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REVIEW ARTICLE

From Gels to Chips: "Minisequencing" Primer Extension for Analysis of Point Mutations and Single Nucleotide Polymorphisms

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In the minisequencing primer extension reaction, a DNA polymerase is used specifically to extend a primer that anneals immediately adjacent to the nucleotide position to be analyzed with a single labeled nucleoside triphospate complementary to the nucleotide at the variant site. The reaction allows highly specific detection of point mutations and single nucleotide polymorphisms (SNPs). Because all SNPs can be analyzed with high specificty at the same reaction conditions, minisequencing is a promising reaction principle for multiplex high-throughput genotyping assays. It is also a useful tool for accurate quantitative PCR-based analysis. This review discusses the different approaches, ranging from traditional gel-based formats to multiplex detection on microarrays that have been developed and applied to minisequencing assays. Hum Mutat 13:1–10, 1999.

KEY WORDS: **minisequencing; single nucleotide primer extension; mutation detection; single nucleotide polymorphism; DNA arrays**

INTRODUCTION

Single base substitutions have been estimated to occur on the average at one out of a thousand nucleotides in the human genome (Cooper et al., 1985; Wang et al., 1998). The Human Genome Project will provide a complete sequence of the human genome by the year 2005 (Rowen et al., 1998). This reference sequence representing a few individuals will make it possible to design assays to elucidate in more detail the genomic sequence variation between a larger number of individuals. Analysis of the genetic variation caused by single nucleotide polymorphisms (SNPs) will be an important tool in the search for genes underlying multifactorial diseases by association studies or genome wide linkage disequilibrium mapping (Schafer and Hawkins, 1998). Most of the known human genetic diseases are caused by point mutations and, as our understanding of the genetic variants that predispose to common, multifactorial diseases increases, a growing numbers of SNPs will be routinely analyzed to diagnose genetic disorders. To assess the influence of sequence variation on the activity of enzymes involved in the metabolic pathways of the cell will be of central importance for the design of new therapeutic drugs (Nebert, 1997). The study of human genetic diversity conferred by the slowly evolving SNPs will provide new insight into the history of human populations. Consequently, improved technology for screening SNPs on a large scale will be required in the near future both in basic research and for routine diagnostics.

Most of the currently used methods for detecting SNPs or point mutations are based on amplification of the target DNA by the polymerase chain reaction (PCR) technique or another amplification method, which allows sensitive and specific analysis of the target DNA sequence. The sequence variants are distinguished in the amplified fragments by hybridization with allele-specific oligonucleotide (ASO) probes or with the aid of nucleic acid modifying enzymes, such as restriction enzymes, DNA ligases or DNA polymerases. Primer extension reactions catalyzed by a DNA polymerase are used either for allele-specific am-

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plification of the target DNA or for allele discrimination in previously amplified targets by single nucleotide primer extension reactions. Hybridization with allele-specific probes is the most widely applied method for detecting SNPs, but the principle of single nucleotide primer extension is used in a rapidly growing number of applications because of its high specificity in distinguishing between sequence variants.

This review discusses the different strategies and assay formats that have been developed to use single nucleotide primer extension reactions for analyzing point mutations and SNPs. Obviously, each assay format can, in principle, be used for detecting any nucleotide variation or mutation. Therefore the emphasis of the article will be on technology, and the reader is referred to the cited references for details on the specific applications. The single nucleotide primer extension assays are known under different names and acronyms, listed in Table 1. In the following "minisequencing" will often be used as a general term for the different variants of the assays.

THE REACTION PRINCIPLE

In the "minisequencing" primer extension assays, the DNA synthesis reaction catalyzed by the DNA polymerases is used to distinguish between sequence variants. The concept of the assays is to anneal a detection primer to the nucleic acid sequence immediately 3′ of the nucleotide position to be analyzed and to extend this primer with a single labeled nucleoside triphosphate that is complementary to the nucleotide to be detected using a DNA polymerase (Syvänen et al., 1990; Sokolov, 1990; Kuppuswami et al., 1991) (Fig. 1).

