

Brief Introduction of Single Nucleotide Polymorphism: Basic Concept and Research Methods

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1. SNPs: Impact on Gene Function and Phenotype

Single nucleotide polymorphism (SNP) is the simplest form of DNA variation among individuals. These simple changes can be of transition or transversion type and they occur throughout the genome at a frequency of about one in 1,000 bp. They may be responsible for the diversity among individuals, genome evolution, the most common familial traits such as curly hair, interindividual differences in drug response, and complex and common diseases such as diabetes, obesity, hypertension, and psychiatric disorders. SNPs may change the encoded amino acids (nonsynonymous) or can be silent (synonymous) or simply occur in the noncoding regions. They may influence promoter activity (gene expression), messenger RNA(mRNA) conformation (stability), and subcellular localization of mRNAs and/or proteins and hence may produce disease. Therefore, identification of numerous variations in genes and analysis of their effects may lead to a better understanding of their impact on gene function and health of an individual. This improved knowledge may provide a starting point for the development of new, useful SNP markers for medical testing and a safer individualized medication to treat the most common devastating disorders. This will revolutionize the medical field in the future. To illustrate the effect of SNPs on gene function and phenotype, this minireview focuses on evidences revealing the impact of SNPs on the development and progression of three human eye disorders (Norrie disease, familial exudative vitreoretinopathy, and retinopathy of prematurity) that have overlapping clinical manifestations.

2. Silent (Synonymous) SNPs: Should We Care About Them?

One of the surprising findings of the Human Genome Project was that single nucleotide polymorphisms (SNPs), which, by definition, have a minor allele frequency greater than 1%, occur at higher rates than previously suspected. When occurring in the gene coding regions, SNPs can be synonymous (i.e., not causing a change in the amino acid) or nonsynonymous (when the amino acid is altered). It has long been assumed that synonymous SNPs are inconsequential, as the primary sequence of the protein is retained. A number of studies have questioned this assumption over the last decade, showing that synonymous mutations are also under evolutionary pressure and they can be implicated in disease. More importantly, several of the mechanisms by which synonymous mutations alter the structure, function, and expression level of proteins are now being elucidated. Studies have demonstrated that synonymous polymorphisms can affect messenger RNA splicing, stability, and structure as well as protein folding. These changes can have a significant effect on the function of proteins, change cellular response to therapeutic targets, and often explain the different responses of individual patients to a certain medication.

3. SNP Databases

Researchers interested in obtaining detailed information on SNPs now work in a golden age of online database availability: never has so much data and such a wealth of information been freely accessible for such a substantial proportion of the 18 million single nucleotide polymorphism (SNP) loci currently characterized in the human genome. This chapter describes the major SNP databases available for human genetics studies. Tools and strategies are outlined that can help researchers properly formulate a database query to be able to access the most appropriate information needed for their research aims, including medical or population genetics analysis – an approach that is getting increased attention given the expanding scale of online SNP data.

4. Mining SNPs from DNA Sequence Data; Computational Approaches to SNP Discovery and Analysis

Single nucleotide polymorphisms (SNPs) are the most abundant form of genetic variation and are the basis for most molecular markers. Before these SNPs can be used for direct sequence-based SNP detection or in a derived SNP assay, they need to be identified. For those regions or species where no validated SNPs are available in the public databases, a good alternative is to mine them from DNA sequences. The alignment of multiple sequence fragments originating from different genotypes representing the same region on the genome will allow for the discovery of sequence variants. The corresponding nucleotide mismatches are likely to be SNPs or insertions/deletions. A large amount of sequence data to be mined is present in the public databases (both expressed sequence tags and genomic sequences) and is free to use without having to do large-scale sequencing oneself. However, with the appearance of the next-generation sequencing machines (Roche GS/454, Illumina GA/Solexa, SOLiD), high-throughput sequencing is becoming widely available. This will allow for the sequencing of polymorphic genotypes on specific target areas and consequent SNP identification. In this paper we discuss the bioinformatics tools required to analyze DNA sequence data for SNP mining. A general approach for the consecutive steps in the mining process is described and commonly used SNP discovery pipelines are presented.

