

Method for preparing single-stranded DNA templates for Pyrosequencing using vector ligation and universal biotinylated primers

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Abstract

In Pyrosequencing, the addition of nucleotides to a primer–template hybrid is monitored by enzymatic conversion of chemical energy into detectable light. The technique yields both qualitative and quantitative sequence information because the chemical energy is released by a stoichiometric split off of pyrophosphates from incorporated deoxynucleotide triphosphates and a defined nucleotide dispensation order is given. Because Pyrosequencing works best if single-stranded DNA templates are used, template generation usually requires PCR with a target-specific biotinylated primer and a subsequent purification involving interaction of the biotin label with immobilized streptavidin. To circumvent the need for numerous and expensive template-specific biotinylated primers, we developed a method that uses the ligation of amplified DNA fragments into a plasmid vector, thereby facilitating subsequent PCR using a universal vector-specific biotinylated primer. This approach allows easy and straightforward isolation of single-stranded templates of any PCR product. As a proof of principle, we used the method for genotyping two single-nucleotide polymorphisms in the human genes *CARD15* and *A2M* and for characterization of four multisite variations in the human *DEFB104* gene.

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The Pyrosequencing technology has been widely used for analyzing short DNA sequences (20–100 nucleotides), for example, in mutation screening and genotyping, clone checking, and species typing [1–3]. In contrast to most genotyping methods such as Sanger sequencing and single-base primer extension, this technique yields both qualitative and quantitative sequence information. It involves a “sequencing by synthesis” strategy in which the incorporation of nucleotides into a primer–template hybrid is monitored enzymatically [4]. Successfully incorporated nucleotides produce quantifiable signals, so that the peak height is proportional to the number of nucleotides added

to the nascent DNA template. Because nucleotides are dispensed individually in a defined dispensation order, both known and unknown template sequences can be determined. Furthermore, it has been shown that Pyrosequencing is suitable for determination of allele frequencies [5].

Although Pyrosequencing of double-stranded (ds)² DNA templates has been performed, the technique works best if single-stranded (ss) templates are used [6]. Those ss templates are routinely isolated by affinity methodologies. Usually, a PCR is performed with a biotinylated primer, followed by alkaline denaturation of the ds amplicon. Subsequently, the DNA strand with incorporated biotinylated

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² Abbreviations used: ds, double-stranded; ss, single-stranded; nt, nucleotides; SNP, single-nucleotide polymorphism; MSV, multisite variation; bt, biotin; bOligo, blocking oligonucleotide.

primer binds selectively to streptavidin-coated surfaces such as magnetic beads and chromatographic resins. In this way, ss templates are generated and the remaining pyrophosphate and excess amounts of unbiotinylated primer and dNTPs from PCR are removed and so do not interfere with the sequencing process. However, the use of biotinylated primers renders Pyrosequencing expensive, especially if one wants to study many sequence variations in different genomic regions requiring a sequence-specific biotinylated primer for each target. Furthermore, if sequence information of both DNA strands is desirable, two biotinylated PCR primers are necessary. Therefore, a method for preparing ss templates with a universal biotinylated primer suitable for any target will have clear advantages.

This problem has been addressed previously. For instance, Pacey-Miller and Henry [7] developed a PCR protocol in which an “11-base GC tag oligonucleotide” is used in a first PCR; the GC tag is targeted in a second PCR employing a GC tag-specific biotinylated primer. However, this approach might not be applicable for Pyrosequencing analyses in GC-rich regions or CpG islands characteristic of mammalian genomes. Mispriming of the GC tag could occur and would require the use of tags that do not interfere with the respective sequence context. Another method using a universal biotinylated primer protocol was described by Colella and coworkers [8]; it involves the use of three primers in one PCR, namely template-specific forward and reverse primers and one universal biotinylated primer. The template-specific reverse primer harbors at its 5' end 20 nucleotides (nt) that comprise the sequence of a universal biotinylated primer. This tag differs from the tag used by Pacey-Miller and Henry [7] in length (20 vs. 11 nt) and GC content (55 vs. 100%) and, therefore, can be used to analyze GC-rich regions. However, laborious optimization of the PCR protocol is needed; for example, ratios of all primers need to be adjusted. A likewise approach was designed by Guo and Milewicz [9]. Here, PCR-amplified DNA is labeled with biotin for Pyrosequencing and with FAM and HEX for microsatellite genotyping. Recently, Aydin and coworkers [10] introduced a universal biotinylated primer method similar to the approach of Pacey-Miller and Henry [7]. It involves a PCR using two target-specific primers and two different universal primers, one of which is biotinylated. Each target-specific primer harbors a sequence corresponding to one of the universal primers at its 5' end. In addition to requirements for template preparation, it is necessary for certain ss templates to prevent folding back of their endings; this entails self-priming and interferes with Pyrosequencing [11]. In conclusion, although published methods greatly reduce the number of biotinylated primers necessary for preparation of ss templates, they always are applicable to distinct analyses only. Thus, the development of a simple methodology would represent a cost- and time-efficient improvement for Pyrosequencing if many different targets need to be analyzed.