TABLE 1. Names and Acronyms for Minisequencing Single Nucleotide Primer Extension Assays

Name/Acronym	Reference
Primer-guided nucleotide incorporation	Syvänen et al. (1990)
Primer extension technique	Sokolov (1990)
Single nucleotide primer extension, SNuPE	Kuppuswami et al. (1991)
Solid-phase minisequencing	Syvänen et al. (1992a)
Allele-specific primer-extension capture, AS-PE capture	Ugozzoli et al. (1992)
Genetic bit analysis, GBA	Nikiforov et al. (1994)
Arrayed primer extension, APEX	Shumaker et al. (1996)
First nucleotide change, FNC	Pecheniuk et al. (1997)
Template-directed dye-terminator incorporation, TDI	Chen and Kwok (1997)
Primer oligo base extension, PROBE	Braun et al. (1997)
The PinPoint assay	Haff and Smirnov (1997)

A major advantage of the minisequencing reaction principle over hybridization with ASO probes is that the distinction between the sequence variants is based on the high accuracy of the nucleotide incorporation reaction catalyzed by a DNA polymerase, instead of on differences in thermal stability between mismatched and perfectly matched hybrids formed with the ASO probes. Consequently, the minisequencing assays allow excellent discrimination between the homozygous and heterozygous genotypes and the assays are robust and insensitive to small variations in the reaction conditions. Moreover, the same reaction conditions can be employed for detecting any variable nucleotide irrespectively of the nucleotide sequence flanking the variable site. These features are of central importance when designing multiplex assays for the simultaneous detection of many SNPs per sample. Because of the high specificity of the primer extension reaction, it is a useful tool for accurate quantitative PCR-based analysis and for detection of sequences present as a minority of a sample.

DNA polymerases without proofreading activity are preferably used in minisequencing assays to avoid 3′-5′ degradation of the primer (Syvänen et al., 1990; Haff and Smirnov, 1997). In principle, any labeled nucleotide analogue that is incorporated sequence specifically by a DNA polymerase can serve as detectable group in a minisequencing assay. The use of labeled ddNTPs is preferable over dNTPs because they will terminate the reaction, and consequently more than one labeled ddNTP can be included per reaction (Nikiforov et al., 1994; Tully et al., 1996) The ddNTPs are accepted as substrates by the Klenow DNA polymerase and the T7 or "Sequenase" DNA polymerase, but the efficiency and specificity of minisequencing reactions with ddNTPs are significantly enhanced by the use of one of the thermostable DNA polymerase that have been engineered for cyclic Sanger sequencing such as the "ThermoSequenase" enzyme (Tabor and Richardson, 1995; Pastinen et al., 1997; Haff and Smirnov, 1997).

Specific detection of SNPs in the complex human genome comprising 3×10^9 base pairs (bp) requires the enrichment of the DNA regions spanning the SNPs by the PCR or another amplification method before analysis. This is a requirement in most of the currently used methods to analyze genomic SNPs or point mutations. Because the principles of PCR and minisequencing are similar, both reaction mixtures contain primers, nucleoside triphosphates, and a DNA polymerase. To obtain

