Multiplex ligation-dependent probe amplification using a completely synthetic probe set

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The recent development of multiplex ligation-dependent probe amplification (MLPA) has provided an efficient and reliable assay for dosage screening of multiple loci in a single reaction. However, a drawback to this method is the time-consuming process of generating a probe set by cloning in single-stranded bacteriophage vectors. We have developed a synthetic probe set to screen for deletions in a region spanning 18.5 Mb within chromosome 3q. In a pilot study, we tested 15 synthetic probes on 4 control samples and on 2 patients previously found to possess a heterozygous deletion in the region 3q26–q28. These synthetic probes detected deletions at all previously known deleted loci. Furthermore, using synthetic probes, the variability of results within samples was similar to that reported for commercially available M13-derived probes. Our results demonstrate that this novel approach to MLPA provides a generic solution to the difficulties of probe development by cloning; such synthetically generated probes may be used to screen a large number of loci in a single reaction. We conclude that the use of synthetic probes for MLPA is a rapid, robust, and efficient alternative for research (and potentially diagnostic) deletion and duplication screening of multiple genomic loci.

INTRODUCTION

There have been several recent advances in PCR-mediated approaches to the detection of genomic copy number changes of multiple loci. Methods such as quantitative fluorescence PCR (QF-PCR) (1,2) and multiplex amplifiable probe hybridization (MAPH) (3) offer a considerable advantage over more established quantitative techniques such as Southern blot analysis and fluorescence in situ hybridization (FISH) because they require only small quantities of DNA and allow for multiple loci to be tested in a single reaction. A more recently devised method is multiplex ligation-dependent probe amplification (MLPA) (4), which has been found to afford cost-effective, reliable, and rapid screening of multiple loci for copy number changes. During MLPA, two sequence-tagged half-probes (one small, synthetic half-probe and one large, M13-derived half-probe) are hybridized to their genomic target sequence and ligated together at 54°C using thermostable DNA ligase. The ligated probes are then amplified by fluorescently labeled universal primers that correspond to the probes' sequence tags. Each probe is designed to be uniquely sized, resulting in a ladder of amplified products that can be visualized and quantified by automated fluorescent electrophoretic analysis. MLPA can currently screen at least 40 loci in one reaction with as little as 20 ng template DNA (4). Both MLPA and the related oligonucleotide ligation assay (OLA) techniques (5,6) utilize DNA ligase, which is exquisitely sensitive to DNA mismatches, to obtain the target specificity and discrimination difficult to achieve by PCR alone. Both techniques have been used to genotype multiple single nucleotide polymorphisms (SNPs) (4,6,7), whereas MLPA can also be used to screen for dosage variation such as the identification of large deletions in the *BRCA1* gene (8) and expression profiling of up to 45 signaling pathway transcripts by reverse transcription MLPA (RT-MLPA) (9). However, for assays in target region(s) for which there are no existing probe

Table 1. Probes Used for Multiplex Ligation-Dependent Probe Amplification (MLPA) Analysis of 3q Region

