METHODS FOR GENOTYPING SINGLE NUCLEOTIDE POLYMORPHISMS

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■ **Abstract**   One of the fruits of the Human Genome Project is the discovery of millions of DNA sequence variants in the human genome. The majority of these variants are single nucleotide polymorphisms (SNPs). A dense set of SNP markers opens up the possibility of studying the genetic basis of complex diseases by population approaches. In all study designs, a large number of individuals must be genotyped with a large number of markers. In this review, the current status of SNP genotyping is discussed in terms of the mechanisms of allelic discrimination, the reaction formats, and the detection modalities. A number of genotyping methods currently in use are described to illustrate the approaches being taken. Although no single genotyping method is ideally suited for all applications, a number of good genotyping methods are available to meet the needs of many study designs. The challenges for SNP genotyping in the near future include increasing the speed of assay development, reducing the cost of the assays, and performing multiple assays in parallel. Judging from the accelerated pace of new method development, it is hopeful that an ideal SNP genotyping method will be developed soon.

INTRODUCTION

As of the end of 2000, over 1.5 million single nucleotide polymorphisms (SNPs) have been found in the human genome and deposited to public databases (20a, 37). The availability of an ultra-high density SNP map opens the possibility of studying by association genetic factors important in complex genetic traits in the human, taking advantage of the fact that genetic markers in close proximity to mutant genes may be in linkage disequilibrium (LD) to them (21, 42). Association studies can be done with a genome-wide approach (without assuming one region of the genome is more likely to harbor the associated genetic factor) or with a candidate gene approach (using some biological knowledge to prioritize the parts of the genome for the study) (11). Because the strength of LD diminishes rapidly with distance, hundreds to thousands of individuals must be genotyped, regardless of the approach used, with a large number of SNPs (25). Genotyping of SNPs will likely be a major part of every genetic

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association study, and the appropriate genotyping method is critical to the success of the study.

The ideal genotyping method must possess the following attributes: (*a*) The assay must be easily and quickly developed from sequence information; (*b*) the cost of assay development must be low in terms of marker-specific reagents and time spent by expert personnel on optimization; (*c*) the reaction must be robust, such that even suboptimal DNA samples will yield reliable results; (*d*) the assay must be easily automated and must require minimal hands-on operation; (*e*) the data analysis must be simple, with automated, accurate genotype calling; (*f*) the reaction format must be flexible and scalable, capable of performing a few hundred to a million assays per day; and (*g*) once optimized, the total assay cost per genotype (including equipment, reagents, and personnel) must be low.

To date, no such ideal genotyping method exists. Further improvements in biochemistry, engineering, and analytical software must occur before SNP genotyping methods closer to the ideal can be developed. In this review, the three aspects of a genotyping assay—allelic discrimination, assay format, and detection methodology—are discussed. Where appropriate, genotyping methods currently in use are described in the context of these three areas.

ALLELIC DISCRIMINATION

SNPs can be detected in either a sequence-specific or sequence-nonspecific way. Sequence-nonspecific detection is based on the capture, cleavage, or mobility change during electrophoresis or liquid chromatography of mismatched heteroduplexes formed between allelic DNA molecules or single-stranded DNA molecules that assume slightly different conformations under nondenaturing conditions (28). Although sequence-nonspecific detection of polymorphisms is the mainstay in polymorphism/mutation discovery, it is not an acceptable approach to genotyping because one is never certain if the inferred genotyping is the true genotype.

Sequence-specific detection relies on four general mechanisms for allelic discrimination: allele-specific hybridization, allele-specific nucleotide incorporation, allele-specific oligonucleotide ligation, and allele-specific invasive cleavage (27). All four mechanisms are reliable, but each has its pros and cons.

Hybridization

With the hybridization approach, two allele-specific probes are designed to hybridize to the target sequence only when they match perfectly (Figure 1). Under optimized assay conditions, the one-base mismatch sufficiently destabilizes the hybridization to prevent the allelic probe from annealing to the target sequence. Because no enzymes are involved in allelic discrimination, hybridization is the simplest mechanism for genotyping. The challenge to ensure robust allelic discrimination lies in the design of the probe. With ever more sophisticated probe
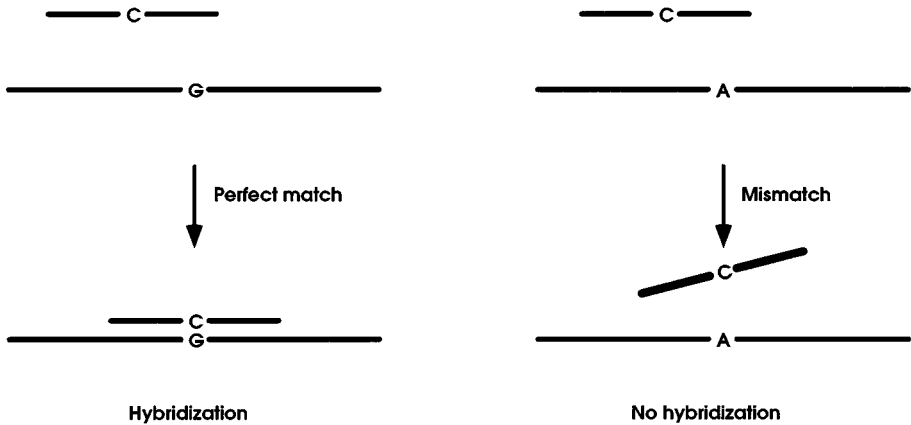
Figure 1   Allele-specific hybridization.

design algorithms and the use of hybridization enhancing moieties, such as DNA minor groove binders, allele-specific probes can be designed with high success rate.

When the allele-specific probes are immobilized on a solid support, labeled target DNA samples are captured, and the hybridization event is visualized by detecting the label after the unbound targets are washed away. Knowing the location of the probe sequences on the solid support allows one to infer the genotype of the target DNA sample. Allele-specific hybridization is also the basis of several elegant homogeneous genotyping assays. These assays differ in the way they report the hybridization event. In the 5′ nuclease assay, a probe annealed to target DNA that is amplified is cleaved during the polymerase chain reaction (PCR) (32). Thus, monitoring the cleavage event is a way to determine whether hybridization has occurred. With molecular beacon detection, hybridization to target DNA opens the stem-loop structure (24, 49). Determining the open-closed status of the stem-loop structure is, therefore, a way to figure out if hybridization has occurred. With "light-up" probes, the thiazole orange derivative linked to a peptide nucleic acid (PNA) oligomer fluoresces only when the PNA oligomer hybridizes specifically to complementary nucleic acids (46). Fluorescence is, therefore, evidence of hybridization.

