ACCESSING GENETIC VARIATION: GENOTYPING SINGLE NUCLEOTIDE POLYMORPHISMS

Ann-Christine Syvänen

Understanding the relationship between genetic variation and biological function on a genomic scale is expected to provide fundamental new insights into the biology, evolution and pathophysiology of humans and other species. The hope that single nucleotide polymorphisms (SNPs) will allow genes that underlie complex disease to be identified, together with progress in identifying large sets of SNPs, are the driving forces behind intense efforts to establish the technology for large-scale analysis of SNPs. New genotyping methods that are high throughput, accurate and cheap are urgently needed for gaining full access to the abundant genetic variation of organisms.

LINKAGE DISEQUILIBRIUM MAPPING
Analysing single nucleotide polymorphism alleles in population-based studies to identify loci that are associated with a particular disease or phenotype.

Department of Medical Sciences — Molecular Medicine, Uppsala University, University Hospital, 75185 Uppsala, Sweden. e-mail: Ann-Christine.Syvanen@medsci.uu.se

Comparison of genomic DNA sequences in different individuals reveals some positions at which two, or in some cases more than two, bases can occur. These single nucleotide polymorphisms (SNPs) are highly abundant, and are estimated to occur at 1 out of every 1,000 bases in the human genome[1,2]. Depending on where a SNP occurs, it might have different consequences at the phenotypic level. SNPs in the coding regions of genes that alter the function or structure of the encoded proteins are a necessary and sufficient cause of most of the known recessively or dominantly inherited monogenic disorders. These SNPs are routinely analysed for diagnostic purposes. Another important group of SNPs are those that alter the primary structure of a protein involved in drug metabolism. These SNPs are targets for pharmacogenetic analyses[3]. Missense SNPs in the coding regions of genes, such as the two SNPs in the apolipoprotein E gene[4] and the factor V Leiden mutation[5], can also contribute to common disease. This type of SNP can be analysed to assess the risk of an individual for a particular disease. In addition, it is likely that SNPs in the regulatory regions of genes might influence the risk of common disease. However, most SNPs are located in non-coding regions of the genome, and have no direct known impact on the phenotype of an individual. These SNPs are useful as markers in population genetics and evolutionary studies[6,7].

The reason for the current enormous interest in SNPs is the hope that they could be used as markers to identify genes that predispose individuals to common, multifactorial disorders by using LINKAGE DISEQUILIBRIUM (LD) MAPPING[8,9]. The rationale would be to genotype a collection of SNPs that occur at regular intervals and cover the whole genome to detect genomic regions in which the frequencies of the SNP alleles differ between patients and controls. It is assumed that the SNP alleles are inherited together with the disease-predisposing alleles through the generations because they are physically close to each other. The disease-predisposing genes could then be localized and isolated, and proteins encoded by them would be valuable targets for developing new therapeutic drugs. As a result of the efforts of the SNP Consortium — a collaboration of 14 major pharmaceutical companies and the Wellcome Trust, as well as members of the Human Genome Project[1] — there are almost 2 million SNPs in public databases, and perhaps twice that number of SNPs in commercial databases, such as that of Celera Genomics[2]. The SNPs are now being verified experimentally, and their distributions between and within populations are being assessed[10,11].

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Table 1 | **Recent or large studies involving SNP genotyping**

Purpose of study	No. of SNPs	No. of samples	No. of genotypes	Method
SNPs in the *ACE* gene[131]	13	1,300	17,000	Restriction-site analysis
NOD2 gene in Crohn disease[132]	13	1,300	17,000	Invader[115] and TaqMan[50] assays
Mapping complex disease traits in mice[34]	109	299	33,000	Allele-specific PCR[60]
Sequence diversity of the *APOE* gene[133]	20	2,200	44,000	Oligonucleotide ligation assay[95]
APOE haplotypes in Alzheimer disease[32]	60	1,000	60,000	TaqMan assay[50]
Ancestral alleles of human SNPs[7]	214	412	99,000	GeneChip[39]
Carrier frequencies of disease mutations[134]	31	4,400	140,000	Primer extension on microarrays[82]
Haplotype tagging in diabetes candidate genes[135]	122	1,500	180,000	Invader PCR assay[115]
Linkage disequilibrium in the 5q31 region[136]	312	681	210,000	Multiplex primer extension[69,71,137]

ACE, angiotensin I-converting enzyme (peptidyl-dipeptidase A)1; *APOE*, apolipoprotein E; SNP, single nucleotide polymorphism.

But how many SNPs are needed? Although recent studies indicate that LD is structured into discrete blocks in the human genome[12,13], the range and distribution of LD in different populations is largely unknown. Furthermore, the effect of genotype on disease phenotype varies between disorders and populations owing to genetic and environmental heterogeneity[14]. For these reasons, it is difficult to estimate the number of SNPs or the number of samples that would be required for a successful genome-wide LD study. The estimates that have been made vary widely, from 1 million[15] or 0.5 million[16] to as little as 30,000 SNPs[17]. If 0.5 million SNPs were to be analysed in, for example, 1,000 individuals, and if the project was to be carried out in a year, ~1.5 million SNP genotypes per day would be produced. At genotyping costs as low as US 10 cents per SNP, this example project would cost US $50 million. The throughput required for this project, which comprises only a modest number of samples, is about 100-fold greater than the capacity of SNP-genotyping technology available now. Both the costs and the throughput would prohibit executing the project in even the largest genotyping centres. For typing 30,000 SNPs in 1,000 individuals in a year, about a tenfold increase in the capabilities of current technology would be enough.

A more feasible alternative to random whole-genome SNP mapping is to use SNP markers in or close to candidate genes, or in candidate genomic regions. For example, the genotyping of 20 SNPs in 50 candidate genes (1,000 SNPs) or of 1 SNP per 10 kb in a 10-million-bp candidate genomic region in 1,000 samples represents a reasonable goal of 1 million genotypes. It would be possible to carry out a project of this size within a few months in an intermediate-sized genotyping centre equipped with modern SNP-genotyping technology. For diagnosis and carrier screening of monogenic disorders and for routine pharmacogenetic applications, tens of SNPs are typically analysed in several thousand samples[3,18,19]. SNP-genotyping technology with acceptable throughput and cost for these routine applications is now available[20], although these fields would obviously benefit from lower genotyping costs. TABLE 1 gives some examples of recent or large studies in which SNP genotyping had a fundamental role. The scale of these projects is modest, and it is certain that larger studies that have not been published have been conducted, or are in progress, in the commercial sector.

Key targets to improve SNP genotyping technology are cost, simplicity of assay design, throughput and accuracy. One approach for increasing throughput is to devise automated assay formats of the generally used reaction principles for genotyping individual SNPs, and to use a 'brute force' strategy by multiplying these automated platforms. This strategy is analogous to the implementation of Sanger's dideoxy method for DNA sequencing[21] by the Human Genome Project[2,22]. A second approach to increase throughput is to multiplex the biochemical genotyping reactions, instead of the platforms. Microarray technology is a typical example of this approach. The most challenging approach is to develop completely new molecular strategies for SNP genotyping. In this article, I review the current state of the art of SNP-genotyping technology, with an emphasis on how amenable the different methods are to multiplexing to increase throughput and bring down the costs of the assays. Several promising new principles and assay formats also discussed.

