# 13

# Application of the Hybridization Protection Assay (HPA) to PCR

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## Introduction

The development of *in vitro* DNA amplification techniques has made detection of specific sequences more sensitive and rapid than ever before. The ability to amplify rare sequences has greatly improved our ability to detect chromosomal translocations, allelic variability, and infectious agents (Innis et al., 1990). Polymerase chain reaction (PCR) is a method of DNA amplification performed by repeatedly denaturing a DNA target, annealing specific oligonucleotide primers, and extending the primers with a DNA-dependent DNA polymerase (Mullis et al., 1986; Mullis and Faloona, 1987; Saiki et al., 1988b). Each cycle theoretically results in a doubling of the number of target sequences. Other amplification systems, such as the transcription-based amplification system, or TAS (Kwok et al., 1987), also give significant amplification of target sequences.

Because it often takes 2–10 times as long to detect amplified product as it does to perform the amplification reaction, rapid, simple, yet sensitive and specific detection systems are needed to allow researchers to take full advantage of the benefits of rapid target amplification, and to allow widespread use outside the research laboratory.

Methods for detection of amplification product generally fall into two categories: those that assay for extension of primers, and those that, by hybridization, assay for amplification of a sequence internal to the primers.

## Primer Incorporation Methods

The most common method used to detect extension of primers is based on detection of a specific size fragment. This is accomplished by gel electrophoresis and ethidium bromide staining, with or without restriction endonuclease digestion. Although widely used, it is recognized that spurious incorporation of primers does occur and nonspecific bands equal in length to the expected fragment can be generated. This is particularly a problem for samples that contain a high level of nontarget DNA, such as cell lysates. The method is also somewhat insensitive, with limits of detection 10- to 100-fold lower than methods using hybridization.

Other methods for detection of primer incorporation have been described (Chehab and

*The Polymerase Chain Reaction*
K.B. Mullis, F. Ferré, R.A. Gibbs, editors

Kan, 1989; Hayashi et al., 1989; Kemp et al., 1989; Triglia et al., 1990). These methods all require a step or series of steps to separate extended from unextended primers. In addition, these methods are highly dependent on the specificity of the primer incorporation as nonspecific incorporation is difficult to distinguish from specific incorporation. For these reasons, hybridization of a probe to the internal region of the amplification product is still considered the method of choice for confirmation of amplification of specific sequences (Abbott et al., 1988; Larzul et al., 1989).

## Radioactive Hybridization Detection Methods

A number of hybridization techniques have been used to confirm the identity of amplified products. By far the most common methods utilize $^{32}$P-labeled DNA probes that can provide sensitive detection of amplified product. These probes must be used in assay formats that physically separate the unhybridized and hybridized probe. In one commonly used assay, the amplification product is attached to a membrane such as nylon either by direct blotting (dot blots) or by transfer after size fractionation by gel electrophoresis (Southern blots) (Keller and Manak, 1989). Blots are hybridized, washed to remove the unhybridized probe, and detected by autoradiography. Quantitation is done visually or by densitometry. This process takes from 1 to 3 days.

Other methods utilize more efficient solution hybridization of amplified product and detection probe. These methods differ in the method used to differentiate hybridized and unhybridized probe. In the detection method described by Kwok et al. (1987), the products were hybridized to a $^{32}$P-labeled oligonucleotide in solution and then hybridized to a second oligonucleotide bound to Sephacryl beads. The beads were washed, centrifuged, and radioactivity measured in a scintillation counter. The authors reported that the method required about 4 hr to complete.

Syvanen and co-workers (1988) simplified this format by performing PCR with 5′-biotinylated primers. Following hybridization of the amplification product to a $^{32}$P-labeled DNA probe, the hybrids were collected on avidin-coated polystyrene particles. Radioactivity was determined following extensive washing steps.

Alternatively, Kwok and co-workers (1987) described an assay in which probe hybridized to product was subjected to restriction endonuclease digestion. The resulting fragments were separated by polyacrylamide gel electrophoresis and the appearance of a specific cleavage product was monitored. Final detection was by autoradiography. This method required several hours to complete.

