J. Microbiol. Biotechnol. (2007), **17**(7), 1079–1082

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Generation of FISH Probes Using Laser Microbeam Microdissection and Application to Clinical Molecular Cytogenetics

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Received: October 26, 2006 Accepted: December 2, 2006

Abstract Chromosome microdissection and the reverse FISH technique is one of the most useful methods for the identification of structurally abnormal chromosomes. In particular, the laser microbeam microdissection (LMM) method allows rapid isolation of a target chromosome or a specific region of chromosomes without damage of genetic materials and contamination. Isolated chromosomes were directly amplified by the degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR), and then the FISH probes labeled with spectrum green- or spectrum red-dUTP were generated by nick-translation. Whole chromosome painting (WCP) probes were successfully generated from only 5 copies of the chromosome. With this method, we produced 24 WCP probes for each human chromosome. We also tried to characterize a marker chromosome, which seemed to be originated from chromosome 11 on conventional banding technique. The marker chromosomes were isolated by the LMM method and analyzed by reverse FISH. We elucidated that the marker chromosome was originated from the short arm of chromosome 5 (5p11→pter). A fully automated and computer-controlled LMM method is a very simple laboratory procedure, and enables rapid and precise characterization of various chromosome abnormalities.

Keywords: Chromosome painting, fluorescence *in situ* hybridization, laser microbeam microdissection, marker chromosome, polymerase chain reaction

The fluorescence *in situ* hybridization (FISH) techniques have played an integral role in overcoming the technical limitation of conventional cytogenetic techniques [5, 16] since it was introduced in cytogenetic analysis for numerical or structural chromosome abnormalities [12].

Whole chromosome painting (WCP) probes have been used for the identification of a derivative or a marker chromosome, an abnormal chromosome in which no part can be identified. To generate the probes for WCP in the laboratory, the flow sorting method and human-rodent hybrid cell lines [8] containing a single human chromosome have been used. In the case of the flow sorting method [9, 12], some of the human C group chromosomes, chromosomes 9, 10, 11, and 12, are hardly separated because of similar chromosome size [1]. A marker chromosome smaller than one-third of the size of chromosome 21 and the chromosomal subregion cannot be collected [16]. FISH probes from human-rodent hybrid cell lines do not hybridize evenly along the chromosome [2, 6].

The chromosome microdissection method is a very powerful approach to generate WCP probes, regardless of the target chromosome size. Microdissection also allows the construction of a region-specific probe. Chromosome microdissection methods allow for the reverse FISH technique, where the target chromosomes or subchromosomal regions acquired by microdissection are amplified and labeled with various fluorochromes, and hybridized to another chromosome preparation. However, conventional microdissection using a glass needle is time-consuming and labor-intensive work [7]. Thus, it requires an experienced person with high-skilled techniques. Furthermore, the glass needle microdissection method cannot avoid physical contact with target chromosomes [3, 18], which has an effect on the specificity of the probe and results in genetic material damage [5].

The LMM method allows to microdissect the multiple whole target chromosomes or subchromosomal regions without any physical contact. Thus, this method can reduce the risk of contamination from unwanted chromosome fragments [13]. In this study, we evaluated and optimized the LMM method, and generated 24 WCP probes for each human chromosome, two region-specific probes (9q31.1→qter region and 22q12.1→qter region). We also characterized a marker chromosome using a probe generated by the LMM method followed by the reverse FISH technique.

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MATERIALS AND METHODS

Preparation of Metaphase Chromosomes

Metaphase slides from a human lymphocyte culture were prepared by standard protocol [15]. The slides were stored at −20°C until used. Slides were stained by G-bands by trypsin using Leishman (GTL) prior to microdissection.

