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RATIONAL SELECTION OF PCR-BASED PLATFORMS FOR PHARMACOGENOMIC TESTING

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ABSTRACT: Background: *Genotyping is crucial to the identification of genetic markers underlying development of neoplastic diseases and individual variations in responses to specific drugs. Cost- and time-effective technologies able to accurately identify genetic polymorphisms will dramatically affect routine diagnostics processes and future therapeutic developments. However, such methods need to fulfill the principles of analytical validation to determine their suitability to assess nucleotide polymorphisms in target genes.*

Approach: *This article reviews the recent developments of technologies for genotyping of single nucleotide polymorphisms (SNPs). For the appropriate choice of any method, several criteria must be considered: i) known or unknown genetic variations in a given cancer gene; ii) needs of testing within pharmacogenomics studies; iii) diffusion and availability of large platforms and required equipments; iv) suitability of tests for routine diagnostics; v) capacity of methods to offer a specific and sensitive detection of mutant alleles within great excess of wild-type alleles in a given sample; vi) suitability for high-throughput implementation.*

Content: *This review is intended to provide the reader with a better understanding of the various technologies for pharmacogenomics testing in the routine clinical laboratory. A brief overview is given on the available technologies for detection of known mutations together with a precise description of the homogeneous technologies and platforms currently employed in genotyping analysis.*

Based on the criteria proposed here, potential users may evaluate advantage and limitations of the different analytical platforms and possibly identify the most appropriate one according to specific operative settings and diagnostic needs.

KEY WORDS: *Genotyping methods, Analytical validations, Molecular diagnostics.*

Abbreviations: Single nucleotide polymorphisms (SNPs); Single-strand Conformational Polymorphism (SSCP); Allele Specific Amplification (ASA); Amplification Refractory Mutation System (ARMS); Restriction fragment length polymorphism analysis (RFLP); Peptide Nucleic Acid (PNA); Matrix-Assisted Laser Desorption/Ionization time of flight (MALDI TOF); Fluorescent Resonance Energy Transfer (FRET); Locked nucleic acid (LNA); Oligonucleotide ligation assay (OLA); Rolling Circle Amplification (RCA); High Resolution Melting (HRM).

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INTRODUCTION

Recent research demonstrates that certain genetic polymorphisms are linked to significant variations among individuals in the response, in terms of activity and toxicity, to a given drug[1]. To confirm the candidate genetic markers emerging from such studies, there is a commensurate need for pharmacogenomic laboratories to design and validate targeted genotyping assays capable of rapidly identify the individual Single Nucleotide Polymorphism (SNP) of interest within confirmatory clinical studies and in the routine clinical practice. In recent years, a number of increasingly complex technologies have been applied to the qualitative and semi-quantitative detection of polymorphisms and mutations in DNA (for simplification, we shall mainly refer to point mutations, though in general, small deletions or insertions can be as efficiently detected by the methods described here)[2].

Traditional techniques for SNP genotyping detection by Single-Strand Conformational Polymorphism (SSCP) and Heteroduplex analysis have now been largely replaced by high-throughput methods including "in silico" discovery platforms. These latter methods generate much more data and are easier to automate.

A recent breakthrough in high-throughput strategies is represented by DNA chip technology, which allows the combined detection and identification of mutations[3]. However, for many applications appropriate chips will be only available in the forthcoming years. Thus, conventional screening methods for point mutations and small deletions will most probably keep their place in the diagnostic laboratory for a reasonable amount of time. Costs, however, are projected to be high and assay performance and results interpretation will remain strictly dependent on the availability of highly qualified and well-trained personnel. We will highlight some of the most popular homogeneous technologies that are currently used in specialized laboratory, making the transition from the research setting to the clinical laboratory and discuss key aspects in their validation for genotyping in pharmacogenomics[4].

Homogeneous methods are essentially "single-tube" assays in which all of the processes required for target amplification and detection occur in a single "closed-tube" reaction (except for Pyrosequencing), without a solid phase. Combining the thermal cycling system with the signal detection system allows the on-line monitoring of the PCR amplification process[5]. In this review post PCR agarose gel-based detection methods, will be considered as homogeneous.

The advantages of homogeneous methods include reduced risk of cross-contamination, time-effectiveness and practicability. These methods are usually amenable to automation and high-throughput processing with 96-well plates, the current industry standard, but can be further implemented by 384-well plate capabilities. Maximum automation can be achieved by fully integrated systems with robotic processing of 96- or 384-well plates throughout the stages of DNA extraction, PCR set-up, amplification, detection, and data interpretation[6].

Many of these strategies are now commercially available and this field is characterized by intense competition mixed to many examples of productive cooperation and cross licensing[7]. No single genotyping platform stands out as ideal and it is likely that many of the different technologies described in this article will be employed in combined studies aimed to find disease genes and novel drug targets.

NEEDS TO DETECT GENETIC VARIATIONS IN CANCER CHEMOTHERAPY

Pharmacogenomic approaches have been applied to many existing chemotherapeutic agents in an effort to identify relevant inherited variations that may better predict patient response to treatment and toxicity[8]. Genetic variations which can alter the amino acid sequence of the encoded protein, include nucleotide repeats, insertions, deletions, translocations and SNPs. Genetic polymorphisms in drug metabolizing enzymes like Cytochrome P450 family, drug transporters like Multidrug Resistance-1, and other molecular targets have been actively explored with regard to functional changes in phenotype (altered expression levels and/or activity of the encoded proteins) and their contribution to variable drug response[9,10]. Clinically relevant examples of genetic defects highlighting the relevance of cancer pharmacogenomics in optimizing cancer chemotherapy by improving its efficacy and safety are given in Table 1. A new generation of anticancer drugs has been recently designed with high specificity toward tumour cells, providing a broader therapeutic window with less toxicity as compared to conventional chemotherapy; these drugs represent a new and promising approach to targeted cancer therapy[11].