## Nonradioactive Hybridization Methods

Nonisotopic detection systems are particularly attractive, as increased shelf-life and decreased biohazard are desirable. A number of hybridization formats utilizing nonradioactive detection probes have been reported. Both the Southern blot and dot blot formats have been modified to allow the use of detection probes labeled with digoxigenin (Carl et al., 1990), acetylaminofluorene (AAF) (Larzul et al., 1989), or horseradish peroxidase (Saiki et al., 1988a). In the latter, amplified DNA was attached to membranes and hybridized to oligonucleotide probes linked to horseradish peroxidase. This method has the advantage that the probes are already linked to the enzyme, but suffers from a lack of sensitivity. The authors reported that the nonisotopic method gave signals 1–10% of that observed with $^{32}$P-labeled probes after a 15-min autoradiographic exposure. The procedure allowed allele-specific detection to be achieved and took about 3 hr to perform. A similar procedure was used by Bugawan and co-workers (1988) to detect amplified HLA DQ and globin sequences. These authors reported better results with horseradish peroxidase-labeled probes than psoralen-biotinylated probes.

Gregerson and co-workers (1989) describe the use of biotinylated oligonucleotide probes for the detection of amplified $\alpha_1$-antitrypsin gene variant DNA attached to membranes. In a slightly different format, Keller and co-workers (1989) describe hybridization of the amplification product simultaneously to biotin-labeled detection probes and to capture probes attached to the surface of a microtiter dish. This latter method was used for the detection of amplified HIV-1 DNA sequences. Following hybridization and washing steps, hybrids were incubated with an enzyme conjugate and washed extensively prior to addition of the substrate, which allows color development to occur. Enzyme-labeled probes should theoretically give high sensitivity due to the accumulation of signal with time. However, backgrounds are also typically higher. Indeed, the authors mentioned difficulties finding conditions to completely inactivate nonspecific binding sites, which led to backgrounds higher than desired. In addition, lower sensitivity was observed with the nonisotopic probes compared to $^{32}$P-labeled probes. It was necessary to compensate by amplifying product for a larger number of cycles to allow adequate signal detection. This method took more than a day to complete, but allowed allele-specific detection, and so should be useful for those situations when specificity is important and sufficient target sequences are available such that exquisite sensitivity is not required.

A "reverse dot blot" format has been described for analysis of the HLA DQA locus genes following amplification (Saiki et al., 1989). In this procedure, PCR reactions were performed with biotinylated primers. Following amplification, PCR products were hybridized to oligonucleotide probes immobilized on a membrane and streptavidin–horseradish peroxidase conjugate was simultaneously bound. Detection was accomplished by incubation of the membranes with substrate to allow color development. The technique requires 1 to 2 hr to complete and was particularly suited to situations where the number of probes exceeds that of the number of samples to be analyzed.

Another nonisotopic hybridization format, referred to as the hybridization protection assay (Arnold et al., 1989; Nelson et al., 1990; Tenover et al., 1990), has been developed that utilizes DNA oligonucleotide probes directly labeled with a chemiluminescent acridinium ester. This format is completely homogeneous, not requiring any physical separation steps to distinguish hybridized and unhybridized probe, thus greatly simplifying the assay. The system exhibits excellent sensitivity and specificity, and the hybridization and detection steps can be completed in about 30–40 min.

We describe here the application of this assay format to the detection of amplified sequences from viruses, chromosomal translocations, and bacteria.

## Methods of Synthesis and Use of Acridinium Ester-Labeled Probes

Acridinium ester (AE) was synthesized as described previously (Weeks et al., 1983). Oligonucleotide probes were labeled by reacting the *N*-hydroxysuccinimide derivative of AE with a primary alkyl amine on a non-nucleotide-based phosphoramidite linker-arm (Arnold et al., 1988) introduced into the oligomer during DNA synthesis. The acridinium ester-labeled probe (AE probe) was then purified using high-performance liquid chromatography. Chemiluminescence was detected in a Leader I luminometer (Gen-Probe, San Diego, CA) by the automatic injection of 200 $\mu$l of 0.1% $H_2O_2$ in $HNO_3$ (5–400 m*M*), then 200 $\mu$l of 1 *N* NaOH (with or without surfactant). The measurement period was 2–5 sec; chemiluminescence was expressed as Relative Light Units, or RLU.

