Benchmark

Multiplex Universal Genotyping Using a Modified ARMS-PCR Protocol

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DNA mutations underlie the pathology of inherited disease. The spectrum of genetic changes resulting in disease is diverse, often encompassing both point (single-nucleotide) and length (large deletions) mutations: cystic fibrosis (2) and the muscular dystrophies (10) are classic examples. Similarly, mutational events implicated in carcinogenesis include both single nucleotide substitutions and multi-base pair deletions (1).

In contrast to disease-causing mutations, genetic polymorphisms occur frequently in human populations. SNPs, occurring in the human genome with an average frequency greater than 1:1000 bp, account for most of the sequence variation between individuals (5). Their high frequency combined with their relatively even distribution across the entire genome makes them ideal markers for studies of genetic linkage and disease susceptibility. Disease association studies explore the hypothesis that certain allelic variants of candidate genes, arising either from SNPs or length polymorphisms, contribute to the overall risk or severity of disease (7).

The mass identification of new polymorphisms by the Human Genome Project and the private sector has renewed interest in studying the complex genetics of multifactorial diseases. At the same time, the sheer abundance of polymorphisms, even within single genes, imposes methodological hurdles that are hard to overcome, resulting in mounting criticism of such studies (9). In the absence of data pertaining to the phenotypic impact of individual polymorphisms, the definition of polymorphic haplotypes through linkage analysis offers one way of addressing this problem (7). Therefore, linkage and disease association studies, in addition to clinical diagnostics, would benefit from the development of a universal genotyping tool. Despite the recent development of several high-throughput genotyping technologies, there is as yet no single assay that allows the simultaneous analysis of both point and length polymorphisms.

The amplification refractory mutation system (ARMS-PCR) (4), also known as PCR using sequence-specific primers (PCR-SSP), is an established platform for genotyping SNPs. This technique exploits the relative inability of *Taq* DNA polymerase to extend primers mismatched at their 3′-end as well as its intrinsic lack of 3′→5′ exonuclease activity. As SNPs are virtually always biallelic, ARMS-PCR determines the allelic status of any SNP using two duplex reactions: each one employs either allele-specific primer paired with a common companion primer, as well as a primer pair amplifying an irrelevant locus of suitable, fixed length. The latter reaction should proceed independently of the allele-specific reaction; thus, it serves as an internal control.

Here I describe a simple modification of ARMS-PCR that extends its applicability to the study of length polymorphisms. The essence of this modification lies in using the internal control primer pair to amplify a length polymorphism of interest. Since this reaction remains allele nonspecific, it still doubles as an internal control.

I have recently used this modified ARMS-PCR protocol to study linkage disequilibrium in the human *IL1* gene cluster (12) and to identify polymorphisms associated with chronic graft nephropathy (11). Assay development is essentially a three-step process. Initially, primers to amplify the length polymorphism of interest are designed, and all length variants are characterized. In the example illustrated here, a primer pair (5′-TGGCCTTGTTCATTTTCCCTGC-3′ and 5′-TCATCTTCCTGGTCTGCAGGTA-3′) was designed to amplify a minisatellite occurring within intron 2 of the human *IL1RN* gene (6). This particular hexaallelic polymorphism involves a tandemly repeated 86-bp sequence (8) and has been associated with a diverse spectrum of pathologies, ranging from osteoporosis to cardiovascular disease (14). Subsequently, allele-specific primers are designed for the SNP of interest (see Reference 3 for guidelines), along with a common companion primer. The size of the allele-specific product is crucial, as this should be resolvable from the length polymorphism

variants by gel electrophoresis or other separation technologies. In our study of linkage across the 300-kb segment of human chromosome 2q that includes the *IL1B* and *IL1RN* genes, we looked at two SNPs, both C→T transitions: one lying within the same intron as the *IL1RN* minisatellite and the other located in exon 5 of the *IL1B* gene. The respective primer pair sequences have been published (13).

Figure 1 illustrates the final step in assay development, namely optimization of the duplex reactions. In this case, the allele-specific primer pairs were kept at a fixed concentration (0.75 µM), and the primer pair amplifying the minisatellite was titrated into the reactions. The latter primers were used at 1 µM in all subsequent experiments. All primers were HPSF grade (MWG Biotech, Ebersberg, Germany). Each reaction contained 75 mM Tris-HCl, pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% Tween® 20, 5% glycerol, 100 µg/mL cresol red, 200 µM dNTPs, 2 mM $MgCl_2$, genomic DNA (50–200 ng), and 1 U *Taq* DNA polymerase in a final volume of 13 µL. All reagents were from Sigma unless otherwise stated. Thermal cycling conditions were as follows: initial denaturation at 94°C for 90 s; 10 cycles at 94°C for 30 s, 62°C for 60 s, 72°C for 30 s; and 25 cycles at 94°C for 30 s, 58°C for 45 s, and 72°C for 30 s. PCR products were resolved by electrophoresis through 1.5% 0.5× TBE-agarose gels containing 0.5 µg/mL ethidium bromide, at 16 W for 60 min, using 0.5× TBE as the running buffer. Bands were visualized under UV light and imaged using a DV40 camera and Digital Science™ 1D software (Eastman Kodak, Rochester, NY, USA).

Figure 2 provides an at-a-glance assessment of linkage among the polymorphic markers examined here. Given the close physical proximity of the intronic *IL1RN* polymorphisms (<1 kb apart), total linkage disequilibrium was anticipated. Figure 2A shows the resulting potential haplotypes, namely C/*1, C/*3, C/*4, and T/*2. Figure 2B shows multiplex universal genotyping of the *IL1B* and *IL1RN* loci. While no obvious haplotypes are apparent, close inspection of the image reveals that allele T occurred in IL1RN*1 carriers, but not in IL1RN*2 carriers. Statistical analysis of genotyping results from a larger population supported this observation (12).