Based on the idea of Anderson and coworkers [12], who developed a universal method for sequencing of DNA frag-

ments cloned into an ss phage vector, we set out to optimize ss template preparation for sequence determination by Pyrosequencing. Our method involves two steps. In the first step, a PCR-amplified DNA target is ligated into a linearized DNA vector. In the second step, the recombinant DNA serves as a template for a PCR using one vector-specific biotinylated primer combined with a target-specific (unlabeled) primer. We applied this method to genotyping of two known single-nucleotide polymorphisms (SNPs) located in the human *CARD15* and *A2M* genes and for characterization of four multisite variations (MSVs) [13] in the human *DEFB104* gene, which maps to a segmentally duplicated region characterized by a high polymorphism rate [14,15].

Materials and methods

DNA

Genomic DNA was isolated from whole blood using the Blood & Cell Culture DNA Mini Kit (Qiagen, Hilden, Germany) according to standard protocols. Pooled genomic DNA isolated from human blood (buffy coat) was purchased from Roche. The 1 Kb Plus DNA Ladder was purchased from Invitrogen (Karlsruhe, Germany).

PCR

PCR was performed using 10 ng of genomic DNA and standard PCR protocols [16] in 96-well PCR plates (ABgene, Epsom, UK) containing 25 μ l reaction/well with ReadyToGo PCR beads (Amersham, Braunschweig, Germany). PCR primers were as follows: (i) for *CARD15* (forward: 5'-CAGCCATGTGGAGAACATGC-3'; reverse: 5'-CTGGGCAACATAGCAACAC-3'); (ii) for *A2M* (forward: 5'-AAGCACCATAAAAGGCGTTG-3'; reverse: 5'-TTTTCCAGAGACTGAGGCGT-3'); and (iii) for *DEFB104* (forward: 5'-TTCTGTAGCCCCAACACCTC-3'; reverse: 5'-GGTGCCAAGGACATCTAGGA-3'). PCR products were precipitated by adding 85 μ l of 96% ethanol. DNA was pelleted by centrifugation for 60 min at 3500 rpm and 4°C using an Eppendorf centrifuge 5810R and washed with 70% ethanol. Pellets were dried by evaporation using Savant SpeedVac Plus SC210A and dissolved DNA in 100 μ l of water.

Sequencing primers were as follows: (i) for *CARD15* *CARD15f* (5'-GTTGCCGATCTTCACACCGT-3'); (ii) for *A2M* *A2Mf* (5'-GACCTCAACTTTGTTTAGAG-3') and *A2Mr* (5'-TTCCACCCACACAAG-3'); and (iii) for *DEFB104* *DEFB104SNP1* (5'-CACACTGTGCCTTGTTGAG-3'), *DEFB104SNP2* (5'-GACTTGTGCTGCTATTAGCC-3'), *DEFB104SNP3* (5'-CCATTAGTAAATGCAGTATGTGG-3'), and *DEFB104SNP4* (5'-TCCTTGTAGTGCACCTTACAGC-3'). Individual genotypes of all analyzed SNPs were verified by Sanger sequencing of the PCR products using Dye Terminator chemistry (v3.1, Applied Biosystems) and PCR primers according to the

manufacturer's instructions. Universal primers were as follows: bt-f (5'-bt-GTAAAACGACGGCCAG-3') and bt-r (5'-bt-CAGGAAACAGCTATGAC-3') with biotin (bt) modifications at the 5' ends. Primers were purchased from Metabion (Martinsried, Germany).

Topoisomerase reaction

Ligation of PCR products into pCR2.1-TOPO vector was done with the TOPO TA Kit (Invitrogen) according to the manufacturer's protocol.

