Directly incorporating fluorochromes into DNA probes by PCR increases the efficience of fluorescence *in situ* hybridization*

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Directly Incorporating Fluorochromes into DNA Probes by PCR Increases the efficiency of fluorescence *in situ* hybridization

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Abstract

The object of this study was to produce a directly labeled whole chromosome probe in a Degenerative Oligonucleotide Primed-Polymerase Chain Reaction (DOP-PCR) that will identify chromosome breaks, deletions, inversions and translocations caused by radiation damage. In this study we amplified flow sorted chromosome 19 using DOP-PCR. The product was then subjected to a secondary DOP-PCR amplification. After the secondary amplification the DOP-PCR product was directly labeled in a tertiary PCR reaction with rhodamine conjugated with dUTP (FluoroRed) to produce a DNA fluorescent probe. The probe was then hybridized to human metaphase lymphocytes on slides, washed and counterstained with 4',6-diamino-2-phenylindole (DAPI). The signal of the FluoroRed probe was then compared to a signal of a probe labeled with biotin and stained with avidin-fluorescein isothiocyanate (FITC) and anti-avidin (FITC).

The results show that the probe labeled with FluoroRed gave signals as bright as the probe with biotin labeling. The FluoroRed probe had less noise than the biotin labeled probe. Therefore, a directly labeled probe has been successfully produced in a DOP-PCR reaction. In the future a probe labeled with FluoroRed will be produced instead of a probe labeled with biotin to increase efficiency and reduce noise.

Introduction

Fluorescence *in situ* hybridization (FISH) is a technique which allows visual identification of specific chromosomes or portions of chromosomes through fluorescence microscopy. This technique is performed using a DNA probe specific for a desired chromosome and a glass slide which contains a spread of metaphase chromosomes that are prepared by standard cytogenetic methods. The probe is a short piece of DNA, usually between 300-500 base pairs, which is produced from flow sorted chromosomes. Both the target DNA and the probe are denatured by heating which causes the melting of the DNA double helix. The target DNA and the probe are mixed and the strands are allowed to recombine. The probe reanneals to the target at locations where the base pairs are complementary, forming a double stranded hybrid molecule. Usually the probe is an indirectly labeled probe which requires excess washing and staining. But in this experiment the DNA probes are directly incorporated with fluorochrome conjugated bases. Using directly labeled probes decreases the effort required for post-hybridization washings and omits the time consuming staining process that is required for an indirectly labeled probe.

Materials and Methods

The chromosomes were flow sorted and isolated as described previously (Breneman et al., 1993). Briefly, chromosomes were stained with two fluorescent dyes, Hoechst 33258 (HO) and Chromomycin A 3 (CA3). The dyes caused the chromosomes to fluoresce differently when passed through a dual-laser cell sorter, producing a bi-variant, HO vs CA3 flow karyotype. The dye in the chromosomes excited differently because the size and base-pair composition of each chromosome is different. The chromosomes were then

flow sorted at a rate of 5-10/sec. One thousand chromosomes were sorted into each PCR tube.

Metaphase slides were prepared by the method of Evans et al., (1971). Briefly T lymphocytes were cultured from whole blood, arrested in metaphase with colcemid, harvested and fixed to glass slides with 3:1 methanol/acetic acid.

Whole chromosome probes were prepared using flow sorted chromosomes that were amplified by DOP-PCR. The primer (5'OHCCGACTCGAGNNNNNN NATGTGGOH-3') used was a 22 mer universal primer with a 6 nucleotide long degenerated region that represents all possible 6 nucleotide combinations. For the initial amplification the flow sorted chromosomes served as a template and were combined with the following: 50 ul 2 x Master Mix (25 U Taq DNA polymerase in 20mM Tris HCl, 100 mM KCl, 3mM $MgCl_2$, Brij®35, 0.01% (v/v), 0.4mM dATP, 0.4mM dGTP, 0.4mM dCTP, 0.4mM dTTP, final pH 8.3) 5 ul (40 uM) DOP-PCR primer, 45 ul water (Boehringer Mannheim). PCR was performed on a 480 thermal cycle as follows: 5 minutes at 95°C followed by 5 cycles of 1 minute at 94°C, 1.5 minute at 30°C, a 3 minute transition from 30-72°C and a 3 minute extension at 72°C. This was followed by 35 cycles of 1 minute at 94°C, 1 minute at 62°C and 2 minute at 72°C. Followed by a 7 minute extension at 72°C (Telenius et al., 1992). A secondary DOP-PCR was performed using the conditions described above and the following reagents: 50 ul Master Mix, 5 ul DOP-PCR primer, 2 ul primary template, and 43 ul water.