In conclusion, the modified ARMS-PCR protocol described here is a multiplexed universal genotype analysis platform, as it enables the simultaneous analysis of SNPs and length polymorphisms. While, in its present form, this technique carries the obvious limitations associated with the use of agarose gels (low resolution, low throughput), it is inexpensive, easily customizable, and flexible enough to incorporate more advanced technologies in product separation and detection, such as fluorescent primers, PAGE, and capillary

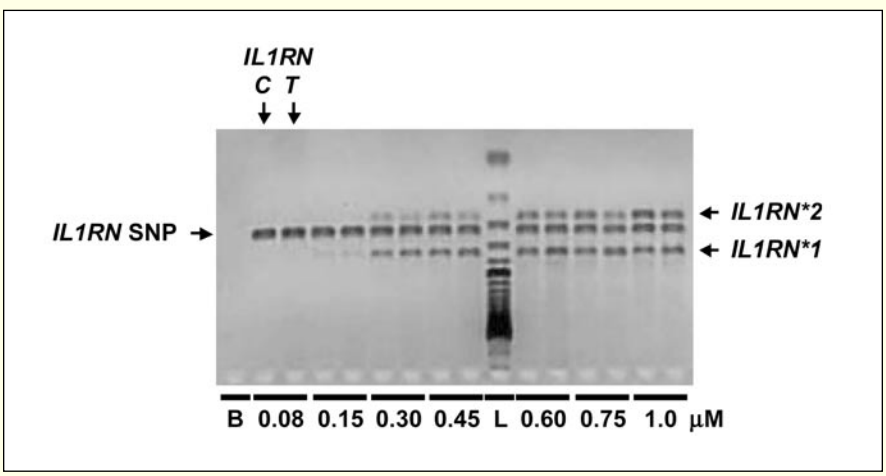

Figure 1. Optimization of the modified ARMS-PCR protocol. This gel image shows co-optimization of the primer pairs amplifying an SNP and a minisatellite polymorphism. Genomic DNA was from an individual heterozygous for both polymorphisms. Concentrations of the primers amplifying the minisatellite are indicated.

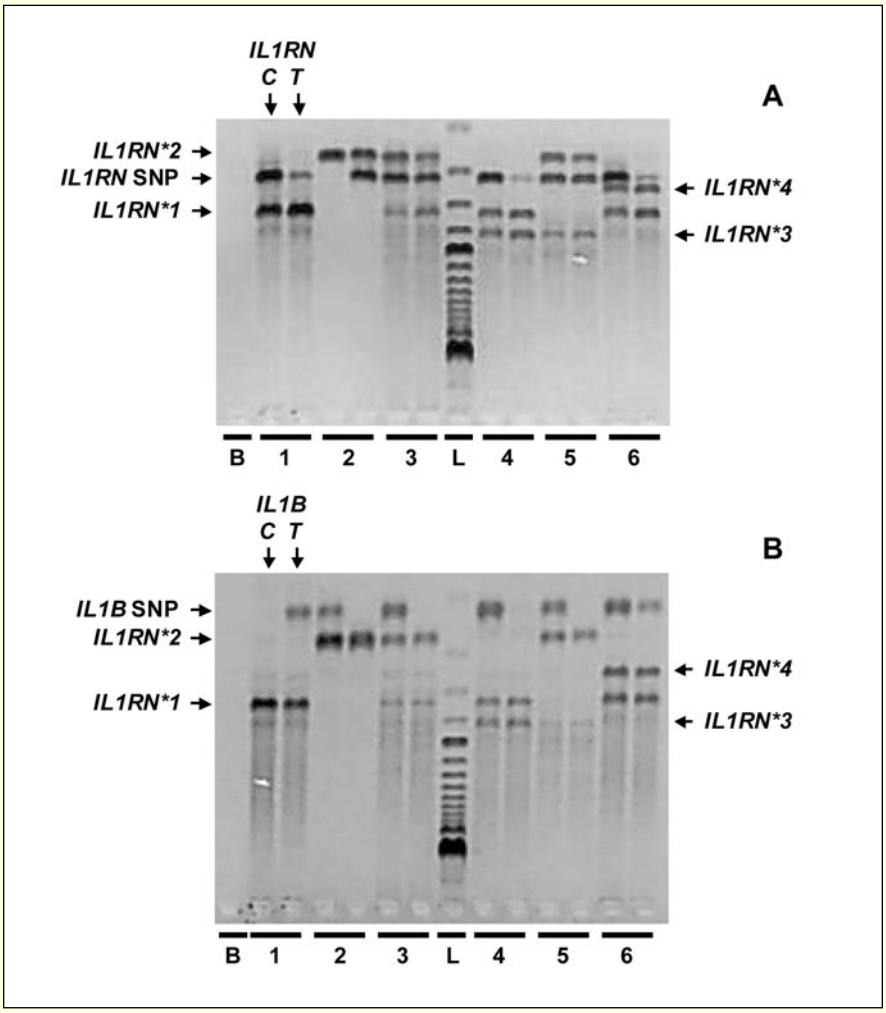

Figure 2. Linkage analyses of polymorphisms in the *IL1* gene cluster using the modified ARMS-PCR protocol. Linkage between two intronic *IL1RN* polymorphisms (A) or one *IL1B* SNP and one *IL1RN* VNTR (B) was investigated. Most of the observed allelic combinations are shown in each case (six individuals/panel). B, DNA-blank control; L, DNA ladder (1 µg, size range 100–1500 bp; Invitrogen, Carlsbad, CA, USA).

electrophoresis. Potential applications of this technique are foreseen in linkage and disease association studies, diagnostics, and forensics.

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