Pyrosequencing

Biotin-labeled PCR products were immobilized on streptavidin Sepharose (Biotage, Uppsala, Sweden) by mixing 20 μ l of the PCR product with 6 μ l streptavidin Sepharose suspension, 10 μ l water, and 40 μ l 1 \times binding buffer (Biotage), followed by shaking at room temperature for at least 10 min. To remove the unbiotinylated DNA strand, the samples were sequentially washed with 70% ethanol and 0.5 M NaOH using the PyroMark Vacuum Prep Tool (Biotage). Immobilized ssDNA was then washed with 1 \times washing buffer for 10 s, transferred to 40 μ l 1 \times annealing buffer plus 4 μ l target-specific sequencing primer (10 pmol/ μ l in water), and kept at 80 $^{\circ}$ C under continuous shaking for 10 min. After equilibration to room temperature, the sequencing reaction was performed using the Pyro Gold Reagent Kit (Biotage) in the PSQ 96MA Pyrosequencing instrument according to the manufacturer's instructions.

Results

Fig. 1 outlines our novel Pyrosequencing protocol. In contrast to previous protocols, it contains an additional step of ligating the PCR-amplified target DNA into a vector that bears at least one universal primer binding site. Without transformation of bacteria, the recombinant DNA is then used as a template in a second PCR employing a universal, vector-specific biotinylated primer and a target-specific unlabeled primer. After affinity purification of the biotinylated DNA strands, their respective 3' ends harbor the annealing site for the sequencing primer. In our approach, the target-specific PCR primer is also used as sequencing primer. Notably, its binding to the very 3' end of the ss template circumvents folding back of the template and thereby prevents possible self-priming; that is, the sequencing primer works simultaneously as blocking oligonucleotide (bOligo) [11].

In most plasmid vectors, universal primer sequences flank the multiple cloning site; for example, many vector systems contain M13 primer binding sites that work perfectly well for sequencing [17]. We used the plasmid pCR2.1-TOPO, a topoisomerase-activated T-overhang vector, provided in the TOPO TA Kit. This enabled the insertion of A-overhang PCR products within a few min-

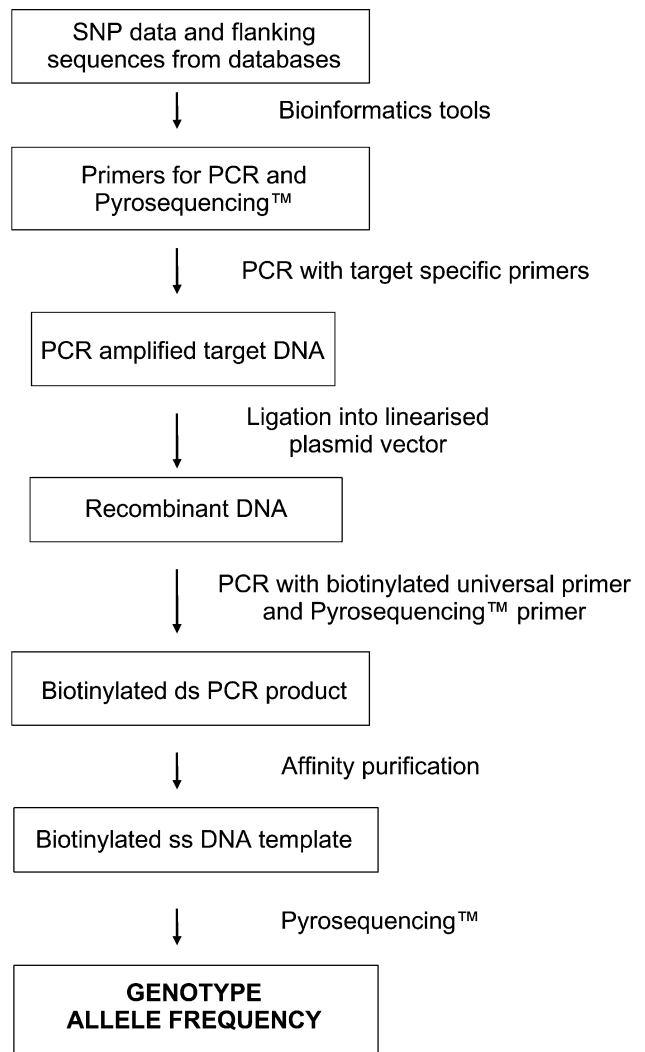


Fig. 1. Outline of the vector ligation/universal primer labeling method applied for the preparation of ss template for Pyrosequencing.

