REVIEW

# Reading Bits of Genetic Information: Methods for Single-Nucleotide Polymorphism Analysis

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Information about the genome of our species is accumulating along two dimensions: While the latitudes of our chromosomes are being charted, crudely at first, but with the expectation of a complete nucleotide sequence of one reference human genome early next millennium, the longitudes of sequence variation at specific locations in the genome are also explored, revealing how different we all are. This variation consists mostly of sequence differences in single nucleotide positions, referred to as single nucleotide polymorphisms or SNPs. An expanding panel of known sequence variants, along with greatly improved methods to monitor them, now promise to offer fresh insights into human biology and pathophysiology. We will discuss some of the advantages of these markers as a background for a presentation of high-throughput techniques to analyze them with an outlook toward the future of such techniques.

Extremely efficient methods will be required for analysis of large numbers of sequence variants in many patient samples to identify subtle genetic risk factors that go undetected in current genome scans by use of fewer markers and limited sample sizes. Moreover, as more and more genetic factors of known relevance for diseases are being identified, extensive panels of disease-associated markers are likely to be routinely applied to secure diagnoses of patients. SNPs are expected to take the place of simple tandem repeat polymorphisms—microsatellites—as markers in disease gene mapping, just as the microsatellite markers rapidly replaced the restriction fragment-length polymorphism markers. There are four main reasons for the increasing popularity of SNPs as markers in genetic analysis. (1) They are far more prevalent in the genome than microsatellites, furnishing large sets of markers near or in any locus of interest. (2) Some of the SNPs located in genes can be expected to directly affect protein structure or expression levels, and these may, therefore, represent candidate alterations for genetic mechanisms in disease. (3) While repeated sequences exhibit some instability, that is, mutations occasionally alter the size of an allele complicating analysis of the inheritance (Weber and Wong 1993), SNPs are much more stably inherited. (4) Finally, there are high hopes that increasingly efficient SNP typing systems will become available with very large throughputs, offering sufficient power in genetic analyses to scan for quite subtle genetic risk factors also.

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## Genetic Properties of SNPs

In every 1000 bases along the human chromosomes, on average approximately one nucleotide position is estimated to differ between any two copies of that chromosome (Cooper et al. 1985; Hofker et al. 1986; Kwok et al. 1996). A locus is viewed as polymorphic when it exists in at least two variants and the allele frequency of the most common variant is <99% (Li and Grauer 1991). Extensive sets of polymorphic sequences are now being identified in the course of genome research, in studies of human genome diversity, and in the process of establishing mutation databases that may ultimately include all common variants of human genes (Cotton et al. 1998).

Increasingly efficient methods for typing SNPs open new possibilities in genetic studies. A full genome linkage scan of power equivalent to a 400 marker microsatellite analysis could be performed with 900 SNPs or, to extract more of the available information from the family material, preferably with 2000–3000 SNPs (Kruglyak 1997). In association studies, more suitable to study complex disorders in which many loci contribute to the risk of developing disease, far more markers could be tested profitably (Risch and Merikangas 1996).

Applications of SNP analyses thus extend from investigations of small numbers of sequence variants known to be associated with a specific disease to investigations of markers across the genome, per-

haps with markers corresponding to several variants of each of the 100,000 or so genes. A large number of methods are now becoming available, aimed to monitor anything from single up to thousands of SNPs per assay.

## Methods for SNP Analysis

A handful of molecular strategies are in use for SNP analysis. All current methods involve target sequence amplification, and this is followed by distinction of DNA sequence variants by short hybridization probes or by restriction endonucleases; discrimination of mismatched DNA substrates by polymerases or ligases; or by observing the template-dependent choice of nucleotide incorporated by a polymerase. Recently, these various techniques have been adapted to assay formats that simplify scale-up in SNP analysis (Table 1). Although it is too early to conclude how the assays all compare in through-put and running costs, we will discuss important properties of some of the assays below.