Primer Extension

Primer extension is a very robust allelic discrimination mechanism. It is highly flexible and requires the smallest number of primers/probes. Probe design and optimization of the assay are usually very straightforward. There are numerous variations in the primer extension approach that are based on the ability of DNA polymerase to incorporate specific deoxyribonucleosides complementary to the sequence of the template DNA. However, they can be grouped into two categories.

First is a sequencing (allele-specific nucleotide incorporation) approach where the identity of the polymorphic base in the target DNA is determined. Second is an allele-specific PCR approach where the DNA polymerase is used to amplify the target DNA only if the PCR primers are perfectly complementary to the target DNA sequence.

In the sequencing approach, one can either determine the sequence of amplified target DNA directly by mass spectrometry (29) or perform primer extension reactions with amplified target DNA as a template and analyze the products to determine the identity of the base(s) incorporated at the polymorphic site (allele-specific nucleotide incorporation; see Figure 2). A number of ingenious ways have been devised for primer extension product analysis in homogeneous assays. Most of these approaches combine novel nucleic acid analogs and monitoring of interesting differences in physical properties between starting reagents and primer extension products.

In the allele-specific PCR approach, one relies on the DNA polymerase to extend a primer only when its 3′ end is perfectly complementary to the template (Figure 3). When this condition is met, a PCR product is produced. By determining whether a PCR product is produced or not, one can infer the allele found on the target DNA. Several innovative approaches have been utilized to detect the formation of specific PCR products in homogeneous assays. Some are based on melting curve analysis, and some are based on hybridization of target specific probes. A variation of this approach is the allele-specific primer extension. Here, the PCR product containing the polymorphic site serves as template, and the 3′ end of the primer extension probe consists of the allelic base. The primer is extended only if the 3′ base complements the allele present in the target DNA. Monitoring the primer extension event, therefore, allows one to infer the allele(s) found in the DNA sample.
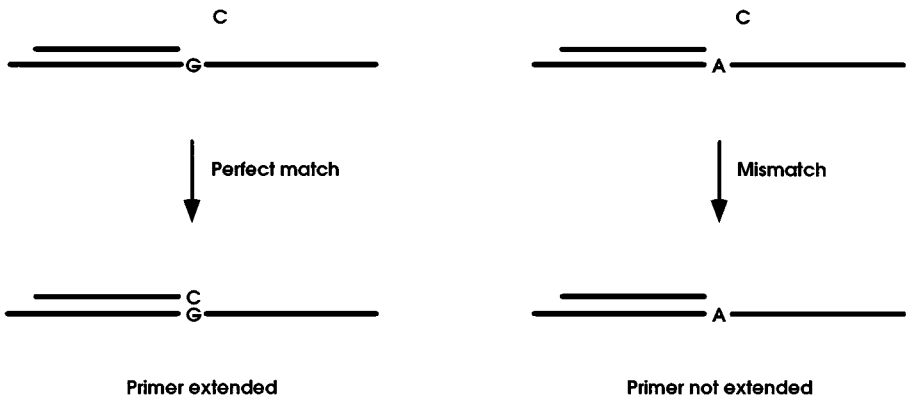

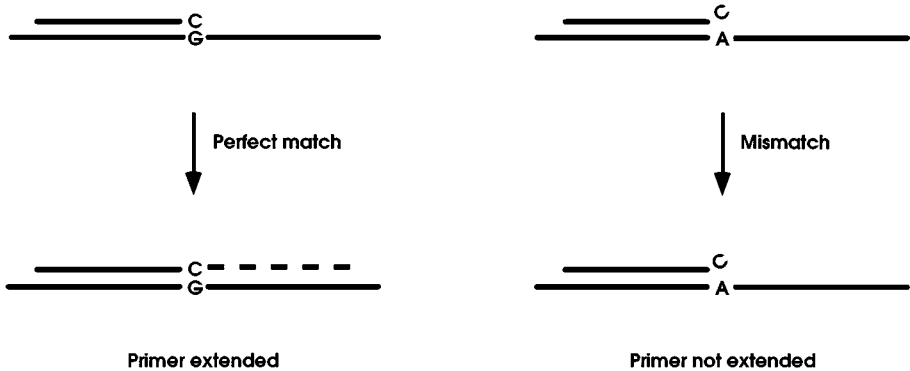**Figure 2**   Allele-specific nucleotide incorporation.

Figure 3   Allele-specific primer extension.

Ligation

DNA ligase is highly specific in repairing nicks in the DNA molecule. When two adjacent oligonucleotides are annealed to a DNA template, they are ligated together only if the oligonucleotides perfectly match the template at the junction (Figure 4). Allele-specific oligonucleotides can, therefore, interrogate the nature of the base at the polymorphic site. One can infer the allele(s) present in the target DNA by determining whether ligation has occurred. Although ligation has the highest level of specificity and is easiest to optimize among all allelic discrimination mechanisms, it is the slowest reaction and requires the largest number of modified probes. However, ligation as a mechanism has the potential of genotyping without prior target amplification by PCR. This can be accomplished either by the ligation
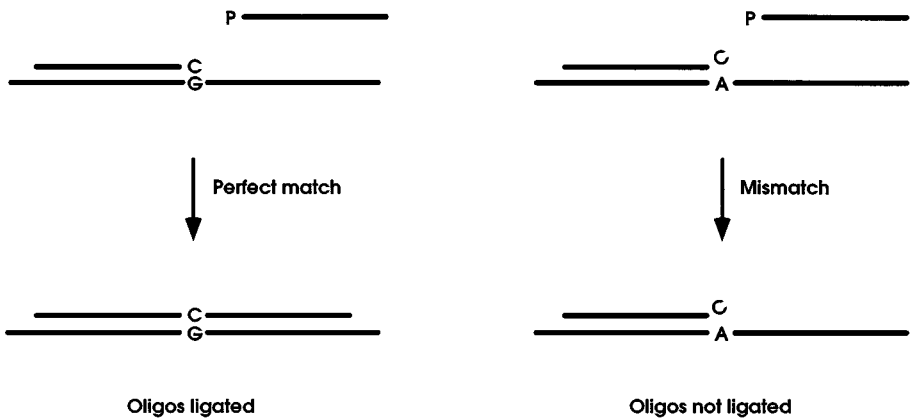

Figure 4   Allele-specific oligonucleotide ligation.

chain reaction (LCR) (3) or by the use of ligation (padlock) probes that are first circularized by DNA ligase followed by rolling circle signal amplification (2, 33).

