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# Universal Fluorescent Labeling (UFL) Method for Automated Microsatellite Analysis

Masashi Shimizu,[1,2] Natsuki Kosaka,[1,2] Takashi Shimada,[1,2] Takemitsu Nagahata,[1] Hironori Iwasaki,[1] Hisaki Nagai,[1] Tadayoshi Shiba,[2] and Mitsuru Emi[1,*]

*Department of Molecular Biology, Institute of Gerontology, Nippon Medical School, 1-396 Kosugi-cho, Nakahara-ku, Kawasaki 211-8533, Japan*[1] *and Department of Molecular Biology, Kitasato University, 1-15-1 Kitasato, Sagamihara 228-0829, Japan*[2]

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**Abstract**

We have devised a novel method for automated microsatellite analysis using "universal" fluorescent labeling. This system is based on polymerase chain reactions driven by sequence-specific primers and a reporter primer labeled with a fluorescent dye at its 5′ end. The forward sequence-specific primer is designed with a tag region bearing no homology to any human genomic sequence. Complementary tag sequences act as templates for the 6-carboxyfluorescein-labeled reporter primer, and those products can be analyzed with an autosequencer. The results we achieved with this assay system were consistent with the results of conventional assays using radioisotope-labeled primers, and diagnosis required less time. Furthermore, the fluorescent-labeled reporter primer is "universal" in that it can be used with different sequence-specific primers designed to carry the appropriate tag sequence at their 5′-ends. Our observations suggest that the "universal" fluorescent labeling method is an efficient tool for analyzing sequence variations in human DNA.

**Key words:** genomic alteration; fluorescent labeling; tagged primer; microsatellites

## 1. Introduction

Genome-wide analysis for DNA sequence polymorphism is a potentially effective approach to identifying genes involved in various diseases or for pinpointing distinct chromosomal areas that harbor genes contributing to carcinogenesis. Methods for detecting human genomic polymorphisms have generally invoked restriction fragment length polymorphisms (RFLPs), variable number of tandem repeat loci (VNTRs), and more recently, microsatellites. RFLP analysis of genomic alterations, which involves radioactive labeling, has many drawbacks such as the length of time required to produce results, the expense of restriction enzymes and isotopes, the possible ambiguity of results, safety aspects associated with the use of isotopes, artifacts due to incomplete digestion, and a high frequency of uninformative (homozygous) cases. Microsatellites, on the other hand, contain multiple copies of short, tandemly repeated sequences and tend to exhibit length polymorphisms[1,2] that make microsatellites useful as markers in linkage analyses, i.e., studies of the tendency of specific alleles of certain genes to be inherited together in families. Microsatellites occur throughout the genome and are highly informative. Genomic sequences containing microsatellites can be amplified from sample DNAs by polymerase chain reactions (PCRs) in which one of the primers is $^{32}$P-labeled; the PCR products are fractionated on denatured polyacrylamide gels and rendered visible by autoradiography. Primers labeled with fluorescent dyes are a common alternative for safety reasons, and are convenient for automated DNA sequencing. Fluorescently labeled primers are stable and safe, but a specific primer must be synthesized separately for the detection of any given microsatellite, although some preliminary use of tags and universal sequences, such as FITC-labeled M13 primer, has been carried out.[3]

In the present study, we established a novel assay system based on universal fluorescent labeling (UFL) for detection of microsatellite markers. We compared the UFL method with a conventional assay using radioisotope-labeled primers, with consistently good results.

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* To whom correspondence should be addressed. Tel. +81-44-733-5230, Fax. +81-44-733-5192, E-mail: memi@nms.ac.jp

## 2. Materials and Methods

### *2.1. Preparation of genomic DNA*

Breast-tumor specimens were obtained from the Cancer Institute Hospital, Tokyo after informed consent in the formal style of the hospital was obtained from each patient prior to surgery. Tumors and corresponding non-cancerous tissues were excised, frozen immediately, and stored at −80°C. Genomic DNAs were extracted from the frozen materials using QIAamp DNA Mini Kits (QIAGEN K. K., Tokyo) according to the manufacturer's instructions.

