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Multiplexed Pharmacogenetic Assays for SNP Genotyping: Tools and Techniques for Individualizing Patient Therapy

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1. Introduction

In this article, we provide an overview of the cytochrome P450 drug metabolism system, a major target for pharmacogenetics assays. We discuss briefly the major enzyme subfamilies and highlight some of the important members of each. We then delve into the currently available methodologies that are used for genotyping including single base (primer) extension, hybridization, ligation, and sequencing. The various methods have distinct requirements but all can be used for the interrogation of single nucleotide polymorphisms. These genetic differences may confer altered properties in the encoded enzymes including differences in the ability to metabolize drugs. Methods to identify such differences can help select subsets of patients who may or may not be able to effectively utilize particular medications. In such a manner, these techniques allow for the appropriate triage of patients to therapies that are targeted for their genotype, allowing for a tailored, individualized treatment regimen. Pharmacogenetic testing of this nature can help to usher in the era of personalized medicine.

2. Genotypic variation in cytochrome P450s and effects on drug metabolism

Adverse drug reactions are important causes of morbidity and mortality, and have been reported to result in significantly increased healthcare costs and longer hospital lengths of stay. Adverse drug reactions can result from comorbid diseases that affect drug metabolism such as renal or hepatic insufficiency, from drug-drug interactions, and from genetic factors affecting drug pharmacokinetics. Reduction of adverse drug reactions associated with comorbid conditions and drug-drug interactions is potentially achievable through increased awareness and reporting; however prevention of adverse drug reactions due to individual genetic differences requires a different approach - efficient and cost-effective determination of individual genotypic profiles of the enzymes involved in drug metabolism.

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Drugs are metabolized through a series of reactions, the majority of which are carried out by cytochrome P450 (CYP), a monooxygenase superfamily of enzymes with over 60 members. The CYP genes are highly polymorphic in humans, with hundreds of single nucleotide polymorphisms (SNPs), insertions and deletions, and copy number variations described to date. These genetic polymorphisms give rise to different metabolic phenotypes: ultrarapid metabolizers (UM), extensive metabolizers (EM), intermediate metabolizers (IM) and poor metabolizers (PM). Individuals with the EM phenotype have two normal alleles and have normal metabolism; those with the IM phenotype have one defective allele and may have reduced drug metabolism; and those with the UM phenotype have gene duplications and have increased drug metabolism. The PM phenotype is characterized by two defective alleles, resulting in markedly decreased drug metabolism and in particular situations, higher levels of drugs and increased risk for adverse drug reactions.

Of the many isoforms of CYP, CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4, are responsible for the metabolism of the majority of clinically important drugs. (Table 1)

	#SNPs	Clinically significant alleles or %poor metabolizers	Examples of substrates
CYP1A2	>30	N.D.	Caffeine, estradiol, clozapine, olanzapine, theophylline
CYP2B6	>70	CYP2B6*6: 15-40% Asians, >50% African-Americans	Bupropion, methadone, ifosphamide, efavirenz, selegiline
CYP2C9	>50	CYP2C9*2: 8-19% Caucasians, 3.2% African-Americans CYP2C9*3: 8.3% Caucasians, 3.3% Asians	NSAIDs, angiotensin receptor blockers, sulfonylureas, warfarin
CYP2C19	>30	PM: 3-5% Caucasians, 15-20% Asians	Proton pump inhibitors, anti-epileptics, clopidogrel
CYP2D6	>100	PM: <1% Asians, 2-5% African-Americans, 6-10% Caucasians	Tricyclic antidepressants, SSRIs, opioids, anti-psychotics, tamoxifen, beta blockers, anti-arrhythmics
CYP2E1	10	N.D.	Anesthetics
CYP3A4	>30	N.D.	Macrolide antibiotics, benzodiazepines, anti-retrovirals, anti-histamines, calcium channel blockers, HMG CoA reductase inhibitors

Table 1. Common CYP polymorphisms affect the metabolism of clinically important drugs. N.D.= not determined.

CYP1A2 metabolizes several drugs including clozapine (used in the treatment of schizophrenia), theophylline (used to treat respiratory disorders such as COPD and asthma), and caffeine. Greater than 30 SNPs have been identified to date, but a genotype-phenotype

relationship has not yet been established. Similarly, a genotype-phenotype relationship has not yet been established for CYP2E1, a CYP protein responsible for the metabolism of most anesthetics.

CYP2B6 is highly polymorphic (>70 SNPs) and metabolizes approximately 8% of clinically important drugs including the anti-retroviral drugs efavirenz and nevirapine, which are used in the treatment of HIV infection. The CYP2B6*6 allele, which is found commonly in Asians and African-Americans, results in decreased metabolism and response to efavirenz.

Greater than 50 SNPs have been identified for CYP2C9, which metabolizes approximately 10% of all clinically prescribed medications. One of the most important drugs metabolized by CYP2C9 is warfarin, a widely used anticoagulant. CYP2C9*2 and CYP2C9*3 alleles which are relatively common in Caucasians (approximately 8%) have been implicated as playing a large role in the interindividual variation in the metabolism of this drug. These alleles have been demonstrated to reduce enzymatic activity *in vitro*, *2 by 70% and *3 by 30%, respectively.

CYP2C19 plays a role in the metabolism of several drugs, but perhaps has been best studied for its role in the metabolism of proton pump inhibitors which are used to treat gastroesophageal reflux disorders. 3-5% of Caucasians and 15-20% of Asians are CYP2C19 poor metabolizers. PMs have reduced metabolism of proton pump inhibitors, leading to increased plasma levels of drug and increased response to treatment. Recently, 2C19 has become popular because of its involvement in the metabolism of Plavix, an antiplatelet drug used to prevent strokes and heart attacks. Important alleles include *2 and *3 which reduce enzymatic activity, and *17 which produces an ultrarapid metabolizer phenotype. Of great interest, 2C19 shares homology with 2C9. In fact, >90% of the amino acid sequence is identical between these two isoforms. Despite their near identity at the amino acid level, the active site of the two enzymes differs, and thus accounts for the differences in substrate specificity.

CYP2D6 is highly polymorphic with greater than 100 SNPs thus far characterized. These genetic polymorphisms play a significant role in affecting the metabolism of ~20% of clinically important drugs including anti-depressants, anti-psychotics, anti-arrhythmics, and beta blockers. These features make CYP2D6 an attractive target for pharmacogenetic assays. The PM phenotype is found in < 1% of Asians, 2-5% of African-Americans, and 6-10% of Caucasians. Interestingly, only six alleles of CYP2D6 account for >99% of the poor metabolizers in the Caucasian population (Roberts et al. 2006). Hence, a targeted approach to interrogate these six SNPs could provide a useful assay to identify such individuals in this limited cohort.

The CYP3A family of isoforms are crucial drivers of drug metabolism in the liver. In fact, CYPs 3A4 and 3A5 are responsible for 40-50% of all such activity. CYP3A4 metabolizes a large range of clinically important drugs, and over 30 SNPs have been described. However, no significant interindividual variability has yet been reported, suggesting that genetic variation may not play a large role in regulating CYP3A4 activity. Interestingly however, the *3 allele which results in a variant with a reduced metabolism phenotype is found in ~30% of Caucasians. The 3A5 isoform is less well characterized but shares overlapping substrate specificity with 3A4.

In summary, hundreds of SNPs have been identified within the multiple members of the SNP superfamily and other genes involved in drug metabolism, making these genes an important target for SNP genotyping in pharmacogenetics and personalized medicine. Various techniques for SNP genotyping are described in the following section.

