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Generation of Chromosome Paints: Approach for Increasing Specificity and Intensity of Signals

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ABSTRACT

Chromosome painting is a widely used technique, and the two principal means of generating probes for such experiments involve DNA isolation by chromosome flow sorting and by chromosome microdissection. Frequently, chromosome paints are bright and specific; however, on occasion, signals can be weak and nonspecific, particularly for microdissected probes. Reasons for this have been attributed to co-amplification of non-target DNA and the formation of primer concatamers during degenerate oligonucleotide primed (DOP)-PCR. Here we describe a technique of circumventing this problem by sequence enrichment. It involves co-hybridization of DOP-PCR biotinylated microdissected material and linked genomic DNA. Biotinylated DNA fragments captured on strepta-

vidin-coated paramagnetic beads are eluted and amplified by PCR using a single primer complementary to the linker arm.

INTRODUCTION

Chromosome painting has a wide range of applications including clinical cytogenetics, genome organization studies, hybrid characterization, and comparative genomics. Chromosome microdissection and flow cytometry are both well-established means of generating chromosome painting probes (1,2). Following isolation by either of these approaches, chromatin can be amplified and labeled by degenerate oligonucleotide primed (DOP)-PCR to produce a paint (1,3). Flow cytometry is the preferred approach for isolating whole chromosome paints, while microdissection is the method of choice for generating sub-regional paints and whole-chromosome paints when individual chromosomes cannot be resolved in a flow karyotype; both have found specific utility in developing chromosome paints for nonhuman species (2,4,5). With the near completion of the human genome project, paints can often be generated by selecting a library of clones from the region of interest (6); however, such a strategy is less practicable in nonhuman species, as fewer clones are available. In our own laboratory, we have found both flow cytometry and microdissection to be invaluable for generating chromosome-specific paints from chicken chromosomes (2).

Frequently, both approaches produce bright specific paints; however, on occasion, experiments result in nonspecific, weak signals. Reasons for this have been attributed to co-amplification of non-target DNA and the formation of primer concatamers during DOP-PCR amplification of the template DNA. This is likely to be more common in paints generated by microdissection, but, even for flow cytometry, if conditions are suboptimal (e.g., a slow-growing cell line from which the chromosomes are prepared or excess debris in the chromosome suspension), this can lead to chromosome paints that are not always as bright and specific as expected. Indeed, these problems tend to be more evident when smaller templates are used (e.g.,

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10–20 isolated chromosomes represent about the limit of a microdissection experiment, whereas flow sorting routinely isolates 400 chromosomes) and can be exacerbated when further amplifications are made from the original primary DOP-PCR product. When a suboptimal paint is generated, often the only solution is to repeat the experiment with no guarantee of success. In this study, therefore, we devised a means of circumventing this problem by enriching for sequences in the region of interest. It involves co-hybridization of DOP-PCR biotinylated microdissected material and linker genomic DNA. Biotinylated DNA fragments captured on streptavidin-coated paramagnetic beads are eluted and amplified by PCR using a single primer complementary to the linker arm. Chromosome paints were compared before and after application of this strategy both for microdissected and flow-sorted paints. In all cases, chromosome paints were demonstrably brighter and more specific following the inclusion of this enrichment step.

MATERIALS AND METHODS

Cell Lines and Chromosome Preparation

Hamster and chicken chromosome preparations were generated from fibroblast primary cultures. Metaphase chromosomes were prepared by standard protocols.

Microdissection of chromosomes. Briefly, preparations on coverslips were stained with 10% Giemsa (Sigma-Aldrich, Poole, UK) and placed on the stage of a Leica inverted microscope (Leica Microsystems, Milton Keynes, UK). Individual chromosomes were isolated from the coverslip using a glass needle driven by an electronically controlled micromanipulator attached to the microscope. The needle was then broken in a tube containing 10 μ L sterile distilled water before PCR amplification (7).