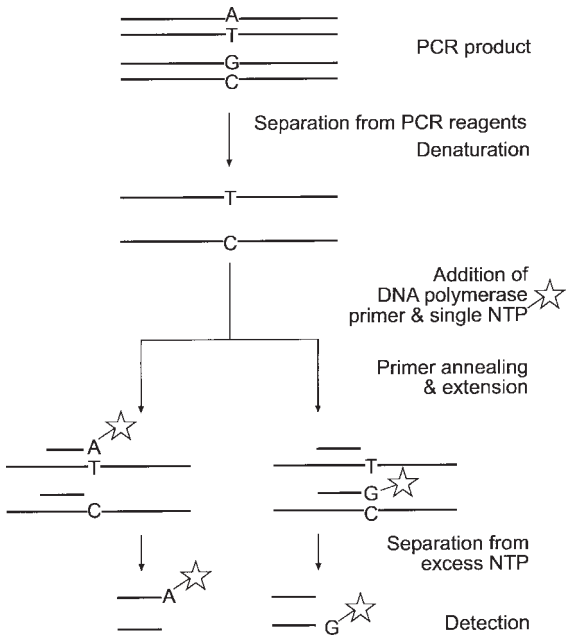

FIGURE 1. **Principle and steps of the minisequencing single nucleotide primer extension assays exemplified by analysis of a G-to-A transition. In the case of a homozygous genotype, a signal is obtained in only one of the reactions and for a heterozygote genotype, signals are obtained in both reactions. The ratio between the labeled nucleoside triphosphates incorporated in the two reactions reflects the initial ratio between two sequences in a sample when they are present in other than homozygote (2:0) or heterozygote (1:1) ratios. This is the basis for quantitative analysis by minisequencing. NTP with a *star* denotes labeled dNTPs or ddNTPs.**

specific extension of the minisequencing detection primer with only a single nucleotide the excess of PCR reagents have to be removed before the reaction. A second separation step is usually required after the minisequencing reaction to separate the labeled primer from the unincorporated labeled nucleotide analogue before measurement. In practice, the choice of label and separation method determines the format of a minisequencing assay.

ASSAY FORMATS

Gel-Based Methods

A traditional approach used in molecular biology laboratories is to separate the PCR products from the PCR primers and dNTPs by gel electrophoresis before the minisequencing reaction. After isolation of the PCR product from the gel, the minisequencing reaction is performed in solution, followed by a second gel electrophoretic step to separate the labeled minisequencing primer from free labeled dNTP or ddNTP (Kuppuswami et al., 1991; Krook et al., 1992; Singer-Sam et al., 1992). Nucleoside triphosphates labeled with ^{32}P and ^{33}P are available both as dNTP and as ddNTP analogues. In the gel-based assay format, radioisotopes ^{32}P or ^{33}P are suitable as detectable groups. The minisequencing primers that have become extended with ^{32}P- or ^{33}P-labeled nucleotides are visualized in the gel by autoradiography or using a phosphorimager instrument, of which the latter gives the advantage of a quantitative numeric result (Greenwood and Burke, 1996). A disadvantage of the gel-based assays is that they are tedious, involving multiple processing steps such as the excision of bands from gels, centrifugation and ethanol precipitation. An advantage of the size separation step is that minisequencing primers of varying length can be used to analyze more than one SNP per minisequencing reaction (Krook et al., 1992). Later this concept has been combined with solid-phase formats and used in multiplex, fluorescent minisequencing assays based on detection of primers of different length using DNA sequencing instruments (Pastinen et al., 1996; Tully et al., 1996; Shumaker et al., 1996; see below).

Solid-Phase Assays

For use on a large scale, solid-phase assay formats in which the immobilization of one of the reactants on a solid support, followed by washing of the support serves as separation method, have a significant advantage over methods that involve gel electrophoretic size separation in that the procedure involves simple manipulations that can be fully automated (Syvänen et al., 1990, 1992a; Nikiforov et al., 1994). In most solid-phase minisequencing assays, the biotin–avidin interaction is used for immobilization. Usually a biotin residue is introduced into the PCR product via one of the primers, and the PCR products are captured in microtiter plate wells (Syvänen et al., 1992a; Jalanko et al., 1992; Livak and Hainer, 1993), on magnetic microparticles (Syvänen et al., 1992b; Nyren et al., 1993), or on manifold supports (Pastinen et al., 1996) coated with avidin or streptavidin. Before the minisequencing reaction, PCR products immobilized on a solid support are conveniently denatured by treatment with alkaline or heat, after which the second strand of the product is removed by a simple washing step. Solid-phase minisequencing in a microtiter plate format with dNTPs labeled with ^{3}H, which has the advantage of weak β-emission and long half-life and can be quantitatively measured by liquid scintillation counting is applied in routine diagnostics and for genotyping of a variety of SNPs (Syvänen, 1994; Hietala et al., 1996; Pajukanta et al., 1998).