Invasive Cleavage

Structure-specific enzymes cleave a complex formed by the hybridization of overlapping oligonucleotide probes. When probes are designed such that the polymorphic site is at the point of overlap, the correct overlapping structure is formed only with the allele-specific probe but not with the probe with a one-base mismatch. Elevated temperature and an excess of the allele-specific probe enable the cleavage of multiple probes for each target sequence present in an isothermal reaction. In an innovative application of this method, the cleaved allele-specific probes are used in a second reaction where a labeled secondary probe is cleaved (Figure 5). This signal amplification step helps boost the amount of labeled cleavage product produced to 10^6–10^7 per target sequence per hour, an amount sufficient for detection without the need for a target amplification process such as PCR (17).

The major advantages of this approach are the isothermal nature of the reaction and the potential for genotyping without PCR amplification. There are a number of technical issues that need further refinement. First, the amount of genomic DNA needed in the reaction is high. Second, the purity of the marker specific probes must be extremely high or nonspecific reactions become a nuisance. Third, probe design is somewhat tricky because the sequential reactions have to work under the
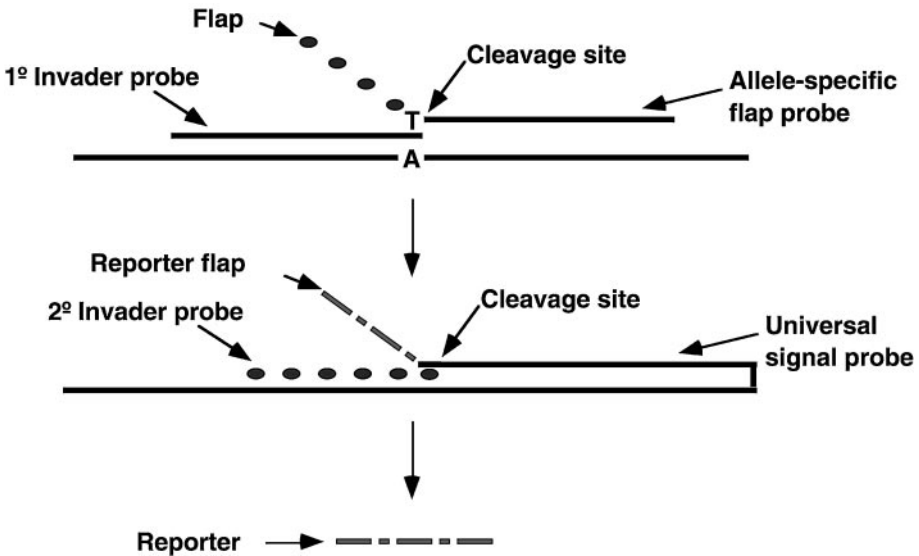

Figure 5 Invader[2] assay.

same conditions, but the sequence context of the SNPs is fixed. With improvements in probe design algorithms and further development, these technical concerns will likely be overcome.

REACTION FORMATS

Starting with genomic DNA, each genotyping method undergoes a series of biochemical steps and a product detection step. The reaction format mostly reflects the requirements of the detection modality. In general, biochemical reactions are more robust in solution, but capturing the reaction products on solid support allows for detection in parallel and increases the throughput substantially.

Homogeneous Reactions

A number of innovative genotyping methods are done in solution from beginning to end and are therefore designated as homogeneous reactions. Some of them require no further manipulations once the reaction is set up initially. Others call for a number of reagent addition steps, but no separation or purification steps are needed. Homogeneous assays are usually robust, highly flexible, and not labor intensive. The major drawback is the limited amount of multiplexing one can do with homogeneous assays.

Solid Phase Reactions

Solid supports used in genotyping can be a latex bead, a glass slide, a silicon chip, or just the walls of a microtiter well. In some cases, marker specific oligonucleotides are placed on the solid support, and the allelic discrimination reaction is done on the support (Figure 6); in other cases, generic oligonucleotides are placed on the solid support, and they are used to capture complementary sequence tags conjugated to marker specific probes (Figure 7). In the former strategy, the oligonucleotide arrays act as a collection of reactors where the target DNA molecules find their counterparts, and the allelic discrimination step for numerous markers proceeds in parallel. In the latter, the arrayed oligonucleotides are used to sort the products of the allelic discrimination reactions (also done in parallel) performed in homogeneous solution. In both cases, the identity of an oligonucleotide on a latex bead or at a particular location on the microarray (on a glass slide or silicon chip) is known, and the genotypes are inferred by determining which immobilized oligonucleotide is associated with a positive signal. The major advantage of performing genotyping reactions on solid supports is that many markers can be interrogated at the same time. Besides saving time and reagents, performing numerous reactions in parallel also decreases the probability of sample/result mix-ups. The drawback of performing genotyping reactions on solid support is that design of the arrays and optimization of the multiplex reactions requires substantial capital and time
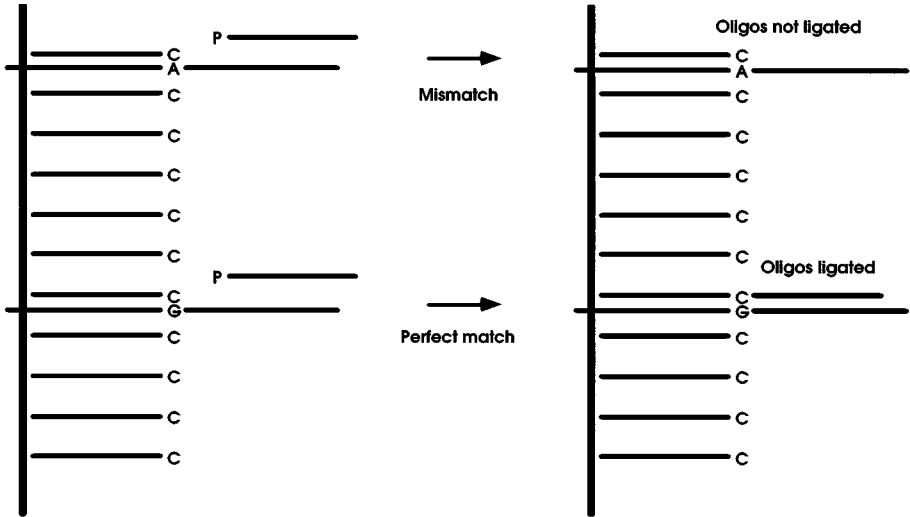
Figure 6   Ligation on solid support.

investment. With better algorithms for multiplex PCR design, this limitation may be alleviated in the near future.

DETECTION MECHANISMS

Detection of a positive allelic discrimination reaction is done by monitoring the light emitted by the products, measuring the mass of the products, or detecting a change in the electrical property when the products are formed. Numerous labels with various light-emitting properties have been synthesized and utilized in detection methods based on light detection or electrical detection. In general, only one label with ordinary properties is needed in genotyping methods where the products are separated or purified from the excess starting reagents. For homogeneous reactions, where no separation or purification is needed, the property of the label has to be changed when a product is formed. This usually requires interaction of the label with another component of the reaction when a product is formed. A number of elegant genotyping methods have been developed to take advantage of certain physical characteristics of the labels.