3. SNP genotyping methods

Many SNP genotyping strategies have been developed, ranging from small scale, low-throughput approaches to interrogate one of few SNPs, to large scale, high-throughput approaches that can genotype hundreds of SNPs. Both small and large scale approaches have been applied for pharmacogenetics studies. These approaches generally detect SNP alleles using one of the following strategies: primer extension, hybridization, ligation, or sequencing.

3.1 Single base (primer) extension

Single base (primer) extension is a process that involves the use of a SNP probe with the 3' end a single base upstream of the SNP of interest. The SNP probe is then extended by a single base, and the incorporated base is detected. Detection can be either through fluorescence, if a fluorophore is incorporated into the dideoxynucleotides and an appropriate detector is used, or can be done based on sizing of fragments if a size separation technique, such as mass spectrometry, is used.

As an example, we have developed an assay using this technology to determine the genotype profile of genes affecting the metabolism of warfarin. Warfarin is a widely used anticoagulant. However, the combination of variable, genetically-based, individual responses to warfarin and a narrow therapeutic window with potentially serious complications, make this an ideal situation in which pharmacogenetics testing could be beneficial. As described above, the CYP2C9*2 and CYP2C9*3 alleles have been shown to be important in the metabolism of warfarin. In addition, warfarin inhibits Vitamin K epoxide reductase complex subunit 1 (VKORC1), an enzyme complex that reduces vitamin K 2,3 epoxide to its active form. Multiple SNPs have been identified in VKORC1, leading to either low-dose or high-dose phenotypes. VKORC1 polymorphisms, which are found commonly in many populations, have been estimated to account for approximately 25% of the variability in warfarin dose requirement. Finally, gamma-glutamyl carboxylase (GGCX) is an enzyme that catalyzes the post-translational modification of vitamin K-dependent proteins and has been reported to have a modest effect on warfarin metabolism.

Four SNPs for these genes were examined simultaneously in a multiplexed assay. Genomic DNA was isolated from whole blood and the region of interest was amplified by PCR. SNP primers (each of different length) were designed with the sequence ending one nucleotide upstream of the SNP of interest. The primer was then extended a single base with a fluorescently labelled dideoxynucleotide terminator (ddNTP). The reaction product was then separated by capillary electrophoresis and analyzed. (Fig 1)

This method has several advantages. It is an accurate procedure that can be performed with minimal hands-on effort. It lends itself to custom design and is flexible in that oligonucleotide probes used to detect SNPs of interest can be added or removed quickly from an existing panel. In our hands, this method gave results that were 100% concordant with traditional sequencing results. This method has the additional benefit of a short turnaround time- the entire analysis may be performed in less than 24 hours, the majority of which is needed for incubation steps and for the automated electrophoretic separation.

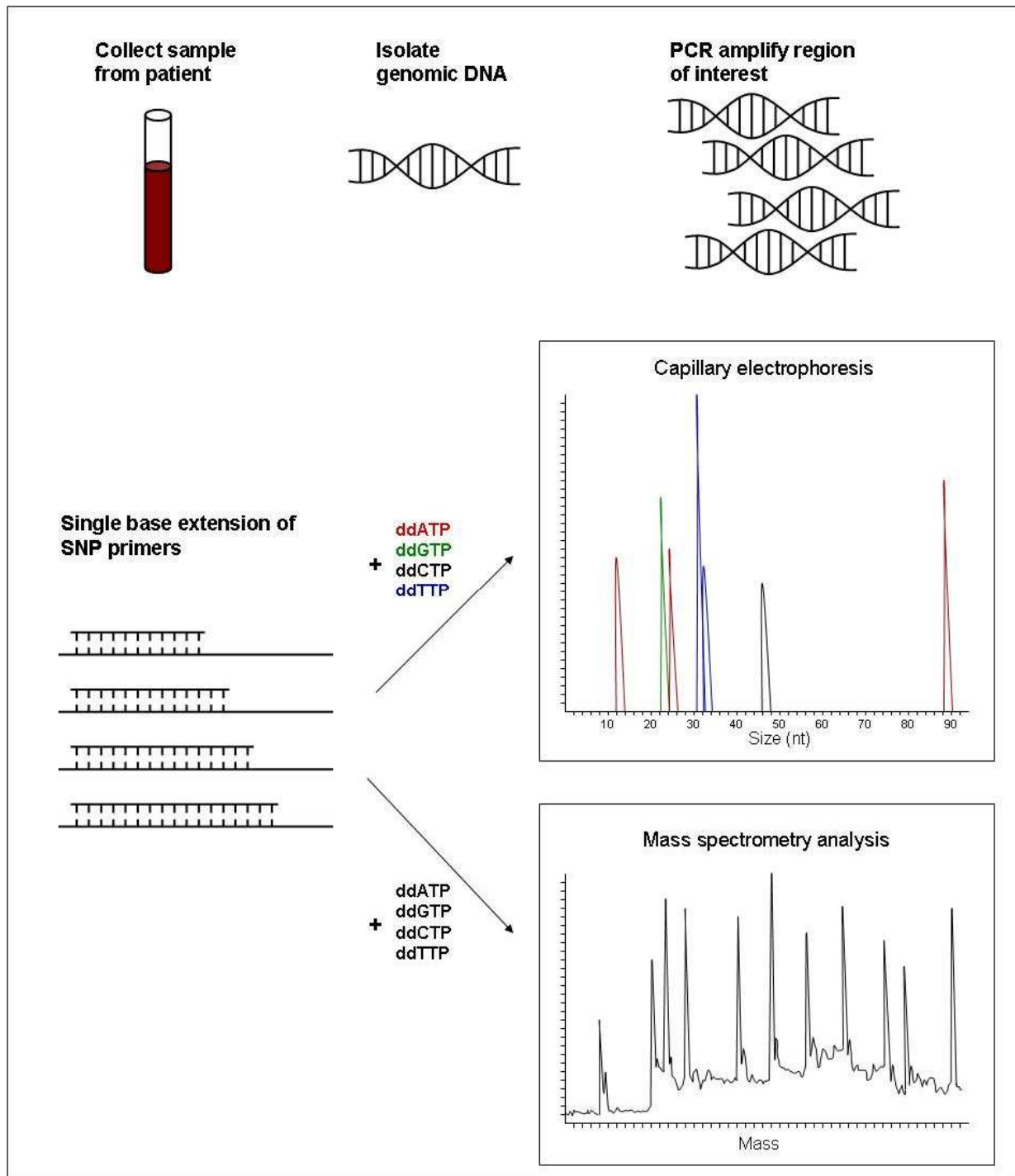


Fig. 1. Detection of SNPs by single base primer extension. Blood is collected from a patient and genomic DNA is isolated from lymphocytes. A multiplex PCR reaction is performed to amplify DNA fragments containing the SNP of interest. This is followed by a multiplex SNP reaction whereby oligonucleotides ending one base pair upstream of the SNP of interest are added and then extended with nucleotide terminators. In the case of capillary electrophoresis, detection is based on fluorescence whereby the ddNTPs are tagged with various fluorophores. In contrast, for mass spectrometry detection is based on accurate sizing of modified nucleotide terminators.

In addition to the assay described above for assessing warfarin metabolism, we have successfully used the same approach to interrogate 8 SNPs of CYP2D6. As described in the previous section, CYP2D6 is highly polymorphic and plays a role in the metabolism of approximately one-fourth of clinically important drugs. We sought to interrogate these SNPs to characterize the PM phenotype. One limitation of this approach (and capillary electrophoresis in general) is in the resolving capacity of this technique, which under our conditions is ~2 nt. In our procedure, we are resolving and visualizing oligonucleotides for SNP interrogation in a window from 10-95 nt, the maximum number of fragments that can be resolved is ~12. This resolving capacity is inherent to the capillary electrophoresis methodology and commercial analyzers of which several are available, all share this limitation.