### *Array Hybridization Assays*

Hybridization assays rely on the differences in hybridization stability of short oligonucleotides to per-

**Table 1. Methods for SNP Analysis**

| Method | Allele discrimination by | Format | Instrumentation | References |
|---|---|---|---|---|
| MASDA | ASO | format 1 array + gel | none | Shuber et al. (1997) |
| Whitehead–Affymetrix SNP microarray | ASO | format 2 array | array scanner | D.G. Wang et al. (pers. comm.) |
| TaqMan | ASO | homogeneous closed tube[a] | TaqMan instrument | Livak et al. (1995) |
| Molecular beacons | ASO | homogeneous closed tube[a] | TaqMan instrument | Tyagi et al. (1998) |
| MADGE | Restriction cleavage | gel | none | Day and Humphries (1994) |
| Allele-specific PCR | 3′ matched/mismatched primer | gel | none | Liu et al. (1997) |
| OLA | ligation | solid-phase microtiter readout | fluorospectrophotometer | Samiotaki et al. (1994); Tobe et al. (1996) |
| OLA multiplex gel | ligation | gel | automated sequencer | Grossman et al. (1994); Day et al. (1995) |
| DOL | ligation | homogeneous closed tube[a] | TaqMan instrument | Chen et al. (1998) |
| Solid-phase minisequencing | nucleotide incorporation | solid-phase microtiter readout | scinitillation counter | Syvänen et al. (1993) |
| Minisequencing on array | nucleotide incorporation | format 2 array | PhosphorImager | Shumaker et al. (1996); Pastinen et al. (1997) |
| Minisequencing multiplex gel | nucleotide incorporation | solid phase + gel | automated sequencer | Pastinen et al. (1996) |
| Minisequencing with FRET | nucleotide incorporation | homogeneous reagent addition[b] | TaqMan instrument | Chen et al. (1997) |
| Pyromini-sequencing | nucleotide incorporation | homogenous reagent addition[b] | luminometer | P. Nyrén and M. Ronaghi (pers. comm.) |

[a]Reagents for amplification and allele distinction are mixed together at the start, and results are recorded without opening the reaction vessel.
[b]After amplification, detection reagents are added to the reaction vessel, but no separations are required before recording the results.

fectly matched and mismatched target sequence variants (Wallace et al. 1979). Allele-specific oligonucleotide (ASO) hybridizations are frequently performed with either the DNA samples or the oligonucleotides arrayed on a surface, sometimes referred to as formats 1 and 2, respectively. Format 1 is suitable for screening many patient samples for the presence of a few disease-causing alleles, as in the multiplexed allele-specific diagnostic assay (MASDA), in which a pool of mutation-specific oligonucleotides are hybridized to spots of pooled PCR products from individual patients, and positive reactions are eluted and sequenced to determine which probe bound to a particular patient sample (Shuber et al. 1997).

Format 2, on the other hand, involving high-density microarrays of oligonucleotides (Fodor et al. 1991; Southern et al. 1992; Yershov et al. 1996), is more suitable to analyze many SNPs in parallel. In a collaborative effort between the Whitehead Institute and Affymetrix, 2000 SNPs are being characterized and an oligonucleotide chip is designed for them. Because resequencing by hybridization on oligonucleotide arrays has been worked out sufficiently well (Chee et al. 1996), the key to the success of this method is the ability to prepare thousands of PCR products for parallel detection on arrays. To this end, very short PCR products are generated for each SNP to ensure robust multiplex amplification. In addition, the PCR primers contain a universal tail such that a second round of PCR amplification can be performed with a single set of primers to render amplification efficiency for different PCR products more similar. Following amplification, many multiplex PCR mixtures, each containing tens of SNPs, are pooled for hybridization with custom-designed DNA chips containing oligonucleotides complementary to the sequence in the vicinity of the SNPs. The hybridization of fluorescent PCR products is recorded through confocal microscopy. Comparison between the images for a test sample and a control sample can yield the genotype of the test sample for thousands of SNPs being tested (D.G. Wang et al., pers. comm.).

Potential problems with this approach are the limitations of multiplex PCR and oligonucleotide hybridization to fairly complex DNA samples, and the lack of flexibility caused by hardwiring of markers on a chip that is not easy to redesign.

### *Homogenous Hybridization*

Two recently developed assays allow hybridization-based allele-discrimination during PCR, with no need for separations or washes. The TaqMan assay takes advantage of the 5′-nuclease activity of *Taq* DNA polymerase to digest a DNA probe annealed specifically to the accumulating amplification product (Holland et al. 1991). TaqMan probes are labeled with a donor-acceptor dye pair that interact via fluorescence resonance energy transfer (FRET). Cleavage of the TaqMan probe dissociates the donor dye from the quenching acceptor dye, greatly increasing the donor fluorescence (Fig. 1A; Lee et al. 1993). All reagents necessary to detect two allelic variants can be assembled at the beginning of the reaction and the results are monitored in real time during PCR (Livak et al. 1995).

