

Pharmacogenetic Testing

How to Choose a Method to Analyze Genetic Changes

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Personalized medicine aims to provide the right treatment to the right patient at the right time. Although still an emerging area, personalized medicine includes some applications that are already accepted in clinical practice, such as molecular tests for HIV drug resistance and *Her2* status for breast cancer, as well as other applications that are just now becoming part of treatment strategies.

The primary driving force behind the move towards personalized medicine is the correlation between drug levels and various polymorphisms of drug metabolizing enzymes, including TPMT, cytochrome P450, and UGT1A1. Molecular tests to determine which polymorphisms are present in a patient's genome promise to increase both the safety and efficacy of various drug therapies (1).

Further driving the advance of this emergent health care strategy, the Food and Drug Administration (FDA) has embarked on several efforts that will likely encourage the practice of personalized medicine. In addition to clearing two different pharmacogenetic assays for drug metabolizing genes, the agency has implemented a voluntary genetic database for genotypes involved with drug distribution, absorption, metabolism, and excretion. The agency also now requires package inserts for certain drugs to include pharmacogenetic information.

As a result of these developments, laboratories have seen an explosion in the availability of different pharmacogenetic test methodologies, and more laboratories are now making pharmacogenetic tests part of their menus (Table 1). To benefit from these new testing options, however, labs must

overview of pharmacogenetic testing technologies and what type of genetic mutations these methods detect.

Non-Amplification Methods

Among the methods to detect large gene deletions or duplications are fluorescent

target or signal. While Southern analysis is not commonly used for pharmacogenomic testing because of the cost and time required to generate a result, the FDA has approved a FISH test for breast cancer that quantifies amplification of the *Her2* gene and is used to identify patients most likely to respond to the cancer drug Trastuzumab (Herceptin). This technology could also potentially be used to detect gene duplications associated with drug metabolism.

Target and Signal Amplification Methods

In amplification methods, either the target or the signal of a particular nucleic acid sequence is increased. Amplification methods require less nucleic acid starting material, provide shorter turn-around times, and reduce hands-on time compared to non-amplification methods.

Target amplification methods such as the polymerase chain reaction (PCR) use enzymes and gene-specific primers to increase the number of target molecules. PCR methods can be further categorized based on the method for detecting the product: end-point detection or real-time detection. For end-point PCR detection, the final product is analyzed using a variety of approaches to identify the genetic variant based on either the size of an amplified nucleic acid target (fragment analysis) or the detection of a fluorescent signal arising from the labeled target. For real-time detection, the method detects and quantifies a fluorescent reporter whose signal increases in direct proportion to the amount of PCR product in a reaction.

End-point PCR Detection

One type of end-point PCR detection is fragment analysis, in which DNA changes in the amplified target product are identified using a variety of methodologies such as allele specific primer reactions, RFLP analysis, oligomer ligase assays, and sequence analysis.

first determine which methods to use. The nature of the genetic polymorphism associated with a drug metabolizing enzyme largely dictates which method a lab should employ. Some genetic variants result from single-nucleotide changes, deletions, or even gene duplications, and labs must determine which molecular method is the most appropriate for the type of genetic variation being analyzed. To aid laboratorians in this selection process, this review will provide an

in-situ hybridization (FISH) and Southern analysis. Mutations that generate or disrupt a restriction enzyme recognition site can also be identified by Southern analysis combined with restriction fragment length polymorphism (RFLP) analysis.