### *2.2. Microsatellite analysis using tagged forward primers and a fluorescently labeled reporter primer*

Tagged primers were designed by linking a tag sequence, 5′-GGTGGCGACTCCTGGAG-3′, to the 5′-end of the forward primer designed for each of three microsatellite markers on human chromosome 1. This tag sequence was derived from base sequences of λgt11, which does not hybridize to any sequence in the human genome. To detect the PCR products, we labeled the 5′-end of the tag sequence with fluorescent dye (FAM). We used this fluorescent-labeled primer as the reporter primer, i.e. the base sequence of the reporter primer is 5′-6-carboxyfluorescein (FAM)-GGTGGCGACTCCTGGAG-3′. Microsatellite sequences in the specimen DNAs were amplified by PCR using 10 ng of genomic DNA, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM deoxynucleotide triphosphate, 5 pmol each of tagged forward primer and FAM-labeled reporter primer, 10 pmol of reverse primer, and 0.5 units of Taq polymerase in a total volume of 20 μl. Cycling conditions were 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, with a final extension step of 3.5 min at 72°C in a Gene Amp PCR 9600 System (Perkin-Elmer, Norwalk, CT). PCR products were electrophoresed according to the manufacturer's instructions for the ABI Prism 377 system (Applied Biosystems, Foster City, CA), and the data were analyzed using GeneScan Analysis Software version 2.1 (Applied Biosystems).

### *2.3. Microsatellite analysis using radioisotope-labeled primers*

DNAs from matched tumor and non-cancerous tissues were examined for polymorphism with respect to three microsatellite markers, D1S1612, D1S1597, and D1S552, that we selected from a comprehensive genetic map of the human genome.[4] Each microsatellite sequence was amplified by PCR using 10 ng of genomic DNA, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphate, 2 pmol each of [$\gamma$-$^{32}$P]ATP-end-labeled forward primer and unlabeled reverse primer, and 0.25 units of Taq polymerase in a total volume of 10 μl. Cycling conditions were the same as above, but the PCR products were electrophoresed in 0.3-mm-thick denaturing 6% polyacrylamide gels containing 36% formamide and 8 M urea at 2000 V for 2–4 hr. After transfer of the gel patterns to filter papers, the filters were dried at 80°C and exposed to autoradiographic films at room temperature for 16–20 hr.

### *2.4. Definition of loss of heterozygosity*

In the case of radioisotope-labeled primers, signal intensities of polymorphic alleles were quantified by a Hoefer GS-300 scanning densitometer; peak areas corresponding to each signal were calculated by electronic integration using a GS-370 electrophoresis data system (Hoefer Scientific Instruments, San Francisco, CA). When FAM-labeled primers were used, signals were analyzed by the ABI Prism 377 system and GeneScan Analysis Software ver. 2.1 (Applied Biosystems). When the signal intensities of alleles of DNAs from tumor tissue were compared with those of DNAs from corresponding non-cancerous tissues, we interpreted a reduction in signal intensity of $> 50\%$ as loss of heterozygosity (LOH).

## 3. Results and Discussion

### *3.1. Principle of the UFL system*

Our novel system for the detection of microsatellites is based on PCRs driven by tailed sequence-specific primers and a fluorescent reporter primer. This principle is illustrated in Fig. 1. One of the two primers for conventional PCR, the forward primer, is designed to carry a tag sequence at its 5′-end that bears no sequence homology to any part of the human genome. Under appropriate conditions the tagged sequence-specific primer will anneal to its target sequences but the tag region will not. The annealed mixture is then incubated with Taq polymerase and four deoxynucleotide triphosphates so that regions of DNA downstream from the primer are selectively synthesized (step 1). At the next step, the extension product of the sequence-specific primer acts as a template for extension by a sequence-specific reverse primer (step 2). The reverse extension product will carry a complementary tag sequence at its 3′-end, so the reporter primer can anneal to this complementary tag sequence. Because the reporter primer does not contain any human genomic sequence, it anneals only to the extension product of the complementary sequence-specific primer (step 3). When the procedure is repeated, at least half of the complementary products of the tagged sequence-specific primer serve as templates for the tagged primer, and within a few cycles the predominant product is a single species of DNA fragment whose length corresponds to the distance between the two original primers. In practice, 30 cycles of reaction are required for effective amplification. Amplified products can be detected with a DNA autose-