Preparation of Coverslip for Laser Microbeam Microdissection

In order to collect target chromosome fragments easily, two types of membranes were used; PEN membrane (1.35 μm thin polyethylene; P.A.L.M. GmbH, Germany) and POL membrane (1.00 μm thin polyester membrane; P.A.L.M. GmbH, Germany). The former was used for generating whole chromosome painting probes, and the latter was used for region- or band-specific probes. A few drops of 0.1% poly-L-lysine (w/v) (Sigma, St. Louis, U.S.A.) were applied onto the coverslip (76×26 mm), and the membrane was mounted on the coverslip and wiped with filter papers to facilitate membrane mounting without wrinkles. The coverslips were exposed to UV (254 nm) for 30 min for decontamination. The metaphase preparation was made on the membrane side of the coverslips.

Laser Microbeam Microdissection (LMM)

Microdissection of a target chromosome was performed with a commercially available UV-Laser microbeam system, Robot-MicroBeam (P.A.L.M. GmbH, Germany), attached to an inverted microscope under ×100 oil immersion lens. Every microdissection procedure was controlled with a computer system and monitored with CCD camera images. The laser microbeam cut around the target chromosome with 75–80 energy level (0.4 μJ/pulse). For isolating, the laser was focused slightly below the membrane. The isolated chromosome-membrane was ejected from the object coverslip with a single laser-beam with 100 energy level (0.5 μJ/pulse) and catapulted onto a collection device (cap of 0.5-ml tube).

Degenerate Oligonucleotide-primed Polymerase Chain Reaction (DOP-PCR)

To amplify the dissected genetic materials, the degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) was performed as described by Telenius *et al.* [14] with 5 to 10 copies of a whole chromosome or a band region of a chromosome. To determine the adequate copy number of target chromosome for the PCR template, we selected a middle-sized human chromosome (chromosome 12) as a standard, and 5 to 50 copies of chromosome 12 in series with increasing 5 copies were dissected and DOP-PCR amplified. The PCR products were examined in 1% agarose gel electrophoresis.

Nick-Translation and FISH Analysis

One μg of PCR product was labeled with spectrumGreen or spectrumRed-16-dUTP (Vysis Inc., Downers Grove, U.S.A.) using a nick-translation labeling kit (Roche, Germany). Labeled products were recovered by ethanol precipitation, resolved in hybridization solution (50% formamide, 2× SSC, 10% dextran sulfate), and 200 ng of labeled product was hybridized to a normal metaphase slide for overnight. The slide was washed and counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). FISH signals were captured by a Zeiss Axioskop epifluorescence microscope with an integrating CCD camera and analyzed by the CytoVision system (Applied Imaging Corp., San Jose, U.S.A.).