5. Next-Generation Sequencing Methods: Impact of Sequencing - Accuracy on SNP Discovery

The advent of next-generation sequencing technologies has spurred remarkable progress in the field of genomics. Whereas traditional Sanger sequencing has yielded the first complete human genome sequence, next-generation methods have been able to resequence several human genomes. In this manner, nextgeneration approaches have powerful capabilities for understanding human variation. The throughput for these approaches is often measured in billions of base pairs per run, astounding numbers when compared with the millions of base pairs per day generated by automated capillary DNA sequencers. However, unlike traditional Sanger dideoxy sequencing, these methods have lower accuracy and shorter read lengths than the dideoxy gold standard. Are these limitations offset by the higher throughputs? An in-depth look at the

single read and composite accuracy of these methods is presented. The stringent requirements for single nucleotide polymorphism (SNP) discovery utilizing these approaches is discussed along with a review of studies that have successfully employed next-generation sequencing methods for large-scale SNP discovery. Ultimately, the application of these ultra-high-throughput sequencing methods for SNP discovery will open up new horizons for understanding human genomic variation.

6. Scanning Probe and Nanopore DNA Sequencing: Core Techniques and Possibilities

We provide an overview of the current state of research towards DNA sequencing using nanopore and scanning probe techniques. Additionally, we provide methods for the creation of two key experimental platforms for studies relating to nanopore and scanning probe DNA studies: a synthetic nanopore apparatus and an atomically flat conductive substrate with stretched DNA molecules.

7. Pyrosequencing for SNP Genotyping

Pyrosequencing is a real-time DNA sequencing method. It is based on the transformation of pyrophosphates, released during DNA elongation by DNA polymerase, into measurable light. During DNA elongation, a single pyrophosphate molecule is released following incorporation of a single nucleotide. In the pyrosequencing reaction, released pyrophosphates are then rapidly converted by sulfurylase to adenosine triphosphate, which in turn is utilized by luciferase to produce light. Within standardized conditions, this reaction is accomplished in a few milliseconds and the light produced can be registered with a CCD camera. Therefore, it becomes possible to quantitatively measure the nucleotides incorporated. This approach has been automated in different platforms and can be used for a wide variety of applications, such as single-nucleotide polymorphism (SNP) genotyping, DNA sequencing, loss of heterozygosity analysis, and CpG methylation studies. Here we describe the entire process, focusing our attention on SNP genotyping, and giving examples of some other applications.

8. Single Nucleotide Polymorphism Screening with Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is a powerful technique for identifying DNA sequencebased differences. The method relies on the fact that double-stranded DNA molecules have unique denaturation rates that are based upon the specific nucleotide composition of the DNA sequence(s). While DGGE is typically used to screen for polymorphisms that vary by multiple nucleotides, it is equally useful for screening single nucleotide polymorphisms (SNPs). For most applications, it is possible to use computer software in advance to determine if SNPs can be differentiated using DGGE. The software can also model the effect of attaching a GC-rich clamp to the PCR primer to improve detection of SNPs. Once feasibility has been confirmed, a perpendicular DGGE can be used to identify the optimal denaturing gradient for the sequences

of interest. Parallel gels can then be used to screen large numbers of samples at one time, eliminating the need for cloning and sequencing or direct sequencing of PCR products. This chapter provides step-by-step instructions on the use of DGGE and illustrates its application for detection of SNPs, as well as multiple nucleotide polymorphisms, in the major histocompatibility complex.

9. Temporal Temperature Gradient Electrophoresis for Detection of Single Nucleotide Polymorphisms

The presence of single nucleotide polymorphisms (SNPs) in nuclear DNA and mitochondrial DNA (mtDNA) can be detected using a range of electrophoretic techniques, of which temporal temperature gradient electrophoresis (TTGE) is often the most user-friendly and reproducible. The technique operates on the same principle as denaturing gradient gel electrophoresis, but does not require a chemical gradient in the gel. Instead, TTGE relies on a steady and gradual increase in temperature during electrophoresis to denature and separate DNA sequences that differ by as little as one base pair. TTGE can be easily accomplished using DNA of high quality and it is a rapid-throughput method for SNP screening once conditions have been optimized. Detection of SNPs is, for example, important for the diagnosis of mitochondrial disorders such as heteroplasmy, the presence of more than one type of mitochondria within a cell or tissue. Here we describe the basic steps for TTGE and illustrate its utility for the detection of heteroplasmy in mtDNA control region sequences.