A similar approach can be used to multiplex a larger number of SNPs, and can thus overcome the limitations described above. Such a genotyping approach needs to exploit methods that are of higher resolution (relative to capillary electrophoresis), such as mass spectrometry. Using this technique, genomic DNA is isolated, the region of interest is amplified by PCR, and SNP primers are hybridized, as described above. These SNP primers are extended by a single base with unlabelled dideoxynucleotide terminators.

The SNP allele is then detected by the mass of the extension product, as a function of the time required to traverse the time-of-flight tube. (Fig 1) We have recently used this technique to interrogate 11 SNPs simultaneously from a single sample. This technique is (theoretically) capable of resolving up to 35-40 SNPs in one well, and thus a greater number of SNPs can be interrogated (relative to capillary electrophoresis), whether they reside in one gene or many genes. Such a methodology is ideal when the number of SNPs of interest is within these parameters.

Several commercial platforms offer larger scale SNP genotyping using single base extension as an approach. The MassARRAY system (Sequenom), an example of a mass spectrometric based platform as described above, can interrogate ~35-40 SNPs simultaneously. The SNPstream assay (Beckman Coulter) is able to interrogate either 12 or 48 SNPs simultaneously in a 384-well plate. A fluorescently labelled nucleotide is added to a tagged SNP probe by single base extension. Each well of the 384-well plate contains tagged oligonucleotides at specific positions within the well. These tagged oligonucleotides are complementary to one of the 12 or 48 tagged SNP probes. The genotype of the SNP is identified by determination of the position of fluorescence in the well.

3.2 Hybridization-based approaches

Hybridization-based approaches for SNP genotyping depend on stringent hybridization conditions (conducive to the ability or inability to form Watson-Crick base pairs) as a means to distinguish one or more alleles. As compared to single base primer extension, hybridization assays are sensitive to variations on length and sequence of both probe and target oligonucleotides. Similar to primer extension, hybridization assays can also interrogate many SNPs simultaneously within the same sample. For example, the Affymetrix GeneChip is an array of oligonucleotides that allows genome-wide interrogation of SNPs.

In hybridization-based approaches, genomic DNA is isolated, regions of interest are amplified, cleaved, and then tagged, for example with biotin. The tagged products are subsequently hybridized under stringent conditions to allele-specific oligonucleotides on a

solid matrix, such as a bead or array. These allele-specific oligonucleotides differ by only one or few bases, and correspond to the various alleles of the DNA fragment of interest. The reaction is performed under conditions whereby mismatched targets, i.e. those that do not hybridize perfectly, can be washed away. This leaves only the stably hybridized DNA fragments, i.e. those that have a perfect match to their corresponding target, that are fluorescently labelled. Subsequent detection of the fluorescent signal allows for the determination of the SNP genotype. The specificity of the assay can be increased by using multiple probes for each SNP allele (Figure 2).

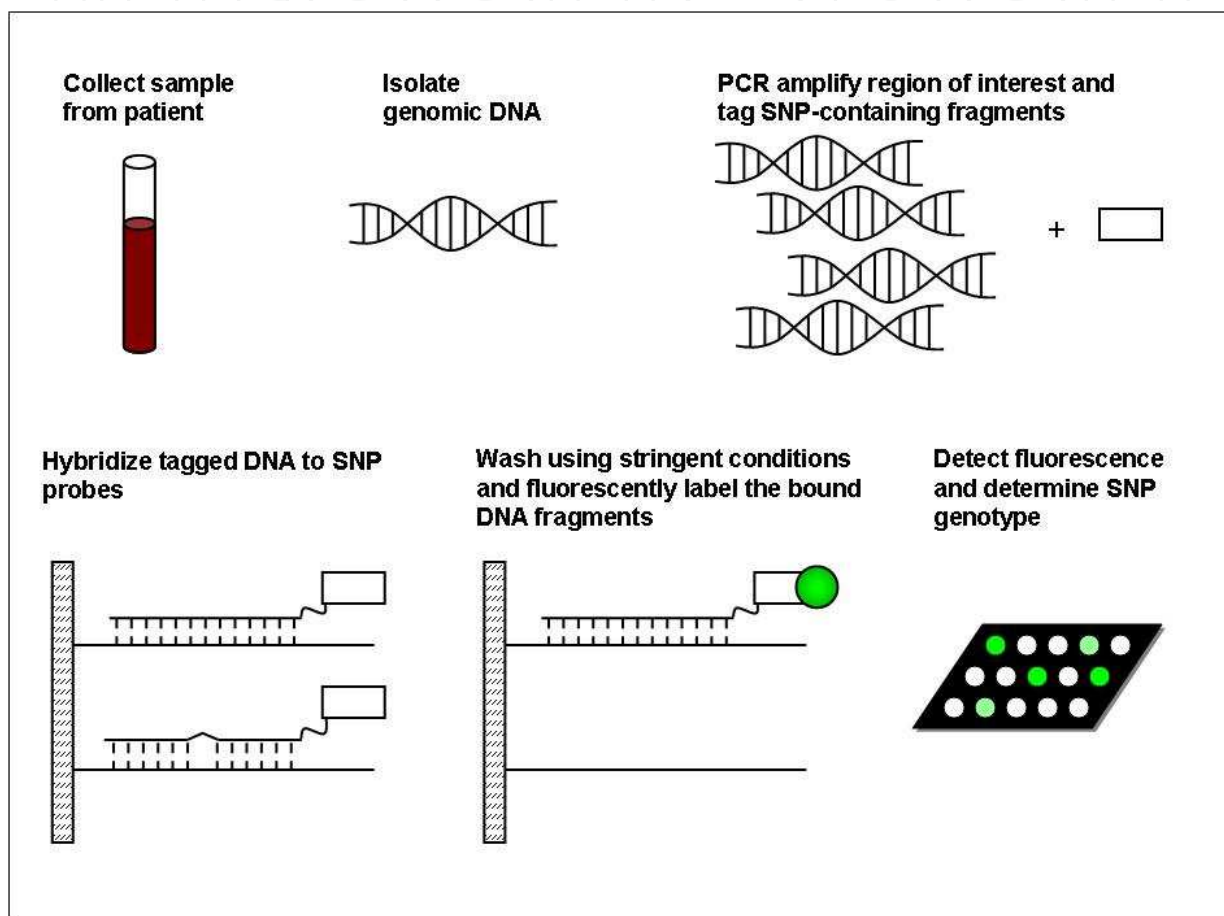


Fig. 2. Detection of SNPs by hybridization. Blood is collected from a patient and genomic DNA is isolated from lymphocytes. A PCR reaction is performed to amplify and tag DNA fragments containing the SNP of interest. Tagged DNA fragments are hybridized to SNP probes bound to a solid matrix, and mismatched fragments are washed away. Hybridized fragments are fluorescently labelled and detected to determine the SNP genotype.

Dynamic allele-specific hybridization (DASH) is another assay that utilizes differential hybridization for SNP genotyping. In DASH, genomic DNA is isolated, and the region of interest is amplified using a biotinylated primer. The biotin tag allows for the attachment of a single stranded DNA fragment to a bead. This is then hybridized with a SNP-specific oligonucleotide. Now, a fluorescent molecule that intercalates into the double-stranded DNA is added, and the fluorescence signal is measured over an increasing temperature

gradient. A melting curve can thus be established. (Fig 3) A complete match between the genomic DNA and the SNP-specific oligonucleotide results in the expected melting temperature curve, whereas mismatches result in a curve showing lowered melting temperatures. This technique was used in a study of 13 SNPs in the adiponectin gene which has been linked to type 2 diabetes.