Fluorescence-activated chromosome sorting. Chromosomes were prepared for flow sorting as described previously (1), centrifuged briefly at 100 $\times$ *g* for 1 min to remove any debris, and then the supernatant was stained with 2 μ g/

mL Hoechst 33258 and 40 μ g/mL Chromomycin A3 (both from Sigma-Aldrich). Bivariate flow karyotypes were generated on a FACStar Plus (BD Biosciences, Oxford, UK) dual-laser flow cytometer equipped with two 5-W argon ion lasers. Approximately 400 chromosomes from each peak in the flow-karyotype were flow-sorted into a 0.5-mL Eppendorf® tube containing water.

DOP-PCR. A primary round of DOP-PCR amplification was performed on these chromosomes (1,3). From each of these primary DOP-PCRs, 1–2 μ L were used as a template for a secondary DOP-PCR incorporating biotin-16-

dUTP or digoxigenin 11-d-UTP (Roche Applied Science, Lewes, UK). This enabled amplification and labeling of the relevant chromosome, thus producing a chromosome paint (1,3).

Enrichment of Region-Specific Sequences

Total genomic DNA from either hamster or chicken was isolated by standard protocols, sonicated to a size of 200–800 bp, and ligated to a linker arm (BD Biosciences Clontech, Basingstoke, UK) containing the *Sa*I restriction site using T7 DNA ligase (sequence 5'-CC-

Figure 1. Schematic of the isolation procedure described.

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TCTGAGGTTCCAGAATCGATAGG-TCGACCGCGGTCGACCTATCGA-TTCTGGAACCTTCAGAGGTTT-3'). Independently, both the chromosome paint material (100 ng) amplified and labeled by DOP-PCR and the linked genomic DNA (1 μ g) were co-hybridized with cot-1 DNA (8,9) from hamster or chicken, in 50 and 10 times excess, respectively. The hybridization mixture contained 50% formamide, 1 $\times$ Denhardt's solution, 12.5% dextran sulfate, 0.1% SDS, 1.6 $\times$ SSC, and 40 mM phosphate buffer, pH 6.8. In both cases, co-hybridization involved the separation of dsDNA at 75°C for 10 min and then re-annealing at 37°C for 3 h. Thereafter, both solutions of DOP-PCR product and linked genomic DNA were combined and allowed to re-anneal overnight. Biotinylated DNA was captured in solution on streptavidin-coated paramagnetic beads (Promega, Sunderland, UK). Preparation of beads, capture of biotinylated DNA, washing, and elution were performed following the bead manufacturer's protocol. Amplification of the resulting linked DNA was achieved by using a single primer complementary to the linker arm (5'-CCTCTGAAGGTTCCAGAATCGATAG-3'). Briefly, the PCR mixture contained 200 μ M each dNTP, 0.5 μ M primer, and 1.5 U *Taq* DNA polymerase (HT Biotechnology, Cambridge, UK). The PCR profile was as follows: 5 min at 94°C and then 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, with a final elongation of 8 min at 72°C. Products were checked using agarose gel electrophoresis. In case of a faint signal, extra polymerase and primers were added and amplification was performed for a

further 30 cycles. For FISH, biotin or digoxigenin were incorporated in 25 cycles of PCR using as template 2 μ L of the primary products. Figure 1 illustrates this protocol.

FISH

Metaphase preparations were aged for 3 h at 55°C. Labeled probe (100 ng) was dissolved in hybridization buffer (containing 50% formamide, 2 $\times$ SSC, and 10% dextran sulfate). Chromosomes and probe were brought into contact under a 18 $\times$ 18 mm glass coverslip, sealed with rubber cement, and denatured together on a hot plate for 5 min at 68°C. The hybridization was carried out for 12–16 h.