Principles of SNP-genotyping methods

The ability of hybridization with allele-specific oligonucleotides (ASO) to detect a single base mismatch was first shown in 1979 (REF. 23), and then used to detect the sickle-cell mutation in the β-globin gene by Southern blot hybridization to human genomic DNA in 1983 (REF. 24). Identification of a single base change in the 6×10^9 bp of the diploid human genome is, however, a demanding task. Not until the PCR technique was invented[25,26], did it become possible to design useful assays for genotyping SNPs in complex

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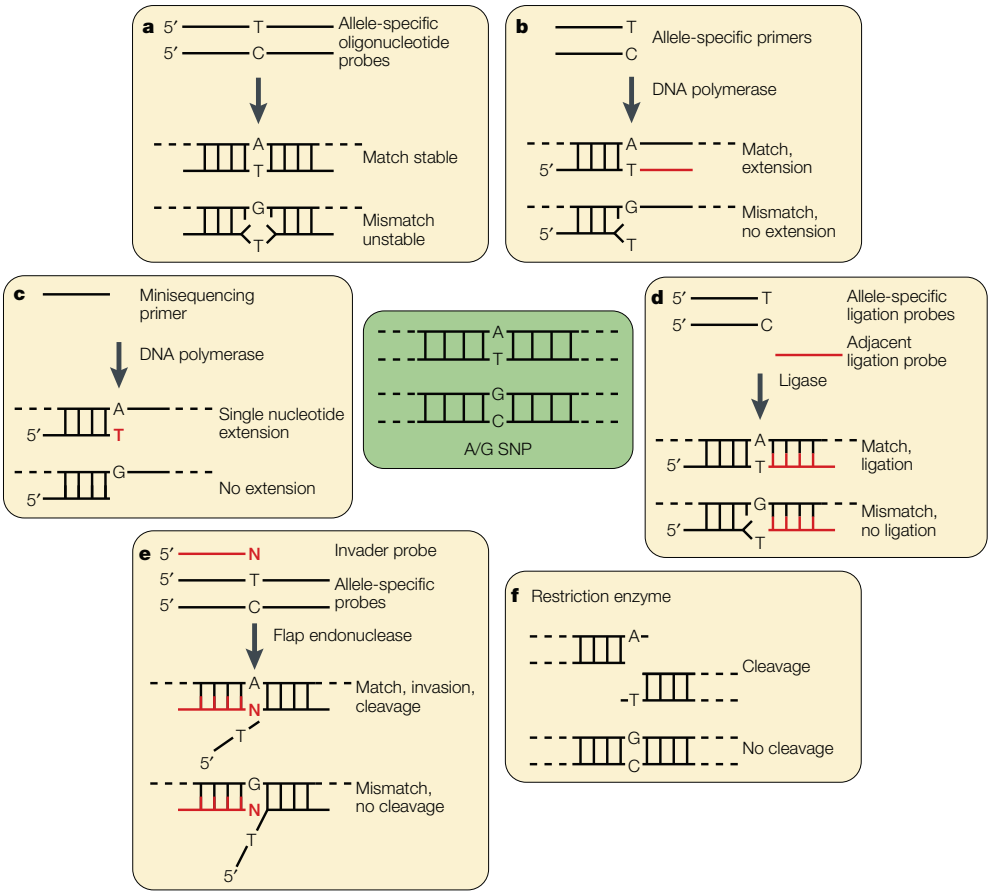
REVERSE DOT BLOT
A genotyping method based on hybridization between allele-specific oligonucleotide probes that have been immobilized on a membrane, and amplified DNA fragments in solution.

genomes. BOX 1 summarizes six central biochemical reaction principles that underlie SNP-genotyping methods, and FIG. 1 illustrates how some of the current SNP-genotyping platforms have been devised by combining a reaction principle with an assay format and a detection strategy.

Some of the early SNP-genotyping assays used for PCR products were based on ASO hybridization in dot blot[27] or REVERSE DOT BLOT[28] formats. The reverse dot blot format can be viewed as the precursor of the high-density microarray-based methods for multiplex genotyping of SNPs by ASO hybridization[29,30]. Before long, the PCR technique was developed further by several groups to allow allele-specific amplification and genotyping of SNPs[31]. The use of ASOs as hybridization probes or as PCR primers is the basis for the SNP-genotyping assays that are referred to as 'homogeneous', because they contain no separation steps and are monitored in real time during PCR. These assays are frequently used for large-scale genotyping of SNPs today[32–34]. The solid-phase assays for enzyme-assisted genotyping, using a DNA ligase[35] or a DNA polymerase[36], were also introduced more than a decade ago. Because the enzyme-assisted methods have proven

Box 1 | **Biochemical reaction principles underlying SNP genotyping**



The panels illustrate detection of the A-allele of an A-to-G transition. The G-allele would be detected analogously in a parallel reaction. In panel a, hybridization with allele-specific oligonucleotides (ASOs) is shown. Two short ASO probes are used, usually with the nucleotide complementary to the allelic variant of the single nucleotide polymorphism (SNP) in the middle position of the probe sequence. The probes are allowed to base pair with the target DNA that contains the SNP at conditions in which only perfectly matched probe–target hybrids are stable, and hybrids that contain a mismatch are unstable. In panel b, allele-specific primer extension is shown. Two primers that anneal to their target sequence adjacent to the SNP and have the nucleotide complementary to the allelic variant at their 3′-end are used in primer extension reactions catalysed by a DNA polymerase. Only primers with perfectly matched 3′-ends will be extended. In panel c, 'minisequencing' single nucleotide primer extension is shown. One primer that anneals to its target sequence immediately adjacent to the SNP is extended by a DNA polymerase with a single nucleotide that is complementary to the nucleotide at the site of the SNP. The identity of the nucleotide by which the primer becomes extended defines the genotype. In panel d, oligonucleotide ligation is shown. Pairs of oligonucleotide probes that anneal to their target sequence adjacent to each other and have an allele-specific 3′- or 5′-nucleotide at the junction between the probes are used. When the probes are perfectly matched to their target sequence, they will be joined by a ligase, whereas a mismatch at the junction inhibits ligation. In panel e, invasive cleavage is shown. Pairs of allele-specific oligonucleotide probes are used, but the sequence 5′ of the SNP is unrelated to the target. In addition, an upstream (invader) oligonucleotide is used that is complementary to the sequence 5′ of the SNP. When the allele-specific oligonucleotide is perfectly matched to its target, it is displaced at the SNP site by the upstream invader oligonucleotide, and the formed structure is specifically recognized and cleaved by a FLAP endonuclease, which releases the 5′-part of the probe. In panel f, restriction site cleavage is shown. Restriction endonucleases are used for allele-specific cleavage of the target DNA when a SNP alters the recognition sequence for the enzyme. Target molecules with intact recognition sites will be cleaved, whereas target molecules with altered sites remain uncleaved.