Monitoring light emission is the most widely used detection modality in genotyping, and there are many ways to do so. Luminescence, fluorescence, time-resolved fluorescence, fluorescence resonance energy transfer (FRET), and fluorescence polarization (FP) are useful properties of light utilized in a host of genotyping methods.
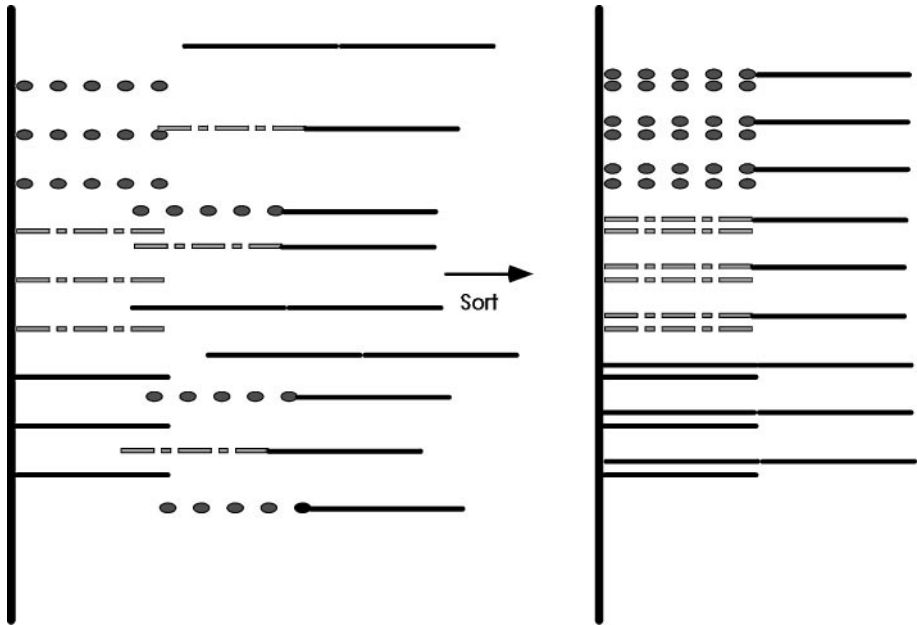
Figure 7   Multiplex homogeneous assay with assortment on solid support.

Luminescence Detection

Luminescence is emitted in an ATP-dependent luciferase reaction. When ATP production is coupled with a primer extension reaction, luminescence is observed every time a deoxyribonucleoside is added in the primer extension reaction. Because the background is extremely low, luminescence has a very good signal to noise ratio. However, the additional enzymatic steps and substrates required complicate the experimental procedure and increase the cost of the assay.

Fluorescence Detection

Fluorescence detection is straightforward and easy to implement. Besides using it to capture fluorescent labels on a solid support or separate the fluorescent product from the label by gel or capillary electrophoresis, fluorescence detection can be used to monitor the formation of double-stranded DNA with a DNA intercalating dye that only fluoresces in the presence of double-stranded DNA. Direct fluorescence detection is very versatile and can be done in multiplex to a certain extent. However, the need for product purification or separation when fluorescent labels are used and the interference by nonspecific double-stranded DNA species when intercalating dyes are used are some of the drawbacks of direct fluorescence detection.

Time-Resolved Fluorescence Detection

Time-resolved fluorescence as a detection approach is feasible when the emission half-life of the fluorescent dye is long. With this class of dyes (mostly compounds of rare earth elements, such as Lanthanides), the fluorescence reading is done sufficiently long after excitation, such that autofluorescence (which has a very short half-life) is not observed (18, 23). The background in time-resolved fluorescence detection is almost nonexistent; thus, this is a very sensitive detection modality. The drawback is that the lanthanides are inorganic compounds that cannot be used to label nucleic acids directly. An organic chelator conjugated to the probe must be used to bind the lanthanides in the reaction.

Fluorescence Resonance Energy Transfer

Fluorescence resonance energy transfer is a popular detection method in homogeneous genotyping assays. FRET occurs when two conditions are met. First, the emission spectrum of the fluorescent donor dye must overlap with the excitation wavelength of the acceptor dye. Second, the two dyes must be in close proximity to each other because energy transfer drops off quickly with distance. The proximity requirement is what makes FRET a good detection method for a number of allelic discrimination mechanisms. Basically, any reaction that brings together or separates two dyes can use FRET as its detection method. FRET detection has, therefore, been used in primer extension and ligation reactions where the two labels are brought into close proximity to each other. It has also been used in the 5′ nuclease reaction, the molecular beacon reaction, and the invasive cleavage reactions where the neighboring donor/acceptor pair is separated by cleavage or disruption of the stem-loop structure that holds them together (17, 24, 32, 49). The major drawback of this method is the cost of the labeled probes required in all the genotyping approaches with FRET detection. In the cleavage approaches, the probes are doubly labeled, further increasing the cost of probe synthesis.

Fluorescence Polarization

Fluorescence polarization (FP) has been used in clinical diagnosis and numerous binding assays for years, but its use as a detection method for SNP genotyping has a very short history. This is because instruments sensitive enough for detecting small amounts of dyes were not available until recently. When a dye is excited by plane polarized light, the emitted fluorescence is also polarized. The degree of polarization is determined by the temperature, the viscosity of the solvent, and the molecular volume of the fluorescent molecule. All these factors affect molecular motion, and in general, the faster a molecule tumbles and rotates in solution, the less polarized is its fluorescence. Because molecular volume is proportional to molecular weight, fluorescence polarization is, therefore, a good method to detect changes in molecular weight. In principle, any genotyping method in which the product of the allelic discrimination reaction is substantially

larger or smaller than the starting fluorescent molecule can use FP as a detection method. Indeed, FP is used as the detection method in the primer extension reaction where small fluorescent dye terminators are incorporated into a larger probe (8). Furthermore, FP is a good detection method in the 5′ nuclease reaction where small fluorescent molecules are formed when large fluorescent probes are cleaved in the reaction (30). Our group also found that FP can be used as a detection method for the invasive cleavage reaction where the large fluorescent signal probe is cleaved, producing a small fluorescent tag (T.M. Hsu, S. Law, S. Duan, B. Neri, & P-Y. Kwok, unpublished results). The advantages of the FP detection method include the much smaller amount of fluorescent dyes needed compared to FRET or direct fluorescence detection methods, the cheaper probes used, and the potential for utilizing the full visible spectrum in multiplex reactions. The drawback is mainly that any nonspecific products will increase the noise in the signal.