10. Zn(II)–Cyclen Polyacrylamide Gel Electrophoresis for SNP Detection

We introduce a method for the detection of single-nucleotide polymorphisms (SNPs) by polyacrylamide gel electrophoresis (PAGE) with an additive Zn²⁺–cyclen complex (cyclen is ,4,7,10-tetraazacyclododecane), called “Zn²⁺–cyclen–PAGE.” The method is based on the difference in mobility of mutant DNA (of the same length) in PAGE, which is due to Zn²⁺–cyclen binding to thymine bases accompanying a total charge decrease and a local conformation change of target DNA. In combination with a polymerase chain reaction based heteroduplexing technique, the method is more accurate than many other conventional PAGE-based methods, as shown by clear gel-shifting bands because of the separation of heteroduplex and homoduplex DNAs. We demonstrate SNP mapping and heterozygosity screening in a human cardiac sodium channel gene (i.e., SCN5A) that relates to inherited arrhythmia syndromes using Zn²⁺–cyclen–PAGE.

11. Phosphate-Affinity Polyacrylamide Gel Electrophoresis for SNP Genotyping

We introduce a genotyping method which relies on the use of a 1:1 mixture of 5'-phosphate-labeled and nonlabeled allele-specific primers for polymerase chain reaction (PCR). The method is based on the difference in mobility of the phosphorylated and nonphosphorylated PCR products (possessing the same number of base pairs) during phosphate-affinity polyacrylamide gel electrophoresis (PAGE). The

phosphate-affinity site in the gel is represented by an immobilized phosphate-binding tag molecule [i.e., a polyacrylamide-bound dizinc(II) complex], which selectively captures the 5'-phosphate-labeled allelespecific product compared with the corresponding nonlabeled one. The DNA migration bands obtained can be visualized by ethidium bromide staining. We demonstrate the genotyping of a single-nucleotide polymorphism reported in a human cardiac sodium channel gene, SCN5A, using the phosphate-affinity PAGE.

12. Estimation of SNP Allele Frequencies by SSCP Analysis of Pooled DNA

The single strand conformation polymorphism (SSCP) method is a sensitive technique used to detect subtle sequence differences in PCR-amplified DNA fragments as separated peaks in electrophoretic analysis. In this chapter, we focus on SSCP analysis for quantifying polymorphic alleles rather than scanning for mutations. Short fragments carrying single nucleotide polymorphisms are amplified from individual and pooled DNA samples, then the products are labeled with fluorescent dyes and analyzed by automated capillary electrophoresis under nondenaturing conditions. Dedicated software, QSNPlite, interprets trace data of the electrophoresis to identify alleles of individuals and quantify these alleles in the pool. The software can also incorporate sequencing data to assign alleles at the nucleotide level. The procedures described here are being used in association studies that compare allele frequencies between cases and controls to identify genes responsible for common diseases.

13. Phenylethynylpyrene Excimer Forming Hybridization Probes for Fluorescence SNP Detection

Excimer formation is a unique feature of some fluorescent dyes (e.g., pyrene) which can be used for probing the proximity of biomolecules. Pyrene excimer fluorescence has previously been used for homogeneous detection of single nucleotide polymorphism (SNP) on DNA. 1-Phenylethynylpyrene (1-1-PEPy), a photostable pyrene derivative with redshifted fluorescence, is able to form excimers (emission maximum about 500–510 nm) and is well suitable for nucleic acid labeling. We have shown the utility of 1-1-PEPy in the excimer-forming DNA probes for detection of 2144A/G and 2143A/G transitions, and 2143A/C substitution in the 23S ribosomal RNA gene of *Helicobacter pylori* strains resistant to clarithromycin. The phenylethynylpyrene pair can be generated either from 1-1-PEPy pseudonucleoside 4-[4-(pyren-1-ylethynyl)phenyl]-1,3-butanediol or from 2'-O-(1-PEPy) modified nucleosides – 2'-O-[3-(pyren-1-ylethynyl)benzyl]uridine and 2'-O-[4-(pyren-1-ylethynyl)benzyl]uridine.