As an alternative to the PCR products, the de-

tection primers may serve as the immobilized reactant in minisequencing assays. Primers have been immobilized on a hybridization membrane through a capturing hybridization reaction (Ugozzoli et al., 1992), in microtiter plates taking advantage of the biotin–avidin interaction (Pecheniuk et al., 1994) or covalently via 5′-disulfide groups (Nikiforov et al., 1994). In the recently developed array-based minisequencing assays, multiple primers are immobilized covalently through their 5′-ends on a small glass support (Shumaker et al., 1996; Pastinen et al., 1997,1998; Head et al., 1997; see below). In methods based on immobilized primers, the PCR product cannot be denatured on the solid support. Instead, single-stranded templates for the minisequencing reaction can be prepared by degrading one strand of the PCR product by digestion with an exonuclease enzyme, which requires that the other strand of the PCR product be protected against degradation by a 5′-phosphothioate group introduced via the PCR primer (Nikiforov et al., 1994). Alternatively, single-stranded templates are prepared by affinity capture of biotinylated PCR product on an avidin-coated support, followed by denaturation and collection of the unbiotinylated strand for use in the minisequencing assays (Shumaker et al., 1996; Pastinen et al., 1997). Denatured double-stranded PCR products may serve as templates in minsequencing reactions without removing the second strand of the product (Kuppuswami et al., 1991; Pecheniuk et al., 1997), presumably with some reduction in reaction efficiency due to competition between the strand reannealing and primer annealing reactions. The necessity to prepare single-stranded templates may also be circumvented by performing cyclic minisequencing reactions (Chen et al., 1997).

Homogeneous Detection

With detection based on the scintillation proximity assay principle using scintillating microtiter plates as the solid support, minisequencing primers extended with ^{3}H-labeled dNTPs can be measured without separation from free ^{3}H-dNTPs. This approach has been applied to minimize the steps of the minisequencing procedure when large sample materials are analyzed (Ihalainen et al., 1994; Hietala et al., 1996). Another semi-homogeneous minisequencing assay is based on measurement of pyrophosphates released from incorporated dNTPs by a homogeneous enzymatic luminometric method (Nyren et al., 1993). This "pyro-sequencing" principle (Ronaghi et al., 1996) has the potential to be further developed into a completely homogeneous assay if the excess of PCR primers and dNTPs present as a mixture with the PCR product can be degraded in solution prior to the minisequencing reaction. Recently, a fully homogeneous minisequencing assay has been designed (Chen et al., 1997). In this format the PCR primers and dNTPs are inactivated enzymatically with alkaline phosphatase and *Escherichia coli* exonuclease I before the minisequencing reaction, and the separation step after the reaction is avoided by using fluorescence resonance energy transfer (FRET) as detection principle (Chen and Kwok, 1997). FRET is observed when two fluorescent dyes are in close proximity to each other and one fluorophore's emission spectrum overlaps the others excitation spectrum. In the homogeneous minsequencing assay, FRET between a 5′-FITC-labeled primer and the incorporated ddNTPs modified with the dyes ROX and TAMRA is measured during a cyclic minisequencing reaction in the "TaqMan" sequence detection instrument.

ELISA Formats

Haptens attached to dNTPs or ddNTPs are often used as detectable groups in minisequencing reactions. The incorporated haptens are detected indirectly with the aid of antibodies conjugated to alkaline phosphatase or peroxidase using colorimetric or chemiluminescent substrates. The concept of using dNTPs modified with haptens as label in the solid-phase minisequencing assay was presented in two early studies (Syvänen et al., 1990; Harju et al., 1993). This approach was not generally applicable, however, until all four nucleotides became available as fluorescein (FITC)-modified analogues that are can be detected with anti-FITC antibodies conjugated to alkaline phosphatase and a chromogenic substrate (Livak et al., 1994; Pecheniuk et al., 1997; Sitbon et al., 1997; Tuuminen et al., 1997). The "genetic bit analysis" uses double labeling with FITC and biotin in combination and sequential detection of the incorporated ddNTPs with anti-fluorescein and anti-biotin antibodies conjugated to alkaline phosphatase and horseradish peroxidase, respectively (Nikiforov et al., 1994). The use of biotin as detectable group is feasible in this assay, because the detection step primer is immobilized covalently in the microtiter plate well. When microtiter plate wells are used as solid support, the format of the minisequencing assay with haptens as detectable groups is identical to that of enzyme-linked immunosorbent assays (ELISA) that

are widely used in clinical routine laboratories. Unfortunately, the detection sensitivity of standard fluorescence readers for microtiter plates is not sufficient for reliable direct detection of the incorporated FITC-labeled ddNTPs.