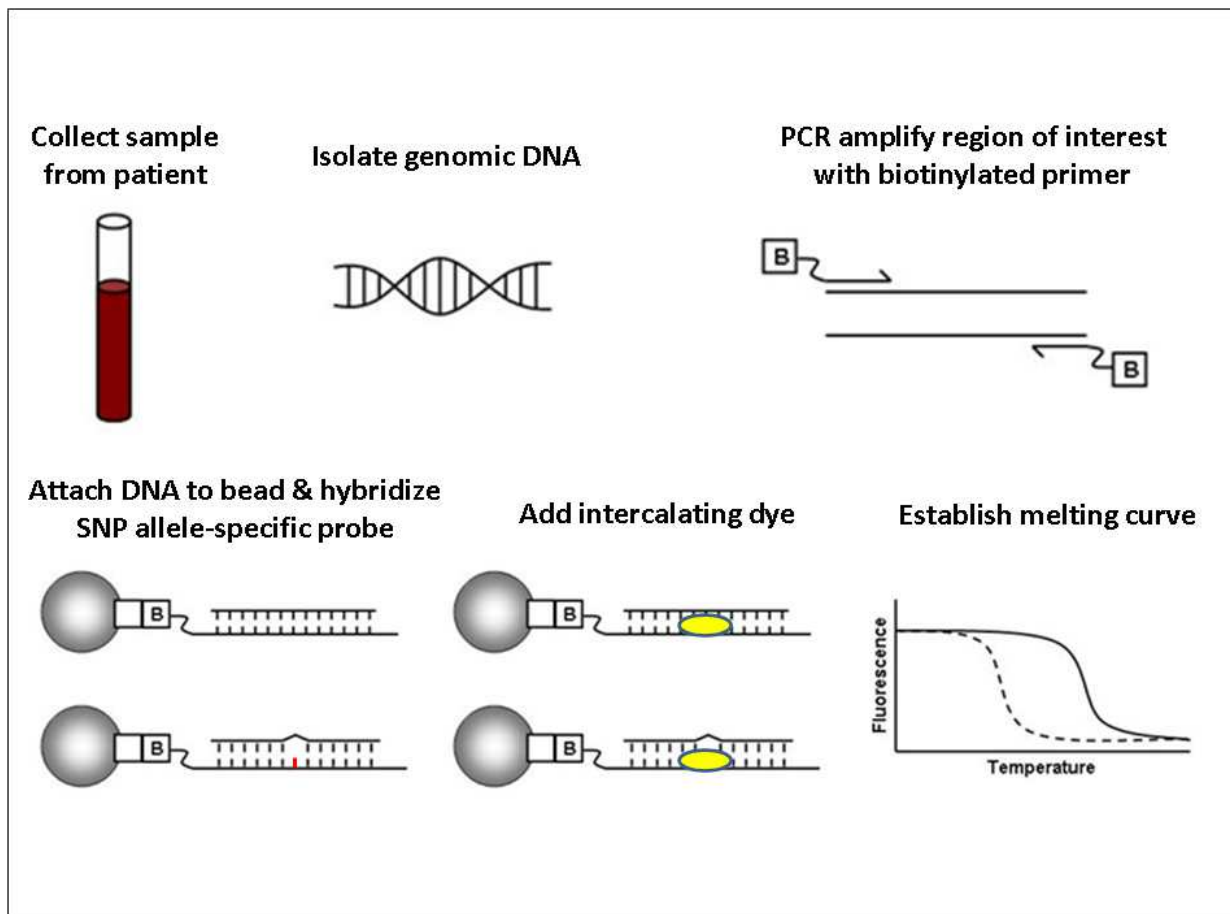


Fig. 3. Dynamic allele-specific hybridization (DASH). Blood is collected from a patient and genomic DNA is isolated from lymphocytes. A PCR reaction is performed to amplify and tag DNA fragments containing the SNP of interest. The DNA fragment is attached to a streptavidin bead, and is hybridized to a SNP probe. A fluorescent intercalating DNA dye is added, and a melting curve is determined. A mismatch (dashed line) will result in a lower melting temperature.

A unique take on the use of hybridization for SNP genotyping involves the use of molecular beacons. A molecular beacon is an oligonucleotide hairpin with a fluorophore at one end and a fluorescence quencher at the other end with a sequence complementary to the SNP allele nested in the middle. In the unbound state, no fluorescent signal will be emitted as the fluorophore and the fluorescence quencher are in close proximity at the stem of the hairpin structure. When the molecular beacon hybridizes with a perfect match to a genomic DNA fragment, the hairpin structure of the molecular beacon is linearized, separating the fluorophore and the fluorescence quencher, allowing fluorescence signal to be emitted (Figure 4).

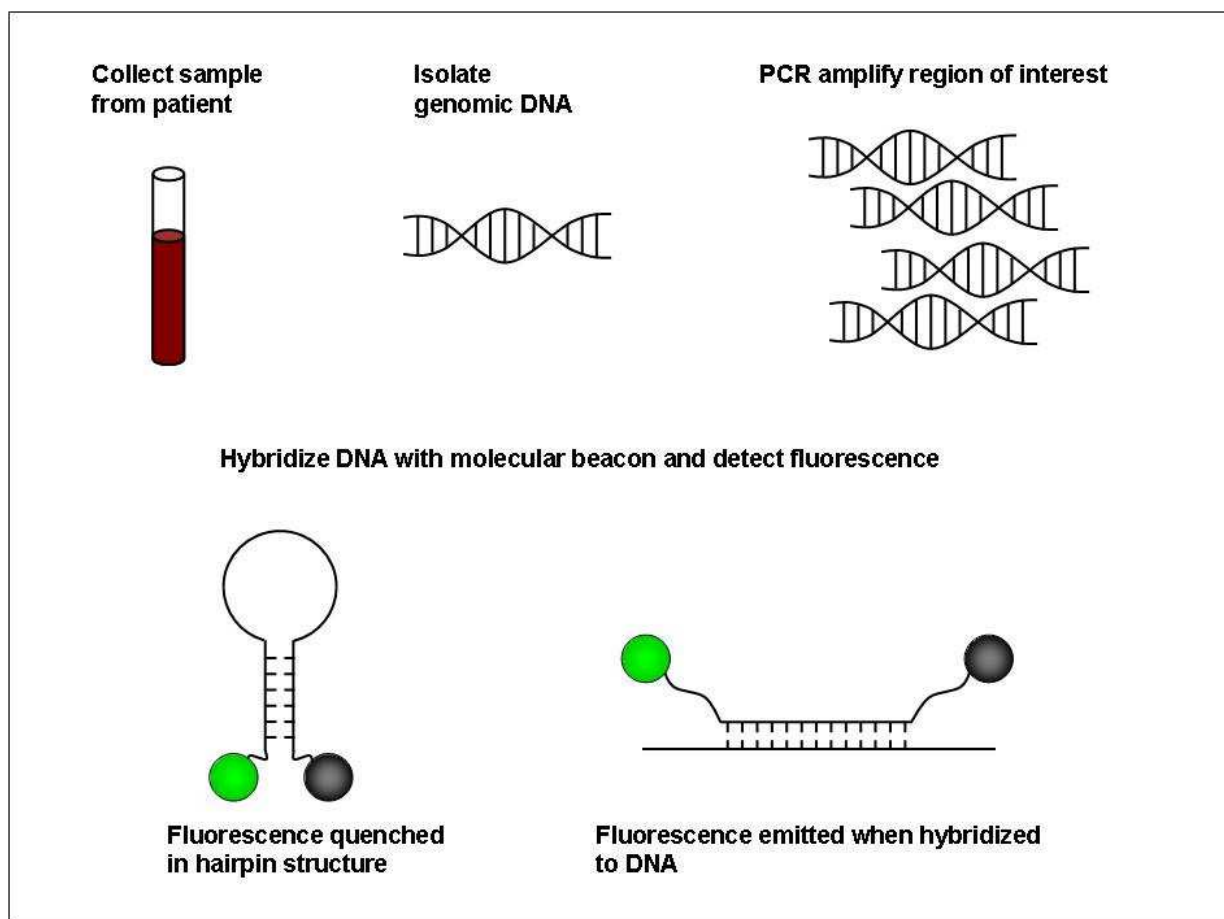


Fig. 4. Interrogation of SNPs using molecular beacons. Blood is collected from a patient and genomic DNA is isolated from lymphocytes. A PCR reaction is performed to amplify DNA fragments containing the SNP of interest. The DNA fragment is hybridized to a molecular beacon. When the molecular beacon is not bound to the DNA fragment, the fluorophore and fluorescence quencher are held in close proximity to each other and no fluorescence signal is emitted. When the molecular beacon hybridizes with the DNA fragment, the fluorophore and fluorescence quencher are separated and fluorescence is emitted.