Probe No.	Size	Position (UCSC)	P1	P2	5′ Half-Probe Sequence	3′ Half-Probe Sequence
1	117	3q: 176952180–297	?	?	5′-GTGTGATTTGCATTAACAATAT-CATTGGTAACC**A**-3′	5′-**T**GGTGAAGTGAATTGTCCACATTTGGTGT-GAGATCACTGTG-3′
2	97	3q: 178811796–822	?	?	5′-CAAGGAGGTGAGTCAAGCACTT-GCT**A**-3′	5′-**A**TAATGGTAGAGAGGGAGGATTGCTGGTC-TAG-3′
3	121	3q: 182751280–315	-	-	5′-GCGCCTGGGCGCCGAGTG-GAAACTTTTGTCGGAG**A**-3′	5′-**C**GGAGAAGCGGCCGTTCATCGACGAGGC-TAAGCGGCTGCGAGCG-3′
4	124	3q: 185428242–286	-	-	5′-CCTCTACTCCCACCCCCAC-TACCTCTGGGAACCACAGCTC-CAC**A**-3′	5′-**A**GGGGGAGAGGCAGCTGGGCCAGACCGAG-GTCACAGCC-3′
5	101	3q: 189132773–874	-	+	5′-GGAGTAAGTTCTTCCCCTAAT-GATTG**T**-3′	5′-**G**AAGCCCAAGGCAAGTATATAAAATGAGA-CCC-3′
6	105	3q: 191448724–47	+	+	5′-GACACAAGGGTGTAAAATG-CAC**G**-3′	5′-**T**TTCAGGGTGTGTTTGCATATGATTTAAT-CAATCAGTATG-3′
7	109	3q: 182014040–78	-	-	5′-GCTGAGTCTCAGAGCAGA-CAAAGAAACCTCCCAAGGG**A**-3′	5′-**A**ACTTTGGCTAAAAACAAGAAAGAAATGG-3′
8	113	3q: 184217895–8008	-	-	5′-GAGGAGCCCGAGGGGTGTCCT-GGGTGCGCGCCTAGCTCCG**C**-3′	5′-**A**CGGGGGACCTCGGAGCTGCTCTAAGGC-GC-3′
9	128	3q: 184868265–313	-	-	5′-GGAGGTGGCTCTAAGTAAAACT-GGGATTGGACAGTAGTGGTG-CATCTGGTC**C**-3′	5′-**T**TGCCGCCTGAGAGCCCCAGGAGACATCG-GCTAG-3′
11	136	3q: 185210004–59	-	-	5′-CCTAAAGCTGTAGTCGCCTC-CAATAGCCATCCATGCCATCCCT-GCCTGTGCCTA**G**-3′	5′-**A**TCAGAGGCCCAGAGGGCCCCCTCAGTT-GCCTGAGCAGC-3′
13	144	3q: 186650385–1347	-	-	5′-CAAAGGCTGGGGGGAAGAG-CAAAATCACGGAGTCAAAACAACT-CAGTTTGTTCCAAAACAA**T**-3′	5′-**A**TAGACTTGTTGAAACAAGGGGACCTCTAT-GTTCATTGAG-3′
16	156	3q: 188313702–858	-	-	5′-GTGAGGTGGGGGAGTCT-GAGCCATTCTCAGGCCAAA-CAGTGCCAATCCTCCCTG-GCTCTCCCTCCCA**G**-3′	5′-**A**CACAATTCCAGACTCTTCCCTCCCAC-CCCACCCCCCAAGCCCAGC-3′
17	140	2p: 12401742–802	+	+	5′-CAATACTTCTCTGCTGCATAT-ATTTCTGTAGTTTATGACCCTT-GAATCAAATGAAACCT**A**-3′	5′-**C**CGTGCTTTCCTGGTGTTGGTTAACTG-GCCTAGAAG-3′
18	147	17q: 48647980–8033	+	+	5′-GTGCAGCCATCTGTAGAGAGA-CCTGGACTGGGCATGAAGGGT-CAGACACCCC**T**-3′	5′-**G**GGCAGAGGGTGAGATTAAGCCAGG-GGCAGTTGGGGAAGGGGGCTCTTTCAG-3′
19	143	195176776–826	+	+	5′-GGTTTGCTTTCCTCATTCCCAAC-GGGGCCTTCGCGCACAGCG-GCCCTGTC**A**-3′	5′-**T**CCCCGTCTACACCAGCAACAGCG-GCACCTCCGTGGGCCCCAACGCAGTG-3′

The 5′ half-probes are preceded by the 5′ universal primer tag (see Materials and Methods). The 3′ half-probes are preceded by a 5′ phosphate group and followed by the 3′ universal primer tag as described in Materials and Methods. Probe sizes (in nucleotides) show the total length, inclusive of universal primer tags. Nucleotides adjacent to the ligation site are indicated in bold type. Minus and plus signs indicate the deleted and nondeleted loci of the patients (P1 and P2), respectively; and a question mark indicates that the deletion status was unknown before this study.

sets, the main disadvantage of MLPA is the time-consuming process of creating a library of half-probes by cloning into a family of M13 phage vectors.

A recent publication described two individuals with deletions in the region 3q26–q28, which is associated with anophthalmia and microphthalmia (10). These two individuals had overlapping deletions in the same region of chromosome 3, with a common deleted region estimated to be 6.7 Mb in size at the time. In addition to eye abnormalities, both patients displayed craniofacial malformations and other dysmorphic features.

To investigate such deletions, without the laborious probe-cloning process required for conventional MLPA, we created a completely synthetic MLPA probe set up to 156 nucleotides in size that corresponded to multiple loci in this region and tested them on DNA from these patients. The use of synthetic probes has been successfully demonstrated in a similar multiplex technique that couples ligase detection reaction and

PCR (LDR/PCR) for mutation detection and to assess gene copy number in tumor cell line DNA (11,12). This study represents a novel extension of synthetic probe use in the context of MLPA analysis to assess target copy number.

