular ASO does not give a clear hybridization signal, washing at slightly different temperatures or trying hybridization with the reverse complement can help. The conditions of the ASO hybridization are optimized for a length of 18 nucleotides, so be prepared to try different washing conditions for ASOs with different length.
27. Optional: to check for the efficiency of the labeling reaction, load the sample on a spin column (e.g., Quick Spin Oligo Column, Roche) after T4 polynucleotide kinase inactivation. Spin the sample through the column and measure the incorporation of radioactive label using a Geiger counter.
28. Hybridization signals can be improved by increasing the prehybridization time. This is especially true for dot blots being hybridized for the first time.
29. Optimal exposure times differ between ASOs. We routinely expose overnight, followed by a separate 1 h exposure if needed.
30. Blots can be stored up to 2 years without significant loss of signal.
31. A method for further dissecting mixed molecules, applicable for both COs and NCOs, is described in ref. 10.
32. S1 digestion can be included if desired. Follow the steps as described for the CO assay (see Subheading 3.6).

Acknowledgements

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