The DOP-PCR fragments were either directly labeled in a tertiary DOP-PCR reaction that contained fluorochromes or by random priming. The fragments that were directly labeled in a tertiary DOP-PCR reaction used the conditions described above and the following reagents: 5 ul 10 x buffer (Perkin Elmer), 2.5 ul 40 uM DOP-PCR primer, 30.5 ul water, 5 ul secondary template,

5.5 ul dNTPs with FluoroRed(200 uM each of dATP, dCTP, and dGTP, 100uM dTTP plus 0.04nM of FluoroRed), 1.5 ul Taq. The total reaction volume was 50 ul. The DOP-PCR fragments were indirectly labeled by random priming using the BioPrime DNA labeling system (Gibco BRL) to prepare biotinylated. That was done by combining 25-100ng of DNA with 19 ul of water and denaturing the solution for 5 minutes at 100°C. Immediately following the denaturing 5 ul of 10 x dNTP, 20 ul of 2.5 x random primers solution, and 4 ul of water was added. This mixture was mixed briefly and then 1 ul of Klenow fragment (40 U/ul) was added. This solution was incubated for 1-4 hours at 37°C. Then 5 ul of stop buffer was added to stop the reaction.

The hybridization was performed as described previously (Lucas et al., 1992). Briefly, metaphase chromosomes were denatured in 70% formamide, 2 x SSC at 70°C for 2-10 minutes. The slides were then dehydrated in a 70-85-100 % ethanol series and air dried. The probe mix was prepared by combining 2 ul of probe, 7 ul of probe master mix (50% formamide, 10% dextran sulfate, 2 x SSC, pH 7.0), and 1 ul cot-1 DNA. This probe mix was denatured for 5 minutes at 70°C. The probe mix was applied to a slide, covered with cover glass and sealed with rubber cement. The slide was then placed in a 37 °C incubator overnight. The cover glass was then removed and the slide was washed three times at 45°C in 50 % formamide/2xSSC, pH 7, once in 2xSSC and once in PN buffer. The slide was washed in each solution for 5 minutes. The directly labeled probe was then counterstained with DAPI, covered with cover glass and viewed under a fluorescent microscope (see figures 1 and 2). The probe that was indirectly labeled by random priming had to be stained with one layer of FITC-conjugated avidin and one layer of anti-avidin-FITC. (Avidin and anti-avidin were obtained from Vector Laboratories Inc., Burlingame CA.) The indirectly labeled probe was then counterstained with

DAPI, covered with cover glass and viewed under a fluorescent microscope (see figures 3 and 4).

Results

In this experiment our objective was to produce a directly labeled whole chromosomes probe in a DOP-PCR reaction. We began the experiment trying to indirectly label using bio-16-dUTP in a PCR reaction. But we never obtained a good, clean signal using biotin conjugated to dUTP. The next thing we tried was directly labeling with FluoroGreen (fluoroscein-11-dUTP) in a PCR reaction. Again, we never obtained a good, bright, clean signal using FluoroGreen. However, the signal obtained using FluoroGreen was brighter and cleaner than the signal obtained when the biotinylated probe was used. The final type of fluorochrome that we tried was FluoroRed. When FluoroRed was attached to the probe in PCR the signal was bright and clean. The next experiment conducted was to determine the optimal amount of FluoroRed needed for each reaction. We tried using the following ratios of FluoroRed to dTTP: 1:5, 1:6 and 1:7. Both the 1:5 and the 1:6 ratio had good clean signals. We concluded that the 1:6 ratio was more cost efficient, because the signal looked the same, but used less FluoroRed. Finally, we compared a directly labeled FluoroRed probe, generated by PCR, to an indirectly biotinylated probe generated by random priming. We compared the signal that each probe gave. The probe with FluoroRed gave a good, bright, clean signal with less noise than the indirectly biotinylated probe.