utes. The vector also bears M13 forward and reverse primer binding sites. Furthermore, the insertion of amplicons occurs statistically in either direction. Thus, there are four primer combinations possible in the second PCR (Fig. 2A), which entails amplification of different pools of recombinant DNA; either one of the biotinylated universal primers will work in combination with one of the respective sequencing primers (Fig. 2A, right panel), or one sequencing primer needs to be combined with each universal primer (Fig. 2A, left panel).

Using our method for ss template preparation, we performed genotyping by Pyrosequencing of two known SNPs located in the genes for caspase recruitment domain family, member 15 (*CARD15*, rs2067085), and α -2-macroglobulin (*A2M*, rs226379) in 10 individuals. In addition, allele frequencies were estimated in pooled genomic DNA. Fig. 2B depicts PCR products obtained by amplification of the SNP rs2067085 containing region (*CARD15*) in 4 individual samples and a pooled DNA sample. Both universal biotinylated primers were combined with the same target-

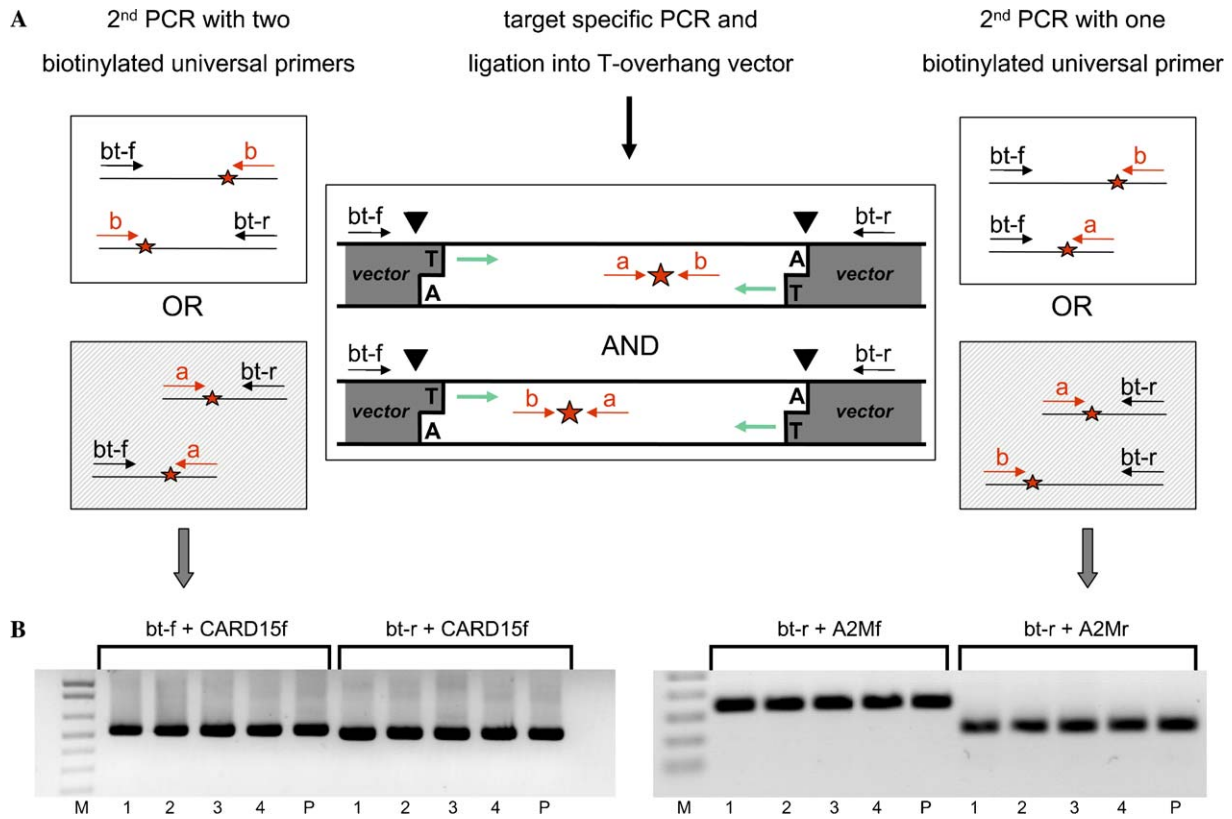


Fig. 2. General labeling procedure of DNA templates for Pyrosequencing using vector ligation and universal biotinylated primers. (A) Possible primer combinations for amplification of biotin-labeled DNA after bidirectional ligation of a PCR fragment into a T-overhang plasmid vector. Red star: SNP in the target sequence; black triangle: vector insertion site; green arrows: target-specific primers used in the first PCR; red arrows (a and b): second PCR and Pyrosequencing primer; black arrows: universal biotinylated forward (bt-f) and reverse (bt-r) primers. (B) Agarose gel electrophoresis of biotin-labeled PCR products generated for genotyping and determination of allele frequency: both strands of SNP rs226379 in the *A2M* gene using one universal biotinylated primer and two target-specific primers (right) and one strand of SNP rs2067085 in the *CARD15* gene using two universal biotinylated primers and one target-specific primer (left). The biotin-labeled PCR products correspond to the primer combinations shown in the shaded boxes above, which represent the schematic second PCR. Results are shown for 4 individuals (lanes 1–4) and pooled genomic DNA (P). M = 1 Kb Plus DNA Ladder.