Because these non-amplification methods use labeled probes to directly identify the presence or absence of a particular genetic sequence, they do not require the use of enzymatic reactions to enhance the



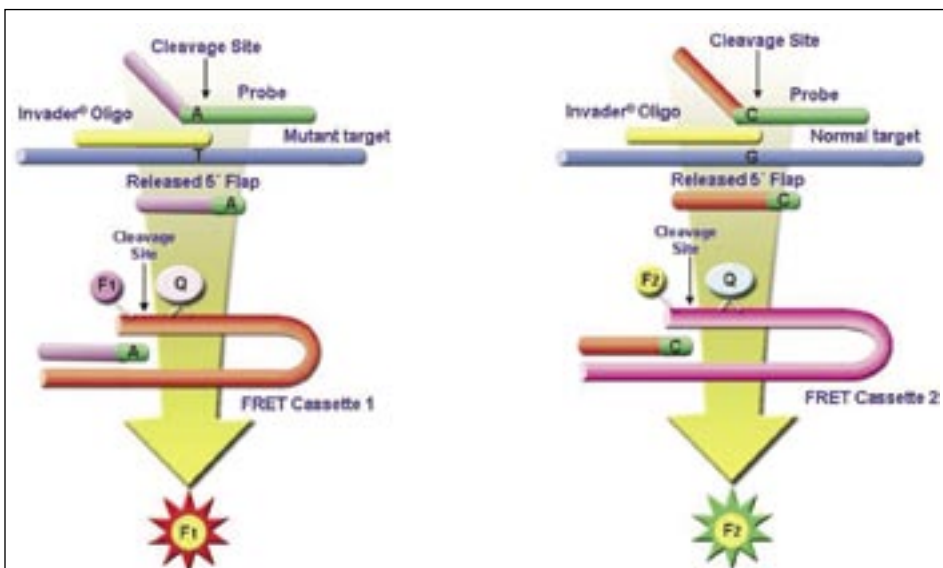


Figure 1. **Thirdwave Technologies' Invader assay. The Invader assay detects specific nucleic acid targets using structure-specific recognition and cleavage with the cleavase enzyme, signal amplification, isothermal reactions, and fluorescence detection.**

Used with permission of Thirdwave Technologies, Madison, Wisc.

Allele-Specific Primers. For detection of single-nucleotide changes, allele-specific primers can be synthesized that are complementary to the variant sequence. After binding to the target gene, these primers are extended in a PCR reaction to generate a PCR product. A primer whose 3'-end is not complementary to the single-base mutation will not be extended, however, and therefore will not generate a PCR product. The presence or absence of a PCR product in a PCR reaction that uses allele-specific primers therefore identifies the homozygous and heterozygous gene carrier (2). Similarly, if the reaction contains a chain-terminating fluorescently-labeled dideoxynucleotide, rather than a dideoxynucleotide, extension of the primer will reflect the variant base. Since each fluorescently-labeled dideoxynucleotide is a different color, the color of the extended primer restriction fragment shows the specific base of the mutation.

Length Analysis: RFLP and OLA. Two other methods of fragment analysis that detect genetic changes in a DNA sequence employ restriction enzymes or ligases following the initial PCR. In RFLP analysis, as with Southern analysis, the variant sequence must either generate or remove a restriction enzyme site. But unlike Southern analysis, the DNA used in RFLP analysis is first amplified and then digested using restriction enzymes. The presence or absence of an additional fragment is diagnostic for the specific genetic changes (3, 4).

Although similar to allele-specific PCR analysis, oligomer ligation assays (OLA) employ a sequence-specific oligomer that is enzymatically joined to another oligomer that has been designed to anneal next to it on the target sequence. If the sequence-specific oligomer does not anneal to the target sequence, ligation will not occur and a ligation product will not be generated. Conversely, if the sequence-specific product anneals to the target sequence, then it will be ligated to the adjacent oligomer and a larger product will be generated. By varying the length of the target specific oligomer, the size of the ligation products can be identified by capillary electrophoresis.

Sequence Analysis. The gold standard for determining and confirming the presence

of a single nucleotide change, a small deletion, or an insertion in a target sequence is sequence analysis of both the forward and reverse strands of a DNA molecule. The sequencing reaction uses a single primer and a mixture of dideoxynucleotides and fluorescently labeled chain-terminating dideoxynucleotides to determine the order of the nucleotides of the DNA template. At least two reactions—a forward reaction and a reverse reaction—are needed to confirm the sequence of both strands of the PCR product.