The TaqMan (Applied Biosystems) assay is a widely used assay that utilizes hybridization to determine SNP genotypes. This assay takes advantage of the 5' nuclease activity of Taq polymerase to detect SNP alleles. In this assay, the region of interest is amplified by PCR. In addition to the forward and reverse primers, an allele-specific probe is also hybridized under stringent conditions to the template DNA. The allele-specific probe has a fluorophore at the 5' end and a fluorescence quencher at the 3' end. When the allele-specific probe perfectly complements the template DNA, it is stably bound, whereas when there is a mismatch, the probe will not hybridize stably with the template DNA and will not be a substrate for the 5' nuclease activity of Taq polymerase. When Taq polymerase reaches the allele-specific probe as it extends DNA from the primer, the 5' fluorophore is released by the 5' nuclease activity of Taq polymerase, and the probe is displaced. Release of the 5' fluorophore separates the fluorophore from the 3' fluorescence quencher, allowing fluorescence to be emitted and subsequently measured (Figure 5). The Taqman assay was recently used to interrogate 121 SNPs to analyze Y-chromosome variation in 264 samples.

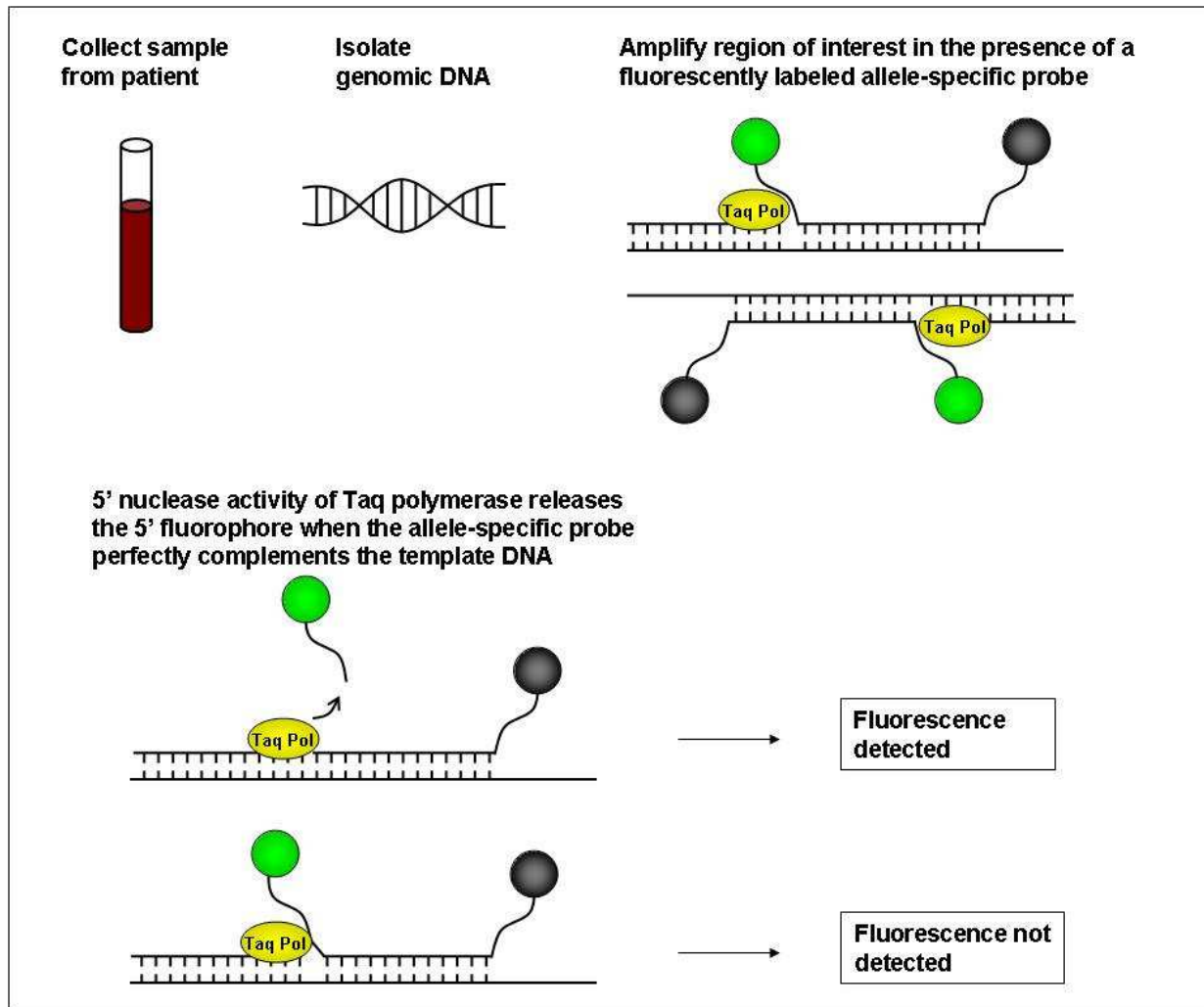


Fig. 5. SNP interrogation utilizing the 5' nuclease activity of Taq polymerase. Blood is collected from a patient and genomic DNA is isolated from lymphocytes. A DNA fragment containing the SNP of interest is amplified by PCR in the presence of an allele-specific probe labelled with a fluorophore at one end, and with a fluorescence quencher at the other end. When the allele-specific probe complements the DNA, the 5' nuclease activity of Taq polymerase will release the fluorophore, and fluorescence signal will be detected.

3.3 Ligation-based approaches

The ligation-based approach to interrogate SNPs exploits the ability of DNA ligase to ligate two adjacent oligonucleotides bound to a template DNA. In this assay, two oligonucleotides are required; an allele-specific oligonucleotide which has its 3' end complementary to the SNP nucleotide to be interrogated, and a second oligonucleotide with its 5' end designed to anneal immediately adjacent to the 3' end of the first oligonucleotide. Both oligonucleotides are hybridized to the target DNA. DNA ligase is then added to the reaction. Only if the 3' end of the first oligonucleotide is indeed complementary to the SNP allele, will ligation occur, as DNA ligase is sensitive to 3' mismatches. The ligated and unligated products are of different sizes and can thus be detected using a separation technique, for example capillary electrophoresis or mass spectrometry analysis. This approach can also be scaled up for high throughput analysis. (Fig 6)