Mass Spectrometry

Unlike all other detection methods that infer the identity of the products generated in the allelic discrimination reaction by monitoring the fate of some label, mass spectrometry (MS) measures the molecular weight of the products formed and is, therefore, the most direct method of detection. Because MS determines the fundamental property of the DNA molecule, no labels are needed. High resolution MS can easily distinguish between DNA molecules that differ by only one base (4, 5, 31, 44). A further advantage of MS is that it takes only milliseconds to analyze each sample, so even though MS analyzes each sample serially, the throughput is still very high. Furthermore, by appropriately designing the probes, moderate multiplexing is possible (44). The main disadvantage of the MS detection method is the exquisite purity required of the analyte for it to work. With further refinement of the product purification process, it may be possible to overcome this drawback.

Electrical Detection

A promising detection method is one that monitors a change in the electrical properties of the products of the allelic discrimination reaction. Currently, this is done on solid support where oligonucleotides are deposited on electrodes (12, 51). The electrical property of the probe is altered when the DNA complementary to the probe is annealed to it. This is exaggerated if a ferromagnetic label is used. Electrical detection combines semiconductor technology with biochemistry and eliminates the need for light detection or extensive product processing. This area is still in its infancy, and there are still a number of biochemical and engineering obstacles to overcome before the throughput of genotyping methods based on this detection mechanism is high enough and the cost low enough for its wide acceptance.

EXAMPLES OF GENOTYPING METHODS

A number of SNP genotyping methods are discussed in some detail to highlight how the allelic discrimination mechanisms, reaction formats, and detection modalities can be combined in various ways to produce the many SNP genotyping approaches in use today. Because of space limitations, the list of genotyping methods is not exhaustive. Interested readers are encouraged to examine the primary references for in-depth description of the methods.

Microarray Genotyping: Hybridization on Solid Support with Fluorescence Detection

The first large-scale genotyping method was developed jointly by the Whitehead Institute and Affymetrix, Inc. (50). The GeneChip HuSNP Mapping Array contains 1494 SNPs that can be genotyped in one experiment (35). The major breakthrough of this approach is the degree of multiplexing achieved by designing PCR assays that amplify very small products and a second round of PCR with a common set of primers. In the current version of the assay, the entire set of 1494 SNPs is amplified in just 24 multiplex reactions (average of 62 SNPs in one multiplex reaction). In a rather long protocol, the PCR products are pooled, concentrated, hybridized to the DNA microarray, stained, and visualized. Because each SNP is interrogated by a set of "tiling" oligonucleotides, the genotypes called are quite accurate. Other advantages of this approach include the low requirement for starting genomic DNA (120 ng total for all 1494 markers), the large number of SNPs that are typed in one experiment, and the minimal manual steps. The major drawback of this approach is that because the design and manufacture of the microarray are quite expensive, the set of markers selected cannot be changed quickly or arbitrarily. Furthermore, it is a common experience that about 20% of the SNPs on the HuSNP chip do not yield confident results (J. Fan, U. Surti, T. Taillon-Miller, G. Kennedy, T. Ryder, & P-Y. Kwok, unpublished results). This level of failure rate is too high for many applications.

Another assay based on hybridization on solid support is the DASH (dynamic allele-specific hybridization) assay (19). Instead of monitoring the hybridization at a constant temperature, this approach looks for the melting temperature differences between the allele specific probe when it is annealed to the matched and mismatched targets by monitoring the hybridization over a range of temperatures. Using a DNA intercalating dye, such as Sybr Green I, that fluoresces in the presence of double-stranded DNA, fluorescence is observed only when hybridization occurs and double-stranded DNA species is formed. With recent improvements in probe design, DASH assays can now be designed for nearly 100% of SNPs that can be uniquely amplified by PCR (41). The specificity and robustness of the assay come with a price. Because double-stranded DNA species will cause the dye to fluoresce, the PCR products have to be rendered single-stranded. This is accomplished by utilizing a biotinylated PCR primer and capturing the biotinylated

PCR product on solid support, followed by denaturing and washing away the unlabeled strand of PCR product. Furthermore, the single reporter used means that two reactions must be run in parallel for each SNP.

Molecular Beacon Genotyping: Homogeneous Hybridization with FRET Detection

Molecular beacons are stem-loop structures that hold a fluorescent reporter in close association with a universal quencher such that fluorescence is only observed when the stem-loop structure opens up (24). With proper design, a DNA target that is perfectly complementary to the sequence of the loop portion of the molecular beacon hybridizes to the molecular beacon and forces open the stem, leading to the emergence of fluorescence. The molecular beacon with a one base mismatch will not hybridize to the target strongly enough to disrupt the stem-loop structure, and no fluorescence is observed (49). Because the presence or absence of fluorescence reflects the open or close status of the stem-loop structure, no purification or separation steps are needed. In fact, once the assay is set up, no more manual manipulations are needed. As long as one can monitor the fluorescence, one can infer the genotype of the DNA target. This "closed-tube" system has real advantages because cross contamination is minimized and automation is easily achievable. An added advantage is that when real-time fluorescence monitoring is possible, the assay can be used to quantify the amount of DNA present in an unknown sample. With many fluorescent dyes available in the visible spectrum, multiplex analysis is possible to some extent. The one drawback of this approach is the cost of the two doubly labeled molecular beacons needed for each SNP marker. Until design algorithms are perfected, a fraction of molecular beacons will not work without optimization.

5′ Nuclease Assay: Homogeneous Hybridization with FRET or FP Detection

Taq DNA polymerase possesses a 5′ nuclease activity that displaces and cleaves oligonucleotides hybridized to a DNA segment undergoing replication. Based on this observation, the TaqMan assay was developed with a doubly labeled probe consisting of a reporter fluorescent dye and a minor groove binder (MGB)/universal quencher complex (32). During the extension phase of PCR, the TaqMan probes hybridize only to the perfectly matching DNA target and not to those with a one-base mismatch. Cleavage of the hybridized probe separates the quencher from the reporter, and fluorescence is observed. One can, therefore, infer the genotype of a test sample by monitoring the fluorescence of the reaction mixture. It was shown recently that fluorescence polarization is a good detection method for this assay (30). Because the starting probe has a much higher molecular weight than the cleavage products, the fluorescence polarization changes drastically in a positive reaction. Just like the molecular beacon assay, the TaqMan assay is a closed-tube

system and can be used for quantification of unknown DNA samples. This assay has been in use and thoroughly tested over several years now. With a number of improvements in assay design, such as the incorporation of the minor groove binder that enhances the discriminating power between the TaqMan probes and more reliable primer design algorithms, the assay is easier to optimize. The cost of the labeled probes is the main obstacle to the widespread adoption of this method by the average laboratory.