Multiplex Size-Based Detection

DNA sequencing instruments allow sensitive detection of minisequencing primers extended with fluorescent ddNTPs. Since the primers are separated by size during the electrophoresis, multiple SNPs can be analyzed simultaneously in a single reaction using primers of different size (Pastinen et al., 1996; Tully et al., 1996; Shumaker et al., 1996). The size of the primers defines the position of the polymorphism, and the fluorescent ddNTP by which the primer becomes extended gives the identity of the nucleotide at each site. Solid-phase purification of the PCR products in combination with this gel-based detection strategy has been employed both using the ALF instrument (Pharmacia Biotech) that is based on detecting a single fluorphore (Pastinen et al., 1996) and using the ABI four-fluorophore instruments (Perkin-Elmer/Applied Biosystems) (Tully et al., 1996; Shumaker et al., 1996). These multiplex assays were set up to analyze 8–12 SNPs per sample, but the resolution of the gel electrophoresis would permit a larger number of primers for more SNPs to be included in the analysis. In an analogous method separation by capillary electrophoresis and detection by laser-induced fluorescence (CE-LIF) is used for analyzing the extended minisequencing primers (Piggee et al., 1997).

A related approach, in which the products of primer extension reactions are separated by their differences in mass using mass spectrometry (MALDI-TOF) has been suggested for multiplex detection of point mutations or SNPs (Braun et al., 1997; Haff and Smirnov, 1997). A potential advantage of minisequencing assays with mass spectrometric detection is that the four ddNTPs without label can be included in one reaction to serve as detectable groups. It remains to be seen whether the assays based on MALDI-TOF will reach a sensitivity of detection required for typing SNPs in practice.

Multiplex Analysis on Primer Arrays

A promising approach toward highly multiplex analysis of genomic sequence variation is to use arrays of oligonucleotides ("DNA chips") as solid support in miniaturized assays. Sophisticated technology has been developed for the production of high density arrays of oligonucleotides (Pease et al., 1994). Hybridization with ASO probes has so far been the most frequently applied reaction principle for detecting SNPs and point mutations in a DNA chip format (Hacia et al., 1996; Wang et al., 1998). There are, however, problems related to these assays that originate from the limited specificity of differential hybridization with ASO probes. These problems have led to the design of complex chips for the assays, in which tens of probes with all possible combinations of the sequence flanking each SNP to be analyzed are used (Hacia et al., 1996; Chee et al., 1996). To facilitate discrimination between homozygous and heterozygous genotypes, a reference sequence must be added to each sample, which leads to complex algorithms for interpretation of the results (Hacia et al., 1996).

Minisequencing in a chip format is a more straightforward approach than ASO hybridization, as only a single primer is required per SNP to be analyzed. Experimental comparison between these two reaction principles in the same array based format revealed that the power of discriminating between homozygous and heterozygous genotypes is at least one order of magnitude better with minisequencing than using hybridization with ASO probes (Pastinen et al., 1997) (Table 2). Multiplex minisequencing tests in primer array formats have been used with ^{32}P- or ^{33}P-labeled ddNTPs and detection in a phosphorimager instrument (Shumaker et al., 1996; Pastinen et al., 1997) or using hapten-labeled ddNTPs and a fluorogenic precipitating substrate (ELF) visualized by UV illumination (Head et al., 1997). Oligonucleotide arrays can be produced by chemical coupling of 5′-modified primers as miniaturized spots on an activated glass surface using high-capacity printing robotics (Lamture et al., 1994; Schena et al., 1996). Using this technology, minisequencing primer arrays with spots of 100 μM in diameter at a spacing of 500 μM on glass slides have been manufactured using a custom built printing robot (Pastinen, 1998b). The reproducibility the arrays was demonstrated by successful genotyping of two case-control materials comprising about 300 samples each (Pastinen et al., 1998a,b).