New agents are designed to interfere with a specific molecular target, usually a protein with a critical role in tumour growth or progression (i.e. a tyrosine kinases). There are multiple types of other targeted therapies already clinically available, including monoclonal antibodies, antisense inhibitors, proteasome inhibitors, enzyme-activity modifiers and immuno-modulatory drugs. Obviously, any of these new agents may exert a selective pressure on tumour cells that elaborate

TABLE 1. MOST COMMON GENETIC ABNORMALITIES IN CANCER GENES AND THEIR EFFECT IN CHEMOTHERAPY OUTCOMES.

GENE	Polymorphism (nucleotide translation)	Molecular effect	Drug	Effect on therapy
Cytochrome P450 family	Various nucleotide translation	Decreased enzyme activity	Cyclophosfamide Etoposide Paclitaxel	Inter-individual variability in Pharmacokinetics
TPMT2, 3A, 3C	Various Polymorphism	Rapid degradation	6-MP Thioguanine	Hematopoietic toxicity
UGT1A 28	TA repeats in 5' promoter	Low expression	Irinotecan	Neutropenia toxicity
MDR1	(C3435T)	Low expression	Various	Drug resistance
TYMS	3 tandem repeats	High expression	5-FU, Methotrexate	Drug resistance
DHFR	(T91C)	Increase enzyme activity	Methotrexate	Drug resistance
MTHFR	(C677T)	Decreased enzyme activity	Methotrexate	Toxicity
c-KIT	(T1982C) (T81421A)	Constitutive signal activation	Imatinib	Desensitizes activity in GIST
c-KIT	D816V		Imatinib Semaxinib	Good response in t(8;21)-positive AML
EGFR	L858R		Gefitinib Erlotinib	Good response in NSCLC
ABL	T(9;22) BCR/ABL fusion gene	Constitutive signal activation	Imatinib Dasatinib Nilotinib	Good response in CML
ABL	T315I M351T		Imatinib	Drug resistance
RARα	T(15;17) PML/RARα fusion gene	Block of maturation of Myeloid cells	All Trans Retinoic acid (ATRA)	Good response in AML-M3 subtypes

Abbreviations: TPMT = thiopurine methyltransferase; UGT1A1 = UDP-glucuronosyltransferase 1A1; MDR1 = multidrug resistance 1; TYMS = thymidylate synthase; DHFR = Dihydrofolate reductase; MTHFR = 5,10-methylene tetra hydrofolate reductase; EGFR = Epidermal Grow Factor Receptor; 5-FU = 5-fluorouracil; 6-MP = 6-mercaptopurine; GIST = Gastro-intestinal Stromal Tumor; AML = Acute Myeloid Leukemia; NSCLC = Non-Small Cell Lung Cancer; CML = Chronic Myeloid Leukemia

strategies to survive and proliferate in their presence. The same basic principle applies to protein kinase inhibitors; the best understanding of this problem at a molecular level derives from studies on imatinib resistance in Chronic Myelogeneous Leukemia (CML) patients carrying the BCR/ABL fusion gene. These imatinib-resistant leukemic cell clones, develop following a single nucleotide mutation in ABL kinase domain (with consequent amino acid substitution), but can be efficiently suppressed by second-generation tyrosine kinase inhibitors (i.e. dasatinib, nilotinib bosutinib)[12,13]. These latter agents maintain a full antineoplastic activity on almost all imatinib-resistant mutant leukemic cell clones[14].

Imatinib is also able to inhibit the activity of other tyrosine kinase such as those encoded by c-KIT and FLT3 genes in patients with Acute Myelogeneous Leukemia and in Gastrointestinal Stromal Tumours (GISTs)[15,16].

Similarly to imatinib, two other biological drugs (gefitinib and erlotinib) showed a clinical activity in a subset of patients affected by Non Small Cell Lung Cancer (NSCLC). The mechanism of action of both these agents is the selective inhibition of kinase activity of Epidermal Growth Factor Receptor (EGFR). Recently, it has been reported that detection of specific point mutations of the EGFR gene in tumour cells may allow to discriminate gefinitib-responding patients (EGFR mutated), from non-responders (EGFR wild type), in NSCLC[17]. The availability of this kind of biomarkers could then represent a useful tool for investigating drug resistance to specific types of targeted therapies.

GENOTYPING METHODOLOGIES

New in vitro diagnostic assays and the multiplex assay technologies have been developed to respond to rapid advances in the understanding of genomic variation affecting drug responses. These molecular assays guide the therapeutic treatment of many diseases because they give information

about: molecular subtypes of disease (that require differential treatment), which drug has the greatest possibility of managing the disease, and which patients are at the highest risk of adverse reactions to a given drug therapy[18].

Mutation-detecting technologies can be divided into two major categories depending on the capacity to screen for new mutations or to identify already known mutations.

Until a short time ago the only platform cutting edge considered was Matrix-Assisted Laser Desorption/Ionization time of flight (MALDI TOF), able to fulfill both discrimination between alternative alleles and detection of both alleles in a single step assay. Alternatively, there were several allele discrimination methods that combine PCR-based methods with hybridization probes. The most used homogeneous platforms for the detection of known SNPs can be operatively classified in two major categories of PCR-based methods (Table 2): i) agarose gel-based detection; ii) fluorescence-based detection[19]. For the unknown mutations, platforms must be able to perform both screening and detection. The techniques listed in Table 2 show many overlaps and attempts to compare each other may result difficult and unproductive. In almost all assays, DNA amplification is required.