Amplifications were performed with AmpliTaq polymerase (Perkin-Elmer/Cetus) using the conditions recommended by the manufacturer. Hybridizations with AE probes were performed at 60°C in 0.05–0.1 *M* lithium succinate buffer, pH 5.2, containing 1–10% lithium laurel sulfate, 2–10 m*M* EDTA, and 2–10 m*M* EGTA. Hybridization volumes ranged

from 50 to 200 μl and incubation times ranged from 10 to 20 min. Differential hydrolysis was performed at 60°C in 0.15–0.20 *M* sodium tetraborate, 1–5% Triton X-100, pH 7.5–8.5.

Ester hydrolysis rates were determined as follows: AE probe (typically 0.1 pmol) was hybridized with an excess of target (typically 0.5–1 pmol). Aliquots of 15 μl containing approximately 100,000 RLU were placed in 100 μl of 0.2 *M* sodium tetraborate, pH 7.6, 5% Triton X-100, and incubated at 60°C. At various times, separate aliquots in individual tubes were removed and measured for chemiluminescence. The resulting data were plotted as log of percent initial chemiluminescence versus time; slopes and associated half-lives were determined by standard linear regression analysis.

The HPA procedure was as follows:

1. Denature—add target (typically in 10 μl) to the reaction tube and incubate at 95°C for 3–5 min.
2. Hybridize—add AE probe (typically in 50 μl) and incubate 10–20 min at 60°C.
3. Hydrolyze—add hydrolysis buffer (typically in 300 μl) and incubate 6 min at 60°C.
4. Detect—put reaction tube in luminometer and measure chemiluminescence for 2–5 sec.

## Characteristics of the Hybridization Protection Assay

The hybridization protection assay (HPA) format is centered around the highly chemiluminescent acridinium ester (AE) shown in Figure 13.1. The AE reacts with hydrogen peroxide under alkaline conditions to rapidly (2–5 sec) produce light at 430 nm, which is easily detected in a standard, commercially available luminometer. Detection is very sensitive, with a limit of approximately $5 \times 10^{-19}$ mol ($3 \times 10^5$ molecules) of AE, and is linearly quantitative over a concentration range of more than 4 orders of magnitude. The rapid reaction kinetics of the AE improve sensitivity since the short read time limits the contribution of background noise, and allow reading a large number of samples within a short time.

To be utilized in a DNA probe-based hybridization assay, the AE is covalently attached directly to the DNA probe using standard *N*-hydroxysuccinimide (NHS) coupling chemistry (Weeks et al., 1983) (Fig. 13.1). Direct labels greatly simplify assay formats since the "capping," binding, and washing steps required for indirect labels (those attached through a biotin/avidin interaction, for example) are not necessary, and separate reagent additions for label coupling or substrate addition are not necessary. An additional, very important aspect of the AE label is that the acri-

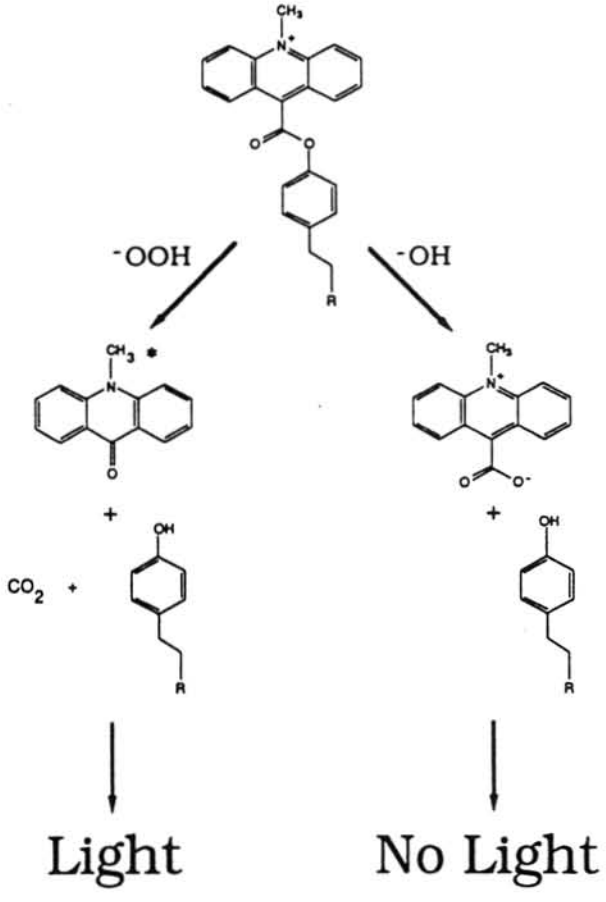


FIGURE 13.1. Structure and reaction pathways of acridinium ester. The *N*-methyl, phenyl acridinium ester reacts with alkaline peroxide to produce light, and with hydroxide to yield nonchemiluminescent ester hydrolysis products. The R group represents the *N*-hydroxysuccinimide (NHS) moiety, which reacts with primary amines, thus providing a means to specifically label DNA probes (see text); *electronically excited state.