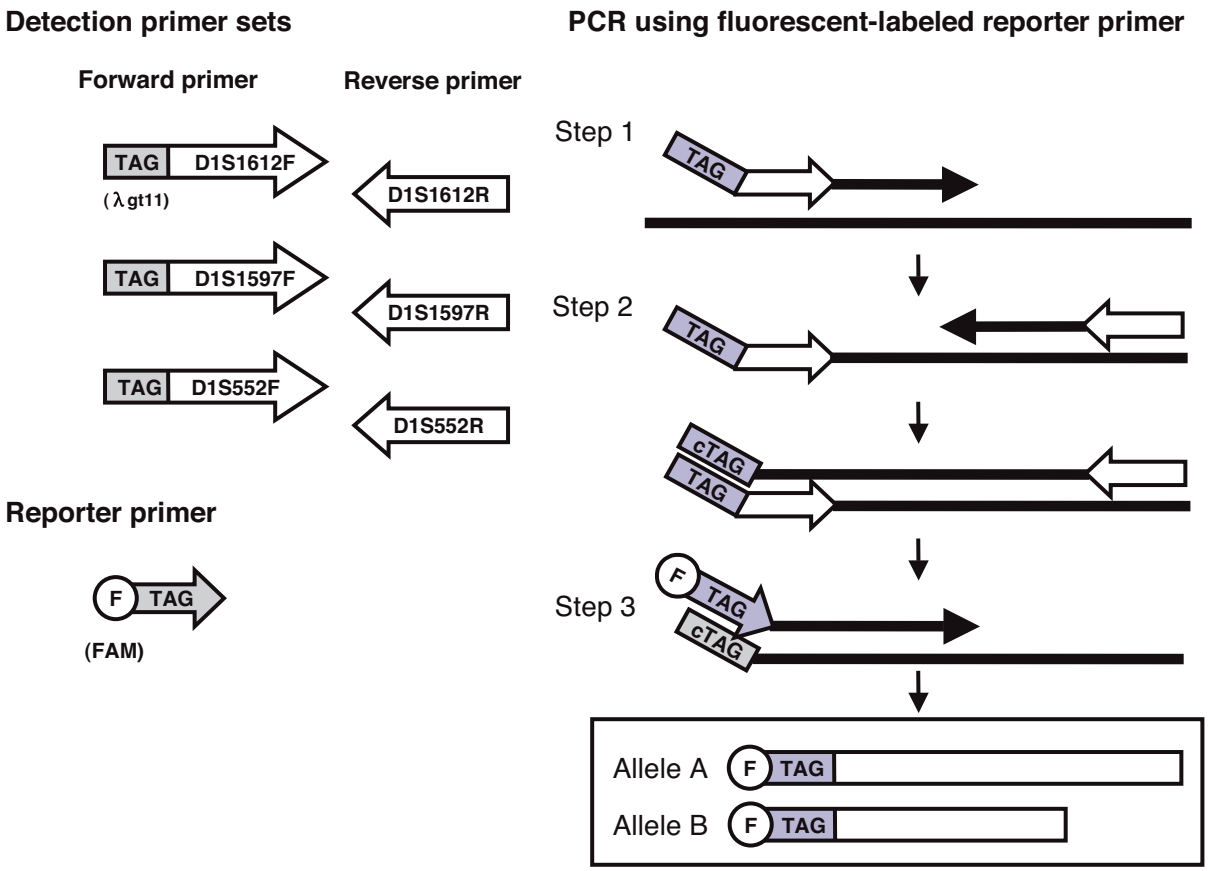


**Figure 1**. Principle of the assay using a fluorescently labeled reporter primer. The forward primer is designed with a tag sequence at its 5′-end, which bears no sequence homology to the human genome. The reverse extension product will have a complementary tag sequence at its 3′-end. The fluorescent reporter primer can anneal to the tag sequence, and because the reporter contains no human genomic sequence it anneals only to the complementary extension product of the sequence-specific primer.

quencer such as the ABI prism 377, because the 5′-end of the tagged primer is now labeled by fluorescent dye (FAM).