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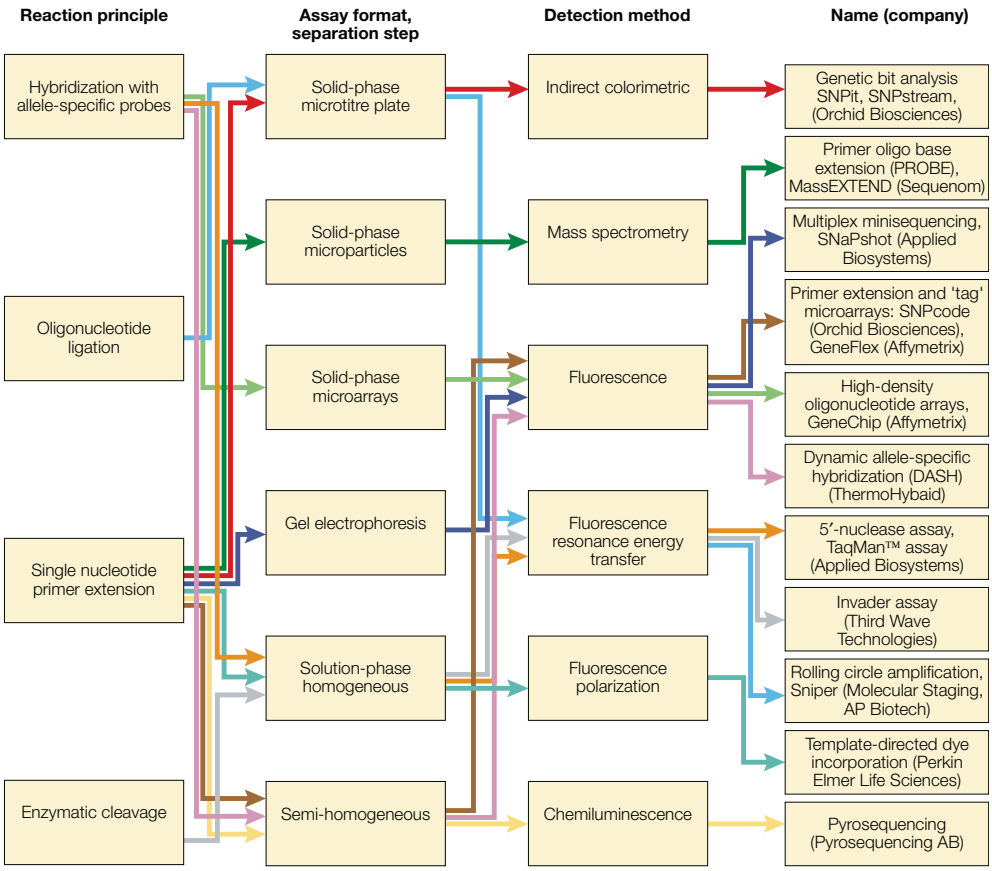

Figure 1 | **'Modular' design of some of the assays for SNP genotyping.** Coloured arrows are used to show the reaction principles, assay format and detection methods that make up a particular genotyping method. For example, the TaqMan™ assay involves hybridization with allele-specific oligonucleotides, a solution-phase assay and detection by fluorescence resonance energy transfer. The figure illustrates principles for assay design, and the list of assays is not intended to be comprehensive.

to be more robust and to provide more specific allele distinction than ASO hybridization[37], these methods have been multiplexed, automated and adapted to various detection strategies, and they provide most of the current high-throughput SNP-genotyping platforms.

All methods used for genotyping SNPs in large diploid genomes depend on PCR amplification of the genomic regions that span the SNPs before the actual genotyping reaction. The PCR provides the required sensitivity and specificity for distinguishing between heterozygous and homozygous SNP genotypes in large, complex genomes. The difficulty of designing and carrying out multiplex PCR reactions is an important factor that limits the throughput of the current SNP-genotyping assays. In the following sections, these methods are discussed in more detail. TABLE 2 summarizes some of their features.

Hybridization methods

The thermal stability of a hybrid between an ASO probe and its SNP-containing target sequence is not only determined by the stringency of the reaction conditions, but also by the nucleotide sequence that flanks the SNP and the secondary structure of the target sequence[24,38] (BOX 1). However, it is difficult to predict, *a priori*, the reaction conditions or the sequence of the ASO probe that will allow the optimal distinction between two alleles that differ at a single nucleotide position using ASO hybridization. These parameters must be established empirically and separately for each SNP. Consequently, there is no single set of reaction conditions that would be optimal for genotyping all SNPs, which makes the design of multiplex assays based on hybridization with ASO probes an almost impossible task.

A widely known approach to circumvent the difficult problem of assay design is to carry out multiplex ASO hybridization reactions on microarrays that carry tens or even hundreds of ASO probes for each SNP to be analysed. The probes include all possible sequences at the sites of the SNP and a stretch of nucleotide sequence that flanks the SNP[39]. It becomes feasible to have large numbers of ASO probes per SNP when high-density arrays are used that can carry up to 10^6 probes cm^{-2}, and that can be manufactured with sophisticated light-directed combinatorial synthesis[40]. In the GeneChip assay (Affymetrix), a computer algorithm is then used

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PEPTIDE NUCLEIC ACID (PNA). Biopolymer molecule that consists of DNA bases connected by a backbone of peptide bonds instead of phosphodiester bonds as in natural DNA.

LOCKED NUCLEIC ACID (LNA). DNA analogues in which the 2′ and 4′ positions in a furanose ring are connected by a methylene moiety.

to interpret the complex fluorescence patterns formed by the multiple probes and to assign the genotypes of each SNP. However, mapping studies, in which 400–500 SNPs were analysed using high-density ASO microarrays, showed that the assay failed to distinguish between heterozygous and homozygous SNP genotypes for a large fraction of the SNPs despite a redundancy of 40–50 probes per SNP[41,42].

One approach to carrying out ASO hybridization at conditions that allow more robust SNP genotyping is to monitor the duplexes between an ASO probe and its target over a temperature gradient, during which the optimal stringency for discriminating between the SNP alleles will be achieved at one point of the gradient. This concept is used for parallel analysis of duplexes on microarrays with probes immobilized in miniaturized polyacrylamide gel pads[43] and in a dynamic allele-specific hybridization (DASH) method in a microtitre plate format[44]. A related approach is to use electric field strength instead of temperature to denature the ASO-probe–target hybrids[45]. Other approaches to increase the power of ASO hybridization are to use PEPTIDE NUCLEIC ACID (PNA) analogues[46,47] or LOCKED NUCLEIC ACIDS (LNA)[48] that have very high affinities for complementary DNA. The high affinity makes it possible to use shorter PNA and LNA probes than the natural ASO probes to improve the discrimination between the SNP alleles.