MATERIALS AND METHODS

Probes and Oligonucleotides

All patient and control DNA were donated or obtained from Guy's Hospital. Fifteen uniquely sized synthetic probes were designed to investigate the deleted region (Table 1). Nine synthetic probes (3–5, 7–9, 11, 13, and 16) were designed to lie between 182 and 189 Mb on chromosome 3 from the ENSEMBL database (http://www.ensembl.org/datasearch.html), covering the largest known deletion, that in patient 1. Two probes (6 and 19) were designed to be distal to the deletion, one of which (probe 19) was known to be present by FISH analysis using bacterial artificial chromosome (BAC) clone 135A1 (GenBank® accession no. AC080129). The two probes proximal to the deletion (1 and 2) had an unknown copy number because the position of BAC clone 134F2, which had defined the proximal side of the deletion, had been revised. Finally, two control probes, 17 and 18, from chromosomes 2 and 17, respectively, were also included.

The synthetic 5′ or 3′ half-probes were designed to contain a unique target sequence plus the direct and complementary universal primer sequences at their 5′ or 3′ ends, respectively (Table 1). Each target sequence was designed to be of unique size, and, unlike conventional MLPA, contained no noncomplementary "stuffer" sequence in the 3′ half-probe. Genomic sequences were retrieved from ENSEMBL build number v17.33.11 (probes 18 and 19), v16.33.1 (probes 7, 8, 9, 11, 13, 16, and 17), and v14.31.1 (probes 1–6) (http://www.ensembl.org/Homo_sapiens/). Ideally, the GC content of the hybridizing sequence was chosen to be between 40%–60%; however, three of the probes (3, 4, and 5) had a GC content of approximately 65%, and one (probe 7) had a significantly lower GC content of 28%.

Each probe sequence was compared to the entire human genome using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/BLAST/) and BLAT at the University of California Santa Cruz (UCSC; http://genome.ucsc.edu/cgi-bin/hgBlat) to check its position and uniqueness (particularly across the ligation site). The probes were designed to avoid repeat sequences to improve the likelihood of obtaining a unique target site. Where synthetic probe sequences were not wholly unique, the two-nucleotide ligation site was chosen either in a unique area or where there was at least one mismatch across it. The probe sequence, especially across the ligation site, was checked against the SNP databases at NCBI and UCSC to minimize false-positive results. Finally, the ligation site was chosen to be flanked by no more than three guanine and/or cytosine bases to ensure efficient ligation (4).

Probe sizes were designed to avoid electrophoretic overlap and to differ by four nucleotides in length (inclusive of the universal primer tags) from their neighbors. A and T bases were never permitted to be adjacent to the universal primer tags because this can compromise amplification efficiency (4).

All half-probes except probe 17 were synthesized at a 0.2-µmol scale by Sigma-Genosys (Haverhill, UK), with the 3′ half-probes synthesized with a 5′ phosphate group essential for ligation. Purification of half-probes was performed by desalting, except for the larger half-probes, 11F (74 nucleotides), 13F (81 nucleotides), and 16F (87 nucleotides), which were polyacrylamide gel electrophoresis (PAGE)-purified to reduce contamination with incompletely synthesized oligonucleotides that might reduce reaction efficiency. Probe 17 was synthesized at a 200 nmol scale (5′ half-probe, PAGE-purified) and 40-nmol scale (3′ half-probe, no purification) by Biolegio (Malden, The Netherlands).

MLPA Reaction

MLPA kit reagent EK1 was obtained from MRC-Holland (Amsterdam, The Netherlands), and all reactions were

performed essentially as previously described by Schouten et al. (4), using a thermal cycler with a heated lid. Briefly, hybridization was performed using 0.5 µL of a 4-nM synthetic probe mixture with 200 ng of DNA overnight and then ligated. One quarter of the ligated probe mixture was used then for amplification, according to alternative protocol 2 (http://www.mrc-holland.com/mlpa_dna_protocol.htm), with universal 5′ N-(3-fluoranthyl)maleimide (FAM)-labeled primer (GGGTTCCCTAAGGGTTGGA) and the 3′ primer (TCTAGATTGGATCTTGCTGGCAC). One microliter of the products was dissolved in 15 µL of deionized formamide, 0.2 nM GeneScan®-ROX 350 size standards, and 0.5 µL loading dye (all from Applied Biosystems, Foster City, CA, USA) and denatured for two minutes at 95°C. The products were electrophoresed on an ABI PRISM® 3100 Genetic Analyzer model capillary sequencer (Applied Biosystems) in the GeneScan mode. Analysis of the products was performed using GeneScan 3.7 and Genotyper® 3.7 software (Applied Biosystems) consecutively.

