Single Nucleotide Polymorphism (SNP) Genotyping Techniques—An Overview

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INTRODUCTION

Single nucleotide polymorphisms (SNPs) are individual base positions in the genome that show natural variation in a population. They represent the most abundant form of genetic variation in humans, accounting for more than 90% of all differences between unrelated individuals. SNP patterns are likely to influence many human phenotypes; therefore large-scale association studies based on SNP genotyping are expected to help identify genes affecting complex diseases and responses to drugs or environmental chemicals. SNPs play a major role in all stages of the drug development process—from target identification to clinical trials. The analysis of SNPs may also help to tailor drugs and drug regimes to particular genotypes—the underlying principle of pharmacogenomics. Given the potential impact of SNPs on healthcare, the biotechnology industry has focused urgently on the development of high-throughput methods for SNP genotyping. All genotyping methods are a combination of distinct methods for allele discrimination and signal detection, and these methods are the subject of this article.

SNP GENOTYPING

High-throughput SNP genotyping methods have been developed in order to exploit the many healthcare benefits which will arise from a detailed knowledge of genetic variation. SNP genotyping technologies have two components—a method for determining the type of base present at a given SNP locus (allele discrimination), and a method for reporting the presence of the allele(s) (signal detection). There are three general allele discrimination methods: hybridization/annealing (with or without a subsequent enzymatic discrimination step), primer extension, and enzyme cleavage. In each case, the technology platform may be homogeneous (in solution) or heterogeneous (involving both a liquid and a solid phase, such as a microarray). Some of the assays require prior amplification of the genomic target, whereas others are sensitive enough to work directly on genomic DNA or cDNA. There are many signal detection platforms, and most of these follow the fate of a label either in real time or at the assay end point. Uniquely, mass spectrometry can be used to detect the allele-specific product of a discrimination assay without the need for a label, by distinguishing the masses of DNA molecules containing alternative bases.

ALLELE DISCRIMINATION METHODS

Allele-Specific Hybridization

The simplest method for discriminating between alleles at an SNP locus is hybridization using allele-specific oligonucleotide (ASO) probes. Two probes are required—one specific for each allele —and stringency conditions are employed such that a single-base mismatch is sufficient to prevent hybridization of the nonmatching probe (Fig. 1a). The ASO probes can be used as labeled/unlabeled pairs, in which case two separate assays are required to genotype each SNP, one in which probe 1 is labeled and probe 2 is unlabeled, and one in which these roles are reversed. Alternatively and preferably for high-throughput assays, distinct labels can be used for each probe (e.g., different fluorophores or mass tags), so that each SNP can be genotyped in a single reaction. More sophisticated assays, such as TaqMan, use allele-specific hybridization as the primary discriminating reaction, but additional enzymatic steps are required to detect the signal.

Allele-specific polymerase chain reaction (PCR) is a modification of allele-specific hybridization in which discrimination is achieved by allele-specific primer annealing (followed by PCR amplification) rather than the direct detection of a hybridized people (Fig. 1b). Although it is possible to use a strategy in which the variable base position is in the middle of one of the primers, which is directly analogous to the ASO technique, this allows the extension of a mismatched primer/template if stringency conditions are not optimized. A more sensitive approach is to place the 3’ end of one of the primers at the variable base position because extension is dependent on perfect complementarity at the 3’ end of the primer.
Another modification of allele-specific hybridization is allele-specific ligation, in which two oligonucleotides are annealed at adjacent sites and DNA ligase is used to join them together. One of the oligonucleotides has two allele-specific variants, and the discriminatory base is at the most 3' position. If there is perfect complementarity between the ASO and the template, it will be successfully ligated to the common oligonucleotide. If not, ligation will fail and the two oligonucleotides will remain separate. The assay in itself is not very sensitive, but sensitivity can be increased using additional steps such as the ligase chain reaction (in which the ligation reaction is reiterated with further oligonucleotides complementary to the first ligation product) or rolling circle amplification technology (RCAT; in which two ASOs about 80 nucleotides in length are designed to form a circle on the template, generating a closed loop or padlock probe when ligated). Primers annealing to this circle can be extended with a strand-displacing DNA polymerase, so that when the nascent strand completes the circle and encounters itself, it is continually displaced, generating a long concatemer that is easy to detect using fluorescence methods.\[8\]

Allele-Specific Single-Base Primer Extension

In allele-specific single-base extension (also called mini-sequencing), primers that anneal one nucleotide upstream of the polymorphic site are designed, and allele discrimination depends on the ability of this perfectly annealed primer to be extended (Fig. 1c).\[9\] This is distinct to allele-specific PCR where the discriminatory position lies within one of the primers and extension depends on the ability of the primer to anneal to its template. Allele-specific primer extension methods are more adaptable than hybridization/annealing assays because a much greater diversity of labeling strategies can be used. For example, the free nucleotides in solution can be labeled with four different

Fig. 1  Principles of allele discrimination methods. (a) Allele-specific hybridization. (b) Allele-specific PCR. (c) Allele-specific single-base primer extension. (d) Allele-specific invasive cleavage. In each case, the SNP position and the variable base position in the probe are shown. (View this art in color at www.dekker.com.)
fluorescent tags, mass tags, or haptens, allowing the same mix to be used in the detection of multiple SNPs in parallel (e.g., on a microarray). Primer extension methods for genotyping have been extensively patented as Genetic Bit Analysis™ (GBA) technology (Orchid Biosciences, Inc.) and form the basis of many popular commercial genotyping systems (e.g., Snapshot; Applied Biosystems).