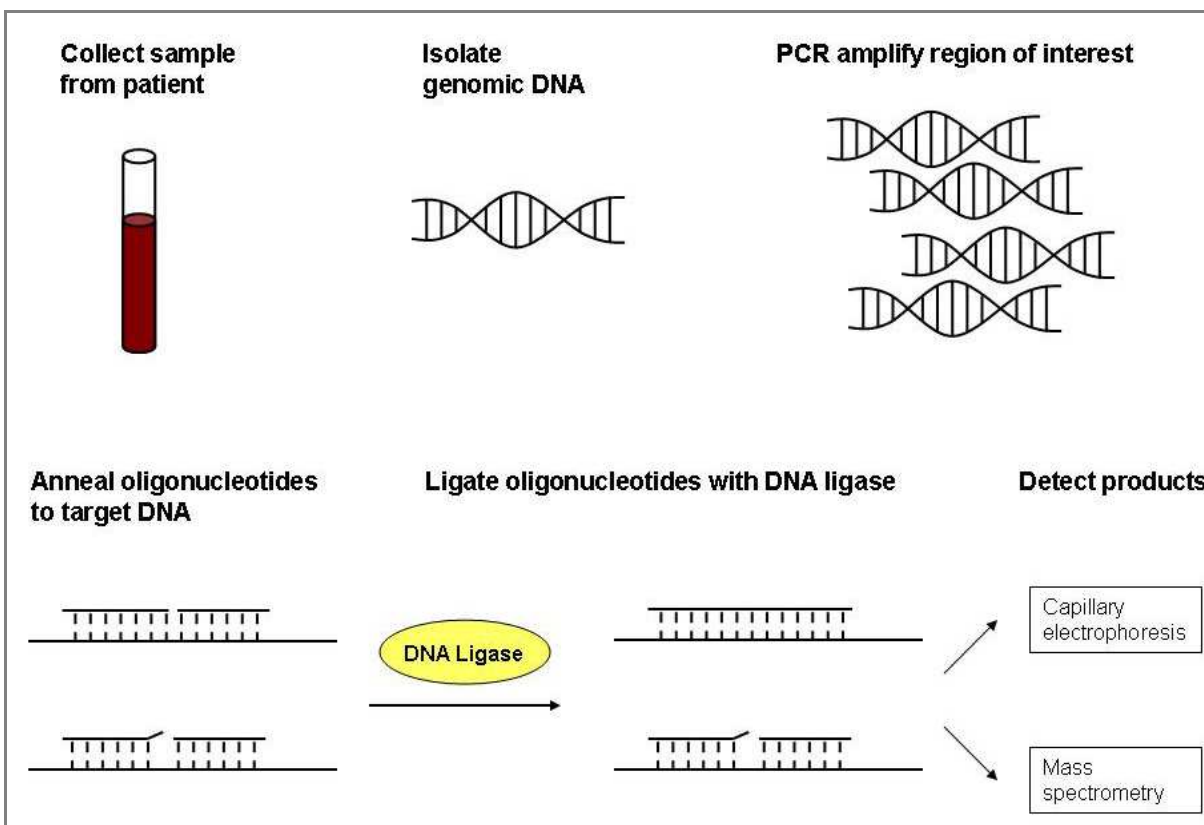


Fig. 6. SNP allele detection by ligation. Blood is collected from a patient and genomic DNA is isolated from lymphocytes. A DNA fragment containing the SNP of interest is amplified by PCR. Two oligonucleotides are annealed to the DNA fragment, flanking the SNP site to be interrogated. If the allele-specific oligonucleotide is complementary to the SNP allele, DNA ligase will be able to ligate the oligonucleotides, and the ligated product can be detected by capillary electrophoresis or mass spectrometry.

The oligonucleotide ligation and capillary electrophoresis method is used in the SNPLex assay (Applied Biosystems), a platform which allows multiplexing for the detection of multiple SNPs simultaneously. The SNPLex assay has been used in multiple studies; in a recent study it was used to detect SNPs in 528 members of families with sarcoidosis.

3.4 Sequencing based strategies: Pyrosequencing and other next generation sequencing methods

Conventional capillary electrophoresis based Sanger sequencing was developed in the (late) 1970s and became widely adopted thereafter. It revolutionized life science research in the subsequent years by providing a critical tool that was fundamental in the elucidation of genetic sequence information. Despite this, the technology suffers from limitations in speed, resolution, throughput and scalability

Next-generation sequencing technologies have recently been developed and have made possible cost-efficient, high-throughput sequencing, that can overcome these drawbacks. An example of next-generation sequencing is pyrosequencing, a sequencing-by-synthesis technique. The pyrosequencing technique sequences approximately 250 bases per read. In pyrosequencing, each added base is detected in real-time by fluorescence. Specifically,

genomic DNA is isolated, and the region of interest is amplified by PCR. A sequencing primer is then annealed to the template DNA, and the reaction components are added: DNA polymerase, ATP sulfurylase, luciferase, apyrase, adenosine 5' phosphosulfate (APS), and luciferin. One of four deoxynucleotide triphosphates (dTTP, dCTP, dGTP, or dATP α S) is then added (dATP α S is used in place of dATP as it can be incorporated by DNA polymerase, but is not a substrate for luciferase). If the added dNTP is complementary to the template DNA, the dNTP is incorporated by DNA polymerase, releasing pyrophosphate (PPi). The released PPi is converted to ATP by ATP sulfurylase with adenosine 5' phosphosulfate as a substrate. The ATP serves to drive the conversion of luciferin to oxyluciferin by the luciferase enzyme. Oxyluciferin generates visible light proportional to the amount of ATP. This visible light is measured and used to determine if a dNTP was incorporated, and if so, the number of dNTPs added. The unincorporated dNTPs and ATP is degraded by apyrase. This cycle of reactions is repeated with the next dNTP. By this process of sequential addition of dNTPs, the sequence can be determined. (Fig 7)