14. The Chemical Cleavage of Mismatch for the Detection of Mutations in Long DNA Fragments

Methods to rapidly scan large regions of DNA that are not dependent on highly specific melting

temperatures or suitable only for large-scale discovery are important to reduce the amount of sequencing

required for DNA samples that appear to contain a mutation. This protocol describes the chemical cleavage of mismatch method to assess if a region of DNA contains a mutation and accurately localize the position of the mutation in the same reaction. To detect mutations, PCR heteroduplexes are incubated with two mismatch-specific reagents. Hydroxylamine modifies mismatched cytosine residues and potassium permanganate modifies mismatched thymine residues. The samples are then incubated with piperidine, which cleaves the DNA backbone at the site of the modified mismatched base. Cleavage products are separated by electrophoresis, revealing the identity and location of the mutation. The chemical cleavage of mismatch method can efficiently detect point mutations as well as insertions and deletions.

15. Mismatch Oxidation Assay: Detection of DNA Mutations Using a Standard UV/Vis Microplate Reader.

Simple, low-cost mutation detection assays that are suitable for low-throughput analysis are essential for diagnostic applications where the causative mutation may be different in every family. The mismatch oxidation assay is a simple optical absorbance assay to detect nucleotide substitutions, insertions, and deletions in heteroduplex DNA. The method relies on detecting the oxidative modification products of mismatched thymine and cytosine bases by potassium permanganate as it is reduced to manganese dioxide. This approach, unlike other methods commonly used to detect sequence variants, does not require costly labeled probes or primers, toxic chemicals, or a time-consuming electrophoretic separation step. The oxidation rate, and hence the presence of a sequence variant, is detected by measuring the formation of the potassium permanganate reduction product (hypomanganate diester), which absorbs at the 420-nm visible wavelength, using a standard UV/vis microplate reader.

16. High-Throughput Methods for SNP Genotyping

Single nucleotide polymorphisms (SNPs) are ideal markers for identifying genes associated with complex diseases for two main reasons. Firstly, SNPs are densely located on the human genome at about one SNP per approximately 500–1,000 base pairs. Secondly, a large number of commercial platforms are available for semiautomated or fully automated SNP genotyping. These SNP genotyping platforms serve different purposes since they differ in SNP selection, reaction chemistry, signal detection, throughput, cost, and assay flexibility. This chapter aims to give an overview of some of these platforms by explaining the technologies behind each platform and identifying the best application scenarios for each platform through cross-comparison. The readers may delve into more technical details in the following chapters.

17. High-Throughput SNP Genotyping: Combining Tag SNPs and Molecular

Beacons

In the last decade, molecular beacons have emerged to become a widely used tool in the multiplex typing of single nucleotide polymorphisms (SNPs). Improvements in detection technologies in instrumentation and chemistries to label these probes have made it possible to use up to six spectrally distinguishable probes per reaction well. With the remarkable advances made in the characterization of human genome diversity, it has been possible to describe empirical patterns of SNPs and haplotype variation in the genome of diverse human populations. These patterns have revealed that the human genome is structured in blocks of strong linkage disequilibrium (LD). Because SNPs tend to be in LD with each other, common haplotypes share common SNPs and thus the majority of the diversity in a region can be characterized by typing a very small number of SNPs; so-called tag SNPs. Herein lies the advantage of the multiplexing ability of molecular beacons, since it becomes possible to use as few as 30 probes to interrogate several haplotypes in a highthroughput approach. Thus, through the combined use of tag SNPs and molecular beacons it becomes possible to type individuals for clinically relevant haplotypes in a high-throughput manner at a cost that is orders of magnitude less than that for high throughput sequencing methods.

18. SNP Genotyping by the 5'-Nuclease Reaction: Advances in High-Throughput Genotyping with Nonmodel Organisms

Population genetics studies play an increasingly important role in the management and conservation of nonmodel organisms. Unlike studies with model organisms, a typical population genetics study of a nonmodel organism may be conducted by analyzing thousands or hundreds of thousands of individuals for several dozen single nucleotide polymorphisms (SNPs). The use of robust, roboticallymediated TaqMan reactions provides substantial advantages in these types of studies. We describe the methods and laboratory setup for analyzing a sustained high throughput of SNP assays in routine university or natural resource agency laboratories with a handful of thermal cyclers. Agencies sustain rates of nearly 150,000 assays per week using uniplex reactions with the Applied Biosystems 7900HT Fast Real-Time PCR System (AB 7900HT). We further describe the medium-density array run on the BioMark from Fluidigm, which increases this rate to over 500,000 assays per week by multiplexing 96 samples for 96 SNPs.