TABLE 2. COMMONLY USED METHODS FOR GENOTYPING AT MOLECULAR LEVEL

Methods for detection and screening for unknown mutations

Screening
- Heteroduplex DNA assay (melting curve)
- Denaturing-HPLC
- Denaturing gradient gel electrophoresis (DGGE)
- Single strand conformation polymorphism (SSCP)

Detection and screening
- Conventional sequencing
- High Throughput sequencing

Methods for detection of known mutations

***Homogenous:* gel based detection**
- Allele Specific Amplification (ASA)
- Restriction Fragment Length Polymorphism (RFLP)
- Peptide nucleic acid-mediated Clamping PCR

***Homogenous:* Fluorescent-based detection**
- FRET probe Allelic Discrimination (Hyb Probe®, TaqMan®, Beacons® Scorpions®)
- Locked Nucleic Acid (LNA) probe
- Oligo ligation assay (SNPlex®)
- Invader® Assay
- Pyrosequencing*
- High resolution melting (HRM)

Heterogenous
- Gene Chip technology
- Maldi-TOF Mass Spectroscopy
- Golden Gate® Assay

*Required pre-PCR step

DNA microarrays and next-generation sequencing (NGS) are the two most important technologies for high-throughput genomic analysis. DNA microarray technology has been developed and consolidated as a routine tool in research laboratories and is now transitioning to the clinic. NGS technologies have emerged that enable the sequencing of large amounts of DNA in parallel and they are suitable to different applications, such as whole or targeted genome sequencing, and RNA sequencing (RNA-seq).

Because of the cost, the last application is still slow to replace DNA microarray transcriptome profiling analysis, especially in our country.

A real progress may soon be reached with the advent of PCR-microarray platforms combining multigenic analysis with real-time detection. Their sample-to-result characteristic and simple use will enable them to bridge the technical gap between research and clinics. The miniaturization, integration and automation of these tools increase accuracy and reproducibility, making them more suitable for routine use. With these advances, genome-based tests have the potential to become a standard tool for mainstream diagnostics, in order to monitor disease onset and progression, facilitate individualized patient therapy and, ultimately, improve patient outcomes[20].

Non-PCR-based technologies such as the ligase chain reaction, Rolling Circle Amplification (RCA) and Invader® assays (Third Wave Technologies, Madison, WI, USA) are able to genotype directly from genomic DNA (i.e. without PCR amplification) and are amenable to be applied as homogeneous detection methods. DNA chip-based microarray, Golden Gate® Assay and mass spectrometry genotyping technologies are the latest development in the genotyping arena. These newer technologies are currently less widely used in the clinical laboratory setting than PCR-based methods. Due to their wide diffusion, special attention will be paid to performance and quality assessment in all of the homogeneous methodologies.

HOMOGENOUS METHODS FOR DETECTION OF KNOWN MUTATIONS

The choice of a specific genotyping detection assay for identification of mutations is strongly dependent from the type of mutation and its allele heterogeneity. In general, homogeneous systems increase throughput, reduce the chance of cross contamination and are amenable to automation, but require more fluorescently-labelled probes, increasing costs and reduced multiplexing capabilities. When a large panel of SNPs assays needs to be developed and budget for instruments are lim-

ited, methods based on conventional PCR followed by a gel-based detection assay should be preferred over fluorescent hybridization-based methods. Another advantage of electrophoretic detection systems is the possibility to direct check for the appropriate size of amplicons. We define these methods as low-throughput, due to their time-consuming and labour-intensive characteristics. However, fluorescent-based detection systems have been developed for application in routine laboratories, due to their high specificity, high sensitivity and medium/high-throughput.

Gel loading-based detection. Low- throughput

Allele Specific Amplification (ASA)

The method is based on a PCR performed in two parallel reactions. In the first reaction, the 5' primer is complementary to the wild-type sequence; in the second reaction, the 5' primer is complementary to the mutant or polymorphic sequence. Assuming that elongation occurs only when primer and target sequence match completely, only one allele of either mutant or wild-type DNA is amplified. Two different approaches have been described. The first approach is based on the lack of primer elongation due to a mismatch at the far 3'-end of the primer. This method has been named ARMS "Amplification Refractory Mutation System" and developed by DxS Diagnostics (Manchester, UK)[21]. In the second approach, the mismatch is located within the primer, preventing primer annealing when mispairing occurs; methods based on this principle are defined AS-PCR "Allele Specific-PCR"[22].

Assuming a homozygous situation, lack of amplification will occur in one of the reactions when PCR is performed with different pairs of 5' primers, one complementary and the other not complementary to the represented allele. By multiplex, PCR developed by ARMS, different alleles can be distinguished in a single PCR, by using two annealing temperatures and four primers[23]. However, elongation of mismatched bases can be avoided when appropriate primers and reaction conditions are applied. Specificity of primer extension may be improved by appropriate adjustment of experimental conditions and a web-based AS primer design application called WASP [http://bioinfo.biotec.or.th/WASP]. This software offers a tool for designing AS primers for SNPs. By integrating the database for known SNPs (using gene ID), it also facilitates the awkward process of getting flanking sequences and other related information from public SNP databases[24].

From these simple PCR-based methods, most of other above described technologies have stemmed: Real Time SYBR Green is currently used to enhance throughput; pyorosequencing of ASA PCR amplicon, could be used to enhance accuracy[25,26]. The specificity and sensitivity of the method is strongly influenced by the ratio of mutant to wild-type DNA. Limit of detection and identification of a homozygous or heterozygous state is the main application of the ASA. Several authors have reported the detection of few tumor cells carrying mutations in the presence of a large number of normal cells[27].