specific primer (CARD15f, corresponding to the lower left box in Fig. 2A). The slightly different size of the amplicons is due to the different position of the vector primers with respect to the plasmid insertion site. Results of genotyping by Pyrosequencing of 10 individuals were identical in both reactions for each individual sample (Table 1); furthermore,

genotypes obtained by Pyrosequencing correspond with genotypes obtained by direct Sanger sequencing. Identical allele frequencies were obtained for both Pyrosequencing reactions in the pooled DNA sample, indicating that there is no bias in orientation of the inserted fragments. All allele frequencies are similar to those reported in the public data-

Table 1
Results of Pyrosequencing versus Sanger sequencing of PCR products from individual samples and pooled genomic DNA

	Sample										Pool
	1	2	3	4	5	6	7	8	9	10	
rs2067085 (<i>CARD15</i>)											
Sanger sequencing	GG	GG	GC	GC	GC	GC	GC	CC	CC	CC	n.a.
Pyrosequencing: CARD15f-bt-f	GG	GG	GC	GC	GC	GC	GC	CC	CC	CC	58.5% C 41.5% G
Pyrosequencing: CARD15f-bt-r	GG	GG	GC	GC	GC	GC	GC	CC	CC	CC	59.4% C 40.6% G
rs226379 (<i>A2M</i>)											
Sanger sequencing	GA	GA	AA	AA	GA	AA	GA	GA	GG	AA	n.a.
Pyrosequencing: A2Mf-bt-r	GA	GA	AA	AA	GA	AA	GA	GA	GG	AA	78.0% A 22.0% G
Pyrosequencing: A2Mr-bt-r	GA	GA	AA	AA	GA	AA	GA	GA	GG	AA	77.5% A 22.5% G

Note. n.a., not available.

base (dbSNP: Perlegen; AFD_EUR_PANEL: 0.417 for G allele).

Our approach also allows the analysis of both DNA strands of the target sequence with only one universal biotinylated primer. An example is shown in Fig. 2B; we analyzed SNP rs226379 in the *A2M* gene using one biotinylated universal primer (bt-r) in combination with two target-specific primers flanking the SNP (corresponding to the lower right box in Fig. 2A). Again, the results of Pyrosequencing are consistent for forward/reverse sequencing reactions in single individuals and were confirmed by Sanger sequencing. The allele frequencies determined for the pooled DNA are identical in the sequencing reactions and correspond with reports in the public database (dbSNP: Perlegen; AFD_EUR_PANEL: 0.25 for G allele).