Because each chain-terminating dideoxynucleotide has a fluorescent label of a different color, each sequencing product will differ in length by one base pair with the end of each product being the fluorescently-labeled dideoxynucleotide. To determine the sequence, these products must then be fractionated by high-resolution methods such as denaturing gel electrophoresis or capillary electrophoresis. Sequencing will not detect large deletions, duplications, or rearrangements and is labor intensive compared to other amplifying methods, but interpreting sequencing results can be facilitated by following the American College of Medical Genetics guidelines (5).

Real-Time PCR Detection

While real-time PCR also uses fluorescence to detect specific genotypes, it differs from the previous applications in that it does not require any post-PCR manipulation. Specifically, PCR products are detected in real time as the PCR reaction proceeds by using variant-specific probes, allele-specific PCR, or a fluorescent intercalating dye (6). This closed-tube detection method decreases hands-on time and the risk of contamination with extraneous DNA.

Signal Amplification

In contrast to the target amplification methods discussed above, signal amplification technology aims to detect the target molecule at the concentrations present in an unaltered sample, without prior amplification. An example of this method is the pharmacogenetic test cleared by the FDA to detect polymorphic UGT1A1 genotypes. Thirdwave Technologies' (Madison, Wis.) Invader

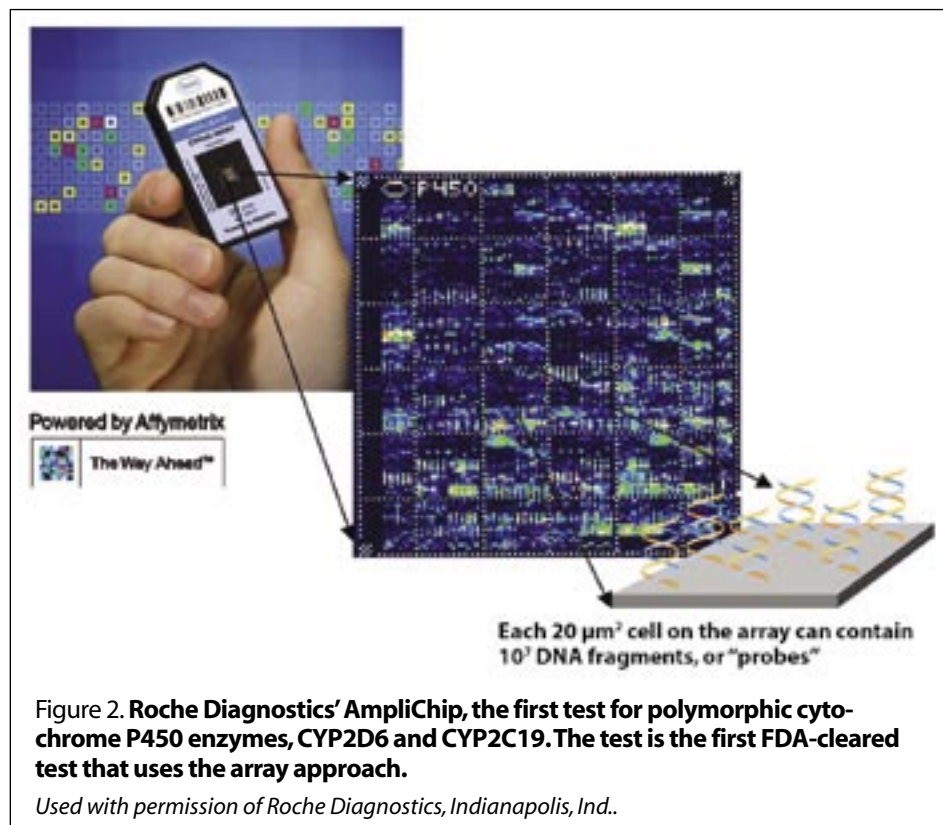


Figure 2. **Roche Diagnostics' AmpliChip, the first test for polymorphic cytochrome P450 enzymes, CYP2D6 and CYP2C19. The test is the first FDA-cleared test that uses the array approach.**

Used with permission of Roche Diagnostics, Indianapolis, Ind..

assay (Figure 1) uses an enzyme that detects three bases overlapping at the same position. A robust, homogeneous assay, the Invader assay is highly sensitive and specific in genotyping single-nucleotide polymorphism (SNP) markers.