Discussion

Directly labeling the probe in PCR was more efficient than random priming of DOP-PCR products. Directly labeling the probe in PCR eliminates the time required for random priming (1-4 hours) and for staining the slide (2-3 hours.). One disadvantage to directly labeling in PCR was that another

PCR program was required to label the DNA. Another, more important, advantage to the use of fluorochrome-labeled DNA was that when hybridized it produced less noise (non-specific signals) than biotin labeled probes. Therefore, a directly labeled probe has been successfully produced in a DOP-PCR reaction. In the future a probe labeled with FluoroRed will be produced instead of a probe labeled with biotin to increase efficiency and reduce noise.

Acknowledgments

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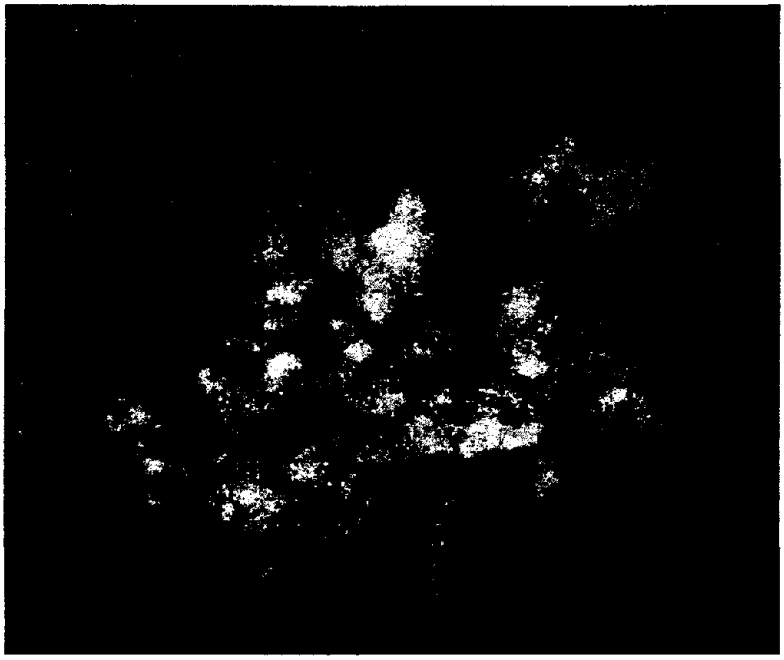

Figure # 1:

Metaphase spread hybridized with DOP-PCR chromosome 19 that was directly labeled in PCR with FluoroRed.

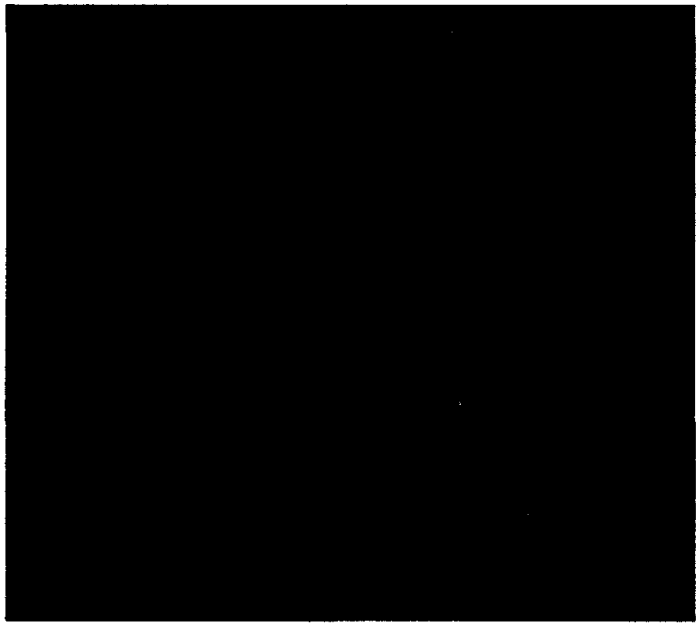

Figure # 2:

Metaphase spread hybridized with DOP-PCR chromosome 19 that was directly labeled in PCR with FluoroRed .