Allele-Specific PCR: Homogeneous Primer Extension with Fluorescence or FRET Detection

Allele-specific PCR has been used for quite some time to genotype SNPs. It is a relatively simple technique, and when coupled with gel analysis, the genotypes can be called easily. Because the products are of the same size, two parallel reactions must be performed for each marker when gel electrophoresis is used as the detection method. Because there is no way to control for false-negative results, allele-specific PCR is not used in large-scale projects. Everything changed when three groups devised novel ways to detect PCR products in homogeneous solution.

Germer & Higuchi took advantage of several recent advances to achieve single-tube genotyping by allele-specific PCR (15). First, a DNA intercalating dye was used to detect the presence of double-stranded DNA. Second, real-time fluorescence detection was used to determine the melting curve of a PCR product. Third, a GC-rich sequence was added to one of the allele-specific PCR primers to increase the melting temperature of one of the PCR products. Fourth, the stoffel fragment of Taq polymerase with two important attributes for allele-specific PCR was used in this method. Under assay conditions, the stoffel fragment of Taq polymerase only extends the primer where the 3′ end matches the target sequence and only yields amplicons <100 bps. Taken together, these four advances allow for highly specific PCR amplification, with the resulting products easily distinguishable by melting curve analysis. Because both alleles can be assayed in the same reaction, this is another closed-tube method where one only has to set up the reaction and the instrument will take care of the rest. The advantage of this method is the low cost of the unlabeled primers and the simplicity of the assay. The only drawback is that not all SNPs can be assayed by this method because the amplicons must be small. Under the best circumstances, kinetic PCR cannot be designed for about 20% of SNPs.

Todd et al. developed a method based on the ability of a DNA enzyme that can cleave an RNA-containing reporter probe (47). Specifically, the antisense sequence of a 10–23 DNAzyme is added to one of the PCR primers for the assay such that the active DNAzyme is formed only if PCR amplification occurs. A DNA/RNA chimeric reporter substrate containing fluorescent and quencher dye molecules on opposite sides of the cleavage site is added to the reaction mixture and is cleaved as the DNAzyme forms during PCR amplification. The accumulation of PCR products is monitored in real time via changes in the fluorescence that is

released by the separation of fluoro/quencher dye molecules as the newly formed DNAzyme cleaves the reporter substrate. The DzyNA-PCR DNA detection is novel and attractive because the only specialty reagent, the energy transfer DNA/RNA hybrid reporter substrate of the DNAzyme, can be used in any assay. The only target specific reagents are the two PCR primers, with one modified with the antisense sequence of the DNAzyme. Because this assay can be monitored in real time, DNA quantification is possible. At this point, the limitations of this assay are that only one reporter substrate is used and SNP genotyping has to be done in parallel reactions.

Myakishev et al. recently described a similar method in which the two allele-specific PCR primers are tailed with sequences that introduce priming sites for universal energy-transfer-labeled primers (36). The energy-transfer-labeled primer contains a reporter dye that is quenched by a universal quencher in its natural stem-loop structure. When allele-specific PCR products are formed, the priming site is formed and the energy-transfer-labeled primer is extended. The extension product serves as a template for the next round of PCR, and the stem-loop structure is opened up as PCR proceeds, thereby releasing the reporter dye from the quencher and fluorescence is observed. Like kinetic PCR, only one reaction is required for each SNP and it is done in a closed-tube format. With one set of universal energy-transfer-labeled primers that can be used for any assay, this approach is quite cost-effective.

All three allele-specific PCR methods have nice features and are relatively inexpensive to develop. However, allele-specific PCR cannot be designed for every SNP because of local sequence constraints and because of the difficulty of performing the reaction in multiplex.

Allele-Specific Primer Extension: Primer Extension on Solid Support with Fluorescence Detection

Instead of performing allele-specific PCR, one can utilize allele-specific primers to detect the presence or absence of a SNP within a PCR product. Because the PCR step is separated from the allele detection step, this method is more versatile and has two levels of specificity. It is, therefore, very robust, and assays can be designed for almost all SNPs. In a recently described rendition of this method, some clever modifications were made to simplify the reaction procedure (40). By attaching a T7 RNA polymerase promoter sequence to one of the PCR primers, researchers generated RNA templates from the PCR products. The RNA templates then serve as the target in an allele-specific primer extension reaction mediated by a reverse transcriptase. Dye-labeled rNTPs are used in the reaction, and they are incorporated when the immobilized allele-specific primer's 3′ base matches the allele found on the RNA template. Because of the large number of RNA templates produced by the T7 RNA polymerase and the multiple dye-labeled rNTPs incorporated, very small amounts of PCR products are needed. This approach solved some of the problems that plagued solid phase primer extension reaction in the past, namely,

the need for PCR product purification and generation of single-stranded DNA template for robust reaction. The good attributes of this approach include simple reaction procedure, small reaction volume, and low requirements for genomic DNA templates. The reaction mixture is complex, however, with multiple enzymes and specialty rNTPs.

Arrayed Primer Extension: Primer Extension on Solid Support with Fluorescence Detection

Unlike allele-specific PCR, which assays for the presence or absence of a PCR product, the "generic" primer extension approach assays for the specific nucleotide that is incorporated onto the primer at the polymorphic site. In the arrayed primer extension (APEX) approach (13, 39, 45, 48), oligonucleotides that sequence correspond to the those neighboring the polymorphic sites of their corresponding SNPs and are immobilized via their 5′ end on glass surface. These probes are extended by one base in the presence of PCR products containing the SNP sequences. With each of the four dideoxyterminators labeled with different fluorescent dyes, the identity of the incorporated base can be inferred easily. Using fluorescence imaging techniques, the genotypes can be determined simply by noting the colors found on the various spots on the array. The APEX assay is quite robust and can be multiplexed. Furthermore, a universal master mix containing the four dye-labeled terminators and DNA polymerase is used for all SNPs, making it a very simple reaction to set up. The challenge is that thermal cycling is generally not easily achieved in solid phase reactions, so single-stranded templates are needed for robust primer extension. This requires a larger amount of PCR products as target and a strand separation step that increases the cost of the reaction. In addition, placing SNP-specific probes on the solid support decreases the flexibility of the approach.

Homogeneous Primer Extension Assays with FRET or FP Detection

These approaches take advantage of the fact that dye-labeled terminators are incorporated covalently onto an oligonucleotide as the reaction proceeds. If a donor dye is found on the probe, excitation of the donor dye will cause the acceptor dye attached to the incorporated terminator to fluoresce (7). Observation of FRET is, therefore, an indication that primer extension has occurred. With two different acceptor dye-labeled terminators, the genotype of a sample can be determined in one reaction (10). When a thermostable DNA polymerase is used and the fluorescence monitored in real-time, the assay is very sensitive and robust. However, the dye-labeled probe is relatively costly.