APPLICATIONS

Analysis of Sequence Variation

Minisequencing methods have been applied in a large number of studies to analyze point mutations for diagnosis of both inherited and somatic genetic disorders as well as for screening for carriers of recessive diseases. The methods have also

TABLE 2. Comparison of Allele Specific Oligonucleotide Hybridization and Minisequencing in an Array Format[a]

	Ratio between signals from normal and mutant alleles[c]					
	Homozygous positions PPT (T/T) FV (G/G)		Heterozygous positions[c] PPT (T/A) FV (G/A)		Power of discrimination[d] between genotypes	
Gene[b]	ASO	Miniseq.	ASO	Miniseq.	ASO	Miniseq.
PPT	7.4	157	1.4	1.1	5.3	142
Factor V	14	85	2.3	1.5	6.1	57

PPT, palmitoyl protein thioesterase; FV, coagulation factor V.
[a]Data are from Pastinen et al. (1997), where details of the experiment are given.
[b]An A→T mutation at position 364 of the PPT gene causes the INCL disease (Vesa et al., 1995), and G→A transition at position 1691 of the FV gene predisposes to thrombosis (Bertina et al., 1994).
[c]Several reaction conditions were applied. Only the best genotyping results for both methods are shown.
[d]Calculated by dividing the ratio between signals from the normal and mutant allele obtained at a homozygous position with the corresponding ratio obtained at a heterozygous position.

been used for analyzing biallelic sequence variation caused by SNPs in the identification of individuals, for tissue typing and in genetic mapping and association studies. Mutation detection by minisequencing is not limited to point mutations only, the method is generally applicable also to both large and small deletion mutations (Järvelä et al., 1996; Pastinen et al., 1997), and it has been adapted for analyzing DNA methylation (Gonzalgo et al., 1997). Table 3 exemplifies some applications of the different assay formats described above. Some of the analyzed mutations or SNPs have served as model systems for development of the assay technology, and others are being used in routine laboratories.

QUANTITATIVE PCR ANALYSIS

The high specificity of the DNA polymerase catalyzed nucleotide incorporation reaction makes the minisequencing method an ideal tool for quantification of DNA or RNA sequences by "competetive PCR." The competetive PCR methods are based on co-amplification of a known amount of standard sequence with the target sequence (Wang et al., 1989). The ratio between the amount of product amplified from the standard and the target sequence allows determination of the initial amount of target, provided that both sequences are similar, and thus have been amplified with equal efficiency. Because the two analyzed sequences in minisequencing are amplified with the same primers, and the sequences differ only at a single nucleotide position, they can be assumed to be amplified with equal efficiency. Hence the ratio between the two nucleotides incorporated in a minisequencing reaction reflects directly the ratio between the two sequences originally present in the sample.

For quantification of the absolute amount of an mRNA species in a sample, a known amount an artificial standard RNA or cDNA that differs only by a single nucleotide from the mRNA to be quantified is added to the samples before the reverse transcription or the amplification reaction, respectively (Ikonen et al., 1992; Hermansson et al., 1997; Paunio et al., 1997). The relative expression levels of two or more homologous genes or allelic transcripts present as a natural mixture in a tissue or cell sample (Singer-Sam et al., 1992; Karttunen et al., 1996; Lombardo and Brown, 1996; Greenwood et al., 1997) or even in a single cell (Shen et al., 1998) can be accurately determined without the addition of standard sequences. The absolute amount of a DNA sequences or the relative amounts of two closely related DNA sequences present as a mixture in a sample can be determined analogously (Table 4). The accuracy of the single nucleotide incorporation reaction catalyzed by the DNA polymerase permits detection of a sequence present as a minority of 0.1–1% in a sample (Singer-Sam et al., 1992; Syvänen et al., 1992a,b), which gives the quantitative assays a wide dynamic range. Quantitative minisequencing assays have a good precision with coefficients of variation ranging from 4% to 20%, depending on the proportion of the two sequences in the sample (Greenwood and Burke, 1996; Karttunen et al., 1996; Lombardo and Brown, 1996; Hermansson et al., 1997).