In this assay, the first strand is the patient sequence, the second strand is a synthetic "invader" oligomer complementary to the variant single-nucleotide polymorphism (SNP), and the third strand is composed of an oligomer containing the complementary base to the SNP in the middle. Two regions, a flap and a gene-specific sequence, flank the variant specific SNP. The flap will not bind to the patient's DNA; however, when the three strands are aligned in such a way that

the SNP is hybridizing to the flap-gene specific oligomer and the invader oligomer, the cleavase enzyme cuts the oligomer, releasing the nonbinding flap portion. The flap portion proceeds to function as an invader oligomer for a fluorescence resonance energy transfer (FRET) cassette. The invading flap oligomer provides the conformational target for cleavase to release a fluorochrome from the cassette. For each genotype, two flap-gene specific oligomers and two FRET cassettes are added to the reaction mixture to enable the detection of both wildtype and variant sequences. Because the invader oligomer and the flap oligomer are not degraded in the reaction, the rate limiting component is the FRET cassettes. Genotype-specific

Table 1 Methodologies for Pharmacogenetic Testing

Method	Company	FDA Cleared or Approved
Sequencing*	Abbott (Abbott Park, Ill.)	yes
Real-time PCR	Applied Biosystems (Foster City, Calif.)	—
PCR Arrays	Autogenomics (Carlsbad, Calif.)	—
Sequencing*	Bayer Healthcare (Tarrytown, N.Y.)	yes
Pyrosequencing	Biotage AB (Uppsala, Sweden)	—
Real-time PCR	Celera Diagnostics (Alameda, Calif.)	—
Real-time, Allele-specific PCR	DxS Genotyping (Manchester, U.K.)	—
PCR	Gentris (Morrisville, N.C.)	—
User-developed PCR Arrays	Nanogen (San Diego, Calif.)	—
Nanoparticles	Nanosphere (Northbrook, Ill.)	—
PCR Arrays	Roche Diagnostics (Indianapolis, Ind.)	yes
Invader Assay	Thirdwave Technologies (Madison, Wisc.)	yes
PCR Bead-based Detection	Tm Biosciences Corp (Toronto, Ontario)	—
FISH	Vysis (Des Plaines, Ill.)	yes

PCR: polymerase chain reaction; FISH: fluorescent in situ hybridization
*Sequencing for HIV drug resistance