19. The TaqMan Method for SNP Genotyping

Single nucleotide polymorphisms (SNPs) are common DNA sequence variations that occur at single bases within the genome. SNPs have been instrumental in elucidating the genetic basis of common, complex diseases using genome-wide association studies, candidate gene case-control association studies, and genome-wide linkage analyses. A key to these studies is genotyping of SNPs. Various methods for SNP genotyping have been developed. For a particular genotyping project, the choice of method is dependent on the number of SNPs (n) and the number of DNA samples (m) to be genotyped. For a genome-wide or

large-scale project with very high n and small m, the Affymetrix SNP GeneChip and Illumina GoldenGate BeadChips assays are the ideal methods. For a project involving a small number of SNPs (small n) and a large population (high m), the TaqMan assay is the preferred technology as it has high throughput and is highly accurate, precise, time-efficient, and cost-effective. Here, we describe the detailed procedures for TaqMan SNP genotyping assay, including preparation of high-quality DNA samples, the operating protocol, clarification of technical issues, and discussion of several cautionary notes.

20. Qualitative and Quantitative Genotyping Using Single Base Primer Extension Coupled with Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MassARRAY)

Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) has developed over the past decade into a versatile tool for the analysis of nucleic acids and especially as a reliable genotyping platform. This chapter summarizes its use in the context of the most widely used MALDI-TOF MS genomics platform, the Sequenom_MassARRAY_ system. MassARRAY_ genotyping is based upon region-specific PCR followed by allele-specific single base primer extension reactions which are then desalted, dispensed onto a silica array preloaded with matrix, and the genotyping products are resolved on the basis of mass using MALDI-TOF MS. The platform is versatile in that it can resolve multiplexed reactions (40t separate loci per reaction), acquires and interprets data quickly, gives a quantitative output which reflects the amount of product generated for each allele within an assay for multiplexed reactions, and is highly sensitive. These characteristics coupled with integrated software for sequence annotation, assay design, data interpretation, and data storage have lead to its wide adoption and use for multiple nucleic acid analysis applications in both the realm of genomics research and molecular diagnostics.

21. SNP Detection Using Trityl Mass Tags

A new method suitable for single nucleotide polymorphism (SNP) detection using differential oligonucleotide probe extension has been developed. Sulfur-linked laser-cleavable trityl labels are implemented in this protocol. The method is based on mass spectrometry and utilizes a single surface for affinity purification of extended probes and matrix-independent desorption–ionization of the cleavable labels. The usefulness of this method for SNP genotyping is demonstrated.

22. Putting the Invader_ Assay to Work: Laboratory Application and Data Management

Choosing a single nucleotide polymorphism genotyping method that suits specific research needs is not much less of a challenge than determining the genetic components underlying the disease and/or trait

being investigated. This is especially true with a long list of tempting methodologies available, as summarized in this book. Here, from an end-user point of view, we discuss how a commercially available genotyping platform, the Invader_® assay, can be utilized to meet the needs and demands of human genomic research in a laboratory.

23. SNP Genotyping Using Multiplex Single Base Primer Extension Assays

Single nucleotide polymorphisms (SNPs) are the most common form of polymorphisms present in the human genome. The single base primer extension (SBE) method is an effective and sensitive tool that can type over 30 known loci scattered throughout an organism's genome in a single reaction. It allows the typing of tetra-allelic SNPs and has been adapted to a broad range of analytical necessities: single-cell analysis, molecular diagnosis of monogenic diseases, forensic mitochondrial DNA analysis on highly degraded human remains, and high-throughput SNP screening for population studies. Every SBE-based assay will need customized optimization efforts that are generally proportional to the number of desired SNPs typed in a single reaction. This chapter offers a detailed outline on which to base the design and optimization of any multiplex SBE assay that can then be tailored to the analytical conditions that characterize each specific application.

