

Rapid Detection of Genetic Mutations Using the Chemiluminescent Hybridization Protection Assay (HPA): Overview and Comparison with Other Methods*

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ABSTRACT: The detection of genetic mutations is of paramount importance for the study, diagnosis, and treatment of human genetic disease. Methods of detection generally fall into one of two categories: those to scan for unknown mutations and those to detect known mutations. This review focuses on methods for the detection of known mutations. The hybridization protection assay (HPA) is described in detail. The HPA method utilizes short oligonucleotide probes covalently labeled with a highly chemiluminescent acridinium ester (AE). The assay format is completely homogeneous, requiring no physical separation steps, and can rapidly and sensitively detect all single-base mismatches as well as multiple mismatches, insertions, deletions, and genetic translocations. When very low copy number targets are assayed, HPA is coupled with transcription-mediated amplification (TMA), an isothermal method that amplifies DNA or RNA targets. Other methods that are described for the detection of known mutations include hybridization with sequence-specific oligonucleotides, hybridization to oligonucleotide arrays, allele-specific amplification, ligase-mediated detection, primer extension, and restriction fragment analysis. The advantages and limitations of each of these methods are discussed. Methods to scan for unknown mutations are briefly described.

KEY WORDS: diagnostics, oligonucleotide, acridinium ester, single-base mismatches, DNA, homogeneous, transcription-mediated amplification, ligase, PCR, allele-specific amplification, primer extension, restriction, arrays, chips.

I. INTRODUCTION

With the large amount of sequence information made available through the Human Genome Project as well as recent rapid advances in molecular genetics, the discovery and identification of genetic mutations that are associated with human disease have increased dramatically. Likewise, similar advances have occurred in plant and animal genetics as well as the molecular pathology of bacterial and viral infectious disease agents. As a result, the need for molecular assays to detect genetic mutations and polymorphisms has grown dramatically. The scientific community has responded to this need, and in the last decade the increase in the number of powerful molecular assays available for the detection of genetic mutations has been impressive.

Molecular assays for the detection of genetic mutations can generally be separated into two categories: those used for scanning large regions of DNA or RNA for unknown mutations and those used for detecting known mutations. There can be overlap between these two categories (i.e., one assay can be used for both objectives), although the requirements of each category are generally quite different. Sequencing is still considered to be the gold standard and clearly can be used for both categories. Indeed, putative mutations detected by scanning methods are often confirmed and/or identified via sequencing. However, full sequencing is not required in many applications, especially detection of known mutations, and due to cost and complexity is often not the method of choice and is not described further here.

The focus of this review is the description of methods for detecting known mutations. In particular, the method based on transcription-mediated amplification (TMA) and the hybridization protection assay (HPA) is described in detail, followed by an overview of the other available methods. An evaluation of the performance of each of these assays is given as a means to compare and contrast the different techniques. A brief description of some of the methods used to scan for unknown mutations is first provided to help direct those interested to the appropriate material.

A. Methods to Scan for Unknown Mutations

Several methods are available to scan nucleic acid targets for unknown mutations. In the single-strand conformation polymorphism (SSCP) approach,¹⁻⁴ single-stranded DNA or RNA is analyzed using nondenaturing

gel electrophoresis. Differences in base composition between nucleic acid molecules, including single-base mutations, yield differences in secondary structure that cause differences in electrophoretic mobility. Therefore, mutated DNA or RNA is detected by a shift in mobility compared with the wild-type sample. Standard electrophoresis as well as capillary electrophoresis have been utilized for analysis. Mutations are identified by sequencing. In a related technique, differences in the melting properties of duplex domains are analyzed using denaturing gradient gel electrophoresis (DGGE),^{5,6} constant denaturant capillary electrophoresis (CDCE),^{7,8} temperature gradient gel electrophoresis (TGGE),^{9,10} or temperature gradient capillary electrophoresis (TGCE).¹¹ As duplex DNA migrates into the gradient of increasing temperature or denaturant, the strands in low-melting domains separate, but the duplex is held together by the annealed strands of high melting domains. This partial opening of the duplex causes a change in electrophoretic mobility. When a mutation is present in the low-melting domain, the melting characteristics and therefore the electrophoretic mobility are changed, and thus wild-type and mutant sequences can be separated. The formation of heteroduplexes between wild-type and mutant sequences prior to electrophoresis enhances separation and is commonly performed. The use of "GC clamps" in the high-melting domains has also been utilized to improve separation.¹² Solution melting prior to electrophoresis has also been used to improve detection of mutations in high-melting domains.⁶ In another related method referred to as heteroduplex analysis (HET),¹³⁻¹⁷ heteroduplexes and homoduplexes display different electrophoretic mobilities under nondenaturing conditions, thus revealing the presence of mutations.

In a different approach, mismatch repair enzymes have been utilized to recognize mutations in heteroduplex DNA.¹⁸⁻²¹ Transversion and transition mutations as well as small insertions and deletions can be detected in DNA as large as 10 kb. A variety of assay formats have been utilized, including cleavage of mismatched duplexes followed by gel electrophoresis¹⁸ and binding of mismatched duplexes to the mismatch binding protein MutS immobilized on a solid support.²¹ In a related method referred to as enzyme mismatch cleavage (EMC),²²⁻²⁴ T4 endonuclease VII or T7 endonuclease I (bacteriophage resolvases) are used to cleave mismatched heteroduplexes formed between wild-type and mutant alleles. Cleavage products are then analyzed using gel electrophoresis. In an improvement on the basic method, heteroduplex is separated from homoduplex prior to gel electrophoresis to improve signal-to-noise ratio.²⁵ Cleavage of mismatched heteroduplexes with RNase followed by analysis

using gel electrophoresis has also been utilized to detect mutations.²⁶⁻²⁸ The chemical cleavage method (CCM)²⁹⁻³¹ is the chemical equivalent of the EMC method in which mismatched bases in heteroduplexes are modified (typically using hydroxylamine or osmium tetroxide) and cleaved (typically using piperidine), then analyzed by gel electrophoresis. One of the earliest methods used for analysis of sequence variation is the restriction fragment length polymorphism (RFLP) technique (described more fully below) that can detect the presence of a mutation if it creates or destroys a restriction site. In a recent variation of this method referred to as the cleavase fragment length polymorphism (CFLP) technique,³²⁻³⁴ sequence-dependent secondary structure in single-stranded DNA is recognized and cleaved by cleavase endonuclease I and other similar enzymes. Thus, mutations lead to different structures and consequently different cleavage patterns after gel analysis.

It should be noted that in many of these procedures target nucleic acid is first amplified, typically with PCR. In these cases polymerization errors must be taken into account in analysis of the data. It has been reported that the mismatch repair enzyme system can be utilized to estimate the frequency of mutations that arise during amplification.¹⁸ For more information about scanning methods, the reader is directed to recent reviews.³⁵⁻³⁷

B. Methods to Detect Known Mutations

There are many instances where there is a need to detect known mutations or polymorphisms. For example, a single point mutation in the β -globin gene has been clearly linked to sickle cell anemia.³⁸ Neonatal screening for this mutation and other hemoglobinopathies is required in many locations. Likewise, a single A to G mutation at position 985 in the dehydrogenase gene leads to medium-chain acyl-coA dehydrogenase (MCAD) deficiency, a metabolic disorder that can result in sudden infant death syndrome.³⁹ This is another application where neonatal screening is very favorable. Hereditary hemochromatosis (HH) is a disorder of iron metabolism that leads to accumulation of excess iron resulting in multi-organ dysfunction. It affects 1 out of every 200 to 400 individuals of Northern European descent, leading to a carrier frequency of 1 in 8 to 10. If detected early, the disease is manageable using periodic phlebotomy to remove excess iron. However, due to a number of factors this disorder has been historically difficult to diagnose and 95% (or greater) of affected individuals have gone undiagnosed. Recently, a point mutation was iden-

tified that strongly correlates with disease.⁴⁰ A nucleic acid-based test that could be used to routinely screen for this mutation would provide an excellent diagnostic tool that would allow affected individuals to be effectively diagnosed and properly treated. Several mutations in HIV have been identified that lead to drug resistance.⁴¹⁻⁴³ A molecular test that could detect these known mutations would be of great value in determining treatment for HIV-infected individuals. Similarly, mutations in *Mycobacterium tuberculosis* that confer drug resistance have also been identified.⁴⁴

These types of assays for the detection of known mutations should be, in general, able to detect all possible mutations, able to detect a small amount of the mutant sequence in the presence of a large amount of the normal sequence (i.e., yield high matched-to-mismatched signal-to-noise ratios), sensitive, simple to perform, rapid, inexpensive, and amenable to high throughput applications and automation. Following is a description of various methods used for the detection of known mutations with a discussion of the general performance characteristics and limitations for each type of method.