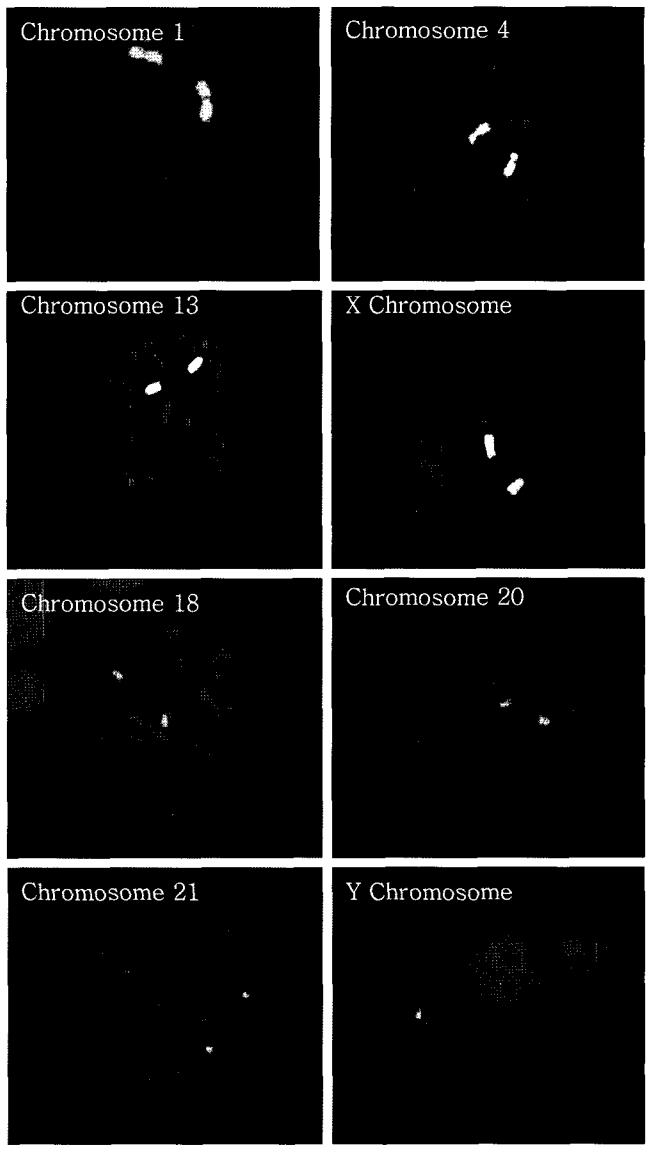

Fig. 1. Some examples of whole chromosome painting probes generated by the laser microbeam microdissection method. The chromosome number of each probe is indicated.

RESULTS

DOP-PCR products showed smearing patterns in the 200-bp to 800-bp range on 1% agarose gel electrophoresis. In the standardizing procedure with chromosome 12, the amount of DOP-PCR products increased with increasing copy numbers of targets up to 20 copies; however, there were no differences between 20 and 50 copies (data not shown). Although as little as 5 copies of the chromosome was enough to generate the FISH probe, we routinely used up to 25 copies of chromosome as a standard protocol.

Whole chromosome painting probes for 24 human chromosomes were generated by microdissection of 25 copies of each chromosome and successfully hybridized to the target chromosomes. Some of the FISH results are shown in Fig. 1. The band-specific probes encompassing 9q31.1→qter and 22q12.1→qter were generated with dual colors. These dual-color probes were applied to normal metaphase (Fig. 2A) and chronic myeloid leukemia (CML) patient metaphase with Philadelphia chromosome (Fig. 2B). The fluorescent signals were as bright and clear as commercial probes.

Reverse painting FISH with probes generated from microdissection of a marker chromosome was carried out. The subject was a 5-year-old boy with multiple congenital anomalies and mental retardation, and his karyotype was 47, XY, +mar on routine G-band analysis. The marker chromosome looked like the short arm of chromosome 11. Ten copies of marker chromosomes were isolated with the LMM method and a FISH probe was generated. When this home-made FISH probe was hybridized to normal metaphases, it was hybridized on the short arm of chromosome 5 (5p11→pter) (Fig. 3B). He was confirmed to be a 5p trisomy patient.

DISCUSSION

The FISH analysis is a powerful technique to identify both complicated structural chromosome rearrangement and numerical aberration [4, 10, 11, 17]. WCP probes for all 24 human chromosomes and locus-specific identifying (LSI) probes for several microdeletion syndromes and hematological malignancies have been developed and are commercially available. Although commercial probes have been used widely, generating custom-made FISH probes is still an open question in many molecular cytogenetic laboratories.

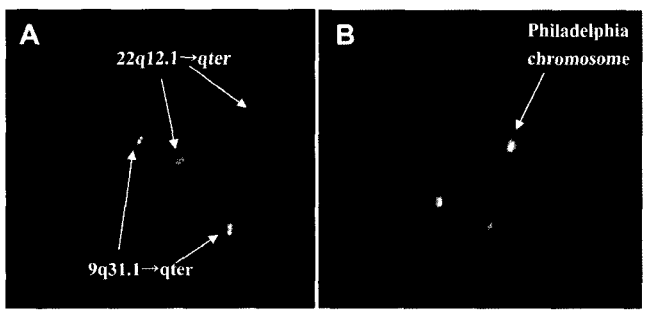

Fig. 2. Band-specific probes generated by the laser microbeam microdissection method.
Dual-color FISH analysis with 9q31.1→qter (Green) and 22q12.1→qter (Red) on normal metaphase (**A**) and CML patient metaphase with Philadelphia chromosome (**B**).