### *3.2. Analysis of microsatellite markers using tagged forward primers and FAM-labeled reporter primer*

We analyzed microsatellite markers at three different loci in DNA from paired normal and tumor DNAs, using tagged forward primers and the FAM-labeled reporter primer. Representative electrophoretic patterns are displayed in Fig. 2A. To compare these results with a conventional method, we performed PCR using radiolabeled primers and the same samples (Fig. 2B). Both parts of Fig. 2 show that one allele was lost in the tumor of patient 47 and judged as LOH. Retention of both alleles was observed in patient 41, and homozygosity (non-informative) was observed in patient 42. Both methods achieved identical results.

Data from fluorescent spots on the gels were automatically analyzed by the GeneScan Analysis Program at the end of each run. Figure 3 shows the fluorescence intensity of each PCR product from a tumor and corresponding non-cancerous tissue. The faster-migrating of the two fluorescent peaks derived from tumor DNA in patient 47 was smaller than the retarded peak, indicating LOH (Fig. 3); the peak area representing the tumor was reduced to 22.8% of normal. Patients 41 and 42 show retained alleles and homozygosity, respectively. The assay was repeated three times, with the same results.

### *3.3. Multiplex PCR for analysis of microsatellite markers*

This novel assay system for the detection of microsatellites described here can compare different marker loci simultaneously, because one FAM-labeled reporter primer can be used with multiple sequence-specific primers that have been appropriately tagged at their 5′-ends. The multiplex PCR described here was performed using one FAM-labeled reporter primer with tagged primers for three separate markers on chromosome 1, and the corresponding reverse primers, in one reaction tube. Results are shown in Fig. 4, where the average PCR product size was 120 bp, 173 bp, and 250 bp for the D1S1612, D1S1597, and D1S552 loci, respectively. The differences

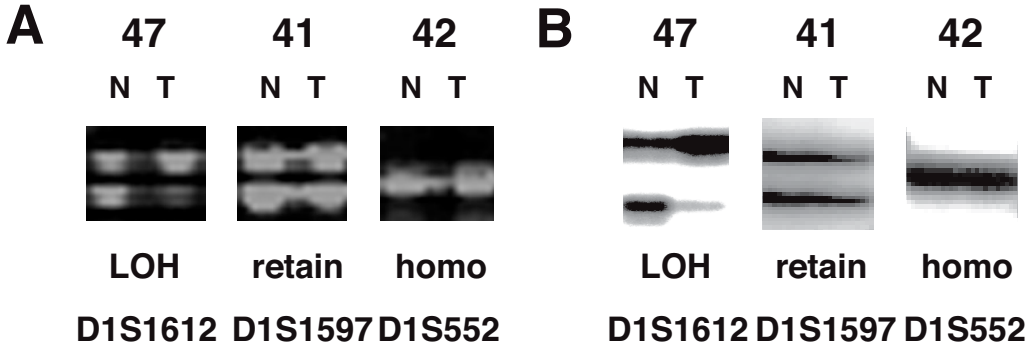


**Figure 2**. (A) Representative fluorescent electrophoretic patterns in the indicated regions of chromosome 1, analyzed with GeneScan. (B) Representative autoradiographic patterns when radiolabeled primers were used to amplify the same microsatellite markers. N and T represent normal and tumor DNAs, respectively, from the same patient in each case; homo, homozygosity.