Table 2 | **Features of SNP-genotyping methods**

Method	Most significant advantage	Disadvantage	Special feature
Hybridization methods	Widely used	Limited genotype discrimination	
Reverse dot blot[28]	Multiplexing possible	Prone to non-specific background	Precursor to microarrays
GeneChip microarrays[39]	Very high probe density	High failure rates[41,42], expensive	High-tech manufacturing process[40]
DASH[44]	Inexpensive labelling method	Complex design rules	Dedicated instrument
PNA[46] and LNA[48] probes	Specific as ASO probes	Not widely available	Innovative chemical design
TaqMan[49,50]	Simplicity of assay	Expensive probes	Quantitative real-time PCR
Molecular Beacons[51,52]	Simplicity of assay	Expensive probes	Versatile stem–loop structure
Allele-specific PCR	Real-time PCR assay	Requires optimization	
Intercalating dye[60]	Inexpensive labelling method	Non-specific products detected	Accurate quantification[138]
FRET primers[61,62]	Simplicity of assay	Expensive primers	Universal FRET primers
AlphaScreen[64]	Detects specific product	Expensive probes	Applicable to ASO hybridization
Primer extension	Accurate genotyping	Similar reagents as in PCR	
SNPstream, GBA[68]	Inexpensive and robust	Multiple detection steps	Automated high-throughput process
Multiplex minisequencing, SNaPshot[69,71]	Multiplexing capacity	Size separation step	Compatible with capillary DNA sequencers
Pyrosequencing[72,74]	Sequencing of up to 50 bases	Expensive, difficult to multiplex	Dedicated instrument
MassEXTEND, MassArray[75]	Labelling method avoided	Expensive instrument	Multiplexing capacity[76]
GOOD assay[77]	Labelling method avoided	Multi-step procedure	Sensitive mass spectrometric detection
Microarray miniseq, APEX[37,70]	Potential for high thoughput	Requires microarray instruments	Four-colour or single-colour detection
Microarray primer extension[82]	Potential for high throughput	Requires micorarray instruments	‘Array of arrays’ format
‘Tag’ arrays[85,86]	Flexible assay design	Requires microarray instruments	Generic ‘tag’ microarrays
Coded microspheres[87]	Multiplexing potential	Microspheres not widely available	Flow cytometric detection
TDI, fluorescence polarization[94]	Simplicity of assay	Difficult to multiplex	Universal detection principle
Oligonucleotide ligation	Variety of assay formats	Multiple labelled probes required	
Colorimetric OLA[95,96]	Robust assay	Multiple detection steps	Resembles ELISA
Sequence-coded OLA[97]	Multiplexing capacity	Size separation step, expensive probes	Polymer for sequence coding
Microarray ligation[100]	Potential for high throughput	Requires microarray instruments	Universal zip code microarray
Ligase chain reaction[102]	Alternative to PCR	Lower efficiency than PCR	Thermostable ligase
Padlock probes[108]	Localized detection	Probes difficult to produce	Avoids target amplification
Rolling circle amplification[98]	Signal amplification	Steric hindrance on solid phases	Based on circularized oligonucleotides
Endonuclease cleavage			
Restriction site analysis	Inexpensive, requires no equipment	Not suitable for high throughput	Traditional method
Invader assay[112]	PCR amplification avoided	Requires large amount of DNA	Interesting FLAP endonuclease

APEX, arrayed primer extension; ASO, allele-specific oligonucleotide; DASH, dynamic allele-specific hybridization; ELISA, enzyme-linked immunosorbent assay; FRET, fluorescence resonance energy transfer; GBA, genetic bit analysis; LNA, locked nucleic acid; OLA, oligonucleotide ligation assay; PNA, peptide nucleic acid; TDI, template-directed incorporation.

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Homogeneous hybridization assays

At present, the most widely used ASO hybridization methods distinguish between the SNP alleles in real time during PCR in homogeneous, solution-phase hybridization reactions with fluorescence detection. The TaqMan™ (Applied Biosystems)[49,50] or Molecular Beacon probes[51,52], which were originally designed for quantitative PCR analysis, can also be applied to SNP genotyping.

The TaqMan™ and Molecular Beacon assays are both based on a principle of energy transfer in which fluorescence is detected as a result of a change in physical distance between a reporter fluorophore and a quencher molecule on hybridzation of the ASO probe to its perfectly matched target sequence (BOX 2). The strong tendency of the Molecular Beacon probes to adopt a stem–loop structure destabilizes mismatched hybrids, increasing their power of allele distinction compared with linear ASO probes[51,53]. Analogously to PNA and LNA probes, TaqMan™ probes modified with minor groove-binder molecules that increase their affinity for the target show improved powers of allele discrimination[54]. The use of two probes, each labelled with a different reporter fluorophore, allows both SNP alleles to be detected in a single tube. The TaqMan™ assay has been multiplexed by using probes labelled with seven different fluorophores[55], and wavelength-shifting Molecular Beacons have been used for multiplex genotyping of up to ten SNPs[56]. Wavelength-shifting Molecular Beacons contain a harvester fluorophore that absorbs energy from a monochromatic light source, an emitter fluorophore with the desired emission wavelength and a quencher. In the absence of a target the probes are dark, whereas in the presence of a target the harvester fluorophore transfers energy to the emitter fluorophore that emits it as fluorescence of its own characteristic wavelength. Because the efficiency of FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET) depends on the distance between the harvester and emitter fluorophore, placing several emitter fluorophores on each probe at different distances from the harvester creates possibilities for the combinatorial design of distinct fluorescence emission signatures for highly multiplexed assays[57].

In the TaqMan™ and Molecular Beacon assays, the increase in fluorescence due to accumulating PCR product is usually monitored in real time in 96-well or 384-well microtitre plates. Alternatively, the fluorescence generated from the two alleles can be measured after completion of the PCR[58]. In this case, the results are expressed as a signal ratio that reflects the hybridization of the two oligonucleotides to the target sequence and so differences in amplification efficiency between samples do not affect interpretation of the genotyping results. Because no post-PCR processing or label-separation steps are required, the TaqMan™ and Molecular Beacon assays are simple to do, which renders them useful for high-throughput genotyping[32,33]. The optimal probes must be designed individually for each SNP, and the TaqMan™ and Molecular Beacon assays are therefore most efficient when a limited number of SNPs are analysed in a large number of samples. The cost of probes modified with fluorescent and quenching moieties might also be a limiting factor in high-throughput application of the TaqMan™ and Molecular Beacon assays. An interesting advantage of the Molecular Beacon probes over the TaqMan™ probes is the possibility of immobilizing them on microarray surfaces for the detection of unlabelled DNA targets[59].

Homogeneous allele-specific PCR

PCR primers with the 3′-end complementary to either of the nucleotides of a SNP can be used in combination with a common reverse PCR primer to selectively amplify the SNP alleles[31] (BOX 1). The simplest approach for monitoring the formation of allele-specific PCR products using a homogeneous assay format is to include a fluorescent dye that intercalates with the double-stranded PCR products in the reaction mixture[60]. The Molecular Beacon probes described above have been adapted to allele-specific PCR primers for homogeneous SNP-genotyping assays[61,62]. The incorporation of the primer into the PCR product releases the fluorescent label from the action of the quenching molecule.

Allele-specific PCR has been rationalized by using primary allele-specific PCR primers that contain a universal 5′-tail sequence that becomes part of the PCR product on amplification. A universal pair of secondary-energy-transfer-labelled, hairpin-structured primers can therefore be used for all SNPs[63] (BOX 2). When using intercalating dyes or labelled allele-specific PCR primers without a consecutive target-specific detection reaction or size-separation step, the specificity of the method might be hampered owing to PRIMER DIMERS and other spurious amplification products that will not be distinguished from the actual PCR products. The homogeneous AlphaScreen (Packard Bioscience) proximity assay avoids this problem as it is based on a pair of bridging energy transfer probes that hybridize to the region between the primers in the allele-specific PCR products[64]. Alternatively, size separation in a high-throughput mode might be accomplished by using 96-channel capillary sequencing instruments, microplate array diagonal electrophoresis[65] or capillary array electrophoresis microplates[66]. A limitation of all variants of allele-specific PCR is that the reaction conditions or primer design for selective allele amplification must be optimized empirically for each SNP. Like the TaqMan™ and Molecular Beacon assays, the homogeneous allele-specific PCR methods are best suited for the analysis of a limited number of SNPs in large sample collections.

DNA-polymerase-assisted genotyping

In methods based on single nucleotide primer extension — minisequencing — the distinction between genotypes of the SNPs is based on the high accuracy of nucleotide incorporation by the DNA polymerases[36,67] (BOX 1). The primer extension reaction is robust, allowing specific genotyping of most SNPs at similar reaction conditions. These features are advantageous for high-throughput applications because the effort required for assay design and optimization are minimized.