DISCUSSION

Limitations of the Minisequencing Assays

Specific and sensitive detection of SNPs in the human genome by minisequencing, as well as by all other currently used methods, requires amplification of the DNA region spanning the SNP of interest before its detection. The difficulty of performing multiplex PCR reactions is the most serious bottleneck for setting up methods for large-scale multiplex detection of human SNPs. In the minisequencing assays, an

TABLE 3. Examples of Applications of the Various Assay Formats

Assay format	Application	Reference
Gel-based methods		
SNuPE	Hemophilia B and cystic fibrosis mutations	Kuppuswami *et al.* (1991)
Multiplex SNuPE	Insulin receptor and GLUT 4 mutations	Krook *et al.* (1992)
RT-PCR SNuPE	Allele-specific phosphoglycerate transcript levels	Singer-Sam *et al.* (1992)
MS-SNuPE	Methylation status of CpG islands	Gonzalgo and Jones (1997)
Solid-phase assays		
Polystyrene particles	Genotyping of apolipoprotein E	Syvänen *et al.* (1990)
Microtiter plate, ^{3}H	Cystic fibrosis mutations	Jalanko *et al.* (1992)
Microtiter plate, ^{3}H	Panel of SNPs for forensics and paternity testing	Syvänen *et al.* (1993)
Microtiter plate, ^{3}H	Mitochondrial mutations in MERRF and MELAS	Suomalaninen *et al.* (1993a,b)
Magnetic microparticles	Minority N-*ras* mutations in acute myeloid leukemia	Syvänen *et al.* (1992b)
Manifold support	HLA-typing using ALF DNA sequencer	Pastinen *et al.* (1996)
Homogeneous detection		
SPA	Carrier screening for aspartylglucosaminuria	Hietala *et al.* (1996)
ELIDA	Drug resistance in HIV reverse transcriptase	Nyren *et al.* (1993)
FRET	CF, HLA-H and RET proto-oncogene mutations	Chen *et al.* (1997)
ELISA formats		
Minisequencing, DNP	α_1-Antitrypsin mutation	Harju *et al.* (1993)
Minisequencing, FITC	Genotyping of apolipoprotein E	Livak and Hainer (1994)
GBA, FITC, biotin	Panel of SNPs for horse parentage verification	Nikiforov *et al.* (1994)
FNC, FITC	Coagulation factor V mutation	Pecheniuk *et al.* (1997)
Multiplex fluorescent size-based detection		
ALF sequencer	Typing 12 HLA-DQA1 and DRB1 polymorphisms	Pastinen *et al.* (1996)
ABI sequencer	Typing 12 mitochondrial sequence polymorphisms	Tully *et al.* (1996)
ABI sequencer	Detection of 8 mutations in the *HPRT* gene	Shumaker *et al.* (1996)
CE-LIF	3 mitochondrial mutations causing Leber's disease	Piggee *et al.* (1997)
Multiplex analysis on primer arrays		
^{32}P manually made arrays	5-base region of HPRT exon 3	Shumaker *et al.* (1996)
^{33}P manually made arrays	9 recessive disease mutations	Pastinen *et al.* (1997)
Hapten-indirect ELF	33-base region of p53 exon 8	Head *et al.* (1997)
^{33}P, printing robotics	Screening of CCR5 and MBL polymorphisms	Pastinen *et al.* (1998)
^{33}P, printing robotics	Case control study of 12 MI associated SNPs	Pastinien *et al.* (1998)

SNuPE, single nucleotide primer extension; GBA, genetic bit analysis,; FNC, first nucleotide change; SPA, scintillation proximity assay; ELIDA, enzymatic luminometric inorganic pyrophosphate detection assay; FRET, fluorescence resonance energy transfer; DNP, dinitrophenyl hapten; FITC, fluorescein isothiocyanate; CE-LIF, capillary electrophoresis with laser-induced fluorescence; ELF, enzyme-labelled fluorescence; RT-PCR, reverse transcriptase polymerase chain reactions.

additional complication is that the PCR primers and dNTPs must be completely removed before the single nucleotide primer extension detection step. This requirement is a drawback particularly in homogeneous minisequencing assays, where a solid phase is not available for convenient separation of the PCR templates from the reagents.