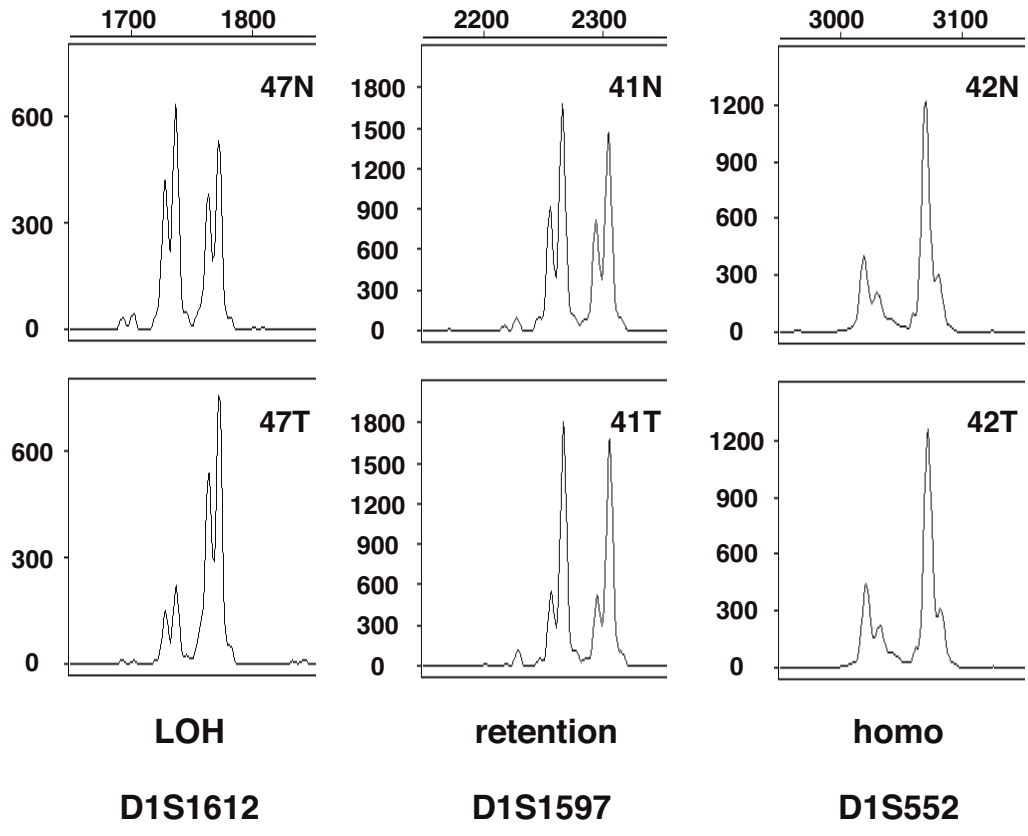


**Figure 3**. Representative electropherograms in the chromosomal regions indicated. N and T represent normal and tumor DNAs, respectively, from the same patients.

in size among these three microsatellites were sufficient for discrimination of the PCR products.

### *3.4. Summary and Conclusion*

Because microsatellites are tandem repeats of short (usually di-or tri-nucleotide) sequences scattered genome-wide and are highly informative as polymorphic markers, thousands of microsatellites have by now been mapped to specific chromosomes and used for diagnostic and research purposes including linkage analysis.[5,6] Current technologies involving the PCR and fluorescent DNA enable us to analyze microsatellites easily using a variety of genetic typing kits that are commercially available. Nevertheless, the high cost of synthesizing many different fluorescently labeled DNAs can be a problem. In the study reported here, we developed a rapid and inexpensive technique for detecting microsatellites, which can be