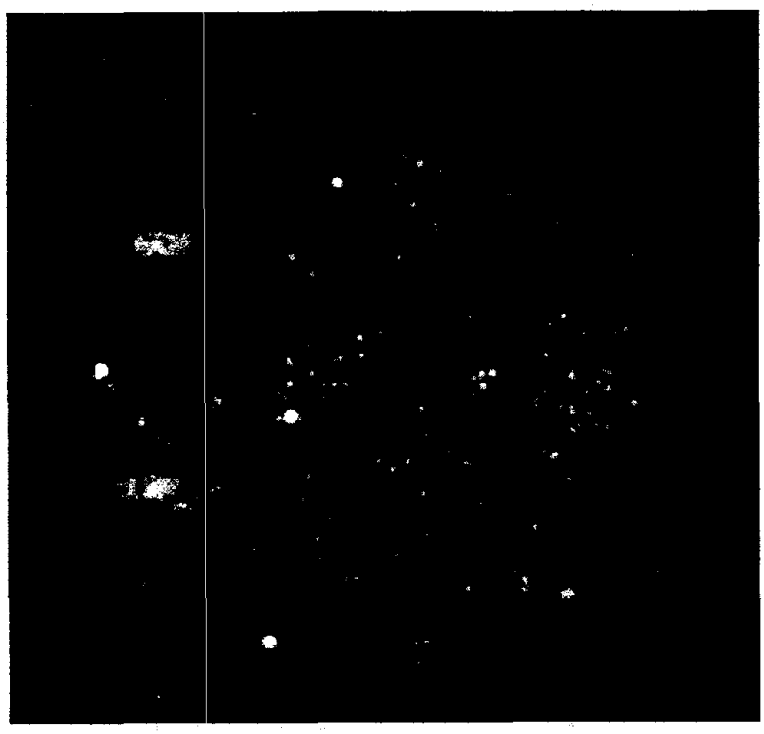

Figure # 3:

Metaphase spread hybridized with DOP-PCR chromosome 19 that was biotinylated.

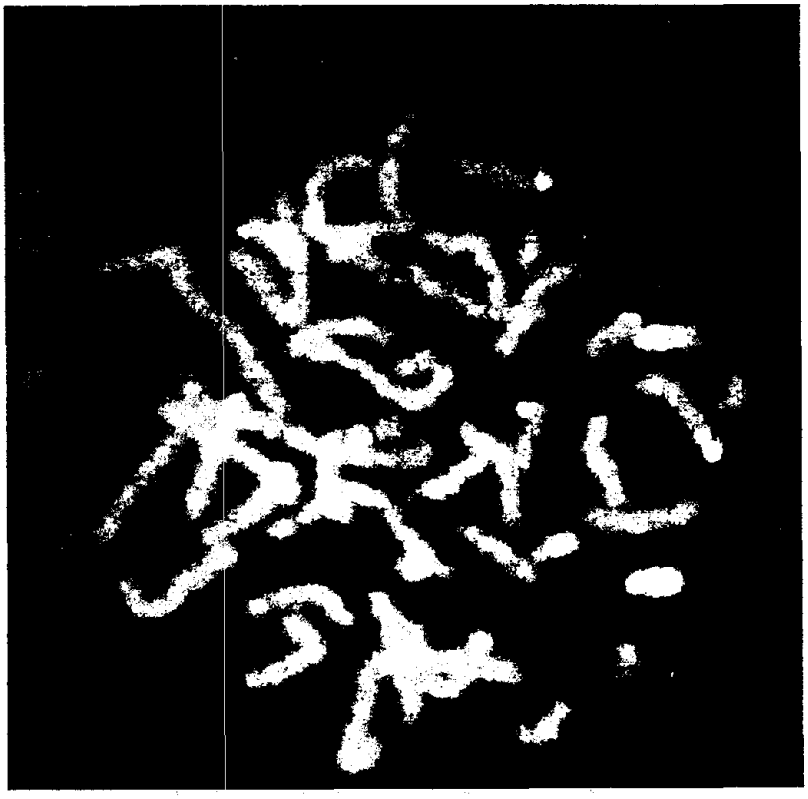

Figure # 4:

Metaphase spread hybridized with DOP-PCR chromosome 19 that was biotinylated.