FLUORESCENCE RESONANCE ENERGY TRANSFER
A phenomenon by which the energy from an excited fluorophore is transferred to an acceptor molecule at short (<100 Å) distances, leading to quenching of the fluorescence. The efficiency of energy transfer depends strongly on the distance between the donor and acceptor molecules.

PRIMER DIMER
Unwanted PCR products formed when two primers interact during the extension phase of PCR, followed by extension of the 3′-end of one or both primers with the other primer acting as a template.

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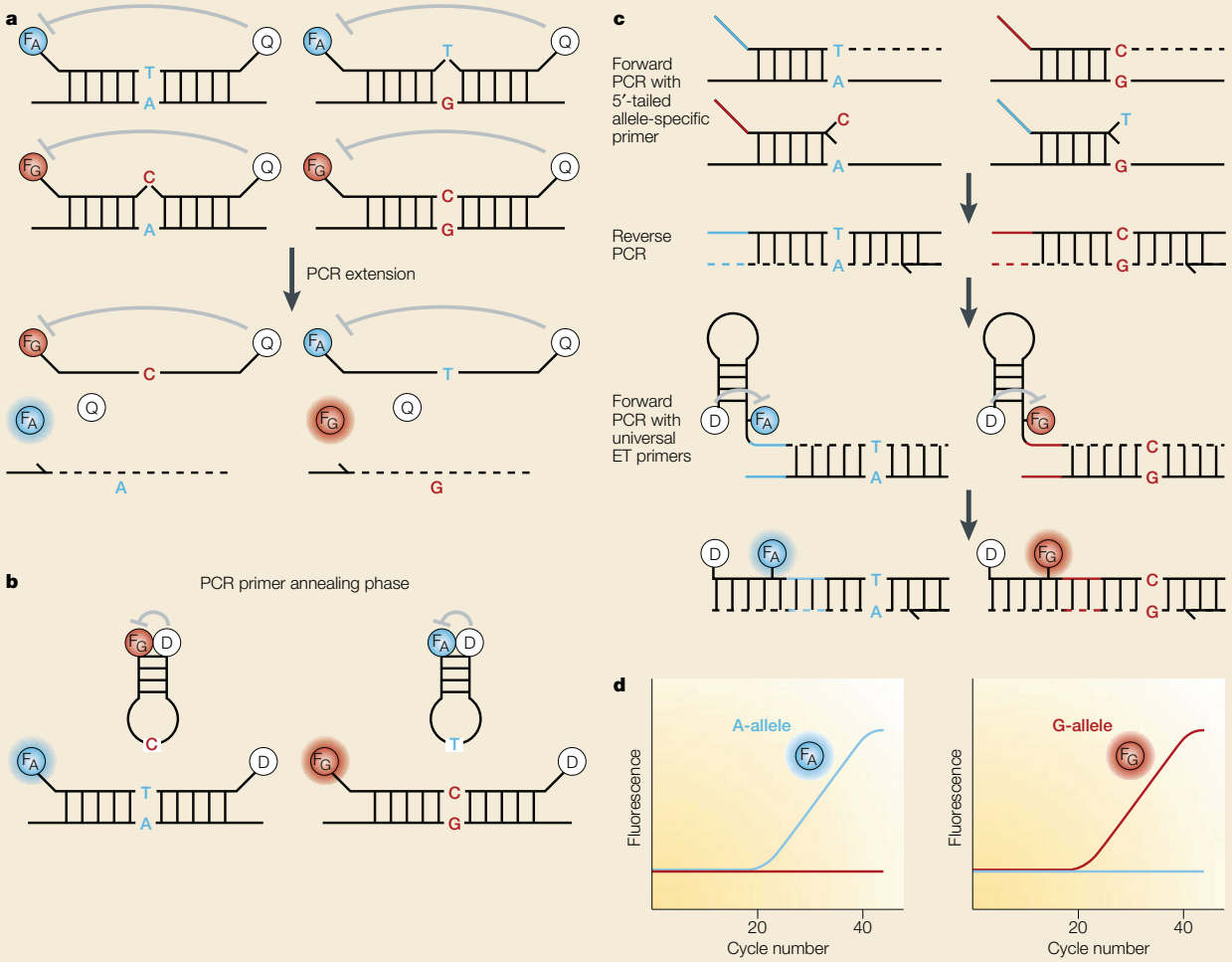
Box 2 | **Principles for homogeneous SNP genotyping by real-time PCR**

The example illustrates the detection of an A-to-G transition. Panel **a** shows the 5′-exonuclease (TaqMan™) assay: allele-specific oligonucleotide probes are labelled with different fluorophores (F_A (blue) or F_G (red)) at their 3′-ends and with a quencher molecule (Q) at their 5′-ends. The quencher interacts with the fluorophores by fluorescence resonance energy transfer, quenching their fluorescence. The allele-specific probes are included in the PCR reaction mixture. During the annealing phase of PCR, the probes hybridize to the strands of the PCR products and during the extension phase of PCR, the 5′–3′ exonuclease activity of the DNA polymerase degrades perfectly matched, annealed probes. The fragmented probes are released into the solution, separating the fluorophore from the quencher, which leads to an increase in fluorescence. Mismatched probes are displaced from the target without degradation. Molecular Beacon probes (shown in panel **b**) consist of a sequence that is complementary to the target sequence and a short stretch of self-complementary 5′- and 3′-nucleotides with a fluorophore (F_A or F_G) at the 5′-end and a DABCYL moiety as quencher (D) at the 3′-end. When free in solution, the Molecular Beacon probes adopt a stem–loop structure that brings the fluorophore and quencher into close proximity. When a Molecular Beacon probe hybridizes to a perfectly matched target during the primer annealing phase of PCR, the stem–loop structure opens, and the distance between quencher and fluorescent molecule increases, which restores the fluorescence. Mismatched probes readily adopt the stem–loop structure. It should be noted that the physical energy transfer mechanism differs between the TaqMan™ probes, with the fluorophore TAMRA as quencher, and the Molecular Beacon probes, with DABCYL as quencher. Universal allele-specific energy transfer primers are shown in panel **c**. The primary allele-specific PCR primers carry different 5′-tail sequences. The secondary primers consist of a 3′-sequence complementary to the tail sequences and a sequence that is held in a hairpin loop conformation by complementary stem sequences similar to the Molecular Beacon probes. During the primary allele-specific PCR, the 5′-tail sequence of the primer becomes incorporated into the PCR product. The energy transfer primer is used to initiate a secondary PCR. On synthesis of the reverse strand of the PCR product, the stem–loop structure of the secondary primers opens, separating the quencher from the fluorophore, and therefore restoring its fluorescence. Fluorescence measurement is shown in panel **d**. The increase in fluorescence at the emission wavelengths for the fluorophores F_A and F_G is monitored in real time during PCR. At a threshold cycle, the fluorescence rises to a detectable level and increases as more PCR products accumulate. Alternatively, the result can be interpreted by measurement of fluorescence intensity at the end point of PCR. (DABCYL, 4-dimethylaminoazobenzene-4′-sulphonyl; ET, energy transfer; TAMRA, 6-carboxytetra-methylrhodamine.)



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Consequently, single nucleotide primer extension is gaining acceptance as the reaction principle of choice for high-throughput genotyping of SNPs, and has been adapted to various assay formats, detection strategies and technology platforms (FIG. 1).