II. HYBRIDIZATION PROTECTION ASSAY (HPA) AND ASSOCIATED METHODS

A. General Assay Strategy

The HPA approach for the detection of genetic mutations (and nucleic acid mismatches in general) is based on sequence-specific hybridization using short oligonucleotide probes covalently labeled with a chemiluminescent acridinium ester (AE). The assay is performed completely in solution with no physical separation steps. The general sequence-specific oligonucleotide hybridization approach for the detection of mutations (see Section III below) is based on the difference in stability between a perfectly matched duplex and a mismatched duplex. Stringent hybridization and/or wash conditions are used to disfavor hybridization of the probes to mismatched targets yet allow hybridization to matched targets. Although HPA utilizes hybridization stringency to aid in discrimination between matched and mismatched targets, the primary basis for discrimination is differential hydrolysis of the AE label, which greatly reduces chemiluminescent signal from mismatched duplexes (described below). Using this approach, the assay can discriminate matched and mismatched targets in about 30 min with a signal-to-noise ratio of 1000:1. Using chemically

modified acridinium ester labels, multiple targets can be simultaneously detected in the same reaction tube (see below).

The assay is sensitive and can often be performed directly in clinical specimen after minimal sample preparation. However, in other applications the target is not present in high enough copy levels to be detected directly (as is the case with various genetic mutations, for example). In those applications the target must be amplified prior to detection using HPA. We have coupled Transcription-Mediated Amplification (TMA; see below) with HPA to provide a rapid and simple method to assay for low copy number targets. TMA, HPA, and detection are all performed in the same tube with nothing ever being removed, which greatly simplifies the assay and reduces the risk of contamination.

The various aspects of the technology are now described in more detail, followed by some examples of use and a description of the performance characteristics and limitations of the method.

B. The Hybridization Protection Assay (HPA)

This utilizes short, sequence-specific oligonucleotide probes to detect nucleic acid targets of interest. One acridinium ester (AE) molecule, a highly chemiluminescent reporter group used in a number of assay formats,^{45–53} is covalently attached to each probe molecule using a non-nucleotide linker arm chemistry that allows the AE to be placed at any position in the backbone.^{54,55} Detection is achieved through reaction of AE with alkaline peroxide (Figure 1), yielding an electronically excited *N*-methyl acridone, which then decays back to the ground state with the emission of a photon of light.^{45,56,57}

Another important reaction of AE is hydrolysis of the ester bond (Figure 1), yielding the permanently nonchemiluminescent *N*-methyl acridinium carboxylic acid.^{45,47} When attached to an oligonucleotide probe, hydrolysis of AE is rapid in mild alkaline solution. However, when the AE-labeled probe (AE-probe) anneals to its perfectly complementary target, the AE hydrolysis rate is slow. This difference in AE hydrolysis rates allows discrimination between hybridized (annealed to target of interest) and unhybridized AE-probe molecules with no physical separation step.

Therefore, the HPA method^{47,48} for the detection of target nucleic acid consists of the following steps, all of which are performed in solution: (1) add AE-probe to sample and hybridize (typically 15 to 30 min); (2) add a mild alkaline solution and hydrolyze AE linked to unhybridized probe

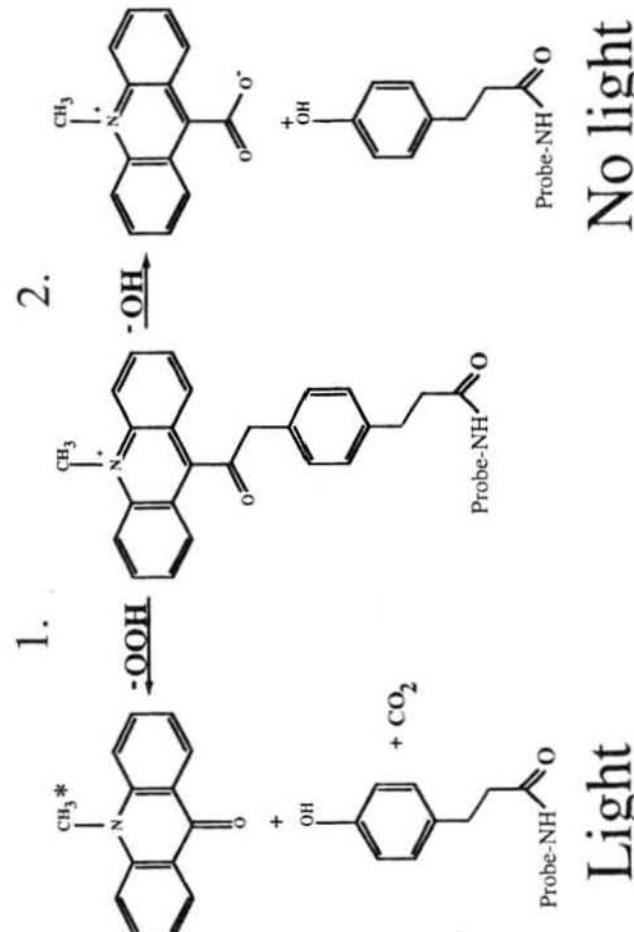


FIGURE 1. Two common reaction pathways of acridinium ester (AE). One molecule of alkaline hydrogen peroxide reacts with one molecule of AE to produce the electronically excited *N*-methyl acridone (*indicates that this molecule is in the excited state), which emits light after collapse to the ground state (reaction pathway 1). In alkaline solution alone, the ester bond of AE is hydrolyzed, yielding the permanently nonchemiluminescent *N*-methyl acridinium carboxylic acid (reaction pathway 2).

(5 to 10 min); (3) detect remaining AE as a measure of the amount of target present (2 to 5 s).

C. Detection of mutations using HPA

When a mismatch is present in the duplex immediately adjacent to the site of AE attachment, protection of AE is disrupted and the AE hydrolysis rate increases back to or near those values observed in single-stranded AE-probe. This difference in AE hydrolysis rate between matched and mismatched duplexes allows rapid, in-solution discrimination between matched and mismatched targets using the HPA format.⁵⁸ Disruption of AE protection is observed with all single-base mismatches and therefore all can be detected. Multiple mismatches, insertions, deletions, and genetic translocations can also be detected. A schematic diagram summarizing the HPA format for detection of mutations is shown in Figure 2.

Mismatch discrimination using the sequence-specific oligomer (SSO) method (see below) is typically achieved using stringent hybridization conditions under which the mismatched duplex is not stable but the matched duplex is stable.^{59–65} In the HPA method, mismatch discrimination obtained through the differential hydrolysis properties of AE can be coupled with normal hybridization stringency to further enhance discrimination. Furthermore, mismatches that are difficult to detect using only hybridization stringency characteristically yield among the highest levels of discrimination from differential AE hydrolysis, and vice versa, which renders the combination of the two approaches very powerful. For example, G · G and G · T mismatches are among the least destabilizing to the duplex and therefore are difficult to detect using stringency alone,⁶⁶ whereas the matched-to-mismatched signal-to-noise ratio for these mismatches are among the highest seen using differential hydrolysis of AE.⁵⁸ Likewise, a T · C mismatch yields lower differential hydrolysis discrimination ratios, whereas it is among the most destabilizing mismatches and therefore discrimination can be increased using stringent conditions.

Typically, an AE-probe is designed to be exactly complementary to each target to be detected, with the AE attached at a location that is immediately across from the site of a known mutation. For example, if a point mutation is to be assayed, a wild-type AE-probe and a mutant AE-probe are constructed with the AE attached at the site of the mutation, such that normal probe will detect the normal target but not cross-react with mutant target and mutant probe will detect the mutant target but not cross-