Performance and quality assessment is crucial, since the possibility of false positive or negative results is the major limitation of ASA. False-positive results may be due to contamination or artificial point mutation introduced by mis-annealing of the primers specific for the mutated allele. Operational guidelines to avoid contaminations and appropriate of assay conditions should be strictly followed. In addition, positive target alleles must be included as controls.

PCR-restriction fragment length polymorphism analysis (RFLP)

This is a commonly used method including a gel electrophoresis-based technique, such as PCR, coupled with RFLP analysis. Specific DNA sequences can be amplified. The PCR products are then digested with appropriate restriction enzymes and visualized by staining the gel after electrophoresis. If the genetic polymorphism produces a gain or loss of the restriction site, a different restriction digestion pattern can be recognized[28]. A major limitation of the PCR-RFLP method is the absolute requirement that the polymorphisms alter a restriction enzyme cutting site[29]. For same point mutation or SNPs that reside in sequences one nucleotide away from endonuclease restriction sites, allele-specific primers introducing a point mutagenesis may be used to generate artificial mutation sites for RFLP[30]. Detection limit of simple RFLP analysis is of one mutant cell out of 50 to 100 non-mutant cells[31].

For RFLP analysis, a specificity of 100% can be achieved when appropriate restriction enzymes are used. As quality controls, different allelic variants or wild type and mutant DNA must be included in each analysis. Recognition sequences may be destroyed by errors of the Taq polymerase. In general, errors due to mis-incorporations will become detectable only when high numbers of PCR cycles and/or sensitive detection methods are used. The method has to be adjusted to conditions such that no false-positive results are obtained when variable

amounts and different proportions of wt and mutant DNA are analyzed. Improvement of specificity and double-checking for questionable results can be achieved by sequencing of the PCR product.

PNA-mediated Clamping PCR

The peptide nucleic acid (PNA)-based PCR procedure has been developed for the selective enrichment of mutant alleles-specific amplicons within a large excess of wild type alleles[32]. PNA is a synthetic DNA analog in which the normal phosphodiester backbone is replaced by a non-polar 2-aminoethylglycine chain, while its attached nucleobases complement DNA or RNA in the A-T and G-C geometry[33]. Two important features make PNA a superior PCR clamp oligonucleotide for specific alleles: i) PNA cannot serve as a primer for polymerization, nor can it be a substrate for exonuclease activities of Taq polymerase ii) the melting temperature (Tm) of a perfectly matched PNA-DNA duplex is higher than that of DNA-DNA of the same length, but a single mismatch destabilizes the PNA-DNA hybrids, causing a Tm shift of 10-18°C. Therefore, PNA can specifically block primer annealing and/or chain elongation on a perfectly matched template without interfering with templates carrying mismatched bases[34]. In this way, a target mutant DNA can be specifically detected in a large excess of wild type DNA. Detection limit of analysis is of one mutant cell over one hundred-thousand wild type cells[35]. To improve sensitivity and throughput, it should be possible to carry out a large-scale screening in an automated manner by using matrix-assisted laser desorption/ionization time of flight (MALDI TOF) mass spectrometry[36]. In addition, the large Tm difference between perfectly matched and mismatched hybrids makes PNA a good sensor of point mutations. For example, a PNA sensor probe has been used to detect GNAS gene mutations after conventional PCR[37].

The use of melting curve analysis in combination with fluorescent probes provides a powerful tool for the detection of single base alterations. The hybridization probe system is most widely used for this purpose. It consist in a pair of oligonucleotides – the anchor and the sensor – each labeled with a different fluorescent dye, such that fluorescence energy transfer occurs between the two when they anneal adjacent sites of a complementary PCR strand. The melting curve profile of the sensor probe (designed to anneal to the variable region), allows homogeneous genotyping in a closed tube[38]. Recently, fluorescent PNA probe was combined with PNA-mediated PCR clamping for detection of variant BCR/ABL allele in leukemia, and of K-Ras mutation in pancreatic cancer[39,40].

The key feature of this procedure is that a PNA oligomer bound with fluorophore serves both as PCR clamp and sensor probe, which allows the discrimination of sequence alterations in mutant codons from the wild-type sequence[38].

Fluorescent-based detection. Medium/high-throughput

Fluorescent Resonance Energy Transfer (FRET) based allelic discrimination

Many of the probe-based systems rely on the principle of FRET for signal generation. FRET involves the non-radioactive transfer of energy from a donor molecule to an acceptor molecule. Briefly, if two fluorophores with an overlapping spectra of emission are physically close, the wavelength of the light emitted from the first fluorophore after excitation, is adsorbed from the second fluorophore, causing its excitation. Using FRET hybridization probes, a donor and an acceptor fluorophore present in two different probes, co-localize, after hybridization, to an adjacent region on the target molecule. The donor fluorophore is excited by instrument light source transferring energy from the donor to the acceptor, and causing an increase of measurable fluorescence of the acceptor fluorophore. Based on this chemistry several platform for genotyping have been developed: Hyb-Probe® (Roche Diagnostic, Indianapolis, IN, USA) TaqMan® (Applied Biosystems, Foster City, CA, USA), Beacons® (Public Health Research Institute, Inc. NJ, USA) and Scorpions® (DxS Manchester, UK).