Detection of Copy Number

Calculation of copy numbers of each probe was adapted from Yau et al. (1). Each probe's copy number was expressed as a dosage quotient (DQ), where a value of 1.0 indicated the presence of two alleles, and values of 0.5 and 1.5 represented a heterozygous deletion or duplication at that locus, respectively. A category table was compiled in Genotyper (peak areas from split peaks were summed). These data were exported into a Microsoft® Excel® spreadsheet (version 97). The raw peak area values were divided by the average peak area values of the control probes (normally 17, 18, and 19, outside the deleted region) to normalize for any probe variability (probe normalization). To eliminate sample variation, the average of each probe's normalized peak area for all control samples (average control peak area) was calculated and used as a dividing factor for the normalized peak area value of each probe, giving the final DQ.

Statistical Analyses

All plots, bar charts, and correlation values were determined using Microsoft Excel (version XP) software. One-way analysis of variance (ANOVA) was calculated using software developed by T. Kirkman at the College of St. Benedicts (St. Joseph, MN, USA)/St. John's University (Collegeville, MN, USA) (http://www.physics.csbsju.edu/stats/).

RESULTS AND DISCUSSION

Fourteen of the fifteen synthetic MLPA probes produced fluorescent peaks within the required intensity range (between 100 and 6000 arbitrary units) (Figure 1). Probe 13 failed to generate any amplification product. The products showed consistent separation with repeated runs. All probes gave one major peak, with the exception of probe 17, which was split into 2 peaks, 1 nucleotide apart. This split peak could be the result of contamination of one or other of the constituent half-probe by incompletely synthesized oligonucleotide that lacked one nucleotide (*n*-1 oligonucleotides) in regions other than the ligation site. The occurrence of such contaminants has been documented (13), and in this case would result in the successful ligation and subsequent amplification of two probe 17 products one nucleotide apart. The split peak in probe 17 might have a greater proportion of *n*-1 contaminants than the other probes because it was synthesized by a different manufacturer. This was also observed in two other probes (data not shown) also synthesized by the same manufacturer. On average, the peaks appeared three nucleotides smaller than their designed size. This approximately consistent size difference may be due to the high negative charge-to-mass ratio of the dye (14).

Probe Quality

A previous report by Kwiatkowski et al. (15) highlighted the problems of partially synthesized contaminants in synthetic probes that lacked the terminal nucleotide required for ligation. Such incomplete oligonucleotides could compete for target sites but could not be ligated, thereby negatively affecting the signal strength of the final product. This was of particular concern with longer synthetic probes, which are more likely to contain a higher proportion of incomplete oligo-