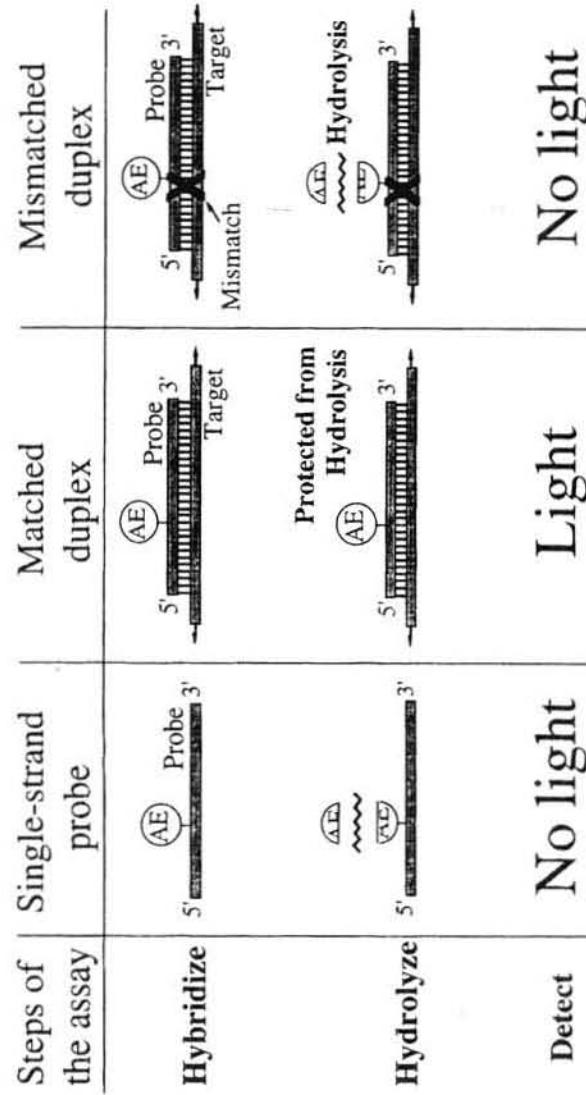


FIGURE 2. Hybridization protection assay (HPA) format for detection of mutations. A schematic diagram summarizing the three steps of the HPA format: hybridization, differential hydrolysis, and detection. Acridinium ester (AE) associated with matched duplex is protected from hydrolysis, whereas AE associated with mismatched duplex is rapidly hydrolyzed.

react with the normal target. These probes can be used in separate reactions to detect their respective targets, or can be used simultaneously in the same reaction tube using the multiple analyte analysis technique described below.

D. Multiple Analyte Analysis Using Chemically Modified Acridinium Esters

The chemiluminescent properties of AE can be altered by chemical modification.⁶⁷ Specific modified AE molecules have been developed that exhibit not only distinct chemiluminescent properties but appropriate differential hydrolysis properties as well. When attached to oligonucleotide probes, these labels can be utilized in the HPA format to simultaneously detect two or more analytes in the same reaction tube.⁶⁸ One mode utilizes differences in the kinetics of the light-emitting reaction to distinguish labels (and therefore analytes). For example, a single methyl group attached to the acridinium ring reduces the rate of light emission, whereas electron-withdrawing groups attached to the phenyl ring increase the rate of light emission.⁶⁸ Using oligonucleotide probes labeled with these two modified AEs, two different analytes can be simultaneously detected using the HPA format as described above. After the hydrolysis step, remaining chemiluminescent signal from either or both labels is collected over multiple time intervals and the signals of the individual labels are resolved mathematically to quantitate the amount of each target present. This assay mode is referred to as the "Dual Kinetic Assay" (DKA).

E. Transcription-Mediated Amplification (TMA)

This is an isothermal, autocatalytic process that amplifies RNA or DNA targets in excess of one billion-fold in less than 1 h.⁶⁹⁻⁷³ The mechanism of TMA is shown in Figure 3, in this case with an RNA target. A primer that also contains a T7 RNA polymerase promoter region (Promoter-Primer) anneals to the target. Reverse transcriptase (RT) then extends this primer, producing a DNA:RNA hybrid. The RNA strand is degraded by RNase H activities, and a second primer (Primer 2) anneals to the DNA strand and is extended by RT, yielding a double-stranded DNA template with an active T7 promoter region. T7 RNA polymerase then produces 100 to 1000 RNA copies of the template, which are the opposite

Transcription-Mediated Amplification (TMA)

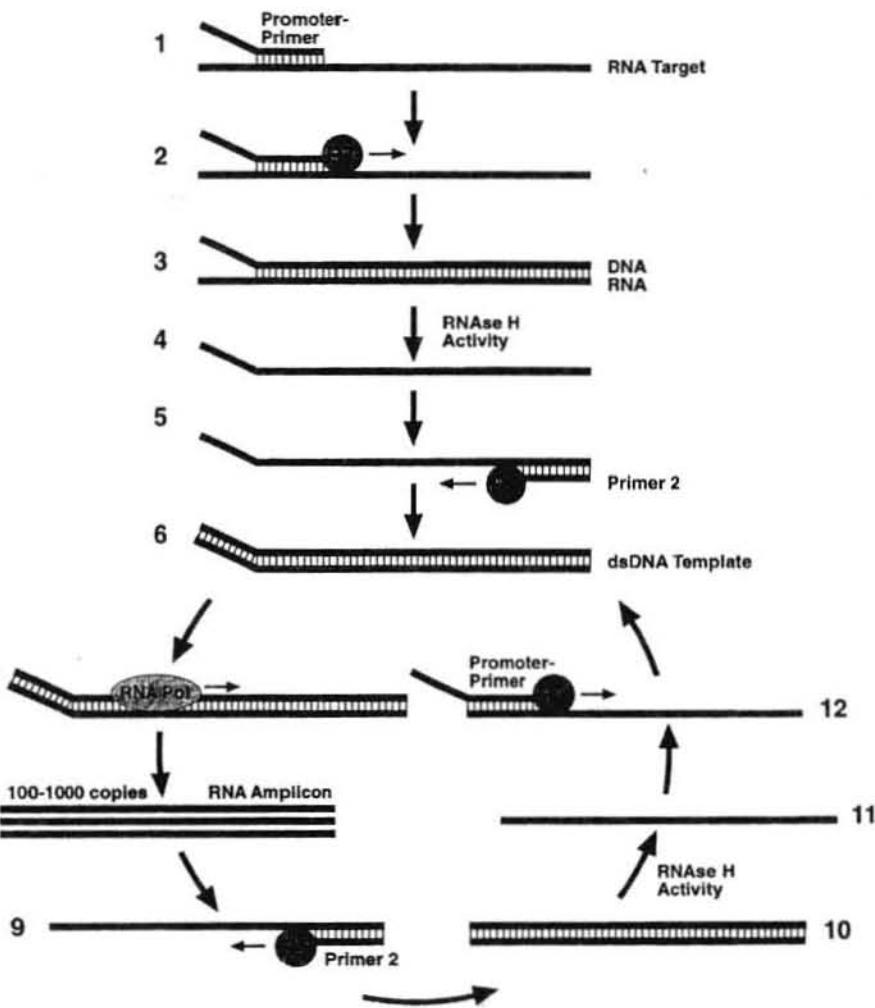


FIGURE 3. Mechanism of transcription-mediated amplification (TMA). The mechanism of TMA is depicted for an RNA target. "RT" is reverse transcriptase and "RNA pol" is T7 RNA polymerase.

sense of the original target (RNA amplicon). Primer 2 anneals to each of these RNA copies, each is extended by RT resulting in DNA:RNA hybrids, and the RNA strands are degraded by RNase H activities. Promoter-Primer then binds to each of the DNA strands and extension with RT produces dsDNA templates with an active T7 promoter. More RNA copies are made, and this cycle continues in an autocatalytic fashion.

TMA is rapid and very simple to perform and consists of the following basic steps (temperatures can vary depending on the application): (1) sample, amplification reagent (primers, buffers, etc.) and oil reagent (which minimizes evaporation and acts as a barrier over the amplification reaction) are added to the reaction vessel and the mixture is incubated briefly at 60°C (primer annealing); (2) enzyme reagent (reverse transcriptase and T7 RNA polymerase) is added and the mixture is incubated at 42°C and amplification is allowed to proceed for 30 to 60 min; (3) amplicon is analyzed using the HPA format as described above (hybridization, differential hydrolysis, detection of chemiluminescence).

F. Applications

Several applications of the TMA and HPA technologies for the detection of mutations have been demonstrated. In one example, a point mutation in the reverse transcriptase coding region of HIV-1 was detected using the HPA mutational analysis technique in the DKA format. An A to G mutation in codon 181 causes broad resistance to non-nucleoside inhibitors such as nevirapine.⁷⁴ AE-probes were designed to be exactly complementary to the wild-type (labeled with AE possessing rapid chemiluminescence kinetics) and mutant (labeled with AE possessing slow chemiluminescence kinetics) HIV sequences with the AE attachment site placed immediately across from the site of the mutation. A mixture of the probes was then used to simultaneously assay various concentrations of the wild type and mutant sequences in the same reaction tube. The signals from the two different AE labels were resolved mathematically and the amounts of wild type and mutant in each sample were quantitated.⁶⁸ Representative results are summarized in Table 1. In one example, the ΔF508 mutation, a 3-base deletion associated with cystic fibrosis⁷⁵ was detected using the HPA format. Wild-type and mutant sequences were accurately detected (Table 2). In another example, HLA typing was performed using HPA.⁷⁶⁻⁷⁸ In one application, TMA and HPA were combined to perform HLA typing of the DRB, DQB and DPB loci. Genomic DNA was

amplified using TMA, and a mixture of AE-probes were utilized in a 96-well microtiter plate HPA format to accurately type each sample. In another example, the chimeric BCR/abl [t(9;22)] sequence associated with chronic myelogenous leukemia (CML) was detected using the HPA format.^{48,51,79}

The technology has been applied to other nucleic acid targets in a variety of assay formats, including FDA-approved kits for the direct detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in urogenital swab specimens (PACE® Assay Kits),⁸⁰⁻⁸⁵ kits for the positive identification of organisms following a culture step (ACCUProbe® Culture Confirmation Kits),⁸⁶⁻⁹² and kits using TMA coupled with HPA for the detection of *Mycobacterium tuberculosis*^{70,71} in sputum specimens and

TABLE 1
Simultaneous Detection of Wild-Type and Mutant HIV-1 Sequences

[Target] (fmol)		Calculated signal (rlu) ^a	
wild-type	mutant	wild-type	mutant
0	0	273	0
0.1	5.0	1,010	102,308
0.1	0.5	1,113	10,504
0.1	0.1	1,570	1,750
5.0	5.0	79,812	116,314
5.0	0.5	68,507	10,832
5.0	0.1	77,682	765

^a Relative light units.