a) Hyb-probe, developed for the use with dedicate instrumentation "LightCycler" (Roche Diagnostic) utilizes a blue-light-emitting diode with the measurement of fluorescence by three photo detection diodes with different wavelength filters. The rapid heating and cooling, allows amplifications to be completed in less than 20 minutes. Using this platform with allele specific primers, Agarwal et al[41] described a meaningful improvement of the MTHFR and TYMS genotyping. Comparing this technique to conventional RFLP, the authors obtained a 100% concordance in test results and concluded that Hyb-probe assay is reliable, economical, and can be performed by less trained technologists.
b) An alternative method for polymorphism detection is the TaqMan-based allelic discrimination assay, which combines the use of a standard pair of PCR primers, designed to am-

plify the region containing the polymorphism of interest, with two different detection probes (one with the wild type sequence and the other containing the mutated nucleotide). Each detection probes is labeled on 5'-end by a "reporter" emitting fluorescent dye (different from the other) and a quencher on its 3'-end. During PCR, the probes specifically annealed to their complementary sequence, are cleaved by Taq DNA polymerase (5' exonuclease), causing the separation of the reporter dye from the quencher. The relative fluorescence emitted from both probes (wt and mutated) is detected by the instrument and plotted in a 2D cluster plot allowing quantitation of the amount of each specific allele present in the analyzed samples. Homozygosity shows increased fluorescence in one channel, while heterozygosity exhibits intermediate fluorescence in both channels.

Generally, in an allelic discrimination assay the default minimum quality value required for an acceptable genotype call is set to 95 (quality range). However, once the accuracy of a genotyping assay employing allelic discrimination is established, the stringency for these quality values can be increased or decreased, as appropriate.

While this method can be considered of medium/high throughput, the ability to analyze multiplex samples in single tube is limited by the restricted number of fluorescent dyes with non-overlapping spectra[42]. An interesting variant of this technique has been obtained by the Fluorescence Polarization detection, which is able to eliminate the need of a quencher dye with the reduction of the probes price[43]. However, probe design is largely empirical and optimization times are significantly increased. Therefore, the optimal use of this platform is probably achieved when a relatively small number of SNPs must be assayed on a large number of samples.

c) Molecular beacons are oligonucleotide probes with two complementary DNA sequences flanking the target DNA sequence and with a donor-acceptor dye pair at opposite ends of each probe. When the probe is not hybridized to the target, it adopts a hairpin-loop conformation so that the reporter and quencher dyes are close together, so that no donor fluorescence is generated. When the probe hybridizes to the target sequence, the two dyes are separated and the fluorescence is dramatically increased[44]. Since the mismatched probe-target hybrids dissociate at a consistent lower temperature than matched ones, the different Tm increases the specificity of molecular beacons. In a typical SNP genotyping, two molecular beacons with sequence matching to the wild-type and variant alleles respectively, are used in the same PCR reaction. The use of two probes labeled with different fluorophores emitting fluorescence at distinct optical wavelengths, allows simultaneous discrimination of the possible allelic combinations.

d) Scorpion is a single bi-functional molecule containing a PCR primer covalently linked to a probe. The molecules are oligonucleotide with a "Stem-Loop" tail containing a fluorophore, which interacts with a quencher to reduce fluorescence. The Stem-Loop tail is separated from the PCR primer sequence by a chemical modification of oligonucleotide, called "PCR stopper" that prevents the copying of the stem-loop sequence during polymerization started by Scorpion primer. During the annealing phase of PCR, the probe sequence in the Scorpion tail curls back to hybridize to the complementary target sequence in the PCR product (as the tail of the scorpion and the amplicon are now part of the same strand of DNA, the interaction is intermolecular). This hybridization event opens the hairpin loop causing the increase of fluorescent signal because the fluorophore is not quenched anymore. The PCR stopper, located between the primer and the stem sequence, prevents read-though of the hairpin loop. Since the hybridization event is generated by a single molecule with two functions (primers and probe), the scorpion system is more effective than the other homogeneous probe systems. The reaction is instantaneous and occurs prior to any other competing or side reactions (e.g. amplicon re-annealing or inappropriate target folding), resulting in stronger signals, more reliable probe design, shorter reaction time and better discrimination. This contrasts to the bimolecular collisions required by other technologies such as Taqman or Molecular Beacons[45].

The possibility to use Scorpion primers for each possible mutations in a single multiplex reaction, reduce scoring mistakes in the presence of a negative results[46]. Moreover, Scorpion chemistry, suitable for several thermal cycling platforms, is cheaper, because it only requires a conventional PCR machine combined to a fluorescent plate reader. Therefore, Scorpion technology can be easily adapted to high throughput analysis for large-scale screening programmes by using 96-well plate formats and kits standardized are likely to become available in the next future.

LNA

Locked nucleic acid (LNA) is a nucleic acid analogue displaying a very high affinity towards complementary DNA and RNA. Structural studies demonstrated that the LNA is a DNA mimic, fitting seamlessly into an A-type duplex geometry. Several reports indicated LNA as a most promising molecule for the development of oligonucleotide-based unambiguous scoring of SNP[47]. Many SNP assays using LNA technology have been designed and implemented. Because the difference of melting temperature between a perfect match and a single-nucleotide mismatch is larger for LNA-DNA heteroduplex than DNA-DNA homoduplex, the discrimination of a SNP is easier using this chemistry. LNA technique allows the sensitive detection of rare mutations in a tissue sample containing an excess of wild-type DNA. During PCR, LNA sequence selectively blocks amplification of wild-type DNA, while allowing the amplification of the mutant codon[48]. Currently, several LNA genotyping assays have been reported for the screening of i) factor V Leiden mutation, ii) apolipoprotein B (apoB) R3500Q mutation and iii) two mutations in apolipoprotein E[49-51]. In these assays, 8mer LNA-capture probes (complementary to either the wild type or the mutated genomic sequence) are covalently attached to individual wells of a microtiter plate and, after hybridization with PCR amplicons, scored colorimetrically with an ELISA-like technique[49]. The assays have been carefully validated and results were highly consistent with DNA sequencing. Immobilized LNA probes may also be successfully used in a multiplex SNP genotyping assay performed on a microarray platform[52].