The primer extension reaction greatly increases the molecular weight of the dye-labeled terminator when it is incorporated onto the oligonucleotide probe. This change is reflected in the observed FP value. When the reaction is driven to completion, the FP value is maximally increased and the genotypes can be determined easily (8). Because the probe is unlabeled and is used only to add

molecular weight to the primer extension product, the start-up cost of the assay is among the cheapest of all genotyping assays in use to date. With highly sensitive instruments readily available for FP detection, the throughput of this assay can be very high.

The major drawback of the homogeneous primer extension assays is the need to degrade the excess PCR primers and dNTPs after the PCR step. This is necessary because the primers and dNTPs will interfere with the primer extension reaction. In the current reaction protocol, the excess primers and dNTPs are degraded enzymatically with exonuclease I and shrimp alkaline phosphatase. After a short incubation, the enzymes are heat inactivated before the primer extension reaction mix is added, and the reaction is allowed to proceed. Although the possibility for multiplex reaction is quite limited, the versatility and simplicity of the assay, with minimal requirements for optimization, make the assay an attractive choice for many applications.

Primer Extension with Detection by Mass Spectrometry

Mass spectrometry (MS), a method well suited for detection of small DNA molecules, has been used as a detection method for a number of primer extension genotyping assays. It is used for both the generic primer extension reaction and the allele-specific primer extension reactions.

To date, the most successful applications of MS detection in the primer extension reaction are found in the biotechnology industry. For example, Ross et al. utilize matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to detect primer extension products in multiplex (44). Because the mass resolution is high, the few mass units that differentiate the primer extension products of the two alleles can be distinguished handily. Careful design of the primers used in the primer extension reaction ascertains that all the extended and unextended primers are in well-resolved mass windows. As a result, researchers can genotype 12 SNP markers in one multiplex PCR/primer extension/MS detection sequence. Buetow et al. (5) describe a variation of the generic primer extension method in which only one dideoxyterminator is used in the reaction such that the primer extension products for the two alleles differ by at least one base (over 300 mass units). Unlike methods that depend on some form of fluorescence detection, no labeling is necessary in MS detection. The intrinsic mass differences between the primer extension products are assayed. Although MS is highly accurate and a moderate degree of multiplex is possible, a number of obstacles need to be overcome. First and foremost, the MS instrument can only handle one sample at a time. Even with multiplex PCR, each MS instrument will probably not be able to genotype more than 10,000 marker assays per day. Second, MS detection requires purified samples that are free from ions and other impurities. This increases both the cost and time required for sample processing. However, many talented groups are working to improve the approach and these obstacles may be overcome in the near future.

Pyrosequencing: Homogeneous or Solid Phase Primer Extension with Luminescence Detection

Pyrosequencing is a new DNA sequencing method based on detecting the formation of pyrophosphate, the by-product of DNA polymerization (43). When DNA polymerase takes a deoxynucleoside triphosphate and incorporates it onto the extending primer, pyrophosphate is formed. By a number of wisely designed enzymatic steps, the pyrophosphate is converted to ATP that fuels a luciferase reaction (1, 38). Therefore, light is observed when a nucleotide is added to the growing chain of DNA. Because stepwise addition of nucleoside triphosphates is needed in this procedure, utilizing this method to give long sequencing reads is difficult. It is a robust method for the primer extension detection, especially if there are a number of closely spaced SNPs (as in HLA typing). The major advantage of this method is the fact that, if needed, multiple bases in the vicinity of the polymorphic site can be determined and so the placement of sequencing primer is more flexible. As in other primer extension methods, excess PCR primers and dNTPs must be removed prior to the pyrosequencing reaction. Furthermore, seven enzymes and two specialized reagents (APS and luciferin) are required in the homogeneous assay (four enzymes are needed in the solid phase reaction format). These requirements make it almost impossible to keep the cost of genotyping with this method lower than other approaches.

Multiplex Primer Extension Sorted on Genetic Arrays: Homogeneous Reaction with Separation/Capture on Solid Support and Fluorescence Detection

To increase the SNP genotyping throughput, a number of groups have devised ways to perform the allelic discriminating primer extension reaction in multiplex in solution and separate the products by capturing them on solid support. This is done by utilizing chimeric primer in the primer extension reaction with 3′ complementarity to the specific SNP loci and 5′ complementarity to specific capture probes on solid support (6, 14). The solid support can be color-coded microspheres or a silicon chip. The dye-labeled terminators provide the identity of the base incorporated, whereas the specific capture probe sequence provides the identity of the SNP being assayed. Flow cytometry is used for sorting the microspheres, and CCD imaging is used for microarray analysis. With this approach, one takes advantage of the more robust homogeneous reaction format in the allelic discrimination step and the capture probes on solid support to allow for multiplex reactions. Because the capture probes are generic, they can be designed in such a way that all of them will anneal optimally to their complementary sequences at the same temperature. Furthermore, the collection of capture probes on microspheres and on the silicon chip or glass slide can be made in high volume, therefore lowering the cost of the reagent. The only drawback of these approaches is that multiplex PCR and multiplex primer extension reaction are still not easy to optimize.

Ligation with Rolling Circle Amplification: Solid Phase Reaction with Fluorescence Detection

Formation of a circular DNA molecule by ligation provides a means to perform SNP genotyping without PCR amplification. Two groups have shown that the circular DNA ligation product serves as a template for a rolling circle amplification (RCA) step that yields a product thousands of times the size of the original circle. If fluorescent nucleotides are used as the building blocks of the RCA reaction, the signal achieved is bright enough to allow one to detect single molecules. This approach has been used to determine the allele found on a single chromosome in FISH (fluorescent in situ hybridization) analysis and to genotype SNPs on solid support (2, 33). The ability to obtain the genotype of SNPs directly from genomic DNA is a major advantage. This is perhaps the only method one can use to determine long-range haplotypes by FISH analysis. The high cost of the probes and the fact that only one level of specificity is involved make this an approach for special applications but not for general use.

Homogeneous Ligation with Fret Detection

When an allele-specific oligonucleotide labeled with an acceptor dye is ligated to an oligonucleotide bearing a donor dye in the presence of the complementary target DNA, FRET is observed. Because PCR and ligation are different reactions, the assay can be done in a closed-tube format by thermally isolating the two reactions (9). The PCR primers are designed to be long and anneal at a higher temperature, whereas the ligation probes are designed to be short and therefore anneal only at a lower temperature. If the PCR reaction is allowed to proceed at high temperature, the ligation probes will not anneal and the 5′ probe will not be extended (and, thus, will not be taken out of the ligation reaction). After the PCR is largely completed, the thermal cycling conditions are changed, and ligation is allowed to run at a lower temperature. FRET is monitored in real time, and the genotypes can be determined quite easily by measuring the rate of emergence of fluorescence for the two dyes found on the allele-specific ligation probes. Because the ligation reaction is very specific, this reaction is probably the easiest of all closed-tube reactions to optimize. However, all 3′ ligation probes are labeled with dyes and the start-up reagent cost is high.