24. High-Throughput SNP Detection Based on PCR Amplification on Magnetic Nanoparticles Using Dual-Color Hybridization

A microarray-based method for detecting single nucleotide polymorphisms (SNPs) using solid-phase polymerase chain reaction (PCR) on magnetic nanoparticles (MNPs) was developed. In this method one primer with a biotin label is captured by streptavidin-coated MNPs (SA-MNPs), and the PCR products are directly amplified on the surface of SA-MNPs in a 96-well plate. The samples are further probed by hybridization with a pair of dual-color probes to determine SNP. The genotype of each sample can be simultaneously identified by scanning the microarray printed with the denatured fluorescent probes. All the reactions can be performed in the same reaction volume without the necessity of purification of intermediates. This approach represents a novel, simple, and labor-saving method for SNP genotyping and can be applied in automation system(s) to achieve high-throughput SNP detection.

25. Restriction Enzyme Analysis of PCR Products

Progress in single nucleotide polymorphism (SNP) detection technologies has provided information for SNP-based studies, such as identification of candidate genes for the complex genetic diseases, pharmacogenetic analysis, drug development, population genetics, evolutionary studies, and forensic investigations. SNP detection is performed by many methods, including hybridization, allele-specific

polymerase chain reaction (PCR), primer extension, oligonucleotide ligation, direct DNA sequencing, and endonuclease cleavage. Each of these methods has its specific advantages and disadvantages. Here we introduce the PCR–restriction fragment length polymorphism (RFLP) method, which has certain advantages over many other techniques used for analysis of SNPs. The PCR-RFLP method allows very rapid, simple, and inexpensive detection of point mutations within the sequences of PCR products. The mutation is discriminated by the specific restriction endonuclease and is identified by gel electrophoresis followed by staining with ethidium bromide. This convenient and simple method is useful in a small basic research study.

26. Allele-Specific PCR in SNP Genotyping

The increasing need for large-scale genotyping applications of single nucleotide polymorphisms (SNPs) in model and nonmodel organisms requires the development of low-cost technologies accessible to minimally equipped laboratories. The method presented here allows efficient discrimination of SNPs by allele-specific PCR in a single reaction with standard PCR conditions. A common reverse primer and two forward allele-specific primers with different tails amplify two allele-specific PCR products of different lengths, which are further separated by agarose gel electrophoresis. PCR specificity is improved by the introduction of a destabilizing mismatch within the 3' end of the allele-specific primers. This is a simple and inexpensive method for SNP detection that does not require PCR optimization.

27. Modified Multiple Primer Extension Method

A multiple primer extension (MPEX) was originally developed for the hybridization, extension, and amplification of a DNA template on a planar substrate by Kinoshita et al. in 2006. Herein we present a modified MPEX method refined by our group for single nucleotide polymorphism (SNP) detection. In this method, hybridization and extension reactions are performed on a plastic S-BIO₁ PrimeSurface₁ substrate, with a biocompatible polymer. Its surface chemistry offers extraordinarily stable thermal properties, as well as chemical properties advantageous for enzymatic reactions on the surface. To visualize allele-specific PCR products on the surface, biotin–dUTP was incorporated into newly synthesized complementary strands during the extension reaction. The products were ultimately detected by carrying out a colorimetric reaction with a substrate solution containing 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. We have further successfully combined this method with multiplex PCR. We demonstrate the advantages of this combined method by analyzing representative SNPs on different linkage disequilibrium blocks of the *m* opioid receptor gene (*OPRM1*), which is a marker gene for pain threshold.

28. Detection of SNP by the Isothermal Smart Amplification Method

The complexity of molecular diagnostic assays is a significant barrier to employment at the point of care, despite the growing need for such technologies. We have developed a sensitive, accurate, rapid, and simple DNA amplification scheme that shows potential for translational medicine from pharmacogenomics-based drug discovery through to point-of-care diagnostics. Called the “Smart Amplification process 2” (SmartAmp 2), the method is isothermal, and employs a unique primer design and background suppression technology that can amplify target sequences from crude cell lysates. The specificity of the SmartAmp 2 assay enables detection of single-nucleotide differences such as somatic mutations in tumors and single nucleotide polymorphism (SNP) variants. Because mismatch amplification can be completely suppressed in SmartAmp 2, a reliable diagnostic result can be achieved based exclusively on amplification alone. Furthermore, mutation detection and SNP genotypes can be obtained in as little as 30–40 min from sample preparation of raw blood or tissue specimens.