PCR-Invader® Assay

The PCR-Invader® (developed by Third Wave Technologies) is a homogeneous assay. It is a robust SNP genotyping method that does not require allele-specific dye-labelled probe for each every SNP marker. The use of two generic dye-labelled probes is sufficient for all SNP markers. This detection method is based on the FRET signal generated by cleavage of a doubly labelled fluorescent probe[53]. Briefly, PCR product is incubated with two allele-specific oligonucleotides (called Invader oligonucleotide) and with the primary probe. The Invader oligonucleotide anneals to the downstream portion of the polymorphic site and while the 3' region of the primary probe is complementary to the upstream region of the polymorphic site. When the polymorphism is complementary to the primary probe (the opposing base), the probe overlaps the 3' end of the Invader oligonucleotide and forms a structural sequence containing a specific site for restriction by the Cleavase® enzyme which release the 5' arm of the primary probe[54]. The cleaved 5' arm of the Invader oligonucleotide, leads to the cleavage of the doubly labelled signal probe by the Cleavase enzyme. Since the signal probe is labelled at the 5' end with a fluorophore and internally with a quencher, the cleavage removes the 5' fluorophore and enhances fluorescence. In the mutant allele primary probe and PCR product do not match to the nucleotide being genotyped, as a result no overlapping flap structure is formed and no cleavage of primary probe occurs. Several properties of PCR-Invader technology make it suitable for high-throughput genotyping, as demonstrated by an association study of 463 SNPs on 33 candidate genes, performed to identify a genetic marker able to predict clinical response to IFN-α therapy[55,56]. This study successfully proved that SNPs in the 5'-flanking region of signal transducer and activator 3 (STAT3) had the most significant association with responsiveness. One of the major disadvantages of the current technology is the need to assay the two alleles of each SNP in separate reaction wells. This reaction format makes this assay time-consuming and labour-intensive. Furthermore, genotype miscalling can occur when one of the two reactions of the sample does not work, leading to a heterozygous individual being mistyped as homozygous.

Oligonucleotide Ligation Assay (SNPlex)

The SNPlex system (developed by Applied Biosystems) uses oligonucleotide ligation assay (OLA) and capillary electrophoresis (CE) to analyze bi-allelic SNP genotypes.

The assay workflow for the SNPlex Genotyping System involves the following seven steps, designed for easy automation, which can be completed within two days http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2291745&rendertype=figure&id=f1: 1) OLA reaction: allele-specific oligonucleotide and locus-specific oligonucleotide probes hybridize to the genomic target sequence and in presence of exactly matched sequence of SNP site; 2) purification of OLA reaction by exonucleolytic digestion of excess probes and linkers. This step is necessary to ensure the efficiency of the subsequent PCR reaction; 3) the reaction involving the simultaneous PCR allowing the amplification of purified ligation products using a single pair of PCR primers, one of which is biotinylated; 4) capturing of biotin-labelled PCR products in streptavidin coated microtiter plates. After a washing step, the non-biotinylated strands

are removed, leaving single-stranded amplicons bound to the microtiter plate; 5) fluorescently labelled universal "Zip-Chute" probes hybridize to the bound single-stranded amplicons. Since each ZipChute probe is complementary to a sequence contained in the ASO (called ZipCode sequence), a double number of ZipChute probe for each SNP in screening is required; 6) the specifically bound ZipChute probes are eluted into CE buffer; and 7) detection is obtained by CE. This approach is well suited for SNP genotyping efforts in which high-throughput and cost effectiveness are essential. The SNPlex genotyping system offers a high degree of flexibility and scalability, allowing the selection of custom-defined sets of SNPs for medium- to high-throughput genotyping projects.

Based on the same principles of the OLA, Faruqi et al[57] used a PCR free Rolling Circle Amplification (RCA) in combination with FRET detection, to genotype 10 SNPs in 192 samples. The method gave quantitative results when a real-time PCR instrument was used, and the specificity of the assay was quite high: matching allele specific probes were ligated 100,000-fold faster than mismatched probes. Genotypes called by RCA were identical to those determined by RFLP or minisequencing with a frequency of 93%. The RCA assay is commercially available as part of the SNiPer™ system (GE Healthcare-Amersham Pharmacia Biotech, UK).

Pyrosequencing

Pyrosequencing detects de novo incorporation of nucleotides. The incorporation process releases a pyrophosphate, which is converted to ATP in the presence of adenosine 5'-phosphosulfate that in turn stimulates luciferase[58]. A charge couple device (CCD) camera detects the light produced by the luciferase-catalyzed reaction. The height of each peak correlates to the light signal and is proportional to the number of nucleotides incorporated. During reaction, after single dNTP incorporation, both ATP and unincorporated dNTPs are degraded by a pyrase, the light is switched off and the cycle re-starts. The novel DNA strand, complementary to the target template, is built up and the nucleotide sequence is determined from the signal peak in the pyrogram. Current instrumentation, produced by PyroMark® (Biotage, Uppsala, S.), can detect 500 SNPs/h post-PCR in a 96-well plates format. Pyrosequencing is highly specific and automated genotype calling is also allowed for the quantitative nature of the assay. Multiplexed reactions (up to 4-plex) have been successfully designed. It has also been described a 3-primer system using a common biotinylated primer, with a further reduction of the cost of each assay[59]. One more advantage of Pyrosequencing is the ability to detect additional sequence flanking the polymorphic site (typically 6-30 bases). Therefore, this method can be applied for discovery of unknown SNPs and turns useful for analyzing those sequence containing complex secondary structures that render difficult the application of conventional sequencing approaches. The major disadvantage of this method lays in the requirement a post-PCR cleanup step for removing unincorporated nucleotides, primers, and salts. Moreover, the presence of >10 nucleotides homopolymer tracts, could complicate the analysis due to the non-linear light response after incorporation of 5-6 identical dNTPs. A further advantage of Pyrosequencing over other genotyping methods is that can it be used to characterize the entire haplotype, not just individual SNPs.