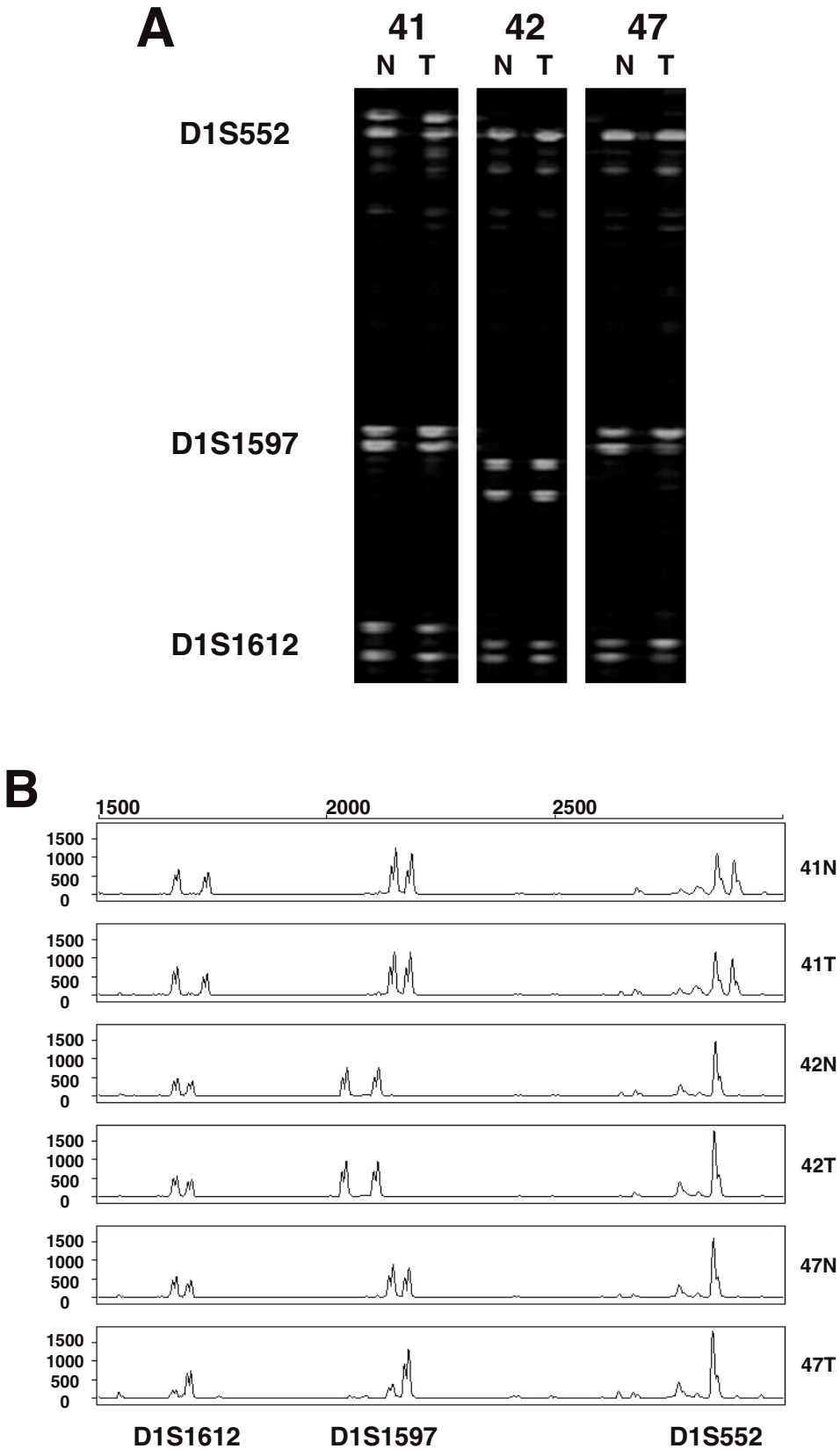


**Figure 4**. (A) Electrophoretic patterns of multiplex PCR products using fluorescently labeled primers in the chromosomal regions indicated. (B) Electropherograms of multiplex PCR products from the indicated marker loci.

applied to any number of primer pairs with results that are, in our experience, consistent with conventional methods. Using fluorescent technology to detect and analyze microsatellite markers makes it much easier to identify and quantify allelic bands automatically than is possible with the radioisotope-labeling method, and throughput is rapid because a DNA sequencer can analyze 96 lanes simultaneously. Moreover, as the fluorescent method of detection is so sensitive that only 1 $\mu$l of a PCR product needs to be run on the gel, the cost of the PCR reagents can be reduced significantly. Once a common fluorescently labeled reporter primer has been synthesized, it can be used for every pair of sequence-specific primers, i.e., appropriately tagged forward primer and corresponding reverse primer.

In summary, we have developed a novel UFL method for amplifying microsatellites in human genomic DNA, which can be applied not only to the detection of allelic loss, but also to linkage mapping and to discovery of genetic alterations such as allelic amplification. Present techniques for linkage mapping that use individual fluorescently labeled primer sets can be replaced by our UFL method.

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