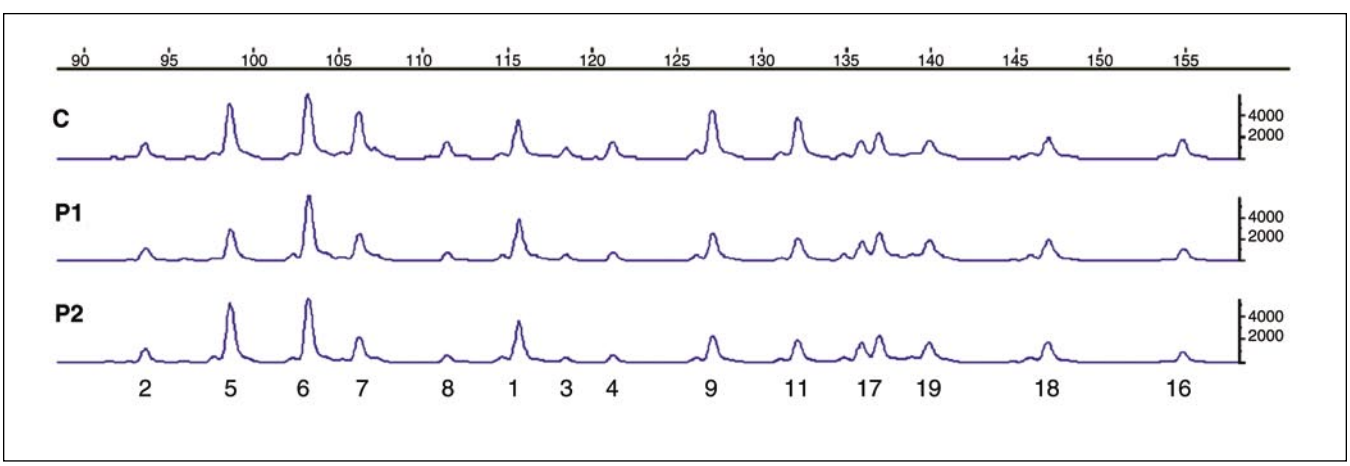

Figure 1. Electropherogram of synthetic probes using control DNA. Probes are indicated below their respective peaks. DNA size scale is shown above the trace. Peak intensity is indicated by the right-hand scale.

nucleotides. To test this, the average peak area of all 14 probes was taken for 4 control samples (56 DQ values in total) and then correlated to their respective sizes. This revealed no statistically significant correlation [r = -0.208; P-(nondirectional] = 0.48) between probe size and signal strength. Further attempts at transforming the data to find other nonlinear correlations also failed to produce any significant relationship (data not shown). This suggests that probe size and purity have little effect on half-probe signal strength. These data corroborate those of Yeung et al. (16), who, in a real-time PCR assay with double-labeled probes, found that probes with at least 20% purity performed as efficiently as those near 100% purity. The explanation for this may be that there is a substantial excess of true half-probe over the partially synthesized competitors, resulting in a negligible difference in reaction efficiency. In addition, as the synthetic probes lack noncomplementary stuffer sequences, the hybridization strength of the longer probes would be enhanced, which might result in increased signal. Several factors have been reported to influence probe signal strength, including the concentration of target sites and the nucleotide composition at or near the ligation site and/or next to the primer tag sites (4). These factors may be more important in influencing signal strength than absolute probe size or purity.

It therefore seems feasible, with judicious design, to extend the probe set with even longer probes. Because the average size limit for a commercially produced oligonucleotide is approximately 130 nucleotides (http://www.sigma-genosys.com/oligo_faq.asp), it may be possible to extend the size of both 5′ and 3′ half-probes, thereby increasing the number of probes in the multiplex reaction.

Evaluation of Synthetic Probe Reliability

Probe DQ values within the 4 control samples ranged between 0.87 and 1.08, within acceptable limits defined empirically to be between 0.80 and 1.20 [standard deviation (SD) less than 10%] from previous repeated experiments. These limits are similar to those of Wallace et al. (http://www.ngrl.org.uk/Manchester/Pages/Downloads/Dosage/MLPA%20analysis%20spreadsheets%20instructions.pdf). However, ANOVA tests were carried out to check that the synthetic probes had equal DQ mean values, which held across different DNA samples. Comparing the DQ values of all 14 probes against each other in 4 control DNA samples revealed no significant difference between the synthetic probes (P = 0.14, see Table S1 at http://www.BioTechniques.com/September2004/SternSupplementary.html). This was reflected by the low standard deviation values of each sample, which ranged from 0.03 to 0.06. A second ANOVA test was carried out to compare how each probe's DQ value varied across

the 4 control samples. Again, no significant difference was found between the probes (P = 1.00, see Table S1), indicating the synthetic probes' DQ values were consistent across different DNA samples. Standard deviations of each probe ranged 2%–10%, which was comparable to those of commercially available cloned probes (4%–10%) (4).

Our experience has shown that to obtain good DQ values, high-quality DNA is of prime importance for assessing copy number change. This has also been reported by other workers (4,8), and, as such, represents a limitation of MLPA. In general, the greater the number of probes used for normalization, the smaller the probe variation across samples. Closely spaced probes across a target region, such that at least two probes are likely to show a change in DQ, is a prudent design strategy in the event of a novel SNP occurring or where samples of unknown quality are to be tested. Alternatively, an abnormal result from a single probe should be confirmed by a separate technique, such as FISH.

Deletion Detection with Synthetic Probe-MLPA

Analysis of the two patient samples revealed that probes 7, 3, 8, 9, 11, 4, 16, and additionally probe 5 in patient 1, yielded DQs ranging from 0.39–0.56, indicating a heterozygous deletion across this region (Figure 2A). No deletion was seen in probes 1 and 2, which are therefore presumably proximal to the deletion. This result was significant to the 0.001 level when patient and control DQs were analyzed by ANOVA (see Table S1). Furthermore, the loci of those deleted probes in the patients corresponded with those previously found by FISH analysis (10), as summarized in Figure 2, B and C. Thus, the synthetic probes accurately detected the known deletions in our two patients.