TABLE 2
Detection of the AEF-508 Cystic Fibrosis Mutation using the HPA Format

[Target] (fmol)	Normal target		Mutant target	
	Normal probe	Mutant probe	Normal probe	Mutant probe
0	1,450	1,330	1,384	1,343
0.5	32,170	1,490	1,284	17,185
2.0	60,757	4,087	1,264	26,457
5.0	152,967	7,597	1,215	58,895
20.0	540,470	2,858	1,425	286,018

*Chlamydia trachomatis*⁷² in male and female urogenital swabs and urine specimens. A quantitative assay for the detection of HIV in plasma⁷² and a TMA/HPA assay for the simultaneous detection of HIV and HCV in donated blood have also been described.⁷² All these assays utilize the HPA method of detection and take advantage of the target specificity properties of AE-probes. The assay for HIV and HCV in donated blood also utilizes a rapid target capture protocol. Specimen is added to the assay tube and virus is lysed with a detergent/buffer reagent. Viral RNA is then specifically captured out of solution onto magnetic particles. Utilizing magnetic collection of the particles, specimen matrix, including potential amplification inhibitors, is washed away. TMA and HPA are then performed as described above. This and other TMA/HPA assays are automated on the Gen-Probe TIGRISTM instrument platform (currently under development), which performs all steps of the protocol, including sample preparation from primary specimens. Time to first result will be 3.5 h, and the instrument will yield a high throughput, completing 500 tests per 8 h and 1000 tests per 12 h.

G. Performance Characteristics

The HPA method can readily detect all single-base mismatches, as well as multiple mismatches, insertions, deletions, and translocations. One of the advantages of this method is that it combines two separate approaches to discriminate matched and mismatched duplexes. First, HPA utilizes differential hydrolysis of the AE label as described above to yield a significant difference in chemiluminescent signal from matched vs. mismatched duplexes. Second, HPA utilizes a sequence-specific oligonucleotide approach, which provides discrimination between matched and mismatched targets through the use of stringent assay conditions. The combination of these two approaches typically yields signal-to-noise ratios of 1000-1 or greater. Therefore a mutation can be detected when it is present at a frequency of only 0.1%. This combined approach is particularly effective when either approach alone yields lower than desired signal to noise.

The assay is very rapid and simple to perform. The HPA protocol takes about 30 to 45 min to complete and there are only three steps in the assay: hybridization, differential hydrolysis, and detection. The assay is completely homogeneous with all reactions occurring in solution, which yields faster hybridization kinetics and higher hybridization efficiency compared

with hybridization on a solid surface (membrane, microtiter well, bead, etc.) and avoids laborious assay steps such as membrane binding and washing and gel electrophoresis. Detection requires only a simple luminometer. The assay is quantitative over a range of approximately 4 orders of magnitude.⁵⁰ Due to its simplicity, HPA is very amenable to automation and high throughput applications.

Using chemically modified AE labels, multiple targets can be simultaneously detected in the same reaction tube using the HPA format. This streamlines the process, reducing the number of assays required and increasing throughput. For example, wild-type and mutant alleles can be assayed in the same reaction tube, so wild-type homozygote, heterozygote, and mutant homozygote can be "called" in one reaction. Additionally, each target can be quantitated, which yields the advantage that the relative amounts of the different targets present in a given sample can be accurately determined. For example, in cancer diagnosis the amount of both wild-type and mutant cells can be quantitated in a single reaction. This feature is especially useful for amplified assays, as the ratio between wild-type and mutant targets can be quantitated in the same amplification reaction (with no transfers), thus eliminating the error from amplification variability if they were assayed from separate amplification reactions.

When target amplification is necessary, TMA is performed on the sample and product is assayed using HPA in the same reaction tube, thus minimizing the risk of carryover and cross-contamination. TMA takes about 60 to 90 min to complete, is simple to perform, and amplification is isothermal, thus not requiring thermal cycling. TMA is very sensitive, will amplify RNA and DNA targets, and can be multiplexed to simultaneously amplify multiple targets in a single reaction tube. TMA can be performed in a qualitative mode or a quantitative mode where the amount of starting target is accurately determined over a concentration range of several orders of magnitude. TMA/HPA-based assays for detection of mutations are very amenable to automation and high-throughput applications. An instrument system is currently under development (TIGRIS; see above).

H. Limitations of the Method

The acridinium ester reacts only once with alkaline peroxide to produce light, at which point the ester bond is cleaved and the system is no longer chemiluminescent. Therefore, questionable samples cannot be re-read but rather must be reassayed. As with any method that relies on

sequence-specific hybridization, a new oligomer needs to be designed for each mutation to be detected. This puts a practical limit on the number of mutations that can be analyzed (although due to the simplicity of the system this number can still be quite high). This is true with most of the methods discussed in this review for the detection of known mutations. This limitation has been overcome to a reasonable degree with DNA probe arrays that have been produced with about 64,000 probes on a single chip, but this technology is still expensive and requires complex instrumentation and data analysis systems (see below). The multiple analyte detection scheme using chemically modified AEs also has a practical limit of approximately 4 or 5 analytes that can be simultaneously detected. As the number of analytes increases, the ratio of the analytes that can be accurately quantitated goes down, although this is typically not an issue with "all-or-none" type signals, as in inherited disorders (100% normal, 100% mutant, or 50% of each). If a clinical specimen is to be assayed directly using the HPA format, any inherent chemiluminescence in the sample will be measured at the end of the assay as nothing is ever removed from the tube. If this becomes a problem, a sample preparation method that removes any interfering substances would be necessary. As simple sample preparation methods are developed, this will become a normal matter of course, especially with target amplified assays.

III. SEQUENCE-SPECIFIC OLIGONUCLEOTIDES (SSO)

Sequence-specific hybridization is a foundational principle in the field of nucleic acids.⁹³ It has long been recognized that a single mismatch in a duplex formed between a nucleic acid target and an oligonucleotide probe can cause a significant decrease in the melting temperature (*T_m*) of the duplex.^{59,94–96} The difference in melting temperature between a perfectly matched duplex and a mismatched duplex (ΔT_m) is the basis by which oligonucleotide probes can be used to discriminate targets differing by as little as a single nucleotide. The magnitude of ΔT_m depends on a number of factors, including the identity of the mismatch, the location of the mismatch in the duplex, the sequence context of the mismatch, the length of the duplex (i.e., the length of the oligonucleotide probe), and the environmental conditions (reagent composition, temperature, duplex bound to a solid phase or free in solution, etc.).^{61,66,97–100} Relatively short probes (15 to 30 nt) are typically used in this approach, as ΔT_m generally decreases with increasing duplex length. Furthermore, probes are usually

designed such that the mismatch(es) are positioned in the interior portion of the duplex (compared with the ends of the duplex), as this generally yields a larger ΔT_m value.