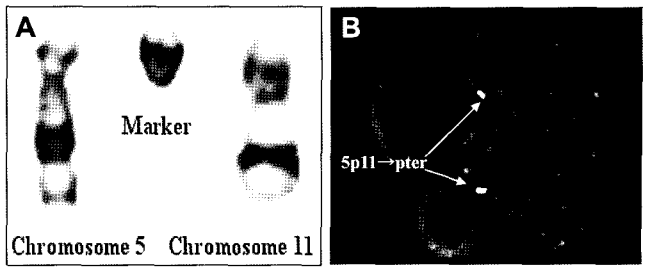

Fig. 3. Identification of a marker chromosome by combined reverse FISH.
A marker chromosome found in conventional G-banding analysis (**A**) and reverse painting FISH analysis (**B**). FISH probes made from microdissected marker chromosomes were hybridized onto the short arm of chromosome 5 (5p11→pter).

Chromosome microdissection is one of the most useful methods for generating a FISH probe. Under a high-power inverted microscope equipped with a micromanipulator and glass needles, several copies of a whole chromosome of interest or a small region of a chromosome can be dissected and collected. In general, the glass needle method needs more number of chromosome copies to generate the FISH probe, not only because of a high risk of chromosome loss during transferring the dissected chromosome to the PCR tube but because of a destruction of genetic materials from unavoidable physical contacts [13]. Compared with the conventional microdissection method with a glass needle, the LMM method provides easier access, finer dissection, and faster recovery of the target chromosomes without any damage of genetic materials and contaminations.

Once the chromosome microdissection and collection have been performed successfully, the next important step to generate a FISH probe is the amplification of target sequences, which has usually been achieved by DOP-PCR. In almost all previous reports, proteinase K and/or topoisomerase I pretreatment have been used to facilitate the amplification of the microdissected chromosomal DNA. However, these additional pretreatment procedures may increase the risk of contaminations from extraneous DNA [3]. In this study, we employed rapid isolation of a target chromosome by the LMM method, which is automatically controlled and monitored by a computer-based system. Furthermore, direct DOP amplification using *Taq* polymerase instead of sequenase could reduce the time and effort, and the risk of contaminations.

Considering the variable size of chromosomes, 10 copies of any small target chromosomes such as a G-group chromosome or small marker chromosomes could be a

sufficient source of FISH probes, although we routinely used up to 25 copies of each chromosome to make WCP probes for human chromosomes.

The reverse painting FISH technique, wherein the probe is directly generated from an abnormal chromosome and hybridized to a normal metaphase slide, is a very useful method for the identification of marker chromosomes. We practically applied the LMM, simplified DOP-PCR, and reverse FISH method to characterize a small marker chromosome. The marker was identified on conventional G-banding analysis. Its size was similar to the G-group chromosome and it looked like a short arm of chromosome 11 (Fig. 3A). From the reverse FISH analysis, the marker was characterized as the short arm of chromosome 5 (5p11→pter) (Fig. 3B).

Furthermore, we generated the band-specific probes encompassing 9q31.1→qter and 22q12.1→qter with dual-color fluorochromes. Although the probes were not characterized finely on the molecular level, the fluorescent signals were as bright and clear as commercial probes when applied to metaphase preparations of a CML patient with the Philadelphia chromosome (Fig. 2B).

In conclusion, we have demonstrated that the LMM method is a very convenient and reproducible method to recover the genetic materials from metaphase preparations. We optimized the chromosome microdissection procedures and reverse FISH protocols with significantly reduced time, effort, and the risk of contamination. This simplified approach for generating diverse FISH probes by the LMM method would complement the limitation of conventional cytogenetics of human genetic diseases, including malignancies with complex chromosomal alterations. The LMM method can be widely used for isolating genetic materials from any other fixed preparations including cancer tissues.

Acknowledgment

This work was supported by grant No. R01-2004-000-10646-0 from the Basic Research Program of the Korea Science & Engineering Foundation. S. H. Shim and J. H. Kyhm equally contributed to this paper

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