This closed-tube, walk-away assay is relatively fast and it minimizes PCR contamination risk. Reagents must be carefully designed to ensure that each of the two allele-specific TaqMan probes anneal only to the perfectly complementary template during PCR but not to the template containing a single mismatched base. The TaqMan assay is currently restricted to simultaneously detecting two probes, because FRET requires specific pairs of donor-acceptor dyes, each of which occupies a rather large portion of the visible spectrum.

In an alternative homogenous hybridization-based PCR procedure, molecular beacons are used for allele discrimination (Fig. 1B; Tyagi and Kramer 1996). These oligonucleotide hybridization probes have two complementary DNA sequences flanking the target-specific sequence, and a donor-acceptor dye pair is present at opposite ends of each probe. When not hybridized to a target sequence, the probes adopt a hairpin-loop conformation, bringing the fluorophore and quencher pair close together, thereby extinguishing the donor fluorescence. On the other hand, when hybridized to the correct target sequence, the two dyes are separated and fluorescence increases by up to 900-fold. A valuable side effect of the preorganized hairpin probe design is that mismatch hybridization is further destabilized, providing increased allele selectivity in SNP analyses (Tyagi et al. 1998). Also, the target-complementary region is designed to hybridize during the annealing steps in PCR, rather than the extension steps as in TaqMan. Accordingly, the lower hybridization stability of the probe further increases its sensitivity to mismatches. Unlike methods based on FRET, the donor and acceptor dyes do not need to have overlapping spectra, probably because they are brought in close contact in the hairpin–loop conformation. As a consequence, four or more differentially labeled molecular beacons can be assayed in the same reaction (Tyagi et al. 1998).

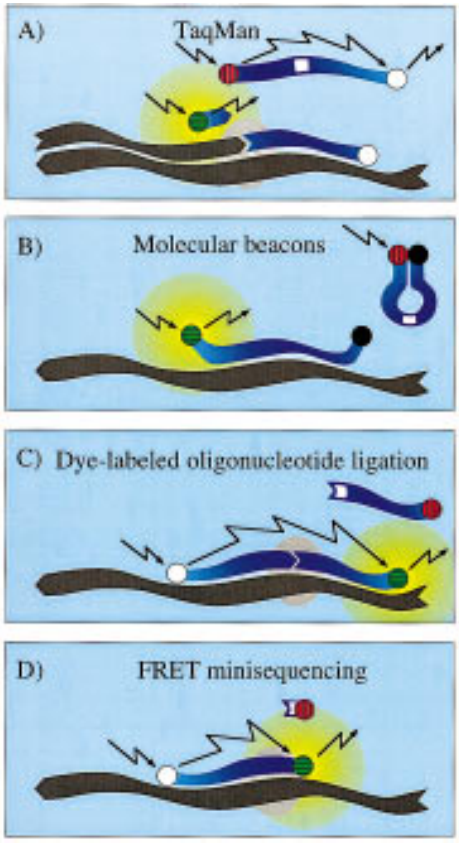


**Figure 1** Molecular strategies applied in fluorescence energy transfer-based SNP assays. Amplified target sequences are shown in black and oligonucleotide probes and nucleotide triphosphates in blue, with the variable positions indicated by small white squares. In these examples, the green circles represent reporter fluorophores on reagents that match the target allele; the red circles represent fluorophores on the mismatched reagents. Jagged arrows indicate excitation of and emission from, as well as energy transfer between the fluorophores. Polymerases and ligases are represented by gray ovals. (*A*) In the TaqMan assay, pairs of fluorophores are conjugated to allele-specific oligonucleotides designed to hybridize downstream of one amplification primer. The donor (red and green fluorophores) fluorescence is quenched in the intact TaqMan probes as a result of FRET to the acceptor (white circle). Fluorescence can be detected when an allele-specific oligonucleotide hybridizes to a target molecule and is digested by the advancing polymerase during amplification. (*B*) Molecular beacon probes are hybridization probes that form hairpin–loop structures in the absence of the correct target sequence. When an allele-specific probe hybridizes to a target sequence, a fluorescent dye conjugated to the end of the probe is brought apart from a chromophore at the other (shown in black), and fluorescence is emitted. (*C*) In dye-labeled oligonucleotide ligation, the ligation of an oligonucleotide probe to another, allele-specific probe hybridizing downstream, results in energy transfer between fluorophores on the two oligonucleotides. (*D*) In FRET minisequencing, an allele-specific fluorophore-labeled nucleotide is added to a primer, resulting in energy transfer between a fluorophore present on the primer and that on the nucleotide.