**Allele-Specific Enzymatic Cleavage**

One of the earliest and most widely used genotyping methods, restriction fragment length polymorphism (RFLP) analysis, works on the principle of allele-specific enzymatic cleavage. An RFLP is generated when an SNP occurs at a restriction endonuclease recognition sequence, and one allele preserves the sequence while the other destroys it. If we consider any DNA fragment with three adjacent restriction sites, with the middle one containing an SNP, then digestion of amplified genomic DNA with the appropriate restriction endonuclease will produce either a single large fragment (if the central restriction site is absent) or two smaller fragments (if the central restriction site is present and cleavage occurs).

RFLP analysis is difficult to adapt for high-throughput genotyping, but this has been achieved for another cleavage-based assay known as Invader.[10] The Invader assay employs a unique method of allelic discrimination involving overlapping probes and an enzyme that specifically recognizes the resulting “flap” (Fig. 1d). An advantage of the Invader assay is that it requires no PCR amplification. Two signal probes are used, one recognizing each allele, plus a third invader probe. The signal and invader probes hybridize in tandem, and the signal probe overlaps the invader probe, generating a flap that is recognized by an enzyme called Cleavase, a modified form of the thermostable FEN-1 enzyme (flap endonuclease). A flap with the appropriate structure is generated only if the signal probe completely matches the template and there is a one-base inversion. The cleaved flap can then be used in a second round invader assay to generate a fluorescent signal, or the cleaved flap can be detected by mass spectrometry.

**DETECTION OF ALLELE-SPECIFIC PRODUCTS**

**Methods Utilizing Fluorescent Labels**

**Direct fluorescence detection**

The most straightforward way to detect an allele-specific product is to label it by incorporating one or more nucleotides conjugated to a fluorescent dye. Direct fluorescence detection is generally used with solid-phase assay formats (microarrays and bead arrays) and where allele-specific products are separated by gel electrophoresis or capillary electrophoresis.

A variation on this approach is to use an intercalating fluorescent dye such as ethidium bromide and to study the melting profile of DNA duplexes. This method for typing SNPs is known as dynamic allele-specific hybridization (DASH).[11] One (unlabeled) ASO is added to a PCR product and allowed to anneal in the presence of an intercalating dye, which fluoresces specifically in the presence of double-stranded DNA. The reaction is then heated, and fluorescence is measured in real time as the temperature rises. As the duplex denatures, fluorescence steadily decreases. When the melting temperature of the probe is reached, denaturation is completed and the fluorescent signal falls to background levels. The thermal melting profile of each allele is different because one is perfectly complementary to the probe and the other contains a mismatch.

**Fluorescence resonance energy transfer**

Fluorescence resonance energy transfer (FRET) occurs where two fluorophores are in close proximity, and one of the fluorophores (the donor) has an emission spectrum that overlaps the excitation spectrum of the other fluorophore (the acceptor). When a lone donor fluorophore is excited, light is produced with a characteristic emission spectrum. However, when the donor fluorophore is excited in close proximity to the acceptor fluorophore, energy is transferred to the acceptor fluorophore with the result that the intensity of emission from the donor is reduced (quenched), whereas that of the acceptor is increased (enhanced).

Although FRET enhancement can be used for SNP genotyping, two of the most popular homogenous assays in current use employ FRET quenching. The TaqMan assay, mentioned earlier, uses the intrinsic 5’ nuclelease activity of *Taq* DNA polymerase to generate a fluorescent signal from a short ASO probe.[12] Two ASO probes are required—one specific for each allele. Each probe contains a unique donor fluorophore and a common acceptor fluorophore, and is short enough for the donor to be quenched when the probe is intact (either hybridized or in solution). However, when the probe hybridizes to the PCR template, the 5’ exonuclease activity of *Taq* DNA polymerase digests it, thus releasing the two fluorophores into solution and eliminating the quenching effect (Fig. 2a). The Molecular Beacon assay[13] involves the use of longer probes that have self-complementary ends labeled with the donor and acceptor fluorophores. Non-hybridized probes will self-anneal, bringing the donor and
acceptor fluorophores together, resulting in quenching. Where hybridization occurs, the donor and acceptor fluorophores are separated such that the quenching effect is eliminated and a fluorescent signal is produced. As with the TaqMan assay, two probes labeled with different donor fluorophores are required—one specific for each allele (Fig. 2b).