In a third example, we characterized four MSVs located upstream, within, and downstream of the first exon of the *DEFB104* gene that lies in the major defensin gene cluster on chromosome 8p23.1. Gene and segmental duplications in the cluster entail extensive polymorphisms in this genomic region; that is, copy numbers of this gene may vary in individual chromosomes, and these copies may harbor

different alleles [15,18–20]. Thus, for a given sequence variation in *DEFB104*, allele ratios may vary considerably. This is demonstrated by direct Sanger sequencing of PCR products containing SNP rs2680507 (Fig. 3). Association studies using conventional qualitative genotyping methods of SNPs in those regions, therefore, are error prone or likely to fail [21]. A quantitative approach similar to allele quantification in pooled DNA samples seems to be a better way to determine individual genotypes of SNPs located in segmental duplications [13]. Therefore, we amplified a 500-bp fragment containing four MSVs (rs17843871, rs2680507, rs17843872, and rs4259430) of *DEFB104* [15] from genomic DNA of 2 individuals. The respective amplicons were ligated into plasmid pCR2.1–TOPO, and subsequently four PCRs using target-specific primers in combination with one universal biotinylated primer (bt-r) and recombinant DNA as template were performed; the resulting fragments are shown in Fig. 4B. We then determined allele frequencies for each MSV (Fig. 4A and Table 2). As is typical for MSVs, these frequencies may vary in the same individual. For example, the respective minor allele frequencies in individual 2 are 38.9% C for rs17843871, 40.6% A for rs17843872,

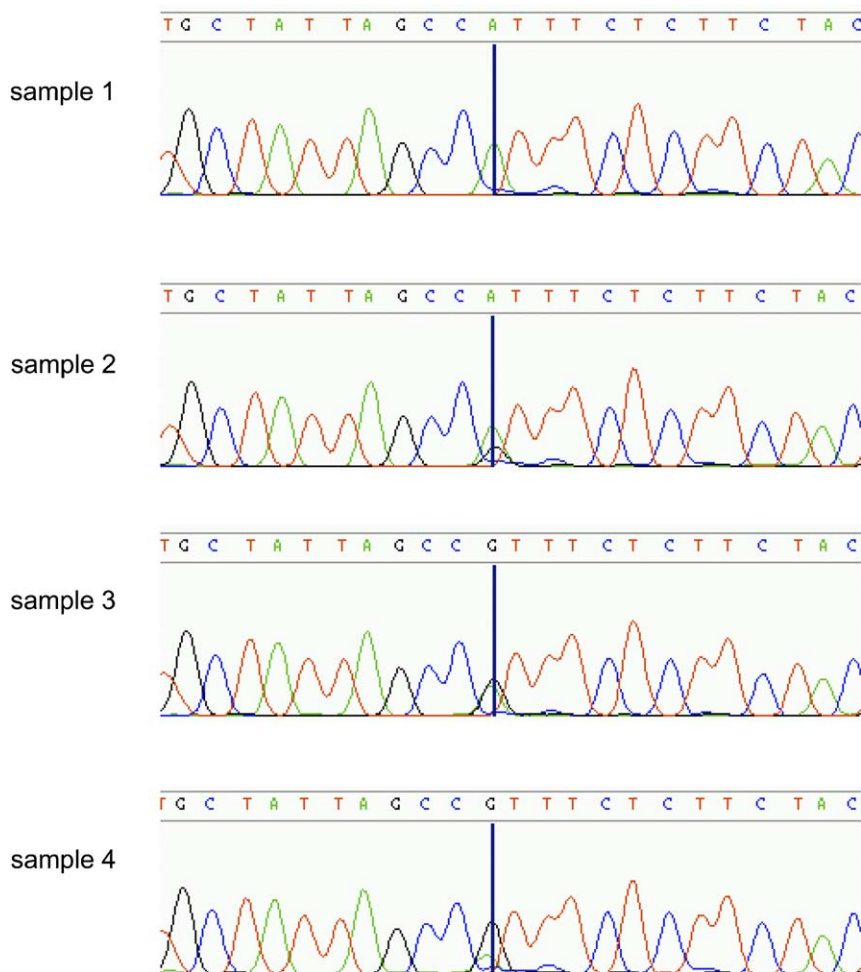


Fig. 3. Genotyping of SNP rs2680507 in the *DEFB104* gene by direct Sanger sequencing in 4 individuals indicating, but not quantifying, differences in allele frequencies/copy numbers of segmental duplications. The black vertical line marks the position of SNP rs2680507 A/G. The A-allele is represented by a green peak, and the G-allele is represented by a black peak. Peak heights reflect different numbers of the alleles.