dinium ring is cleaved from the DNA probe before light emission occurs (Fig. 13.1), thus minimizing intramolecular quenching (Weeks et al., 1983). To provide a site of attachment of the AE within a deoxyoligonucleotide probe, nonnucleotide based alkyl amine linker-arms have been developed, which can be incorporated at any location in the probe during standard phosphoramidite synthesis (Arnold et al., 1988). The amine is reacted with the NHS derivative of AE, yielding a DNA probe with a chemiluminescent AE directly attached through a covalent amide bond.

Acridinium ester attached to a DNA probe displays the same reaction kinetics as the free label, and is detected as sensitively and quantitatively as free chemiluminescent label (Fig. 13.2), demonstrating that performance is not compromised by attachment to the probe. Furthermore, AE probes display hybridization characteristics (thermal stability, rate and extent of hybridization, and specificity) essentially equivalent to their $^{32}$P-labeled counterparts, demonstrating that attachment of the AE label does not compromise hybridization performance. Additionally, hybridization and detection of AE probes can also be performed in the presence of relatively large amounts of clinical specimen material.

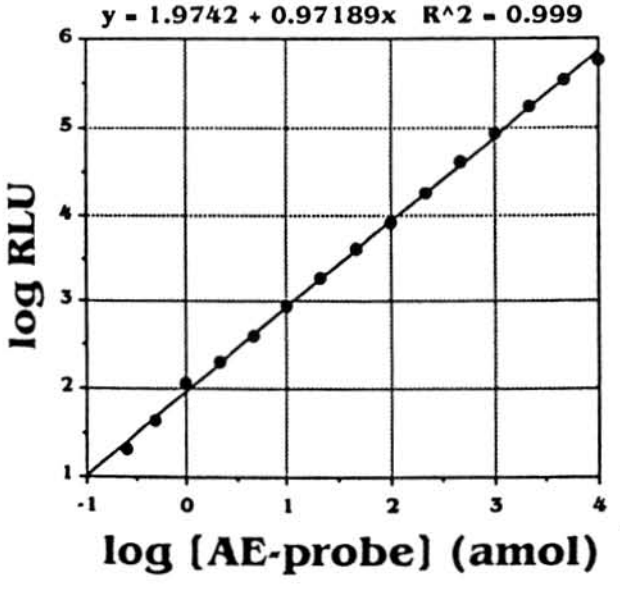


FIGURE 13.2. Sensitivity of detection of acridinium ester-labeled DNA probe. AE probe (see text) was serially diluted and measured for chemiluminescence as described in the text. Each point represents the average of four replicates: background was about 50 relative light units (RLUs) (subtracted from data before plotting). The equation of the line (obtained from regression analysis) appears above the graph.

Several rapid and simple formats have been developed that use AE probes for detecting target DNA or RNA sequences, and a number of these have been incorporated into commercially available assays (Gegg et al., 1990; Granato and Franz, 1989; Kranig-Brown et al., 1990; Rubin et al., 1990; Snider et al., 1990; Tenover et al., 1990; Watson et al., 1990). In these formats, the hybridization and detection reactions were performed in solution, which offers significant advantages compared to standard target immobilization techniques (Keller and Manak, 1989), including faster hybridization kinetics, availability of all the target molecules for hybridization, much better quantitation, fewer steps and less complexity, and much shorter time to result. This in-solution approach is particularly well suited to the analysis of PCR products, because a portion of the amplified sample can be analyzed directly with no further manipulations such as blotting, electrophoresis, binding, or washing steps, which are not only time consuming, but can also result in spread of amplification product throughout the laboratory.