A variety of assay formats have been developed to utilize sequence-specific oligonucleotide probes for the detection of mutations. Early methods utilized gel electrophoresis of target (often fragmented with restriction enzymes), transfer to membranes, and hybridization with radiolabeled probes.^{60,101–102} In more recent applications, dot-blot methodology is used in which target nucleic acid is spotted directly onto the membrane without prior electrophoresis.^{61–62,103} The reverse dot-blot procedure is a further refinement of this general methodology, in which the sequence-specific probes themselves are attached to the membrane and target or a mixture of targets is applied to the surface and allowed to hybridize.^{63,104–105} With this procedure different probes can be applied to different physical areas of the same membrane, and specific targets can be easily identified according to which areas yield a positive signal. Probes have also been applied to surfaces other than membranes, such as microtiter wells,^{98,106} which often simplifies the protocol and renders the assay more amenable to automation.^{107–109} Similarly, target nucleic acid has been immobilized on a variety of surfaces, including streptavidin-coated magnetic particles^{99,110} and a carboxymethylated dextran matrix.¹⁰⁰ In an electrophoresis-based assay termed "profiling of oligonucleotide dissociation gel electrophoresis" (PODGE), target and oligonucleotide probe are hybridized and then electrophoresed on a 20% polyacrylamide gel. An increasing temperature gradient is applied to the gel and target:probe duplexes melt according to stability, thus distinguishing matched (more stable) and mismatched (less stable) duplexes.¹¹¹ In a different approach, artificial mismatches are introduced near the site of the true mismatch to be detected using the base analog 3-nitropyrrrole, improving the ultimate discrimination between wild-type and mutant targets. Using this method the difference in melting temperature (ΔT_m) between wild-type and mutant targets with a single nucleotide polymorphism as increased up to 200%.¹¹² Recently, peptide nucleic acids (PNAs)¹¹³ have been utilized as sequence-specific oligomers for mismatch detection. PNA probes exhibit higher affinity for target nucleic acids than the corresponding DNA probes and an increase in discrimination between matched and mismatched targets has been reported.^{99,110} Another approach that utilizes sequence-specific hybridization as the basis for mismatch discrimination is oligonucleotide array technology. This technology is addressed in a separate section below.

As with the HPA approach, the sequence-specific oligonucleotide approaches may or may not utilize amplification of target nucleic acid prior to the hybridization and detection steps. One of the most commonly used techniques is PCR,^{61,114} which is often used to incorporate chemical moieties (such as biotin) that aid in immobilization of the target or detection.^{63,98,110} A variety of schemes have been utilized for the detection of mutations using the sequence-specific oligomer approach. As mentioned above, early techniques used radiolabels, typically ³²P. Since that time many excellent nonisotopic methods have been developed and utilized in this approach, including alkaline phosphatase-mediated chemiluminescence of dioxetane substrates such as [3-(2'-spiroadamantane)-4-methoxy-4-(3'-phosphoryloxyphenyl-1,2-dioxetane] (AMPPD),⁶³ alkaline phosphatase-mediated color generation from p-nitrophenyl phosphate (pNPP),⁹⁸ DNA-tagged liposomes with an encapsulated red dye for visual discernment of signal,⁶⁴ MALDI-TOF mass spectrometry,^{99,110} surface plasmon resonance,¹⁰⁰ horseradish peroxidase-mediated color generation,¹¹⁴ and fluorescence.¹¹⁵⁻¹¹⁷ Many different mutations have been analyzed using the sequence-specific oligonucleotide approach, including mutations in the human β -globin gene,⁶⁰ tyrosinase gene⁹⁹ and mitochondrial DNA,⁶³ and single nucleotide polymorphisms in the HLA-DRB locus.¹¹²

A. Performance Characteristics and Limitations of the Method

Any method of detecting target nucleic acid that utilizes sequence-specific oligonucleotides enjoys the inherent specificity afforded by Watson-Crick base-pairing. This is in contrast to methods that use nonspecific detection (thus relying on preceding steps in the assay for specificity), such as detecting bands on a gel with ethidium bromide. Thus, product nucleic acid from an amplification reaction, for example, can be positively identified using the SSO approach, thereby distinguishing it from nonspecific amplification products. In regards to detection of mutations, especially single-base changes, the SSO method is generally very good. Matched-to-mismatched signal-to-noise ratios of 100:1 (i.e., 1% sensitivity for detecting mutations in a background of unmutated target) are fairly commonly achieved, although in some systems the signal-to-noise is not this high. Most if not all mismatches can be detected, but some yield much lower signal-to-noise ratios than others. As mentioned above, some mismatches do not destabilize the duplex as much as others and the ΔT_m between

matched and mismatched duplexes is not as high, thus limiting the discrimination between the two species even under optimally stringent conditions. Mismatches that fit this description include G · A, G · G and G · T. The method of artificial mismatch hybridization described by Smith and co-workers¹¹² may be able to improve S/N ratios for these difficult-to-detect mismatches, but not all mismatches have been examined yet.

The assay formats utilized in the SSO approach for the detection of mutations are predominantly heterogeneous, that is, they require some sort of physical separation process. The majority utilize immobilization of either the target(s) or probe(s) onto a surface (nitrocellulose membrane, microtiter wells, microparticles, etc.) and stringent hybridization and/or washing on these surfaces. Although improvements have been made to simplify these methods, in general they are still somewhat tedious and time-consuming, and because material is removed from the tube, there is always the risk of cross-contamination of samples. Furthermore, hybridization on a solid surface is typically slower and not as efficient as in solution, although the use of microparticles overcomes these deficiencies to a reasonable extent. Accurate quantitation is also difficult with blotting techniques. In one particular application, the initial hybridization reaction was performed in solution and then captured onto microtiter wells.¹¹⁵ Automation of these processes, such as in microtiter plates, has significantly improved the ease of use of these assays. Methods to analyze multiple targets have become available, especially coupled with amplification techniques such as PCR. The mass spectrophotometric (MALDI-TOF) method using probes with mass tags⁹⁹ is particularly powerful and elegant, although the cost of the required instrumentation is relatively high.

IV. OLIGONUCLEOTIDE ARRAYS

The fundamental principle utilized in this approach is hybridization of target to sequence-specific oligonucleotides under conditions that discriminate between matched and mismatched duplexes, and in this regard is akin to the methods described in Section III. In array technology different oligonucleotides are attached to a surface in discrete physical locations, and in this regard is akin to the reverse dot-blot methodology described above. However, other aspects of the technology, methods of synthesizing oligos directly on the surface, miniaturization, density of separately addressable elements in the array, large numbers of oligos in the array that are designed to systematically interrogate each nucleotide position in the

target, methods of detection and data analysis, are new and often exquisitely sophisticated. In general, oligonucleotides are immobilized onto or synthesized directly on a surface in high-density arrays, labeled target is hybridized across the surface, and positive signal is visualized at specific locations on the surface. Affymetrix (Santa Clara, California), a leading commercial developer of this technology, utilizes light-directed synthesis of oligomers directly on the surface and epifluorescence confocal scanning microscopy for detection.

Various surfaces have been derivatized, including glass, polypropylene, silicon, and polyacrylamide gel elements fixed on a glass plate; different methods of coupling oligonucleotides to the surface have been utilized, including pre-synthesis using standard techniques and coupling to the surface (using aldehyde/hydrazide and biotin/avidin chemistries, for example) and synthesis directly on the surface (using standard phosphoramidite chemistry and photolithography with photocleavable protecting group chemistries. For example, a series of linkers containing photochemically removable protecting groups are attached to the surface; light is directed to specific areas of the surface through a photolithographic mask, activating those areas toward chemically coupling; a first series of nucleosides, which also contain photochemically removable protecting groups at their 5'-ends, are coupled to the activated areas of the surface via phosphoramidate chemistry; different areas of the surface are activated using a new mask, and the process is repeated until each area of the surface has an oligonucleotide of defined sequence attached. Various detection strategies have been utilized, including 32P and fluorescence, and a variety of array sizes have been utilized, ranging from approximately 25 to 65,000 separately addressable.¹¹⁸⁻¹²⁶

In a typical approach, a series of oligonucleotides of known sequence and of the same length, each shifted one base in the target sequence compared with the preceding oligo, are affixed to the surface. For each target position a set of four oligomers is constructed, each containing one of the four different bases at a given location in the sequence (usually in the middle). This set of four oligos, each attached to a distinct position in the array, is used to "interrogate" the target at this nucleotide position and the assay conditions are adjusted such that only the exact complement will hybridize. Each nucleotide in the target thus can be identified using the series of frame-shifted oligomers, thereby detecting and identifying mutations. Most applications utilize standard assay parameters, such as temperature, target concentration, buffer and salt concentration, to adjust hybridization characteristics, although one system utilizes direct electric

field control to modulate hybridization characteristics, including mismatch discrimination.¹²¹

Oligonucleotide arrays, or DNA chips as they are also called, have been utilized for a variety of applications. Due to the very high number of oligomers that can be attached to specific locations of the chip, unknown mutations can be detected and identified in large targets. Chips have also been used to monitor gene expression.¹²⁴ However, DNA chips can be used to screen for known mutations, such as mutations in the reverse transcriptase coding region of HIV-1, which confer drug resistance.¹¹⁸ Other applications include detection of β-globin mutations,^{120,122} mitochondrial DNA mutations,¹²⁶ and cystic fibrosis mutations.¹²⁷

A. Performance Characteristics and Limitations of the Method

High-density oligonucleotide arrays are a very powerful technology, and the amount of information that can be obtained from one "assay" on a chip is staggering. Arrays can be used for de novo sequencing and resequencing, and as such provide much more information about a target than the other methods described here. Indeed, recently a chip system that can interrogate the entire genomic sequence of *Saccharomyces cerevisiae* has been reported.¹²⁴ Therefore, oligonucleotide arrays are very useful for the analysis of complex targets in which many mutations may be present. For example, there are over 1000 mutations that have been associated with cystic fibrosis. To detect even a reasonable percentage of these mutations with other techniques would be a difficult proposition, but this could be achieved using oligonucleotide arrays. Another example is the detection of HIV mutations that have been associated with drug resistance. Several key mutations could easily be assayed using other techniques, but to assay all or most mutations (and the number of known drug-resistance mutations continues to increase) the oligonucleotide array technology would be very suitable.