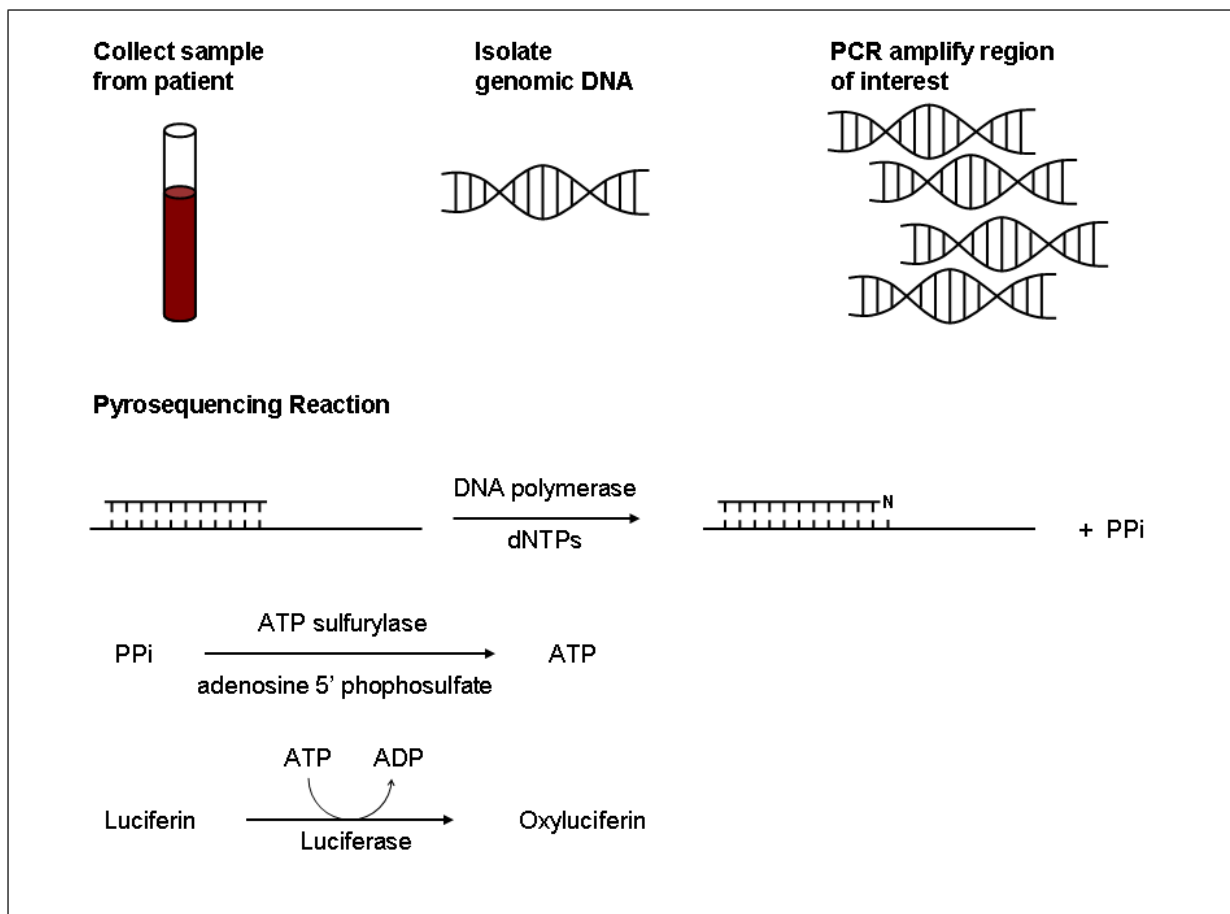


Fig. 7. Pyrosequencing. Blood is collected from a patient and genomic DNA is isolated from lymphocytes. A DNA fragment containing the SNP of interest is amplified by PCR. A sequencing primer is added, as are the sequencing reaction components: DNA polymerase, one of four dNTPs (dTTP, dCTP, dGTP, or dATP α S). If the nucleotide is incorporated, PPi is released, which is then converted by ATP sulfurylase to ATP. The ATP drives the conversion of luciferin to oxyluciferin by luciferase. Oxyluciferin generates visible light proportional to the amount of ATP.

A high-throughput application of pyrosequencing, termed 454 pyrosequencing, was developed in 2005 by 454 Life Sciences. In this technique, the target DNA is attached to a bead and placed in a picoliter-sized well of a fiberoptic slide (containing 1.6 million wells). The substrates for the pyrosequencing reaction are added to the wells in waves via a flow-chamber, the light signal is detected, and the sequence is determined. This parallelized pyrosequencing technology allows the determination of mega- to gigabase amounts of DNA in a fast and cost-efficient manner.

Illumina (Solexa) sequencing is another next-generation sequencing technology that has recently been developed for high-throughput sequencing. DNA is sequenced by repeated cycles of single base extension. As in standard single base extension, DNA is extended a single base with a fluorescently labelled nucleotide terminator. The nucleotide terminator in this case is not a dideoxynucleotide; instead it is a modified, reversible terminator. The identity of the incorporated base is determined by detection of fluorescence. Next, the fluorophore is removed and the terminator is reversed, and the cycle of single base extension is repeated. In this manner, approximately 75 bases can be read at a time.

A third important next-generation sequencing technology is SOLiD sequencing (Sequencing by Oligonucleotide Ligation and Detection), a technology developed by Applied Biosystems. Rather than utilizing a polymerase, this technology utilizes an elegant system of hybridization and ligation steps to determine the sequence of target DNA. In brief, DNA is attached to a flow cell and is hybridized and ligated to one of a set of fluorescently labelled probes. The fluorescence is detected, then cleaved and the process is repeated to extend the sequence. A combination of repeated hybridization and ligation steps and use of primers with different offsets allows not only the sequence to be determined, but also provides a “two-base” read to improve accuracy. This technology can provide sequence data of approximately 50 bases with each individual read. This individual sequencing reaction is performed across millions of templates in a massively parallel fashion. Thus, in a rapid manner, large stretches of DNA can be sequenced spanning entire genomes. In fact, the amount of DNA sequence data that is generated is staggering, exceeding hundreds of gigabytes of information from a single run. A single instrument today can generate more information in a 24 hour period than was possible using multiple instruments with standard technology operating over a decade, in the 1980s, 1990s, and even in the 2000s.

3.5 Other SNP genotyping methods

The Invader assay (Third Wave Technologies) utilizes the ability of Flap endonuclease (FEN) to cleave specific DNA structures to determine SNP alleles. Flap endonuclease is a 5' nuclease that recognizes DNA structures with a single stranded 5' overhang, or flap. Flap endonuclease will cleave DNA at the junction of the single and double stranded DNA. In this assay, genomic DNA is isolated and the region of interest is amplified by PCR. Two probes are hybridized to the target DNA: an Invader oligonucleotide and an allele-specific oligonucleotide. The Invader oligonucleotide is designed to anneal with its 3' end immediately upstream to the SNP site. The allele-specific oligonucleotide, which has a 5' fluorophore and a 3' fluorescence quencher, anneals to the SNP site and to the downstream sequence. In addition, the allele-specific oligonucleotide has additional 5' sequence not complementary to upstream sequence that extends from the bound DNA, forming a structure recognized by FEN. Cleavage of the allele-specific oligonucleotide by FEN separates the 5' fluorophore from the 3' fluorescence

quencher, and allows fluorescence to be emitted. If the allele-specific oligonucleotide does not complement the SNP site exactly, the resulting structure is not recognized by Flap endonuclease and no fluorescence signal will be detected. (Fig 8)