Sivertsson et al[60] used pyrosequencing technique for the simultaneous genotyping and screening for new polymorphisms of 82 samples. Two mutation hot spots containing 5 and 7 known SNPs, respectively, were analyzed in parallel by Pyrosequencing and the more labor-intensive SSCP/cycle-sequencing technique. There was a 100% concordance between the two methods, but the Pyrosequencing assay identified also four novel sequence variation.

High resolution melting (HRM)

HRM represent an extension of previous heteroduplex DNA dissociation or melting analysis. It was recently introduced as a technique to genotype known SNPs within small amplicons. This technique is a closed-tube method, applicable by heteroduplex-detection DNA dyes, which can be used at saturating concentrations without inhibiting PCR steps[61]. These third generation fluorescent dsDNA dyes, such as SYTO 9 (Invitrogen Corp., Carlsbad, CA, USA), LC Green (Idaho Technologies, Salt Lake City, UT, USA) and Eva Green (Biotium Inc, Hayward, CA, USA), devoid of inhibitory ability towards amplification reactions, can be used at higher concentrations resulting in a greater saturation of the dsDNA sample. Greater dye saturation produces a higher fidelity of measured fluorescent signals, apparently because there is less dynamic dye redistribution to non-denatured regions during melting phase and because dyes do not favour higher melting temperature products[61]. The other basic requirement of this technique is the presence of a HRM instrument that collects fluorescent signals with higher optical and thermal precision. Wild type and mutant samples are distinguished by melting temperature (Tm) shift. An altered curve shape, distinguish heterozygous samples from homozygous ones, better than Tm. The advan-

tages of this approach are that labelling of each primer (with dye) is not needed and PCR amplification and melting analysis can be performed in the same tube/capillary, minimizing specimen handling and reducing the possibility of error and sample contamination. HRM is easy, rapid and not expensive and has a relevant accuracy for mutational analysis in clinical practice, mainly for genotyping of genetic disorders and for the identification of somatic mutations in human cancers.

The most relevant screening tests with HRM in cancer studies were the detection of: i) large sequence aberrations of *FLT3* gene in AML; ii) of c-Kit mutation in GIST; iii) mutation of EGFR and HER2 gene, in lung and in head-neck cancer and c-Kit and BRAF gene activating mutations in melanoma[62-65]. HRM was also tested in pancreatic cancer for the mutational analysis of p53, K-ras, BRAF, and EGFR genes, performed on bile duct brushing specimens[66]. Finally, HRM was also proposed as a rapid and sensitive technique for the assessment of DNA methylation[67].

COMPARISON OF GENOTYPING METHODOLOGIES

Homogeneous methods for the detection of known point mutations and small deletions or insertions are summarized in Table 3.

None of the genotyping methods appears ideal for all situations, and then the technique used must be driven by project requirements. Each assay has advantages and disadvantages and reaction conditions must be standardized for each technique.

Fluorescent-based detection systems have been developed for most of the assays described, resulting in an easy application in routine laboratories, due to their high specificity, low detection limit, moderate assay stability (decreasing fluorophore activity) and medium/high-throughput. Furthermore, these systems are specific, sensitive and highly reproducible, but usually require more fluorescently labelled probes, with the increase of cost and limitation in multiplexing capabilities. When a large number of assays must be devel-

TABLE 3. COMPARISON OF METHODS FOR DETECTION OF KNOWN POINT MUTATION IS BASED ON ANALYTICAL VALIDATION.

Genotyping methods	Specificity (Mut/Wt)	Sensitivity	Assay* stability	Equipment# required
ASA	-Medium -High§ (sequencing)	1/100 1/10000	High	Gel electrophoresis system
RFLP	-Medium -High (sequencing)	1/1000 1/10000	Low (restriction enzyme)	Gel electrophoresis system
PNA-mediate PCR calmping	-High -Very High (Maldi-Tof)	1/100000 1/1000000	High	Gel electrophoresis system
FRET Allelic Discrimination **a) LC probe** **b) TaqMan 5'nuclease (End point detection)** **c) Beacons probe** **d) Scorpions probe Hyb probe**	High	1/100	Middle (probe fluoro-labeled)	Dedicate instrumentation and software
LNA probe	Very High	1/10000	Middle (probe fluoro-lebelled)	Common Fluorescent-detecting instrumentation or plate reader
Invader Assay	Very High	1/100	Middle (cleavase enzyme)	
OLA (SNPlex)	Very High	1/100	Middle (probe fluoro-ebelled)	Dedicate instrumentation and software
Pyrosequencing	Very High	1/10000	Middle (luciferase related enzyme)	Pyrosequencer dedicate software
HRM	Medium	1/100	High	Common Fluorescent-detecting

§High/very high specificity, if combined to other detection platform (i.e. sequencer or MALDI-TOF)
*Referred to reagent stability: low = restriction enzyme; Middle = dye-labeled oligonucleotide; High = basic oligonucleotide
#PCR thermal cycler and other common diffuse instrument, are not included in the estimate equipment

oped and optimization time is limited, methods based on simple gel electrophoresis detection are preferable. The flexibility of the system may also be an important factor to be evaluated, if other types of assays or applications are desired. For example, the throughput of Pyrosequencing is limited by the time required for the luminescence cascade after the addition of each nucleotide. On the other hand, this method is more flexible than other assays because it can be used in a wider variety of polymorphisms including, short insertions/deletions, triallelic SNPs and because it can be used for SNP discovery and CpG methylation analysis.