Theoretically, primer extension reactions could be extremely accurate, the error rate of, for example, the *Taq* DNA polymerase that lacks proofreading activity is in the range of 10^{-4}–10^{-5} (Keohavong and Thilly, 1989; Eckert and Kunkel, 1990) and high-fidelity polymerases with proofreading activity, such as the *Pfu* DNA polymerase, can have error rates of $<10^{-7}$ (Cline et al., 1996). In practice, however, the power of discriminating between genotypes and the sensitivity of detecting a minority sequence variant by the minisequencing reaction are at least one or two orders of magnitude lower than the theoretical values predict. Reasons for this discrepancy between theory and practice are probably that the labeled nucleotide analogues may contain other nucleotides as impurities, and that the labeled moieties of the nucleotide analogue affect the specificity of the nucleotide incorporation by the DNA polymerase.

The specificity of a minisequencing reaction is to some extent dependent on the sequence context of the variable nucleotide, although no systematic study on this has been presented. Moreover, template-independent primer extension due to intra- or intermolecular secondary primer structures has occasionally been observed particularly in assay formats based on immobilized primers (Nikiforov et al., 1994; Pastinen et al., 1997). This problem can be avoided by performing the

TABLE 4. Quantitative Analysis of RNA and DNA Sequences by Minisequencing Primer Extension

Application/purpose of analysis	Key reference[a]
Relative levels of allele-specific mRNA	Singer-Sam et al. (1992)
Tissue distribution of specific mRNA species[b]	Ikonen et al. (1992)
Mutant alleles in pooled DNA samples	Syvänen et al. (1992a)
Minority malignant cell populations	Syvänen et al. (1992b)
Tissue mosaicism of variant genes	Picketts et al. (1992)
Heteroplasmic mutations of mitochondrial DNA	Suomalainen et al. (1993)
Population frequencies of SNPs	Syvänen et al. (1993)
Identification of mixed DNA in stain samples	Syvänen et al. (1993)
Proportions of mutant and wild-type viral DNA	Brunetto et al. (1994)
Gene copy numbers[b]	Laan et al. (1995)
Uneven application of alleles in single cells	Paunio et al. (1996)
Haplotype combinations of closely related genes	Schwartz et al. (1997)
Methylation differences at specific sites	Gonzalgo and Jones (1997)
Differential replication of alleles	Xiong et al. (1998)

[a]Many groups have applied the method to the same purpose. A comprehensive list of references is not provided; only the first application is cited.
[b]Quantification with the aid of added standards.

minisequencing reactions at an elevated temperature (Pastinen et al., 1997) and often by analyzing the other strand of the DNA template.

The light-directed combinatorial methods developed for in situ synthesis of high-density DNA chips (Fodor et al., 1991; Southern et al., 1992) are not directly applicable to manufacture minisequencing primer arrays because the solid-phase oligonucleotide synthesis proceeds in the 3′ to 5′ direction and does not leave the 3′-end of the primer free for extension. This limitation could potentially be circumvented by inverting the orientation of the oligonucleotides in situ after the solid-phase synthesis (Kwiatkowski et al., 1998). By contrast, as highly redundant primer arrays are not required in the primer extension assays, primer arrays of intermediate density produced by printing robotics allow high-throughput genotyping of SNPs.

Future Prospects

Because of their high specificity in distinguishing between sequence variants, minisequencing primer extension assays are being used in a growing number of both research and routine applications. In basic research applications, the possibility of accurate quantification of a sequence present as a small minority of a sample is a particular advantage (Gonzalgo et al., 1997; Xiong et al., 1998). For routine genotyping and mutation detection on a large scale, the minisequencing assays are robust and yield unequivocal results (Hietala et al., 1996; Pecheniuk et al., 1997). In the future, high-throughput analysis of SNPs will be performed by rapid, automatic methods based on homogeneous detection principles or by using fully automatic solid-phase assays or, alternatively, using methods in microarray or chip formats. Homogeneous or automated assays formats have a high throughput when a single or a few SNPs are to be analyzed from large sample materials, but array-based solid-phase assays are potentially more efficient for simultaneous detection of multiple SNPs per sample. The minisequencing reaction is a promising principle for both types of assay formats (Chen et al., 1997; Ronaghi et al., 1996; Pastinen et al., 1997).

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