In an ELISA-like single nucleotide primer extension assay, incorporated HAPTEN-labelled nucleotide analogues are detected colorimetrically (genetic bit analysis, Orchid Biosciences)[68]. In this indirect detection procedure, the incorporated haptens are recognized by antibodies, and enzymes that are conjugated to the antibodies catalyse the formation of a coloured product. This detection strategy in a 384-well microtitre plate format has been automated for production-scale SNP genotyping (SNPstream, Orchid Biosciences). DNA-sequencing instruments might also be used as platforms for genotyping SNPs using fluorescently labelled dideoxynucleotides in the minisequencing reactions. The electrophoretic size-separation step facilitates multiplex genotyping in the range of tens of SNPs per reaction using primers modified with 5′-tails of varying length[69–71] (SNaPshot, Applied Biosystems), and by using 96-channel capillary sequencers, this assay can be automated for high-throughput genotyping.

In the PYROSEQUENCING (Pyrosequencing AB) method, primer extension is monitored by enzyme-mediated luminometric detection of pyrophosphate, which is released on incorporation of deoxynucleotide triphosphates[72,73]. The genotype of a SNP is deduced by sequential addition and degradation of the nucleotides using apyrase in a dedicated instrument that operates in a 96-well or 384-well microtitre plate format[74]. Using pyrosequencing, short 30–50-bp sequences of DNA that flank a SNP can be determined. A limitation of the method is that the sequential identification of bases prevents genotyping of several SNPs per reaction in diploid genomes.

Mass spectrometry (MALDI-TOF, matrix-associated laser desorption time-of-flight mass spectrometry) is used to detect the primer extension products in two closely related SNP-genotyping assays — the PROBE (primer oligo base extension) assay[75] (now called MassEXTEND, Sequenom) and the PinPoint assay[47] (Applied Biosystems). Mass spectrometry is particularly useful as a read-out method for primer extension reactions because primers of different lengths can be used in combination with mixtures of deoxy- and dideoxynucleoside triphosphates designed to yield allele-specific primer extension products with clear differences in their molecular mass. Moreover, the assays can be multiplexed if the primer extension products for each SNP have non-overlapping mass distributions[76]. A difficulty with MALDI-TOF is that the primer extension products must be rigorously purified before measurement to avoid background from biological material present in the sample. This limitation is avoided in the so-called 'GOOD' assay[77] by increasing the sensitivity of the mass spectrometric detection so that a small aliquot of the extended primer is sufficient for measurement. The increase in detection sensitivity is accomplished by introducing thiol groups into the 3′-region of the primer, which allows the mass of the allele-specific products to be reduced by enzymatic digestion of the 5′-end of the primer, and by neutralizing the negative charge of DNA by alkylating the thiol groups[77,78].

Owing to its high sequence specificity and robustness, the DNA-polymerase-assisted single nucleotide primer extension reaction is well suited to highly parallel genotyping of SNPs on microarrays. In a comparison with ASO hybridization reactions in the same microarray format, the minisequencing reaction provided tenfold better power of discrimination between genotypes than hybridization with ASO probes[37]. For minisequencing on microarrays[37], also denoted 'arrayed primer extension' (APEX)[70], one detection primer for each SNP is immobilized covalently on a microscope slide. After an extension reaction using fluorescent dideoxynucleotides, the array is analysed by fluorescence scanning[79–81] (BOX 3). Instead of an extension reaction using one primer per SNP and four fluorescent dideoxynucleotides, two immobilized allele-specific primers per SNP can be extended using a mixture of natural and fluorescent deoxynucleotides with RNA[82] or DNA[83,84] as the template for the reaction (BOX 3). An array of arrays format, in which separate reaction chambers are formed on an array by a silicon rubber grid, allows simultaneous genotyping of up to 300 SNPs in 80 samples by minisequencing or allele-specific primer extension[82] (FIG. 2). This assay can yield more than 10,000–20,000 genotypes per microscope slide and the automation of this format would allow extremely high-throughput genotyping. A flexible strategy for SNP genotyping using microarrays is to carry out cyclic primer extension reactions in solution with specific primers that are tailed with 5′-tag sequences, and to use microarrays with complementary oligonucleotide tags for hybridization-based capture and sorting of the products of the cyclic minisequencing reactions[85,86] (BOX 3). This strategy has been used in conjunction with low[85] and high[86] (GeneFlex, Affymetrix)-density microarrays. These microarray-based methods are particularly suited to the analysis of large panels of SNPs.

The concept of generic tags for capturing products of cyclic primer extension reactions has been applied to arrays of MICROSPHERES. Each class of microsphere is embedded with fluorophores that have a characteristic emission wavelength, and the microspheres in each class carry a unique complementary 'tag' sequence for capturing the SNP-specific primer extension products. Measurement of the fluorescence of each individual microsphere in a flow cytometer allows determination of which class it belongs to, and the genotype of the SNPs is determined by the captured fluorescent product[87,88]. So far, this assay has been multiplexed for the detection of tens of SNPs. Microspheres with up to 100 distinct spectral characteristics, generated by mixing fluorophores with red and infrared emission, are available[89] (Luminex).

The accuracy of the primer extension reaction also allows quantitative determination of allelic ratios of SNPs in pooled DNA samples[90]. Particularly accurate quantification is achieved using ^{3}H-labelled

ELISA
(enzyme-linked immunosorbent assay).
A widely used immunochemical method for detecting antigens or antibodies. ELISA methods are carried out in microtitre plates and use colorimetric detection.

HAPTEN
Small molecule that is able to invoke an antibody response when used for immunization of an animal.

PYROSEQUENCING
A method for DNA sequencing, in which the inorganic pyrophosphate (PPi) that is released from a nucleoside triphosphate on DNA chain elongation is detected by a bioluminometric assay.

MICROSPHERES
(also known as microparticles or microbeads). Small 1–100-μm diameter particles used as solid supports in bioassays. They can carry a probe or primer, and can contain internal magnetic compounds to allow magnetic separation or internal fluorescent compounds for labelling.