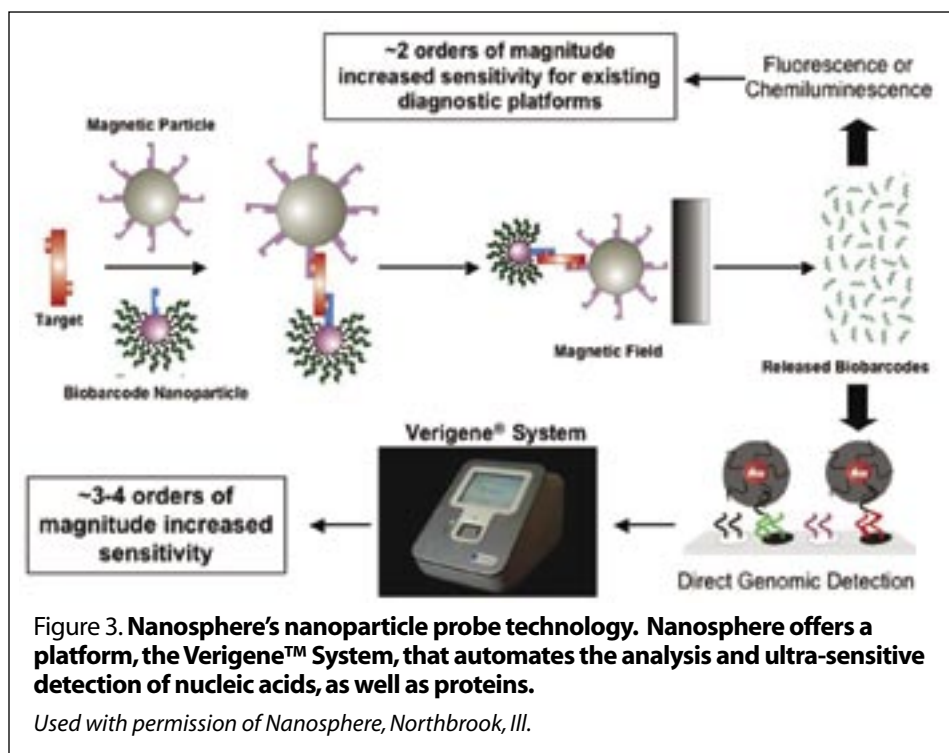


Figure 3. **Nanosphere's nanoparticle probe technology. Nanosphere offers a platform, the Verigene™ System, that automates the analysis and ultra-sensitive detection of nucleic acids, as well as proteins.**

Used with permission of Nanosphere, Northbrook, Ill.

fluorochromes in the FRET cassette permit the identification of the variant and wildtype alleles using a fluorometer.

New Tools for Molecular Testing

In addition to the assay developed by Third-wave, a second FDA-cleared molecular test has been developed by Roche Diagnostics (Indianapolis, Ind.) to evaluate polymorphisms of the cytochrome P450 enzymes. In addition, other companies have developed a variety of tools to aid laboratories that have developed their own molecular assays.

Solid-Phase Arrays. Solid-phase arrays use a variety of approaches to identify numerous genotypes simultaneously. The Roche Diagnostics AmpliChip is the first FDA-cleared test that uses the array approach, and it is also the first test for polymorphic cytochrome P450 enzymes, specifically CYP2D6 and CYP2C19 (7). This assay initially generates PCR products for both of these alleles, as well as CYP2D6 duplication—and deletion—specific products if they are present in the sample. The resultant PCR products are combined into one tube and enzymatically digested with DNase I into fragments ranging in size from 50–200 base pairs (bp). Subsequent reactions label these fragments with

biotin and hybridize them to the sequences on the AmpliChip (Figure 2).

The AmpliChip has 15,000 oligonucleotide probes in the 20- μm^2 format, with 1x10⁷ probes for a particular genotype localized to a site-specific probe cell. Approximately 240 probes are used to identify each genotype in question. Streptavidin-conjugated fluorescent dye binds to the biotin molecules that remain attached to these probes and permits the identification of fluorescing probe cells using a chip reader. Allele-specific probes are present in replicate probe cells. This built-in redundancy provides quality control and assurance for assigning genotypes to the patient sample. The platform also includes interpretative software that further facilitates its use in the clinical laboratory.

Fluorescent-Based Detection: Bead Assays.

In order to increase throughput, some in vitro diagnostic companies have adapted fluorescent-based DNA detection methods to beads. In general, these bead-based methods use a variety of different approaches to physically link a specific DNA sequence to a colored bead. For example, in the detection scheme developed by Tim Bioscience (Toronto, Ontario), an initial PCR product serves as

the template for allele-specific primers designed to detect 14 different genetic variants. When these allele-specific primers match perfectly with the variant, they produce a single-stranded product that incorporates a biotin-labeled nucleotide. Each allele-specific primer extended (ASPE) product or strand is then hybridized to a “tag” that is complementary to a probe sequence located on a genotype-specific colored bead. Finally, the ASPE product is incubated with streptavidin and R-phycoerythrin and detected by laser excitation of the internal dyes on the beads using a Luminex Analyzer (Austin, Texas). Because each gene variant DNA sequence is attached to a uniquely colored bead, the assay can identify the variant present in the patient sample. Current applications of the technology enable labs to identify 14 different genotypes, but the platform has a capacity to detect up to 100 different genotypes.