In conclusion, the use of synthetic probes for MLPA appears to be a robust, efficient, and reliable method for ascertaining copy number changes at multiple loci. The main limitation of this technique is the requirement for good quality and accurately quantified DNA (4,8), a factor that affects cloned and synthetic probes alike. The synthetic probes can be used in any combination and have produced reliable results alone and when multiplexed together with MLPA cloned probes (data not shown). In this study, only one fluorescent label was used, but the use of four fluorescent dyes, conjugated to different universal primers, may allow for many more loci to be analyzed in one multiplex reaction.

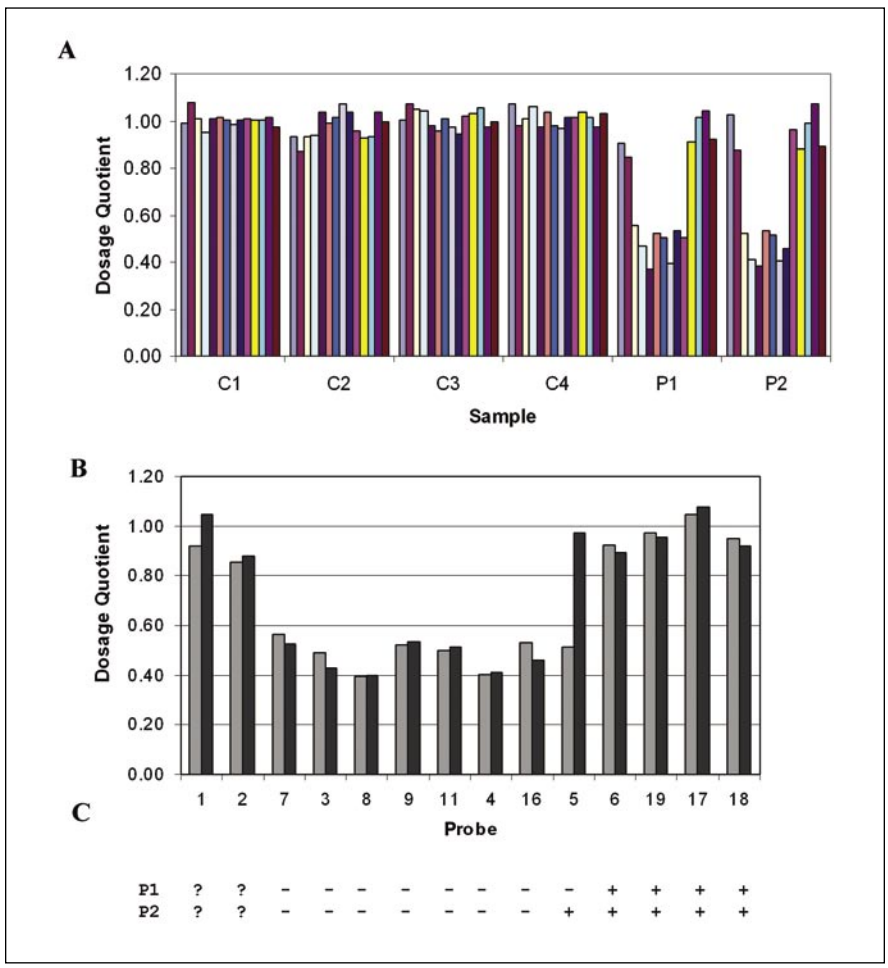

Figure 2. Quantitative multiplex ligation-dependent probe amplification (MLPA) analysis of control and patient DNA samples. (A) The comparison of control DNA dosage quotient (DQ) values (C1–C4) with those of the two patients (P1 and P2). Each probe is represented by a colored bar in genomic order (from left) 1, 2, 3, 7, 8, 9, 11, 4, 16, 5, 6, 19, 17, 18. (B) Data from deleted loci in patient DNA samples; deletions extend between probes 7 and 6 in P1 (gray bars) and 7 and 5 in P2 (black bars). (C) Summary of bacterial artificial chromosome (BAC) clone data previously established by fluorescence in situ hybridization (FISH) analysis (10) that correspond to the probe positions in the patients. Plus and minus signs indicate the nondeleted and deleted clones, respectively, and a question mark indicates loci of unknown deletion status by FISH (10).

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing interests.

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