Following the post-hybridization washes (1 $\times$ 2 min 0.4 $\times$ SSC/0.3% Igelal at 73°C, 1 $\times$ 2 min 2 $\times$ SSC/0.1% Igelal at room temperature), equilibration for higher salt concentration in 4 $\times$ SSC/0.05% Tween[®] 20, and blocking in 4 $\times$ SSC/0.1% Tween 20/2% BSA biotinylated probes were detected with Cy3-conjugated streptavidin (Amersham Biosciences, Little Chalfont, UK) (1:300 dilution in 4 $\times$ SSC, 0.1% Tween 20, 1% BSA), digoxigenin-labeled paints with FITC conjugated anti-digoxigenin (Roche Applied Science) (1:50 dilution). Finally, chromosomes were counterstained with DAPI and mounted in Vectashield anti-fade medium (Vector Laboratories, Burlingame, CA, USA) before viewing. Analysis was performed using a Leica DM epifluorescence microscope (Leica Microsystems), and images were captured using a Photometrics CCD camera attached to Vysis/Digital Scientific (Cambridge, UK) "Smart Capture" software.

RESULTS

Our results clearly demonstrate that chromosome paint material is brighter and more specific following this purification protocol (Figures 2 and 3). In Figure 2a, biotin-labeled chicken chromosome paints gave recognizable but nonspecific and "messy" signals. In our experience, this was not an unusual scenario with avian chromosomes, largely because avian cell cultures from primary embryo tissue tended to have a considerable amount of debris in them and this could clog the nozzle of the flow sorter and lead to suboptimal flow karyotypes. Following isolation, however (Figure 2b), paints were clear, bright, and specific. In the case of the hamster chromosomes, initial microdissection experiments of three bands on the q-arm of chromosome X led to nonspecific signals over the entire q-arm (Figure 3a), we surmise through repetitive elements in the hamster X chromosome. Following our isolation protocol, however (Figure 3b), the individual microdissected fragments were clear (labeled 1, 2, and 3), and this enabled us to address the question of the nature of the chromosome rearrangement in an aberrant hamster cell line.

DISCUSSION

Here we describe an approach based on the work of Chen-Liu et al. (10) and Overmyer et al. (11) who used a similar method for preparing cDNA sublibraries enriched in sequences from specific chromosome regions. Here we make adaptations to the protocol and clearly demonstrate its application for

Figure 2. Chicken chromosome 1 paint (a) before and (b) after isolation.

Figure 3. Three microdissected segments from hamster X chromosome (a) before and (b) after isolation.

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the improvement of the quality of chromosome painting probes. Generation of chromosome painting probes, whether by microdissection or flow sorting, is a very long, involved process and often leads to paints that are suboptimal for the purposes of the investigator. There are a small number of laboratories equipped to perform these specialist techniques and demand for them is often intense. Thus, if suboptimal paints are produced, then this can often lead to an experiment being abandoned or delayed. The use of this protocol may alleviate this problem. Moreover, in our experience, probes generated in this way do not usually require inclusion of cot-1 DNA in the hybridization mixture. We attribute this to the fact that the inclusion of cot-1 DNA in the isolation protocol successfully removes the contaminating repetitive sequences leaving the unique probes free to hybridize.

The ability to negate the effect of contaminating repetitive sequences has revolutionized FISH experiments and paved the way for chromosome painting and physical mapping of large clones such as cosmid, YACs, PACs, and BACs (12). Despite this, probes often need further purification to produce reliable, bright, specific signals, and there are a number of approaches described in the literature to do this. Some simply involve the description of a more efficient washing procedure (13); however, others describe purification of chromosome painting probes. Craig et al. (14) described the removal of repetitive sequences from chromosome painting probes by pre-hybridization of cot-1 DNA to the biotinylated chromosome libraries and capture of the cot-1-containing hybrids using magnetic beads. This preceded purification and reamplification of the unbound fraction, and probes isolated in this way were termed "repeat-depleted" chromosome paints. The authors suggest that hybridization patterns were comparable to those achieved with untreated probes hybridized with cot-1 DNA, and this was confirmed on larger series by the work in References 15–17. For the most part, however, these approaches require high-quality paints from the outset and are designed to obviate the need for competitive in situ suppression in the painting experiment. To the best of our

knowledge, this is the first approach that reliably can take poor-quality chromosome paints and generate bright, specific ones free from repetitive elements, contaminating DNA, primer artifacts, and nonspecific background.

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