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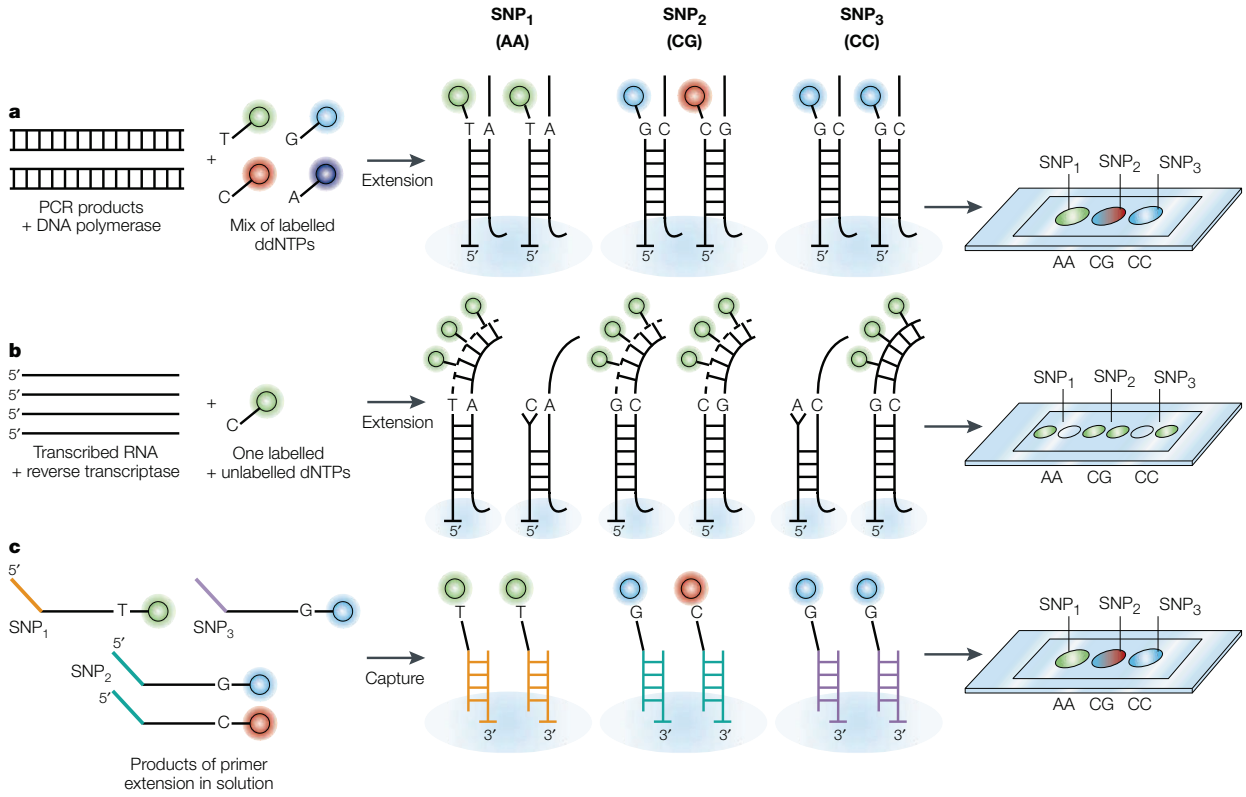
FLUORESCENCE POLARIZATION
A detection method based on excitation of a fluorescent molecule by plane-polarized light, and measurement of the rate of depolarization of fluorescence. This rate is proportional to the rate of tumbling of a fluorescent molecule. As small molecules tumble faster than large molecules in solution, fluorescent molecules of different sizes can be distinguished.

deoxynucleotides that are chemically similar to the natural nucleotides, and are, therefore, incorporated with high specificity by the DNA polymerase[90,91] or using mixtures of unlabelled deoxy- and dideoxynucleotides followed by mass-spectrometric detection[92]. Analysis of pooled DNA samples is beneficial for increasing throughput in association studies and for determining the allele frequencies of SNPs in various populations.

Because both the PCR and primer extension reaction mixtures contain primers, nucleoside triphosphates and a DNA polymerase, a step to remove or inactivate excessive PCR reagents is crucial for the success of all primer extension methods. Immobilizing either the SNP-containing templates or the detection primers on a solid support, such as microtitre plate wells[68,90], microparticles[74,88] or microarrays[37], followed by washing the solid support, provides an efficient way to remove excessive reagents and to render the template single stranded before the primer extension reaction. Alternatively, the PCR primers and nucleotides can be degraded enzymatically with alkaline phosphatase and an exonuclease before the genotyping reaction[93]. The enzymatic degradation step has allowed the design of a technically simple and robust homogeneous SNP-genotyping assay based on cyclic primer extension reactions and detection by FLUORESCENCE POLARIZATION[94].

Box 3 | **Strategies for SNP genotyping by primer extension using microarrays**

The genotyping of three single nucleotide polymorphisms (SNPs) is illustrated (SNP_1 with the nucleotide variation A/G; SNP_2 with G/C; and SNP_3 with C/T) for a sample with the genotypes AA, CG and CC. Panel a in the figure shows minisequencing — arrayed primer extension. One primer for each SNP to be genotyped is immobilized covalently on the surface of a microscope slide. Multiplex PCR products that span the SNP sites, a mixture of fluorescently labelled terminating nucleotide analogues (ddNTPs, dideoxynucleoside triphosphates) and a DNA polymerase are added to the arrays. The primer extension reactions are allowed to proceed on the array surface, and the microscope slides are scanned. The positions of the primers on the microarray surface define which SNP is analysed and the fluorescent nucleotide(s) by which a primer becomes extended defines the genotype of the SNP. Allele-specific primer extension is shown in panel b. Two allele-specific primers with the 3′-base complementary to the two possible nucleotides of each SNP are immobilized on the array. The multiplex PCR products that span the SNPs are transcribed into numerous RNA copies by an RNA polymerase. The RNA molecules act as templates for a primer extension reaction catalysed by a reverse transcriptase, in which several fluorescent deoxynucleotides (dNTPs, deoxynucleoside triphosphates) become incorporated in each product. For homozygous genotypes, a signal is generated from one of the allele-specific primers, and for heterozygous genotypes a signal is generated from both primers. Primer extension using tag arrays is shown in panel c. Cyclic single nucleotide primer extension reactions are carried out in solution in the presence of fluorescently labelled dideoxynucleotides using primers carrying an extra tag sequence in their 5′-end. Generic arrays of oligonucleotides that are complementary to the 'tags' of the primers are used to capture the products of the cyclic minisequencing reactions.



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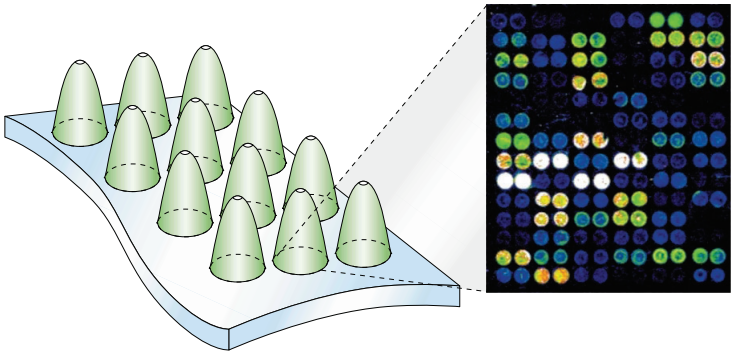

Figure 2 | **SNP genotyping by minsequencing using an 'array of arrays'.** Each small array comprises a set of primers for detecting single nucleotide polymorphisms (SNPs), and each array is isolated by a silicon rubber cone that forms an individual reaction chamber for the array[82]. Reagents are introduced through a hole in the tip of the cone. A standard microscope slide can hold up to 80 arrays. A fluorescence image of one array is shown, in which minisequencing reactions for 84 SNPs with the primers spotted in duplicate have been carried out with TAMRA-labelled ddGTP (U. Liljedhal and A.-C. Syvänen, unpublished data). ddGTP, dideoxyguanosine triphosphate; TAMRA, 6-carboxytetramethylrhodamine.

Ligation methods

The discrimination by DNA ligases against mismatches at the ligation site in two adjacently hybridized oligonucleotides is the basis for genotyping of SNPs by the oligonucleotide ligation assay (OLA)[35] (BOX 1). This assay has been combined with colorimetric detection in microtitre plate wells[95,96] and with multiplex detection using fluorescently labelled ligation probes with different electrophoretic mobilities that can be analysed in a DNA-sequencing instrument[97]. OLA has also been used in microarray formats with one of the ligation probes immobilized[98] or with immobilized single stem–loop probes[99]. Alternatively, ligation can be carried out in solution followed by capture of the ligation products on microarrays[100] or on microparticles[101] that carry a generic set of oligonucleotides that are complementary to a 'tag' sequence on one of the ligation probes.