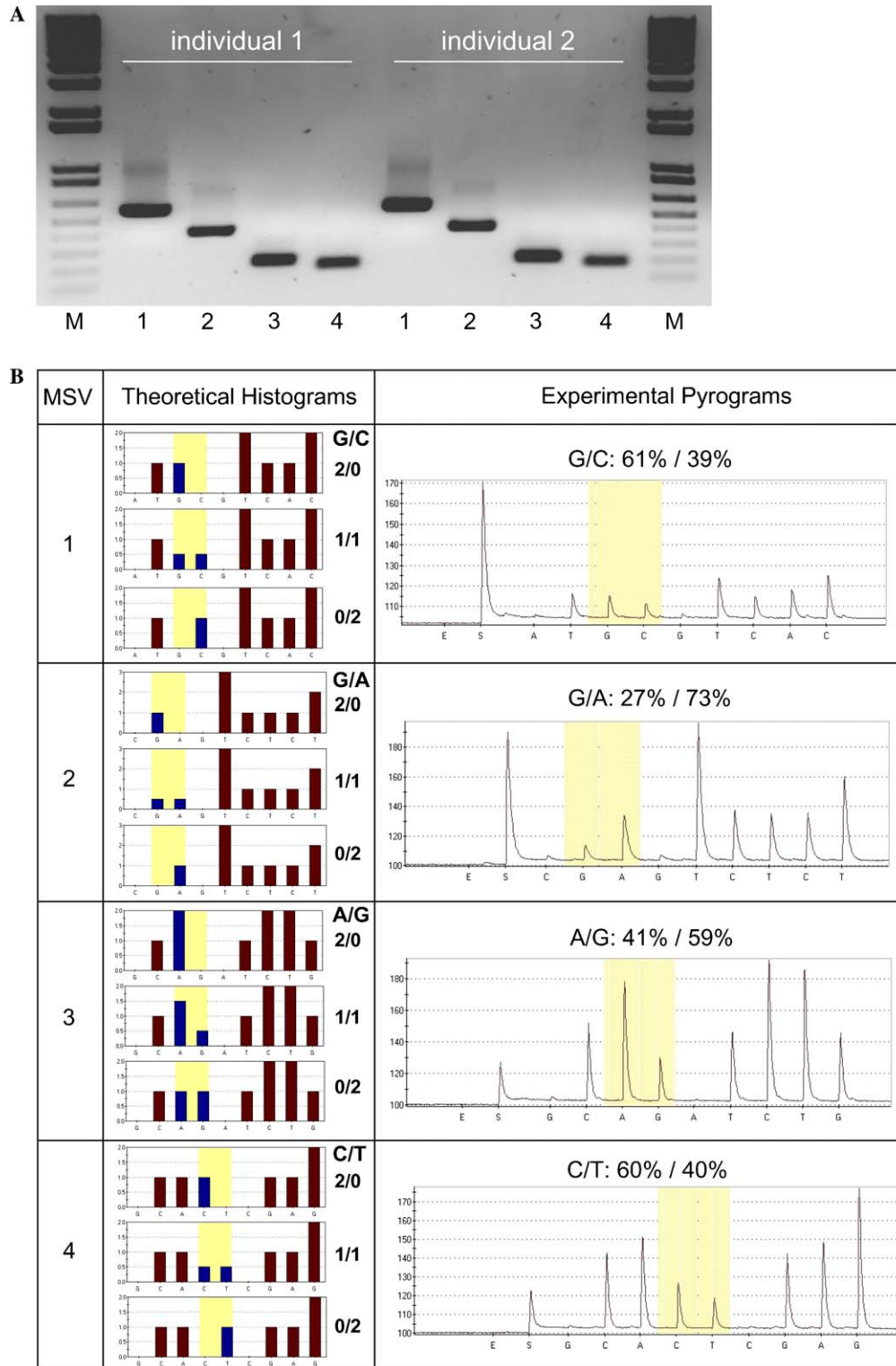


Fig. 4. Allele frequency determination of four MSVs of *DEFB104* by Pyrosequencing using one universal biotinylated primer. (A) Agarose gel electrophoresis of biotin-labeled PCR. Individuals 1 and 2, also given in Table 1, are shown. 1 = rs17843871; 2 = rs2680507; 3 = rs17843872; 4 = rs4259430; M = 1 Kb Plus DNA Ladder. (B) The left column represents theoretical histograms for allele ratios 2:0, 1:1, and 0:2 of respective alleles. The right column represents experimental pyrograms for individual 2 in Table 2. The yellow band highlights the informative alleles. Percentages indicate the calculated allele ratios.