The hybridization protection assay is a completely homogeneous format requiring no physical separation for the discrimination of hybridized and unhybridized AE probe. This format is based on differential chemical hydrolysis of the ester bond of the AE molecule (hydrolysis of this bond renders the AE permanently nonchemiluminescent, as shown in Fig. 13.1). The system is designed such that the rate of hydrolysis of the AE attached to unhybridized probe is rapid, whereas the rate of hydrolysis of AE attached to probe that is hybridized with its target nucleic acid is slow. By adjusting reaction chemistry, the chemiluminescence associated with unhybridized probe is rapidly reduced to low levels, whereas the chemiluminescence associated with hybridized probe is minimally affected. Thus, after this differential hydrolysis process, the

remaining chemiluminescence is a direct measure of the amount of target present.

An example of differential hydrolysis of hybridized and unhybridized probe is shown in Figure 13.3, which illustrates loss of chemiluminescence with time due to ester hydrolysis. From linear regression analysis, the half-lives for hydrolysis were determined to be 53.6 and 0.96 min for hybridized and unhybridized probe, respectively. The theoretical percent remaining chemiluminescent label after a given hydrolysis time can be calculated using the equation

$$(0.5)^{T/t_{1/2}} \times 100 = \text{percent remaining chemiluminescence}$$

where $T$ is the elapsed time of differential hydrolysis, and $t_{1/2}$ is the half-life of loss of chemiluminescence. Using the half-life values given above, the calculated values for remaining chemiluminescence after a 20-min differential hydrolysis step would be 77% for hybridized probe and 0.00005% for unhybridized probe. This is greater than a one million-fold discrimination between hybridized and unhybridized AE probe, in 20 min, with the addition of a single reagent and without any physical separation.

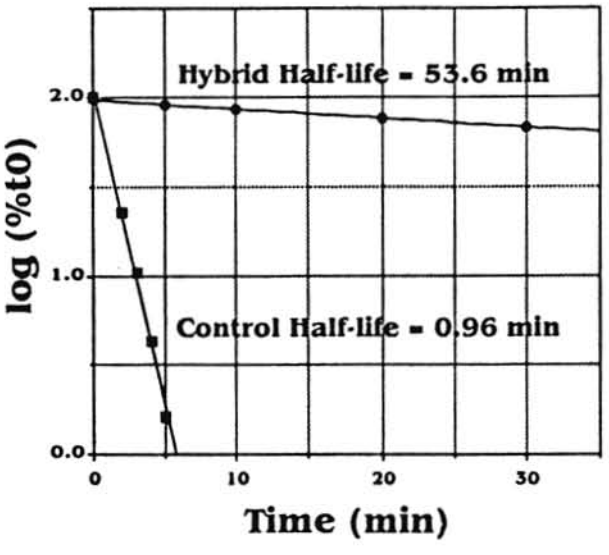


FIGURE 13.3. Differential hydrolysis of hybridized and unhybridized acridinium ester-labeled DNA probe. Rates of acridinium ester hydrolysis were measured for hybridized and unhybridized AE probe. Results were plotted as log of percent initial chemiluminescence versus time, and half-lives of hydrolysis were determined by standard linear regression analysis.

## Application of HPA to the Detection of PCR-Amplified Products

The HPA format has been utilized to specifically detect a variety of PCR amplification products. In one application (Kacian et al., 1990), an overlapping pair of AE-labeled oligonucleotide probes (AE probes) specific for sequences within the *gag* gene of human immunodeficiency virus-1 (HIV-1) amplified by the SK38/SK39 primers described by Ou and co-workers (1988) were designed and synthesized. The performance characteristics of this AE probe pair in the HPA format were first evaluated utilizing known amounts of M13 cloned HIV target DNA (Fig. 13.4A). Detection was linearly quantitative over a concentration range of four orders of magnitude, with a limit of sensitivity of about $10^{-16}$ mol of target.

The detection of PCR-amplified HIV-1 DNA using the HPA format was next evaluated, the results of which are shown in Figure 13.4B. The assay detected serial dilutions of the final PCR product over a range of more than three orders of magnitude. In a separate experiment, an input of three genome equivalents of HIV-1 DNA was easily detected with a signal 22 times over background (data not shown). Ou and co-workers (1990) reported the detection of HIV-1 proviral DNA amplified in the presence of cell lysate containing a constant amount (1 fg) of human genomic DNA utilizing the HPA system described here. They observed a dynamic response over approximately three orders of magnitude of input HIV-1 DNA, with a sensitivity of about four copies. They compared the HPA detection format with a radioisotopic method utilizing a $^{32}$P-labeled SK19 probe (solution hybridization, restriction enzyme digestion, gel electrophoresis, and autoradiography) and found the HPA format gave equivalent (if not more sensitive)

results to the $^{32}P$ method, and the HPA method was complete in less than an hour as compared to the full day required to complete the $^{32}P$ assay. They also performed a comparative study using PCR-amplified DNA from the peripheral blood mononuclear cells of HIV-seropositive and HIV-seronegative patients, and again found the HPA format to perform at least equivalently to the $^{32}P$ method, yet take a small fraction of the time to complete.