In the ligase chain reaction, two pairs of oligonucleotide probes are used in cyclic ligation reactions, together with a thermostable DNA ligase for exponential amplification of genomic DNA[102]. In practice, the thermostable ligases[103] are more frequently used for genotyping SNPs in combination with PCR before the allele-specific ligase detection reactions[104,105]. Because the reaction mechanisms for PCR and ligation are different, the reagents for both reactions can be combined. This feature is used in a homogeneous, real-time PCR assay with ligase-mediated genotyping and detection by FRET[106]. Compared with DNA-polymerase-assisted primer extension methods, a drawback of the OLAs is that detection of each SNP requires three oligonucleotides (BOX 1), of which one is 5′-phosphorylated, and two carry detectable labels, which obviously increases the costs of these assays.

Padlock probes are linear oligonucleotides, the ends of which are complementary to the target and have a central stretch of random sequence[107]. When perfectly hybridized to their target sequence, padlock probes can be circularized by ligation, whereas a mismatch with the target sequence prevents ligation. Padlock probes have been used for colorimetric *in situ* detection of single-base variations in repetitive α-satellite DNA in human metaphase chromosomes[108]. Circularized oligonucleotides can act as templates for DNA-polymerase-assisted rolling circle amplification (RCA)[98,109]. RCA can therefore be used to amplify the number of ligated circularized padlock probes to a level required for detecting single-copy sequences[110]. A homogeneous, isothermal assay for genotyping individual SNPs in a microtitre plate format has been devised by combining exponential amplification of ligated padlock probes using a branched rolling circle amplification reaction with detection by energy-transfer-labelled hairpin primers based on Molecular Beacons[111].

Invasive cleavage of oligonucleotide probes

The Invader assay (Third Wave Technologies) makes use of two target-specific hybridization oligonucleotides — an allele-specific signalling probe with a 5′-region that is non-complementary to the target sequence and an upstream invader oligonucleotide[112] (BOX 1). When the allele-specific probe is perfectly matched at the SNP, the three-dimensional structure formed by these two oligonucleotides and the target sequence at the site of the SNP is recognized and is cleaved by a 5′-endonuclease, called FLAP endonuclease, that is specific for this particular DNA structure. This cleavage releases the 5′-sequence of the signalling probe, which can be detected directly[112] or further amplified by a serial, isothermal Invader assay based on a FRET-labelled probe[113]. The Invader assay has been combined with mass spectrometric detection[46] and with detection by fluorescence polarization[114]. In principle, the serial Invader assay can be applied for identifying SNPs in genomic DNA without previous PCR amplification, but a limitation of the assay is that it requires a large amount of target DNA. Therefore, the homogeneous Invader assay has been applied for genotyping SNPs in DNA fragments previously amplified by PCR[115]. The assay has been adapted to a solid-phase format, which is a prerequisite for multiplex genotyping using the Invader principle in a microarray format[116].

Future trends

In practice, the requirement of a PCR amplification step to achieve sensitive and specific SNP genotyping is the principal factor that limits the throughput of assays today. Multiplex PCR amplification of more than ten DNA fragments is difficult to carry out reproducibly owing to the generation of spurious amplification products[39,41,42,80]. Recently, a PCR strategy based on a single universal PCR primer for amplification of a reduced representation of the human genome that avoids the problem of carrying out multiplex PCR was applied to SNP discovery and validation[117]. Similar generic amplification strategies could also be devised for genotyping panels of known SNPs to circumvent multiplex PCRs with locus-specific primers. Another suggested approach for avoiding non-specific primer interactions during multiplex PCR is to carry out

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the amplifications with primers immobilized at physically distinct locations in 'polonies' (PCR colonies)[118], gel pads[119] or using microelectronic arrays[120]. SNP genotyping by mini-sequencing[67] or pyrosequencing[73] would be compatible with these formats.

The ligation and invasive cleavage methods described above[109–113] make use of two recognition events between oligonucleotides and their targets and so, in principle, these methods have the required specificity for allele-specific SNP detection in unamplified genomic DNA. To achieve the desired sensitivity, the methods rely on enzymatic amplification of the signals. As the ligation and invader methods are based on several enzymes and fluorescent detector probes labelled with multiple fluorophores, it remains to be seen whether they can contest the standard PCR-based methods in terms of reagent cost, throughput and accuracy.

New PCR instruments that use microcapillaries instead of microtitre plate formats have been devised, and offer increased PCR throughput and reduced reagent costs as they use extremely short amplification times and small reaction volumes[121,122]. Fully automated SNP analysis systems could then be designed based on homogeneous detection, or by streamlining the PCR and the subsequent genotyping procedure in microfluidic 'lab chip' devices that operate with submicrolitre reaction volumes. Such microfluidic devices are now under development in several biotech companies[123].

Recent developments of composite materials and fluorescence detection strategies offer increased detection sensitivity and specificity for SNP-genotyping assays. Hybrid gold and silver nanoparticles have been used, instead of fluorophores, as labels on allele-specific oligonucleotide probes[124]. Gold nanoparticles can also replace the organic DABCYL moieties as quenchers on Molecular Beacon energy transfer probes[125], enhancing the sensitivity of the assays by two orders of magnitude. A recently described microvolume detection technique based on TWO-PHOTON EXCITATION can potentially be used to detect individual microparticles in multiplexed bioassays[126]. Another approach to highly multiplexed assays is to use fibre-optic sensors to detect large numbers of coded microspheres in real time[127]. For SNP genotyping, coded microspheres that carry 'tag' sequences would be captured in miniaturized wells at the ends of fibre-optic detectors, and the signals that originate from hybridization, primer extension or oligonucleotide ligation reactions could be monitored (Illumina). Finally, in another very promising strategy for multiplexing bioassays, multicolour optical coding is accomplished by embedding different sized QUANTUM DOTS into polymeric microbeads at precisely controlled ratios[128]. Because of the unique spectral properties of the quantum dots, this technology has the potential for several-thousand-fold multiplexing.

Despite the numerous technical advances in detection and multiplexing strategies, no technique clearly represents a breakthrough. It is difficult to predict which, if any, existing SNP-genotyping technology will facilitate the required 100- or 1,000-fold increase in throughput required for whole-genome SNP analysis in sample collections of relevant size. The next generation technology for SNP analysis should avoid the PCR amplification step, and the technology could be based on the analysis of single DNA molecules, which would allow direct determination of the haplotypes formed by the SNPs. Such futuristic candidate technologies might derive from a further development of the system for haplotyping by carbon nanotube atomic force microscopy probes[129] or a further refinement of the microscopic technology that has recently allowed direct optical mapping of the whole *Escherichia coli* genome[130]. Hopefully, future SNP-genotyping technology will be more elegant than the incremental and brute-force expansion of existing technologies in the way that the Sanger sequencing method has been exploited for genome sequencing. One thing is certain — once very-high-throughput SNP genotyping is available, this technology will have a profound impact on our understanding of the relationship between genetic variation and biological function.

TWO-PHOTON EXCITATION
A detection system in which excitation of fluorophores takes place only in a small three-dimensional focal volume.

QUANTUM DOT
Nanocrystal that consists of a core of cadmium selenide wrapped with multiple monolayers of zinc sulphide that have several times higher extinction coefficients than organic fluorophores. The quantum dots can be excited with light of a single wavelength, and emit very bright fluorescence at several wavelengths that are determined by the size of the cadmium selenide core.

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Acknowledgements

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