The oligonucleotide array approach relies on sequence-specific hybridization for discrimination of mismatched targets, and, as mentioned in Section III above, this approach yields S/N ratios in the range of 100. Like other SSO approaches, some mismatches are much more difficult to assay than others, thus lowering S/N ratios. This can lead to ambiguity in base-calling at certain positions. Also, even though the oligos in the array are of equal length, their Tm values vary depending on base composition.

Compounds such as tetramethylammonium chloride (TMAC) are often included in the hybridization mixtures to overcome this variability,^{125–126} but this also often lowers S/N ratios. Furthermore, hybridization occurs on the surface of a solid support, which as discussed above, leads to slower hybridization kinetics and less efficient hybridization. This has been found to be a problem with array technology, and targets typically need to be fragmented to obtain reasonable hybridization performance. In the high-density array approach, typically the target itself is labeled, usually with a fluorescent tag. This adds steps, time, and complexity to the assay. For example, in one application target is fragmented and labeled with biotin-ddATP using terminal deoxynucleotide transferase, then labeled with streptavidin-phycoerythrin after hybridization of the target to the array.¹²⁴ Furthermore, modification of the target can lead to alterations in its hybridization characteristics.

In general, the assay procedure is complex and requires many steps. Several of these steps are automated, which decreases the amount of labor required and greatly simplifies the assay for the user. The procedure can be rather lengthy as well, with time required for fragmentation and labeling and with some applications requiring overnight hybridization on the array. The assay is relatively costly, especially the initial cost of designing and manufacturing an array for each new application, and the instrumentation required to run the test is sophisticated and costly. Also, the number of samples that can be analyzed at one time is limited, and this procedure is therefore not amenable to high throughput applications.

However, the amount of information that is yielded by each assay is unrivaled by the other procedures described in this review. Therefore, oligonucleotide arrays are best suited and may be the method of choice for analysis of complex targets where a very large number of mutations must be analyzed, whereas less complex, faster, and less expensive procedures that are amenable to high throughput applications are more appropriate for screening and analysis of targets where a smaller number of well-defined mutations must be analyzed in a large number of samples.

V. ALLELE-SPECIFIC AMPLIFICATION (ASA)

In this approach, discrimination between wild-type and mutant or polymorphic sequences is achieved in target amplification by directing binding of the primers to the mutant/polymorphic site in the target. The methods cited in the literature to date are predominantly PCR based,

although other amplification procedures can be used as well. In one group of assays, a primer is designed such that its 3' end is immediately across from the site of a potential mutation. When the primer binds to a target with the mutation to be detected, the 3' end of the primer is mismatched and polymerase extension is attenuated (depending on the identity of the mismatch and conditions of the reaction), leading to a decrease or an elimination of the amplification product.¹²⁸ Therefore, if a point mutation is to be detected, a wild-type primer is made that is mismatched at its 3' end with the mutant target and a mutant primer is made that is mismatched at its 3' end with the normal target, a separate PCR is run for each primer (each paired with the same opposing primer), and product is measured. The wild-type reaction will yield product from the wild-type homozygote and the mutant reaction will yield product from the mutant homozygote. In this configuration, heterozygote yields no signal, so an internal positive control must always be run. In some cases the 3' end of both primers (+ and – sense) are immediately across from the site of a potential mutation.¹³⁴

In one application, multiple primers are used in one reaction such that one allele is amplified in one direction and the other allele is amplified in the other direction, resulting in different sized and/or a different number of products for each allele present.¹³² In this way only a single PCR reaction has to be run to distinguish wild-type, mutant, and heterozygote samples. In another group of assays, the mismatch is located in the central portion of the primer, and the stringency of the annealing reaction is adjusted such that mismatched primers do not anneal and therefore do not support amplification.¹⁴⁰

A number of assays have been designed based on these principles. Systems that utilize 3'-mismatched primers include the amplification refractory mutation system (ARMS),^{129,130} PCR amplification of specific alleles (PASA) and PCR amplification of multiple specific alleles (PAMSA),¹³¹ bidirectional PASA (Bi-PASA),¹³² mutagenically separated PCR (MS-PCR),¹³³ PCR with sequence-specific primers (PCR-SSP),¹³⁴ allele-specific PCR (ASPCR)^{135,136} and mutant-enriched PCR (ME-PCR).¹³⁷ ME-PCR utilizes two rounds of PCR with a restriction enzyme digestion between rounds to increase discrimination between wild-type and mutant sequences, and is discussed further below in Section VIII (restriction fragment analysis). Systems that utilize internally mismatched primers (or a combination of internally and 3'-mismatched primers) include mismatch amplification mutation assay (MAMA),¹³⁸ artificial mismatch PCR,¹¹² tetra-primer PCR,¹³⁹ and competitive oligonucleotide priming (COP).¹⁴⁰

Analysis of amplicon in the vast majority of the allele-specific PCR methods is via gel electrophoresis and detection with either ethidium bromide or 32P. In some cases the primers for different alleles to be detected contain noncomplementary tails of different length to yield different electrophoretic mobility for each allele amplified. Primers labeled with distinguishable fluorescent tags have also been used to identify bands after gel electrophoresis. In one application, product after amplification was immobilized on a membrane and assayed using 32P-labeled allele-specific oligonucleotide probes, which were shown to enhance the specificity of the assay.

A wide variety of mutations and polymorphisms have been analyzed using the general concept of allele-specific amplification. The PCR-SSP system was used to examine polymorphisms in tumor necrosis factor and lymphotoxin alpha;¹³⁴ single-base changes in the human catechol-*O*-methyltransferase gene and the human procoagulant factor V gene were analyzed using the Bi-PASA technique;¹³² ARMS was used to analyze DNA from patients with alpha-1-antitrypsin deficiency¹²⁹ and to detect common mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene.¹⁴¹ The K-ras Val-12 mutation was assayed in colorectal cancer using the ME-PCR technique;^{137,142} a mutation in codon 360 of the apolipoprotein A-IV gene was analyzed using MS-PCR;¹³³ ASPCR was used to determine ABO blood group genotypes¹³⁶ and to detect exonic polymorphisms on the X chromosome genes p55 and G6PD.¹⁴³

A. Performance Characteristics and Limitations of the Method

In general, allele-specific amplification (ASA) is a powerful technique and is very useful in assaying for known mutations. Matched-to-mismatched signal-to-noise ratios of 1000 have been reported, and a signal-to-noise ratio of 5000 was reported for the combination of ME-PCR and competitive allele-specific oligomer analysis.¹³⁷ Indeed, ASA has been utilized widely to detect a variety of mutations in a number of important applications. A drawback for several of these assay modes is that separate reactions have to be run for each allele to be detected. The Bi-PASA method was reported recently that overcomes this drawback by utilizing a set of four primers to generate products of different size depending on the zygosity of the sample. Another drawback of ASA is that some mismatches are not nearly as refractory to amplification as others,¹²⁸ making

these mismatches more difficult to detect and the associated signal-to-noise ratios substantially lower. The degree of refractory activity is dependent on several factors, including sequence context, type of enzyme(s) being used, and amplification conditions. The artificial mismatch hybridization method has been used to increase discrimination between matched and mismatched templates using allele-specific amplification,¹¹² and may prove useful in increasing signal-to-noise ratios for difficult-to-detect mutations.

Methodology for performing ASA can be relatively simple, although the vast majority of the reported procedures utilize gel electrophoresis for analysis. This method is very effective but adds complexity, cost, time, and labor to any assay for a given mutation. Simpler detection methods are necessary for ASA to be a convenient and rapid method for high throughput screening of known mutations. For those mismatches that are more difficult to detect and for protocols used to characterize zygosity in a single reaction, the amplification conditions can be difficult to "fine tune", and the resulting assay may be too delicately balanced and lack the robust character necessary for high throughput screening assays for clinical applications.

VI. LIGASE-MEDIATED DETECTION OF MUTATIONS

A variety of enzymatic ligation-based assay formats have been developed for the detection of known mutations. There are generally two types of assays, one based on the ligation of detection probes and one based on ligation-mediated target amplification. Both assay types rely on the principle that ligation of two oligonucleotides hybridized immediately adjacent to one another on a template is disrupted by the presence of a mismatch at the site of ligation.