Emerging Technologies

In addition to the new technologies that have recently become available, emerging methods for pharmacogenomic testing—including high-resolution melting curve analysis, pyrosequencing, and nanotechnology—promise to reduce labor and reagent cost when compared to standard methods.

High-resolution melting curve analysis is based on the fact that the melting temperature of a PCR product depends on the ratio of G/C to A/T bases. Therefore, high-resolution melting curve analysis following real-time PCR amplification can potentially discriminate between different genotypes by detecting sequence variants that alter the G/C to A/T ratio (8).

Pyrosequencing is an alternative method for identifying the order of bases in a sequence (9). This method measures the light emitted following the release of pyrophosphate from the base that is added to the primer. The pyrophosphate generates ATP, which then mediates the conversion of luciferin to the light emitting oxyluciferin. The resultant visible light is proportional to the amount of ATP used. For example, the incorporation of three adjacent and identical bases will generate a peak that is three times the height of incorporating a single base.

Yet another emerging approach incorporates nanotechnology techniques to molecular testing methods. Nanosphere's (Northbrook, Ill.) technology initially binds the target DNA to a surface. A gold nanoparticle probe binds to the other side of the exposed target in a classic sandwich assay format, and then a catalytic procedure uses silver to amplify the signal by six orders of magnitude (Figure 3).

Final Considerations

So far this article has outlined methods for detecting polymorphic drug metabolizing genes, but the question remains as to which technique is most appropriate. For any particular laboratory, the answer will depend on numerous factors, all of which may influence the decision of which platforms to use. There are many factors to consider, including the laboratory's policy on using FDA-approved tests, ASRs, or user-developed assays. Budget issues, such as acquisition of new capital equipment versus using in-house instruments, as well as reagent costs for assay validation and maintaining proficiency are also important. Other factors that labs should consider are the technical expertise required to perform the test, ease of performing QC and QA, batch size, and turn-around time.

To assure that the appropriate performance characteristics have been considered prior to committing to a platform, laboratorians can review the National Academy of Clinical Biochemistry's proposed practice guidelines on pharmacogenetic testing (<http://www.nacb.org/lmpg/main.stm>), the proposed guidelines for microarrays found in *Clinical and Laboratory Standards Institute MM12-P* (Vol. 25., No. 18), as well as the molecular checklist from the College of American Pathologists. Following identification of the ideal platform(s) for pharmacogenetic testing, laboratorians can then decide if taking the leap into this new era of personalized medicine is right for their lab. **CLN**

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AACC Joins Personalized Medicine Coalition

In recognition of the role that laboratory medicine plays in personalized medicine, AACC recently joined the Personalized Medicine Coalition (PMC), an educational organization of more than 60 pharmaceutical, biotechnology, diagnostics, and informatics companies, along with research institutions, patient advocacy groups, payors, and government agencies. Organized in 2004, PMC's objective is to ensure that society develops an integrated approach to the legal, financial, social, technical, and professional issues that will shape the development and adoption of personalized medicine.

AACC members who work in both clinical laboratories and the IVD industry develop and perform the tests integral to the development and implementation of the targeted therapies that are the goal of personalized medicine. The participation of AACC members in various committees within PMC will make sure that the very important contribution of laboratory medicine will be recognized and included in PMC programs.

Catherine Hammett-Stabler, PhD, of the University of North Carolina (Chapel Hill) has been appointed as AACC's liaison to PMC, and other AACC members will be recruited to serve on PMC Committees. A number of AACC members are already active in PMC on behalf of their own organizations. For more information on PMC, visit its Web site at www.personalizedmedicinecoalition.org/.

This article is available as an 8 1/2" x 11" reprint on the AACC Web site (www.aacc.org). Click on "Clinical Laboratory News," then "Series Articles."