Fluorescence polarization

When a fluorophore is excited by plane-polarized light, the fluorescence emitted by the dye is also polarized. This phenomenon is termed fluorescence polarization (FP). Complete FP occurs only when the dye molecule is stationary. Therefore the degree of observed FP is dependent on how fast a molecule tumbles in solution, and this is in turn dependent on the volume of the molecule, which is related to its molecular mass. Therefore changes in molecular mass (e.g., caused by primer extension, probe hydrolysis, or invasive cleavage) can be detected by changes in FP as long as all other conditions (temperature, viscosity, etc.) remain constant. FP is used as the detection method in the SnaPshot (Applied Biosciences) and Acycloprime (Perkin Elmer) commercial genotyping systems.

Mass Spectrometry

Genotyping assays involving mass spectrometry are distinct from those discussed above in that signal...
detection is direct, based on differing molecular weights of small DNA fragments rather than the behavior of a label.\textsuperscript{[114]} The analysis of DNA by mass spectrometry requires soft ionization (i.e., without fragmentation) and is usually achieved by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) analysis. The MALDI procedure involves mixing the allele-specific products of the discrimination assay with a matrix compound on a metal plate. The mixture is then heated with a short laser pulse, causing it to expand into the gas phase where ionization is achieved by applying a strong potential difference. Ions are accelerated toward the detector and the time of flight (the time taken to reach the detector) is measured, allowing the mass/charge ratio to be calculated.

High-resolution mass spectrophotometers can accurately discriminate between alternative alleles in DNA fragments of 3–20 nucleotides in length. The smallest difference in mass between alternative allelic products is about 9 Da, representing an adenosine/thymidine polymorphism. Advantages of MS-based methods include its accuracy (as long as the sample is very pure) and the fact that each genotyping reaction takes a fraction of a second, allowing thousands of reactions to be carried out serially in a single day.

The majority of current MS genotyping platforms involve the use of DNA chips because the chip can be used directly as a MALDI plate. Thus far, the technique has been used to determine the masses of allele-specific hybridization probes, allele-specific primer extension products, and the products of invasive cleavage reactions. Commercial systems utilizing mass spectrometry include MassArray (Sequenom, Inc.) and PinPoint (PerSeptive Biosystems, Inc.).

Another promising MS genotyping technology involves the use of cleavable mass tags (chemical moieties of known molecular mass) as labels to replace fluorophores.\textsuperscript{[15]} The advantage of mass tags is that, due to the accuracy of MS-based mass determination, the range of labels that can be used is virtually limitless, so ultra-high throughput genotyping is possible. The MassCode system (Qiagen Genomics) uses this technology.

Pyrosequencing

Pyrosequencing is a novel method for sequencing short stretches of DNA based on the detection of pyrophosphate, a normal by-product of DNA synthesis.\textsuperscript{[116]} Although similar in principle to primer extension allele discrimination methods, pyrosequencing is suitable not only for typing SNPs but also for scoring entire haplotypes (groups of linked SNPs). Pyrosequencing works on the basis that pyrophosphate can be used to generate adenosine triphosphate (ATP), which then stimulates luciferase activity, causing the emission of a chemiluminescent signal. To achieve this, the primer extension reaction must include adenosine 5’ phosphosulfate (APS) and the enzyme ATP sulfurylase, which converts APS into ATP in the presence of pyrophosphate. Also present is luciferin (the substrate of luciferase) and the enzyme apyrase, which continuously degrades unincorporated dNTPs and excess ATP. The dNTPs are added to the reaction one by one. Only if the incoming dNTP is complementary to the template will it extend the primer and release pyrophosphate, resulting in the production of an equimolar amount of ATP. Visible light is generated in proportion to the amount of ATP and is detected as a peak on a pyrogram. When the degradation of the present dNTP is completed by apyrase, the next is added. A thioderivative of dATP must be used to avoid constant stimulation of luciferase activity.

CONCLUSION

Association studies aim to find patterns among 0.1% variation in the human genome (mostly represented by SNPs) corresponding to differences in complex phenotypes such as disease susceptibility and response to drugs. The success of such studies relies on accuracy, sensitivity, and high throughput in SNP genotyping. Many of the technologies described in this article are geared toward highly parallel analysis, although MS stands out as a platform where increased throughput is realized by ultra-rapid serial processing.

The sensitivity of most genotyping techniques reflects some form of amplification step either before, during, or after the actual allele discrimination assay. In the majority of cases, this amplification step relies on the PCR, but novel formats such as the Invader assay and RCAT are also becoming popular. Although homogenous assay formats are versatile, array-based technology platforms have the advantage of higher throughput. Recent developments\textsuperscript{[4]} include the use of electrospray ionization arrays, where electrical currents are used to increase the speed and sensitivity of hybridization, and the detection of allele-specific products based on their electrochemical properties when hybridized to oligonucleotide probes attached to minute electrodes. Another emerging technology, bead arrays, combines the flexibility of the homogenous assay format with the convenience and throughput of chip-based detection systems. Bead array systems that employ fluorescence-based detection and sorting are commercially available from Lynx Therapeutics and Illumina, Inc.
REFERENCES