In the first type of assay, referred to as the oligonucleotide ligation assay (OLA),^{144–147} two detection probes are hybridized adjacent to one another on the target nucleic acid (or the amplified product of the region of interest) such that the point of ligation is placed at the site of the potential mutation to be detected. When bound to the exactly complementary target, the probes are ligated with DNA ligase and this long product is detected (see below). However, when a mismatch is present, the probes do not ligate and a long product is not detected. Typically, the sequence of one of the probes is held constant and a probe specific for each of the alleles to be tested is constructed.

In the second type of assay, amplification of the target of interest is performed with the ligase chain reaction (LCR),¹⁴⁸⁻¹⁵⁰ which utilizes thermostable ligase to join two primers that are hybridized adjacent to one another on the target, thereby creating a new template strand for further amplification. Primers are placed at the site of the potential mutation, and ligation is blocked by the presence of a mismatch, thus inhibiting amplification.^{151,152}

In one particular application, β -globin alleles βA , βS , and βC were detected using the OLA strategy.¹⁴⁴ One probe was labeled with biotin and one probe was labeled with ^{32}P . The ligated product of these two probes (which occurred on the matched template) was captured onto a streptavidin solid support, washed, and detected by autoradiography. The authors also report a detection scheme wherein one probe is labeled with a particular fluorophore, a different fluorophore for each target region to be detected, and one probe is unlabeled but contains a noncomplementary stretch of nucleotides attached to its 3' end, a different length for each target region to be detected. Ligated products are detected using gel electrophoresis, each target region yielding a band of unique length and each allele is detected with a unique fluorophore. This assay was automated in a 96-well format, utilizing digoxigenin/anti-digoxigenin-alkaline phosphatase-mediated colorimetric detection; the cystic fibrosis $\Delta F508$ mutation was also detected by this technique.¹⁴⁵ In another application, the cystic fibrosis $\Delta F508$ and $\Delta I507$ mutations were detected using a chemiluminescent OLA system.¹⁴⁶ One probe was immobilized on magnetic particles and the other probe was labeled at its 3' end with a chemiluminescent acridinium ester. After ligation (in the presence of matched target), the spheres were washed in hybridization buffer, resuspended in water and incubated at 65°C to remove hybridized but unligated probe, then washed again. After DNase I treatment, the chemiluminescence of the particle suspension was measured.

In an application using LCR, wild-type and mutant β -globin alleles were detected in a 96-well microplate format. Primers were designed for each allele, and neighboring primers were synthesized to contain a "tail" region used for capture on the microplate. After LCR amplification of the matched sequences, ligated primers were captured onto the wells of a micoplate via hybridization of the capture "tails" and complementary capture oligos coated onto the surface of the microwells. After washing, the captured products were detected colorimetrically via a streptavidin-alkaline phosphatase conjugate. As an amplification control in this assay, human growth hormone gene was simultaneously amplified and captured

using a different "tail" region. Another application¹⁵² used the Gap-LCR method in which DNA polymerase fills in a gap between annealed primers before ligation can occur. This was originally designed to reduce background from target-independent, blunt-end ligation. For detection of mutant sequences, each allele-specific primer was designed to yield a mismatch with the other alleles at either its 3' end or at the penultimate 3' position. Using this procedure, good discrimination between alleles was obtained, as was demonstrated by the detection of an AZT resistance-conferring mutation at codon 215 of the reverse transcriptase gene of HIV-1.

A. Performance Characteristics and Limitations of the Method

Both the LCR and the OLA approaches are excellent methods for detection of known mutations and have been used successfully for a variety of mutations in a variety of applications. All mismatches will cause a decrease in ligation efficiency and therefore can be detected. Matched-to-mismatched signal-to-noise ratios of 1000 have been reported. Simplified formats and automation have also been described. One drawback is that some mutations do not cause a large decrease in ligation efficiency and therefore yield substantially reduced S/N ratios. For example, T · G and G · T (these mismatches were discussed in earlier sections as yielding less destabilization of duplex structure) have been shown to generate reduced S/N ratios.¹⁴⁴ Template-independent blunt-end ligation can also occur in these assays, yielding false-positive results. This problem has been overcome to a large extent by the Gap-LCR procedure.¹⁵² Some reported procedures for ligation-mediated detection of mutations are time-consuming, labor intensive, and not amenable to automation. These shortcomings have been addressed with simplified formats that utilize capture of the ligated product, often in a microtiter plate. These assays still require washing steps, but these steps can be automated, which simplifies the protocol. Finally, to achieve maximal discrimination between matched and mismatched targets, the reaction conditions must be finely tuned, which, as mentioned in the previous section, may decrease robustness of the assay in clinical applications.

VII. PRIMER EXTENSION

There are a variety of ways in which differential primer extension can be utilized to detect mutations. One method is allele-specific amplification

in which a primer with a mispaired 3' end (i.e., directly across from the site of a mutation) is refractory to extension and therefore inhibitory to amplification (described above). A related method is described by Fauser and Wissinger,¹⁵³ in which the region of a known mutation is first amplified using PCR, then a set of primers — one complementary to the wild-type sequence and labeled with FAM (5-carboxy-fluorescein) and one complementary to the mutant sequence and labeled with JOE (2',7'-dimethoxy-4',5'-dichloro-6-carboxy-fluorescein) — with their 3' ends at the site of the potential mutation, are extended for three rounds. The products are run out on a gel and detected on an automated sequencer. Multiple target regions can be simultaneously assayed by engineering different lengths of extension products, which are separated on the gel. This technique was used to assay for three different mitochondrial DNA mutations associated with Leber's hereditary optic neuropathy (LHON).

In another related technique,¹⁵⁴ biotinylated PCR product is immobilized on a streptavidin solid support, denatured, and hybridized with a primer complementary to the wild-type sequence and, in a separate reaction, with a primer complementary to the mutant sequence, again with the 3' end of each primer at the site of the potential mutation. The primer extension rates for each reaction are then accurately measured in real-time using an enzymatic luminometric inorganic pyrophosphate (PPi) detection assay (ELIDA), in which the PPi produced during the reaction is continuously monitored with firefly luciferase. The rate of extension for a matched primer is significantly faster than that for a mismatched primer. This difference in rate is increased when the first base to be incorporated is an alpha-thio-dNTP analog.

Another commonly used technique is referred to as single nucleotide primer extension, or mini-sequencing. In this technique, a primer is hybridized to the target template such that its 3' end is immediately adjacent to the site of the potential mutation. A single nucleotide is then incorporated, and because only the correctly Watson-Crick basepaired nucleotide is incorporated, the identity of the "interrogated" base can be determined. Detection can be achieved in a variety of ways. In one report,¹⁵⁴ four separate extension reactions were run, each containing a different 32P-labeled dNTP. After 30 cycles of extension, the products were run out on a gel. The only dNTP that was incorporated (at the levels of detection used) was the one that was complementary to the target sequence, thus identifying the nucleotide in the position of the potential mismatch. This same technique was used by Kuppuswamy and co-workers,¹⁵⁵ except that they used only two reactions, one corresponding to the normal sequence

and one corresponding to the mutant sequence. They used this approach to analyze mutations corresponding to hemophilia B and cystic fibrosis. Kornher and Livak¹⁵⁶ reported a primer extension assay in which purified PCR product is primed in the region of potential mutations and a defined length segment is synthesized using Taq polymerase with one of the four natural dNTPs replaced with an analog that alters electrophoretic mobility. Therefore, segments of equal length that vary by even a single nucleotide migrate differently after gel electrophoresis. They demonstrated this principle on wild-type and mutant DNA from the human insulin receptor gene.

The primer extension method can also be performed using a solid-phase approach, which simplifies the assay and renders it more amenable to automation. This approach was used to detect the sickle cell mutation by Su and co-workers.¹⁵⁷ The region of interest was first amplified using PCR with one primer biotinylated. The product was then immobilized onto streptavidin coated wells of an 8-well strip, denatured, and hybridized with a primer whose 3' end was immediately adjacent to the site of the potential mutation. The primer was then extended by a single nucleotide using the appropriate fluorescein-conjugated dideoxynucleotide. Fluorescence was measured directly or indirectly using peroxidase-conjugated antifluorescein antibody with colorimetric detection.

A. Performance Characteristics and Limitations of the Method

In one mode of primer extension assays, primer is bound to target (often after an initial amplification of the region of interest) such that its 3' end is across from the site of the mutation, thus forming a mismatch when the mutation is present. Differences in primer extension rates or amount and/or size of the product are measured to determine the presence or absence of the mutation. This mode is akin to ASA, where 3' mismatches in the primer are refractory to amplification, and the two assay systems are similar with respect to performance characteristics and limitations. Primer extension in and of itself is obviously not as sensitive as amplification, although the target is often amplified before the primer extension assay. As with ASA, some mismatches are less refractory to primer extension than others and therefore are more difficult to detect. The S/N ratios for primer extension in this mode are certainly less than those obtained with highly discriminating ASA methods, such as ME-PCR.