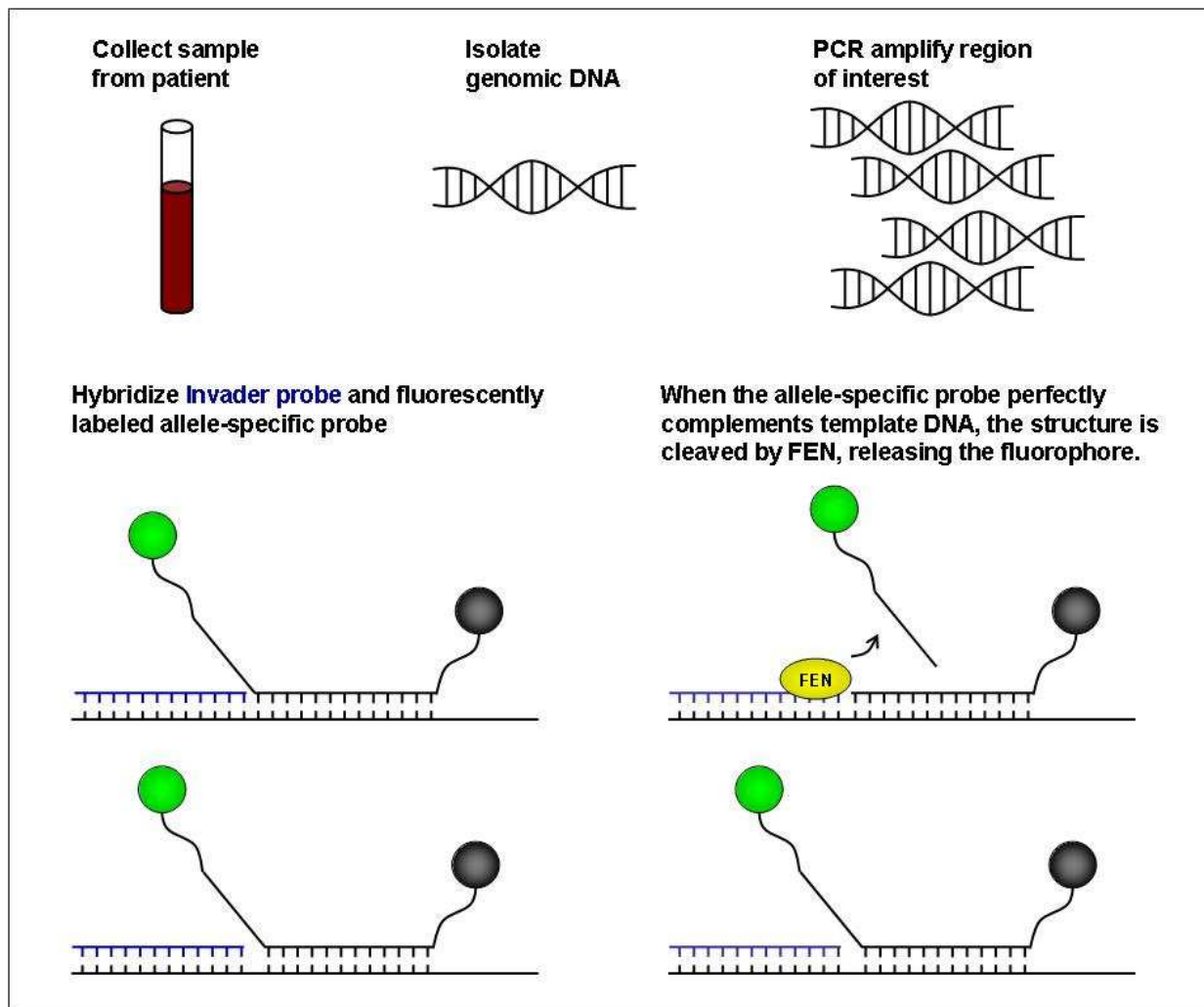


Fig. 8. Invader assay for SNP interrogation. Blood is collected from a patient and genomic DNA is isolated from lymphocytes. A DNA fragment containing the SNP of interest is amplified by PCR. An Invader probe (blue) and an allele-specific probe labelled with a fluorophore and a fluorescence quencher are hybridized to the DNA fragment. FEN recognizes the resulting structure and cleaves it, releasing the fluorophore.

The Invader assay was successfully used (in conjunction with other SNP genotyping methods) to genotype 122 SNPs in 9 candidate genes associated with diabetes. In a study by Ozaki et al., a multiplexed Invader assay was used to interrogate 92,788 SNPs from 94 individuals in a genome-wide association study.

4. Considerations in the selection of the appropriate SNP genotyping tool

Several factors merit consideration and are important for the judicious selection of an appropriate SNP genotyping assay. Accuracy, reliability, and establishment of quality

control are intermingled and readily apparent as important factors. These elements are critical to the development of a robust assay and the validation of such. The accuracy and reliability of results is paramount since these data are used to inform clinical decision making by allowing for optimized selection of therapy for the patient. The inclusion and establishment of an appropriate quality control paradigm ensures the integrity of reagents, test conditions, and experimental and technical workflow. These features form the basis of, and are a prerequisite to, generating an accurate and reliable result.

There are additional factors that will affect more widespread adoption of these assays and make individualized patient therapy available and accessible to most patients. Such considerations include: speed, automation, customizability, and the requirement for specialized equipment and /or technical training. These set of factors deal with the logistics in operationalizing the implementation of such assays.

The speed at which results are obtained and the test's ease of use can directly affect the clinical utility of the assay and further dictate where the assay is performed and who can conduct testing (regulatory requirements differ by country, and within the United States they may also differ by state, e.g. New York). For example, under certain conditions it may be favourable for testing to be conducted by a healthcare provider in an outpatient setting where results are immediately used to make treatment decisions. If an assay is fairly straightforward & easy to use and the chances of obtaining an accurate result are high, it may be advantageous to perform outside the clinical laboratory. On the contrary, it may be beneficial for testing to be sent to a centralized laboratory and performed by a trained, licensed technologist. This is the ideal situation for a higher complexity test. However, in this situation, results may not be immediately available and thus there will be a delay in using the information to change clinical management of the patient.

Assays in which little hands-on effort is required and can be automated will increase speed, throughput, and will help to reduce errors. In cases where numerous SNPs are interrogated, a single, multiplexed reaction allows for the simultaneous investigation of several SNPs, reducing time and cost. The number of SNPs identified and characterized as playing a role in drug metabolism to date has been a relatively manageable size; however, as more SNPs are identified, both multiplexed and high-throughput assays could further reduce costs

Customized assays are advantageous in that they are designed to answer focused questions, and can be tailored to specific patient populations. In addition, they are flexible and can be rapidly changed as new clinically significant SNPs are identified and need to be incorporated. A requirement for specialized training and/or equipment may slow widespread adoption of a SNP genotyping assay but is necessary for conducting high complexity testing of this nature. Finally, cost also can play an important role, particularly in an environment of increasing healthcare expenditures. In recent years, pharmacogenetic testing (and other molecular diagnostics assays) have come under greater scrutiny and the debate over appropriate reimbursement scales by insurance payers continues.

The methodologies described in the previous section have all been used successfully to genotype SNPs, however they have different advantages and limitations that factor into their usefulness in individualized SNP genotyping assays. (Table 2) The ideal assay would combine the advantages of each methodology, and eliminate the common requirement for either large amounts of DNA or for an initial PCR step. New technologies that would

increase sensitivity or reduce the need for multiplexed PCR would be necessary to make the simultaneous interrogation of hundreds or thousands of SNPs faster and more cost-effective.

Methodology	Multiplexed assays?	High-throughput assays?	Advantages	Limitations
Single base (primer) extension	Yes	Yes	High accuracy of incorporation by DNA polymerase	Multiple steps for detection/separation
Hybridization	More difficult	Yes	Widely available; not dependent on enzymatic reaction	Requirement for optimization of probes and hybridization conditions
Ligation	Yes	Yes	High specificity of DNA ligase	Multiple steps for detection/separation
Sequencing	More difficult	Yes	Sequencing of 50-250 bases; speed	Specialized equipment required

Table 2. Comparison of SNP genotyping methodologies.

5. Conclusions

Multiple genetic differences between individuals have been described in the Cytochrome P450 family of drug metabolizing enzymes. These genetic differences may confer altered properties including differences in the ability to metabolize drugs. Methods to identify such differences can help select subsets of patients who may or may not be able to effectively utilize particular medications. Several widely used methods with differing approaches and advantages have been highlighted above. Further technological advances will help these technologies become more widely adopted. These techniques allow for the appropriate triage of patients to therapies that are targeted for their genotype, allowing for a tailored, individualized treatment regimen. Pharmacogenetic testing of this nature will help to usher in this new era of personalized medicine.

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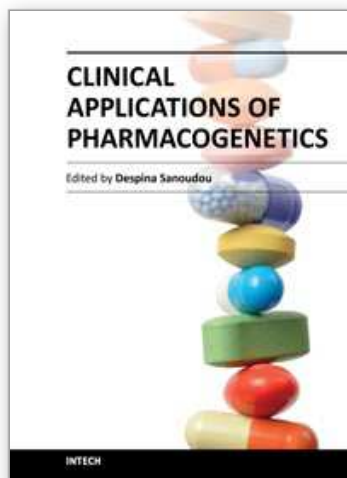
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The rapidly evolving field of Pharmacogenetics aims at identifying the genetic factors implicated in the inter-individual variation of drug response. These factors could enable patient sub-classification based on their treatment needs thus expediting drug development and promoting personalized, safer and more effective treatments. This book presents Pharmacogenetic examples from a broad spectrum of different drugs, for different diseases, which are representative of different stages of evaluation or application. It has been designed so as to serve both the unfamiliar reader through explanations of basic Pharmacogenetic concepts, the clinician with presentation of the latest developments and international guidelines, and the research scientist with examples of Pharmacogenetic applications, discussions on the limitations and an outlook on the new scientific trends in this field.

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