and 40.3% T for rs4259430, whereas the frequency is 27.1% T for rs2680507. For this individual, a minimal copy number of 5 or a multiplicity thereof has been determined previ-

ously [15]. Three of the MSVs (rs17843871, rs17843872, and rs4259430) show equal allele ratios of 2:3, in accordance with the previous findings. However, the allele ratio for

Table 2
Allele frequencies of four MSVs of the *DEFB104* gene in 2 individuals determined by Pyrosequencing using one universal biotinylated primer

	Sample 1	Sample 2
Minimal copy number ^a	8	5
rs17843871 (S)	39.1% C	38.9% C
rs2680507 (R)	46.6% G	27.1% G
rs17843872 (R)	36.3% A	40.6% A
rs4259430 (Y)	39.8% T	40.3% T

^a Minimal copy number was determined previously by the haplotype cloning/counting method [15].

rs2680507 indicates a minimal copy number of 10 (allele ratio of ~3:7). A multiplicity of 10 is unlikely, taking into account the maximum copy number of 12 in human genomes estimated by Linzmeier and Ganz [19] and Hollox and coworkers [18]. Accordingly, Pyrosequencing allows the definition of “gene/allele frequency groups”, representing a novel approach for the determination of genotype–phenotype correlations.

Discussion

Large SNP typing efforts, such as the International Hap-Map Project [22] and complementary initiatives [23,24], have provided invaluable data that are widely used for disease gene mapping and evaluation of genetic differences associated with individual phenotypes. However, even though these millions of SNPs are deposited in numerous public databases and accessible to individual researchers, there is an ongoing need for efficient high-throughput SNP typing. Pyrosequencing is one method used for genotyping; it has the advantage of providing not only a genotype but also information about allele distribution and respective sequence context. Because alleles can be reliably quantified by Pyrosequencing, the method is most suitable for analysis of allele frequencies deviant from the normal ratios of 2:0, 1:1, and 0:2. Such analyses are necessary for the determination of transcript-specific allele frequencies, or allele distributions in duplicated genomic regions, or if pooled DNA samples are to be examined.

Genotyping or allele frequency determination by Pyrosequencing requires ss templates, which usually are generated by PCR and subsequent denaturation and strand isolation employing biotinylated PCR primers and affinity methodologies. Therefore, as many different biotinylated PCR primers are needed as different PCR reactions are to be performed. In our hands, biotinylated PCR primers occasionally differ from the corresponding unbiotinylated primers in PCR performance (data not shown). Therefore, it is indispensable to optimize PCR protocols for biotinylated primers even if respective unbiotinylated primers work well. We find this to be an unnecessary burden, especially if an optimized PCR protocol is available from one’s own work or published data. We offer a solution for this dilemma by adapting the “universal primer” idea of Anderson and coworkers [12], who developed this approach for

convenient Sanger sequencing of ss recombinant DNA fragments, to Pyrosequencing.

The three given examples demonstrate the potential of our universal primer approach for ss template preparation in Pyrosequencing. The method is easy, fast, and straightforward, and it can be adapted to other vector systems and DNA joining techniques. The advantage of our approach, compared with published methods using universal primers [7–10], is that primers and PCR protocols to amplify the target sequence can be defined and optimized regardless of the Pyrosequencing reaction. Because one target-specific oligonucleotide is used in the second PCR and also as the sequencing primer, and because its annealing site is adjacent to the sequence of interest, the 3’ end of the ss template is locked and self-priming of the template is prevented [11]. Another advantage of our approach is the nondirectional insertion of PCR-amplified target into vector, allowing Pyrosequencing of both strands with two target-specific sequencing primers and only one universal biotinylated primer.

An application of our approach to high-throughput genotyping platforms is conceivable, for example, if multiplexed target-specific PCRs are to be performed and more than one amplicon is to be inserted into a plasmid vector in a single reaction. Here, in the second PCR, several target regions can be amplified from the pool of recombinant molecules carrying different inserts by using respective target-specific primers and only one biotinylated primer.

In any case, our method provides a simple and universal way to label ds DNA by PCR with a biotin tag facilitating downstream processing by means of biotin–(strept)avidin interactions for Pyrosequencing. Furthermore, our methodology can be adapted for labeling of ds DNA with other markers, such as FAM, HEX, and NED, useful for mutation screening by ss conformation polymorphism analysis [25] or for microsatellite genotyping [9].

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