Multiplex Ligation Reaction Sorted on Genetic Arrays: Homogeneous Reaction with Separation/Capture on Solid Support and Fluorescence Detection

Like the microsphere/microarray based primer extension described above, the ligation assay can also be done in multiplex and captured/separated on solid support. Once again, chimeric probes are used where the 5′ half of the sequence complements the capture probe and the 3′ half of the sequence is allele-specific for a particular SNP. A reporter dye is used to label the common ligation probe such

that the dye is captured along with the allele-specific ligation probe only if ligated to the probe in the presence of a DNA target with the correct allele (20). In this approach, only one of the three ligation probes is labeled with a dye. The two allele-specific ligation probes are labeled only with inexpensive nucleotide sequences. Furthermore, because ligation is very specific, multiplex ligation requires minimal optimization. The major disadvantage of this method is the need for three ligation probes, compared to just one for the primer reaction.

Invader Assay: Homogeneous Invasive Cleavage with FRET, FP, or Mass Spectrometry Detection

The invader assay is based on the ability of a thermostable flap endonuclease to cleave a structure formed by the hybridization of two overlapping oligonucleotide probes to a target nucleic acid strand (22). By designing the flap probe with the allelic base at the overlapping site, the correct structure is formed only when the probes perfectly complement the DNA target. Upon cleavage, the flap released from the allele-specific probe (an arbitrary sequence unrelated to the SNP) serves as the "invader" probe in the secondary invader reaction. In the secondary reaction, a universal reporter probe is cleaved only when it forms the proper overlapping structure in the presence of the flap from the primary reaction. Taken together, the amplification is squared, and the assay can work from genomic DNA without the need for target amplification (17, 26, 34). The flap design of the universal reporter probe is based on the detection method employed. For example, if mass spectrometry is the detection method to be used, flaps with varying numbers of nucleotides serve as the reporter signal (16). If FRET is the detection method, a reporter dye is placed on the flap while the quencher is placed on the annealed portion of the universal probe (17). For FP detection, the dye reporter is placed at the end of the flap (T.M. Hsu, S. Law, S. Duan, B. Neri, & P-Y. Kwok, unpublished results).

The invader assay is an elegant SNP genotyping method and holds promise for directly assaying genomic DNA without PCR. However, the signal amplification approach suffers from the fact that only one level of specificity is utilized so that a significant fraction of SNPs in genomes with many repetitive sequences cannot be assayed by this method. Furthermore, although the primary probes are unlabeled, they must be exquisitely pure to work well in the system, thus increasing the cost of the starting reagents.

DISCUSSION AND CONCLUSIONS

Clever use of enzymatic and detection methods has produced a number of robust SNP genotyping methods. Despite recent advances in the field, none of the assays is ideally suited for all applications. Which genotyping assay to adopt, therefore, depends on the needs of the projects being pursued. In general, there are three scenarios to be considered. First, in a clinical diagnostic setting or when working

with model organisms where a "canonical" set of markers are being tested on a stream of samples over an extended period of time, one can afford to invest in assay optimization. Here, the closed-tube assays that minimize contamination and sample mix-up are likely to dominate. Second, in a research study where new markers are constantly being identified and genotyped in hundreds of samples, assay development must be simple, and the initial cost of assay development must be low. In this instance, primer extension reactions are the most logical choice. Third, in cases where thousands of markers must be used to type thousands of samples, multiplex assays are needed at very low operating cost. Here, some form of array-based multiplex genotyping method or a high-density reaction vessel capable of handling thousands of homogeneous assays will be needed. Unlike the first two scenarios, the large-scale genotyping studies are currently not feasible.

Reflecting back on the attributes of the ideal SNP genotyping assay listed in the Introduction, it is fair to say that, with optimization, every genotyping method can be made to work robustly and produce accurate results. Furthermore, a number of companies have put together systems for automated SNP genotyping. However, three areas need special attention before an ideal genotyping assay, especially one that can handle large-scale genotpying projects, can be achieved. First, assay development has to be fast and inexpensive. Second, the cost of the assay (from instrumentation to reagents) must be affordable. Third, reactions must be done in a massively parallel fashion.

Although DNA is a simple polymer made up of four bases, the specific sequence of DNA affects its physical property and the way enzymes interact with it. Because we lack complete understanding of the relationship between DNA sequence and physical properties or enzymatic interactions, assay design is not always straightforward, and some optimization is needed for a significant number of SNPs. Furthermore, the redundancy of certain sequence motifs in some genomes (notably the human genome) imposes another level of complexity in assay design. A major challenge for SNP genotyping, in general, is the speed of assay development. If one day is needed to develop 100 assays, 3 years will be required before assays for a set of 100,000 markers (a projected number of markers needed for genome-wide studies) can be assembled for such a project. With increased sophistication in bioinformatics and a better understanding of enzymatic behavior, assay development will be faster and will have a higher rate of success. Given the fact that millions of SNPs have been found just in the human genome alone, this is a significant challenge to all working in the field.

Many of the new assays rely on detection methods that require highly specialized instrumentation. These include high-resolution mass spectrometers, plate readers capable of real-time detection of fluorescence, plate readers that can detect fluorescence polarization at high speed, integrated systems that can perform both the reaction and detection steps, and flow sorting instruments equipped with multiple lasers. These sophisticated instruments are very capable but come at a price too high for most laboratories. Even well-funded institutions cannot afford to acquire more than two or three different systems. Moreover, many assays require

expensive probes or other reagents that keep the genotyping cost high. This double hurdle of high instrumentation and operating costs must be overcome before large-scale SNP genotyping becomes feasible. The hope is that with time, the cost of SNP genotyping will drop sufficiently so that even large studies can be done cost effectively.

The most difficult challenge may be the need to assay thousands of markers simultaneously. At present, multiplex PCR is developed by trial and error, and a 100-plex reaction is almost impossible to achieve routinely. Even with better understanding of the forces behind a successful multiplex reaction and improved software, one still has to synthesize two PCR primers for each marker. Now that the DNA sequences of whole genomes are available for a number of organisms, perhaps a new way of whole genome amplification with concomitant simplification can be achieved such that the target amplification step is done easily and cost effectively. A serious collaboration between molecular geneticists and those skilled in bioinformatics will be needed to make this happen. Judging from the speed with which new genotyping assays are being invented, there is great hope that the ideal assay will be developed in the near future.

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