Nearly all methods require a separate PCR amplification step for the highest specificity and sensitivity. This requirement limits throughput and increases the cost per reaction. Multiplex PCR helps to increase throughput and decrease cost, but it is still not possible to develop robust and highly multiplexed reactions quickly. At present, PNA-mediated PCR Clamping analysis appears the technique best suited for the amplification of low abundance mutated alleles in great excess of non-mutated ones (very high sensitivity). In addition these technique is flexible and could be coupled with Real time-PCR and other post PCR methods like sequencing, MALDI-TOF[68,69]. AS-PCR analysis, if compared to RFLP, is technically simpler and sufficiently specific (higher specific if combined to sequencing of PCR products). Both of them do not require any specialized equipment or reagents, but suffer from a concomitant significant decrease in throughput and are laborious. No attempt has been made to assess the cost per reaction for each of the described methods, due to difference in manufacturer instrumentation model and reagents. Moreover, the costs may consistently vary between different laboratories due to the ability to produce "in house" many of the reagents needed[70].

CONCLUSIONS AND FUTURE PERSPECTIVE

All the homogeneous technologies described here, present advantages and disadvantages as summarized in Table 4. The comparisons among the different genotyping approaches are only made to highlight the differences in performance among these platforms and to draw attention to a core set of selected criteria, before developing SNP assay on a given platform. Decision criteria for the rational selection of on homogeneous platform for SNP detection, mainly depend on specific aims of the different diagnostic laboratories. If the lab is built up to genotype a large number of SNPs in a small number of sample and if it has specialized personnel with a low budget, the most suitable platforms can be those for which the required reagents can be produced "in house" (i.e. AS-PCR, RFLP and PNA). Otherwise, if genotyping testing needs additional information on mutant allele burden quantification, allele-specific detection platform are to be preferred (i.e. HybProbe, TaqMan, Beacons, and Scorpions). Finally, if the genotyping panel is narrow and genotyping samples numerous, high-throughput platforms appear more suitable (Invader, SNPlex). Pyrosequencing is the only platform available for the detection of short sequence context (i.e. nucleotide deletion or insertion). PNA and LNA chemistries are ideal to be used for detection of a rare mutant allele in a large volume of wt allele. Each technology designed to detect genetic abnormalities continues to evolve quickly. All present and future technological implementations for the detecting mutations, which yield to altered drug response, will always be amenable to analytical validation. The homogeneous PCR-based methods described in this review have been validated and are well known in the world of molecular diagnostic. The expected performance of an assay can be estimated and each test can be monitored by validated QCs procedures in the global context of external Quality control assurance programs.

The cornerstone of pharmacogenomics and pharmacogenetics on the future of health care, is the ability to identify genetic variations (SNP, short deletions, translocations and insertions) that alter an individual response to a given drug, and translating SNP test from research to clinical practice. In this scenario, the present process is a multifaceted task that needs the successful cooperation of the diagnostic, pharmaceutical, medical and public health fields.

At the present SNP testing is a small and specialized sector, in the context of global diagnostics industry, comprising less than 5% of molecular diagnostics segment[71]. Over the next few years, the emergence of molecular resistance in the new therapies as results of genomic alteration (i.e. kinase inhibitors), will drives diagnostics company to develop new test able to produce results indicative for tailoring patient's treatment. Hopefully, the future implementation of the methods for genotyping, will result in personalized treatment and eventually, in shifting the balance from disease relapse towards disease eradication[72]. Therefore, pharmaceutical and biotechnology companies should join each other, in order to develop a commercial test suitable for routine diagnostics in pharmacogenomics.

TABLE 4. ADVANTAGE AND DISADVANTAGES OF DESCRIBED PLATFORMS.

Platforms	Advantages	Disadvantages
AS-PCR	Larger diffuse methods Combining capabilities Automation feasible Low cost "in house" set up	Low specificity and sensitivity Low throughput
RFLP	Low cost Simple instrumentation Allelic discrimination	Data interpretation Low specificity and sensitivity Low throughput Intense time-labour
PNA-mediate PCR clamping-	Combining capabilities Automation feasible Low cost "in house" set up High sensitivity	No allelic discrimination Low throughput
FRET Allelic Discrimination **a) LC probe** **b) TaqMan 5'nuclease (End point detection)** **c) Beacons probe** **d) Scorpions probe**	Common instrumentation Medim/high throughput Allelic discrimination Quantification feasible	Low sensitivity Moderate multiplex capabilities Possible data mis-interpretation
LNA probe	Combining capabilities Automation feasible High sensitivity Common instrumentation	No allelic discrimination Labor intense for optimization protocol
OLA (SNPlex)	High throughput Allelic discrimination High sensitivity	Dedicate software and reagents No combining capabilities
Invader assay	High throughput Allelic discrimination (2 tube assay) High sensitivity	Dedicate instrument, software and reagents No combining capabilities
Pyrosequencing	High throughput Allelic discrimination High sensitivity Short sequence context for each sample, good for deletion and insertion	Dedicate instrument, software and reagents No combining capabilities No multiplex
HRM	Common instrumentation Medim/high throughput Allelic discrimination Low cost	Low sensitivity No multiplex capabilities Possible data mis-interpretation

Major selected criteria include: analytical validation, larger diffuse methods and instruments, allelic discrimination, platform combine capabilities, intense time-labor, methods allows the specific and sensitive detection of mutant alleles in great excess of wt alleles, throughput

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Conflict of Interests

The Authors declare that they have no conflict of interests.

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