In another application of the HPA format, the chromosomal translocation product known as the Philadelphia chromosome (Adams, 1985), which is associated with chronic myelogenous leukemia (CML), was amplified and detected (Arnold et al., 1989). In this study chimeric messenger RNA from K562 cells, which carry the Philadelphia chromosome, was converted to DNA and then amplified using PCR. Dilutions of the product were then detected with a DNA probe specific for the Philadelphia chromosome using either the HPA format with AE probe or a standard Southern analysis (Southern, 1985) with $^{32}P$ probe (Fig. 13.5). The Southern blot was sensitive to the 125-fold dilution after a 2.5-hr autoradiographic exposure (a band appeared at

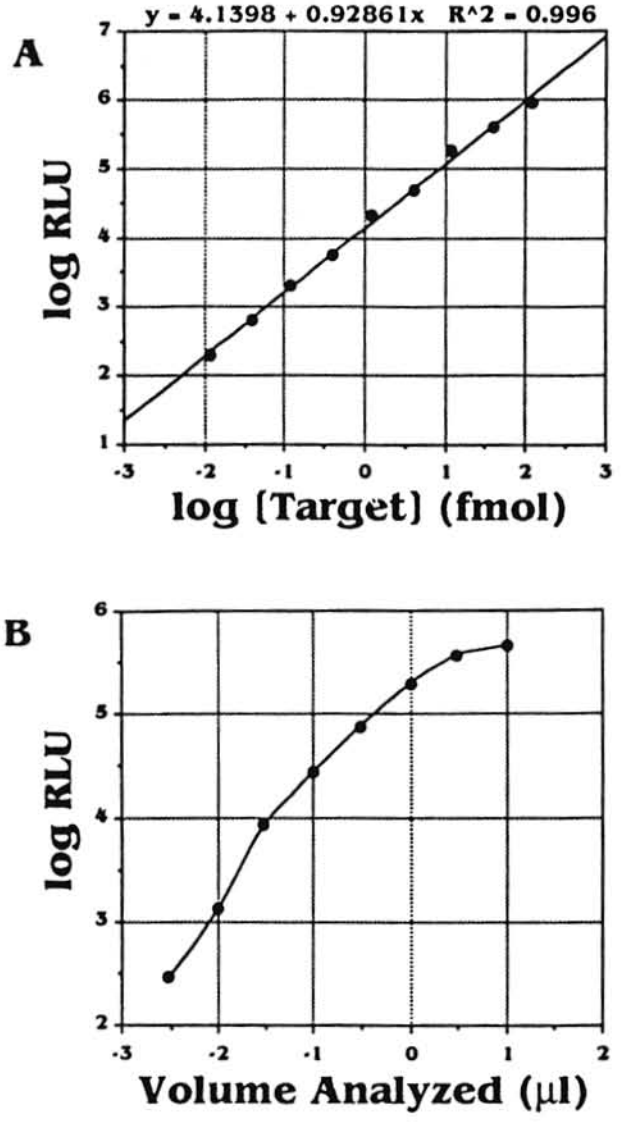


FIGURE 13.4. Use of the HPA format for the detection of PCR amplified HIV-1 DNA. AE probes specific for the *gag* region of HIV-1 (see text) were used to detect HIV-1 DNA. (**A**) Decreasing amounts of M13 cloned HIV-1 DNA were assayed directly using the HPA format. The equation of the line (obtained from regression analysis) appears above the graph. (**B**) HIV-1 DNA was PCR amplified (SK38/SK39 primer set; 30 cycles), and serial dilutions were then assayed using the HPA format as described in the text.