*Restriction Digestion*

An easily scored difference in the electrophoretic migration of a PCR product can be obtained if an SNP alters the recognition sequence for a restriction endonuclease. This principle has been used in the high through put, low-resolving microtiter array diagonal gel electrophoresis technique (MADGE) (Day and Humphries 1994). Here, restriction-digested PCR products are loaded on stackable horizontal gels with wells arranged in a microtiter format. The electric field is applied at an angle relative to the columns and rows of wells in the gel, allowing products from large numbers of reactions to be resolved between the downstream wells. An estimated one-half of all SNPs do not alter any restriction enzyme recognition sequence (Landegren et al. 1988), but this can be overcome by modifying the amplified sequence with mismatched primers to create restriction sites (Cohen and Levinson 1988).

*Mismatch Distinction by Polymerases and Ligases*

The polymerization reactions in PCR places particularly stringent requirements on correct base pairing of the 3′ end of the hybridizing primers. This can be used to selectively amplify one or the other allele of an SNP (Newton et al. 1989; Sommers et al. 1989; Liu et al. 1997). This method also offers a unique advantage in that it permits analysis of the linkage phase of two neighboring SNPs, provided these are located sufficiently close, by use of allele-specific PCR primers at both ends of the amplification product (Sarkar and Sommer 1991).

The joining by a DNA ligase of two oligonucleotides hybridized to a target DNA sequence is quite sensitive to mismatches close to the ligation site, especially at the 3′ end (Landegren et al. 1988; Wu and Wallace 1989). Notoriously difficult G–T mismatches located at a 3′ end to be ligated inhibit the reaction by up to 1000-fold under standardized reaction conditions (Lou et al. 1996). This has been utilized in the oligonucleotide ligation assay (OLA; Landegren et al. 1988) and the ligase chain reaction (LCR; Barany 1991) to discriminate between SNPs. In the OLA, the sequence surrounding the SNP is first amplified by PCR, whereas in LCR, genomic DNA can be used as a template. By use of allele-specific ligation probes differentially labeled with lanthanide chelates, and a multipronged solid support for parallel sample handling (Parik et al. 1993), sets of ligation reactions can be conveniently ana-

lyzed via time-resolved fluorometry (Samiotaki et al. 1994). Dual-color detection of allele-specific ligation products is also possible in a regular spectrophotometer (Tobe et al. 1996).

In a gel-based alternative, sets of a few tens of SNPs can be amplified in multiplex, followed by multiplex ligation, and then analyzed together by electrophoresis on an automated DNA sequencer, distinguishing markers by size and alleles via fluorescence (Grossman et al. 1994; Day et al. 1995). The assay requires careful optimization, but it offers a relatively high through-put.

With the dye-labeled oligonucleotide ligation (DOL) assay, OLA has been rendered homogeneous through the use of FRET (Chen et al. 1998). Thereby all reagents for both PCR and ligation can be added at the beginning of the reaction. The dye-labeled ligation probes are designed to have melting temperatures lower than those of the amplification primers, and a thermostable DNA polymerase without 5′-nuclease activity is used to prevent cleavage of the ligation probes during primer extension. After a number of PCR cycles sufficient to accumulate amplification products, the annealing temperature is lowered to allow ligation probes to anneal and be ligated together in an allele-specific fashion. Ligation joins pairs of fluorophore-labeled molecules and results in FRET that is monitored in real time, permitting automatic scoring of genotypes (Fig. 1C).

### *Minisequencing*

The target-dependent addition by a DNA polymerase of a specific nucleotide to a single primer in a minisequencing reaction (Syvänen et al. 1990) distinguishes more accurately between variable nucleotides located immediately downstream of the primer than is possible by use of the differential stability of hybridization by allele-specific probes (Pastinen et al. 1997). Several markers can be analyzed in parallel by separating locus-specific primers according to size through gel electrophoresis and observing the allele-specific incorporation of labeled nucleotides, while periodically loading new sets of reaction products on the gel for increased throughput (Pastinen et al. 1996). Several groups are currently concentrating on the construction of arrays of extension primers for highly accurate gene analysis of many target sequence positions in amplified DNA samples (Pastinen et al. 1997; Shumaker et al. 1996).