In the single nucleotide primer extension or minisequencing mode, the base at the site of the suspected mutation is identified, and in this respect is obviously very useful and powerful. This method is often limited by high backgrounds due to such occurrences as misincorporation or nonspecific priming and incorporation at a site other than the target of interest. These factors reduce the matched/mismatched S/N ratio that can be achieved with this assay. In one report¹⁵⁷ a protocol to improve S/N was described, but S/N ratios were still less than 100.

The methods of analysis for primer extension assays are typically either gel electrophoresis or a solid phase approach where target (or primer) is bound to the surface, primer extension is performed, and signal from one of various detection schemes (fluorescence, luminescence, etc.; direct, indirect) is measured. The methods are again somewhat tedious and time-consuming, although automation of the solid phase procedure greatly simplifies the assay.

VIII. RESTRICTION FRAGMENT ANALYSIS

Restriction fragment length polymorphism (RFLP) analysis has long been used as a method to determine the presence of polymorphisms and mutations in DNA. Restriction enzyme recognition sites can vary depending on the particular polymorphisms and/or mutations present, thus creating unique fragment patterns (typically analyzed by gel electrophoresis) after enzymatic cleavage. Known mutations can be analyzed in this manner if a restriction site is created or destroyed by the mutation. Applications of this method include the analysis of the Cys282Tyr mutation in the HFE gene recently associated with hereditary hemochromatosis⁴⁰ (mutation of a G to an A at nucleotide 845 creates a Rsa I site),^{158,159} the C677T methylenetetrahydrofolate reductase mutation¹⁶⁰ and mutations in the 21-hydroxylase gene.¹⁶¹ Standard gel electrophoresis was replaced by high-speed microfabricated capillary array electrophoresis in one PCR-RFLP analysis of hereditary hemochromatosis.¹⁶² When no restriction site is created by either the normal or the mutant sequence, a site can be created by PCR mutagenesis. This approach has been termed "amplification-created restriction site" (ACRS),¹⁶³ "artificial introduction of restriction sites" (AIRS),¹⁶⁴ and "PCR-mediated RFLP analysis".¹⁶⁵ Restriction enzyme digestion has also been utilized in an allele-specific amplification procedure termed "mutant-enriched PCR" (ME-PCR).^{137,166} In this method, an initial PCR is performed with low cycle number and the product is

incubated with a restriction enzyme that cleaves the wild-type sequence but not the mutant sequence (at least at one site). Primers internal to the cleavage site are then used to amplify the target in a second round of PCR using a high number of cycles. As the wild-type sequence is cleaved by the restriction enzyme, it does not hybridize with the internal primers and is not amplified, whereas the mutant sequence is, thus enriching the mutant amplicon. This procedure has been used to detect K-ras codon 12 mutations, with a reported sensitivity of 1 mutant cell in 1000 to 5000 normal cells (which represents a substantial improvement in sensitivity compared with normal PCR-RFLP analysis).¹³⁷

A. Performance Characteristics and Limitations of the Method

RFLP analysis was one of the earliest methods available for the detection of mutations or polymorphisms in DNA and has been a workhorse for a number of years. It is still a viable option for a number of applications and continues to be widely utilized. By far the most common method of analysis is gel electrophoresis. As mentioned above, this method is tedious and time-consuming and yields relatively low throughput. Automation is available but costly. However, high-speed DNA microfabricated capillary array electrophoresis chips¹⁶² and other capillary electrophoresis technologies have greatly simplified gel analysis. A past limitation of the RFLP method was that it could not be used if the mutation of interest did not create or destroy a restriction site. This has been solved with PCR mutagenesis, in which a restriction site can be artificially introduced. Regardless of the particular method used, restriction cleavage is slow and often incomplete, thus lowering sensitivity. In ME-PCR, one or two restriction steps are combined with ASA to enrich the amplification product for the mutant target, which then can be detected using the SSO technique. This procedure greatly improves matched-to-mismatched S/N, but it is tedious and time consuming.

IX. CONCLUSIONS

A wide variety of assays are available for the detection of mutations in nucleic acid targets. These assays can be generally separated into two categories: those used to scan for unknown mutations and those used to

detect known mutations. Assays for the detection of known mutations have been reviewed in this article, with the TMA/HPA method being described in detail. A summary of the various methods is given in Figure 4. Each assay has its particular strengths and limitations, and each is well suited for a particular set of applications and not for others. Accordingly, a number of assays are used in the research and clinical sectors for detection of mutations, as no one test can fulfill all requirements of all applications.

The TMA/HPA method exhibits a number of attractive characteristics: it is rapid, simple to perform, sensitive, and homogeneous (after sample processing, nothing is removed from the tube, thus simplifying the assay

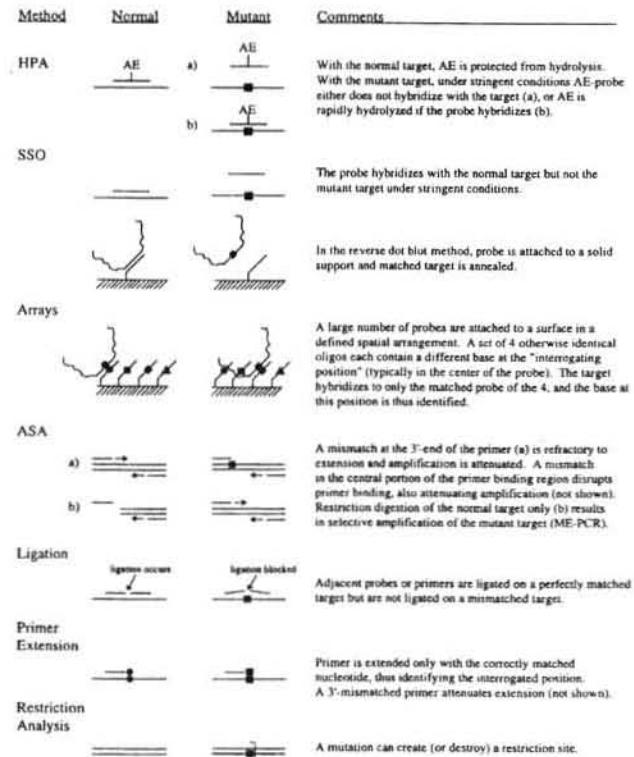


FIGURE 4. Methods for the detection of known mutations. The various methods available for the detection of known mutations are depicted schematically. The filled square symbol represents a point mutation.

and greatly reducing the risk of cross-contamination), yields high matched/mismatched S/N ratios, can detect all single-base mutations, and is amenable to high throughput applications and automation. Detection utilizes AE-labeled sequence-specific probes, and a different probe is synthesized for each target to be detected. With these characteristics the TMA/HPA method is particularly well-suited for high throughput screening assays for known mutations correlated with disease, including neonatal or population screening for such diseases as MCAD deficiency, sickle-cell anemia, and other hemoglobinopathies and hereditary hemochromatosis. Due to its simplicity and its ability to be performed in a multiplex format, this method could also be used for screening a larger number of mutations, such as a subset of HIV mutations or cystic fibrosis mutations. However, to screen for a very large number of mutations, such as the more than 1000 mutations that have been associated with cystic fibrosis, would not be feasible. Due to its high matched/mismatched S/N ratio, TMA/HPA is also well-suited for the analysis of diseases such as cancer and mitochondrial DNA disorders, where mutations can be present at a low percentage in a background of normal cells. It is also well-suited as a tool for research, such as screening for polymorphic markers in genetic research.

Several other methods for detection of mutations exist that possess attractive features as well. ASA and OLA/LCR can yield S/N ratios of 1000 and at these levels are valuable for detecting a wide variety of mutations. Many of these assays utilize gel electrophoresis for detection, which yields relatively low throughput. These types of assays are best suited for research applications and low volume diagnostic applications. Other protocols have been introduced that are simpler to perform and more rapid, which increases throughput and allow higher volume diagnostic testing to be carried out. In the ME-PCR method, ASA is combined with restriction digestion and SSO detection to increase S/N ratios to 5000. This high S/N ratio allows detection of mutations that are present at very low frequency in a background of normal target, as in the case of many cancers. DNA array technology can yield a huge amount of information about a target of interest, and this technique is best suited for low volume applications when highly complex targets with a large number of potential mutations need to be analyzed.

The tools for detection of genetic mutations are plentiful and of high quality. These tools should greatly aid the continued advancement of genetic research, the discovery and identification of new genetic mutations that are associated with human disease, and the rapid diagnosis of genetic disorders.

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