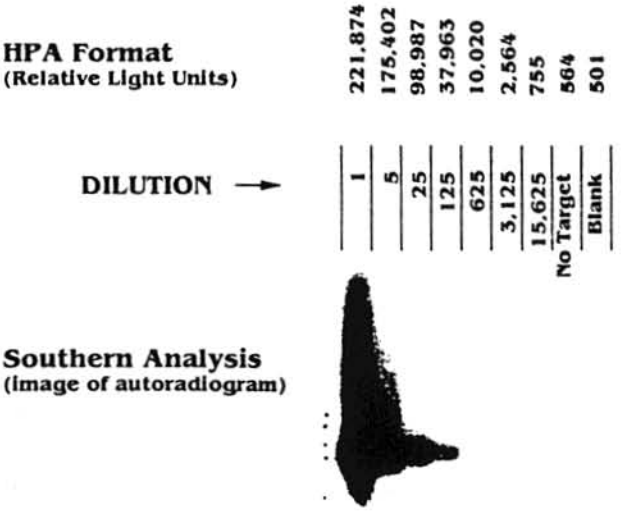


FIGURE 13.5. Comparison of the HPA and Southern blot formats for the detection of PCR amplified Philadelphia chromosome. The Philadelphia chromosome (which is associated with chronic myelogenous leukemia) was amplified from K562 cells and the product was serially diluted in 5-fold increments and detected using either an AE probe in the HPA format or a $^{32}P$ probe in a standard Southern blot format. The HPA format was performed as described in the text (3 min denaturation, 10 min hybridization). For the Southern analysis, probe was 5′-end-labeled with $^{32}P$ immediately before use (specific activity = $3.6 \times 10^6$ cpm/pmol) and $3 \times 10^8$ cpm/ml were used in the hybridization. Autoradiography was performed at −80°C with intensifier screens for 2.5 hr.

the 625-fold dilution after overnight exposure). In the HPA format, the 3125-fold dilution gave a strong, positive signal, and the 15,625-fold dilution gave a significantly greater signal over blank (254 RLU) than the "No Target" sample over blank (63 RLU). Furthermore, the Southern analysis required 2 days to perform (prior to autoradiography) whereas the HPA was complete in less than 30 min.

The HPA has also been used to detect amplified hepatitis B virus (HBV) DNA. Dilutions of cloned HBV (serotype adw) as well as plasma positive for HBV surface antigen (sAg) were subjected to 30 rounds of PCR using the 109/585R primers (SAg region) described previously (Kaneko et al., 1989) and amplification product was assayed using the HPA format (Fig. 13.6). A wide dynamic response, good sensitivity (signal to background ratio of 26 at 6 genomes input), and the ability to perform the assay in the presence of clinical sample were again observed. Comparing these data with the results of PCR detection of HBV reported by Kaneko and co-workers (1989), the HPA format was equivalent in sensitivity to normal PCR coupled with Southern blot analysis or a "double-PCR" method coupled with ethidium-stained agarose gel detection, and 103-fold more sensitive than normal PCR coupled with ethidium staining of gels. Specificity was also equivalent in the HPA and Southern blot formats, whereas in the ethidium staining procedure specificity of amplified bands had to be confirmed by Southern blot analysis. Furthermore, the HPA format was more rapid and easier to perform.

Other PCR-amplified sequences that have been detected using the HPA format include segments of the genes that code for the 16 S ribosomal RNA (rRNA) subunit of *Neisseria gonorrhoeae* (Gegg et al., 1990) and the 23 S rRNA subunit of *Chlamydia trachomatis* (Kranig-Brown et al., 1990), the 7.5-kB cryptic plasmid of *Chlamydia trachomatis* known as pCHL1, the HIV-1 envelope region, and the HIV-2 viral protein X region. The HPA format has also been applied to other systems, including the targeting of rRNA, which provides a natural target amplification (each bacterial cell contains up to 10,000 rRNA copies) as well as increased specificity since rRNA sequences are excellent phylogenetic markers (Enns, 1988; Kohne, 1986; Woese, 1987). Commercially available HPA applications utilizing rRNA targets include detection of *Campylobacter jejuni* (Tenover, 1990), *Haemophilus influenzae* (Snider et al., 1990), and several other organisms isolated in culture, detection