Minisequencing analysis of individual SNPs has traditionally been done by immobilizing amplification products in microtiter wells (Syvänen et al. 1993). However, two different approaches have been used to turn it into a homogenous method. In minisequencing with FRET detection, extension of a dye-labeled primer by one of two fluorophore-labeled nucleotides is observed via fluorescence detection (Fig. 1D; Chen and Kwok 1997). In pyrominisequencing pyrophosphate released from a nucleotide triphosphate, incorporated by a DNA polymerase, is converted to ATP that fuels a luciferase reaction, generating a detectable flash of light (Nyrén et al. 1993). Before a minisequencing reaction, remaining amplification primers and nucleotide triphosphates must be inactivated or removed to prevent interference with the subsequent primer extension reaction. In the FRET method, this is done by use of heat-sensitive alkaline phosphatase and *Escherichia coli* exonuclease I, before the addition of the fluorophore-labeled primer and nucleotides and real-time measurement of fluorescence changes (Chen et al. 1997). In pyrominisequencing, remaining unicorporated nucleotides can be degraded by the enzyme apyrase, allowing sequential detection of several consecutive nucleotide positions by cyclically repeating the addition of individual nucleotides (P. Nyrén and M. Ronaghi, pers. comm.).

## A Partisan View of Future SNP Assays

Despite all the techniques available, there is a pressing need for far greater throughput in analyses of the bits of genetic information represented by SNPs. This requirement is being addressed by a marked trend toward miniaturization to pack more features on analytic devices. Examples of this trend include miniature PCR on a chip with on-board detection (Burke et al. 1997), DNA assays that take place on the surface of microparticles analyzable in a fluid stream (Lövgren et al. 1997), and DNA microarrays.

Of equal or greater importance than assay format, the choice of molecular strategy for high-throughput SNP analysis must ensure highly specific detection of unique DNA sequences, and accurate discrimination of allelic variants. DNA amplification, used in all methods reported herein, elegantly solves the problem of specificity by requiring two target recognition events, one by each primer, to detect a particular sequence, but both specificity and yield suffer when more PCRs are performed together. This is largely the result of a rapidly increasing risk of generating spurious amplification products by all pairwise combinations of oligonucleotide primers (Mullis 1991; Landegren and Nilsson 1997).

On the other hand, without prior DNA amplification, oligonucleotide hybridization in general is not sufficiently specific to unambiguously detect unique genomic sequences without prior target sequence amplification. Longer hybridization probes may identify a unique sequence, but they exhibit poor selectivity for alleles that differ in single nucleotide positions.

A hybrid approach, such as that championed by the Whitehead-Affymetrix group, combining large numbers of maximally multiplexed amplification reactions with allele-specific detection by hybridization to a redundant set of oligonucleotides on a chip, can greatly extend the number of SNPs analyzable at one time. However, to go beyond the barriers defined by the limited multiplexing capacity of PCR, and of oligonucleotide hybridization to complex DNA samples, a molecular procedure will be required that is capable of specifically identifying and distinguishing variants of single-copy sequences in unamplified DNA.

Together with greatly improved methods for scoring sets of SNPs in genomic DNA samples, there will also be a growing need for techniques that directly reveal the linkage phase of SNP variants. With increased genetic testing, questions such as whether two SNP variants in a gene occur in the same or different alleles will assume clinical relevance. Likewise, in genetic disease mapping, haplotype information greatly increases statistical power, but currently it is not possible to determine linkage phase in the absence of family data, except for sequences present on the X and Y chromosomes, and for the haploid genomes of single germ cells (Li et al. 1988) and of complete hydatidiform moles (Taillon-Miller et al. 1997). Again, a highly specific molecular procedure is required to resolve allelic differences in situ in chromosome preparations or along chromatin fibers.

One contender for the role of such a molecular procedure is gene detection by padlock probes (Nilsson et al. 1994). These are linear oligonucleotides of ~90 bases, whose two opposite ends are designed to hybridize to adjacent segments of a target sequence, such that the 5′ and 3′ ends of the probes may be joined by ligation, which will convert the probes to circular molecules. The requirement for target recognition by two separate probe segments appears to ensure adequate specificity to correctly identify unique sequences in DNA samples without prior amplification, and the act of ligation efficiently distinguishes between known sequence variants (Nilsson et al. 1997). Because only intramolecular ligation reactions are scored, cross-reactions between multiplexed probes should not present a problem (Landegren and Nilsson 1997). Furthermore, a ligation-dependent local signal amplification effect may be achieved via rolling circle replication of the circularized probes (Fire and Xu 1995; Liu et al. 1996; P. Lizardi, pers. comm.; J. Banér, unpubl.). Put together, these properties offer some hope that large sets of such probes, immobilized in an arrayed fashion or applied in situ, might be used to interrogate DNA samples and to rapidly access megabits of genetic information.

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