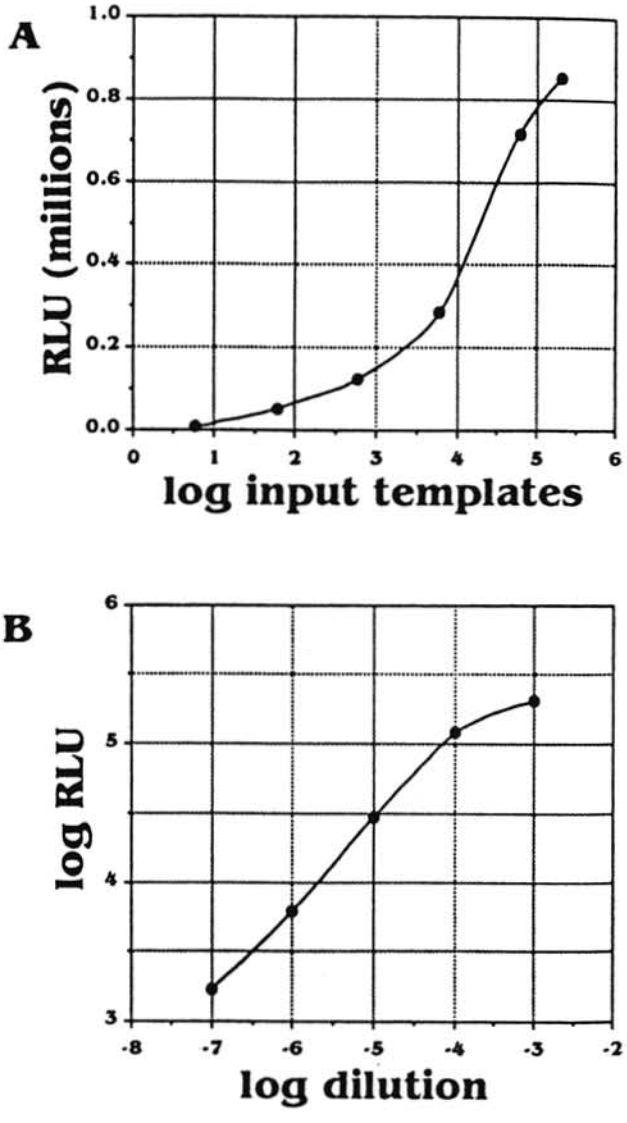


FIGURE 13.6. Use of the HPA format for the detection of PCR amplified HBV DNA. (**A**) HBV cloned DNA was amplified using PCR as described in the text in the absence of clinical sample. (**B**) Plasma positive for HBV surface antigen was diluted in normal serum, alkali treated, neutralized, and amplified by PCR. All products were analyzed by the HPA format utilizing a mix of 2 overlapping AE probes specific for the surface antigen region of HBV.

of bacteria and yeast in urine samples (Watson et al., 1990), and detection of sulfate-reducing bacteria and all bacteria in a research assay. The differential hydrolysis aspect of the HPA has also been combined with a rapid magnetic separation procedure (Arnold et al., 1989) for the detection of *Chlamydia trachomatis* (Kranig-Brown et al., 1990) and *Neisseria gonorrhoeae* (Gegg et al., 1990; Granato and Franz, 1989) directly in patient specimen (urogenital swab in this case). The HPA format has also been used to detect single site mismatches (Arnold et al., 1989).

The HPA format compares favorably with other methods for the detection of PCR amplification products. Sensitivity of the assay is comparable to or better than the other commonly used methods, including those utilizing $^{32}P$. The HPA format exhibits the excellent specificity of detection afforded by other hybridization-based methods such as Southern blots, dot blots, and the $^{32}P$-oligomer:restriction method (Kwok et al., 1989). One distinct advantage of the HPA format is the ease of use and short time to result. In all of the applications cited, the HPA format consisted of the same three steps: (1) solution hybridization (10–20 min; a brief heat denaturation step prior to hybridization is required in most PCR applications), (2) differential hydrolysis (6–10 min), and (3) detection (2–5 sec). This procedure is simpler and faster than any of the other techniques commonly in use, yet retains high sensitivity and the specificity of a hybridization-based procedure. Additionally, the procedure is nonisotopic, thus avoiding the biohazards associated with radioisotopes and does not include steps such as washing, which result in spread of target through the laboratory.

In conclusion, the HPA format is a simple, rapid, sensitive, and specific method for detection and quantitation of amplified DNA from a number of sources.

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