# Advanced methods in PCR product detection.

J G Lazar

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# Advanced Methods in PCR Product Detection

James G. Lazar

Digene Diagnostics, Inc.,
Silver Spring, Maryland 20904

To utilize PCR to its fullest potential, the identification and measurement of PCR products, or amplicons, must be improved compared with traditional methods. Amplicons must be identified by their specific nucleotide sequence to prevent false or ambiguous results caused by primer–dimer formation, nonspecific amplification, or target sequence variation. Quantitation of amplicon levels is an important first step for the estimation of the relative number of initial target molecules.

Until several years ago, PCR was usually performed on clean model systems or on specimens that had been highly purified. Gel-based detection methods worked well for these applications when sample throughput, time, and per-sample costs were not important issues. Over the past several years, PCR has become a mainstream technique, moving from the realm of the molecular biology laboratory to a wide variety of fields that are testing large numbers of samples and a wide variety of sample types. New detection methods are needed that meet the requirements of these users.

## TRADITIONAL DETECTION METHODS

Gel-based methods for the detection of PCR products have a number of drawbacks. Agarose gel electrophoresis with ethidium bromide staining is simple and inexpensive but suffers from a lack of sensitivity and specificity. Typically, at least 10 ng of full-length PCR product in a 5-μl volume must be loaded onto a gel so that a distinct band will be visible against a clean background. For a 200-bp PCR product, this amount translates to nearly $5\times10^{10}$ full-length product molecules and would require an amplification of $5\times10^{9}$ for detection of 10 initial target molecules in a 100-μl amplification reaction. Achieving this level of amplification would require 32 amplification cycles of 100% efficiency, a level of amplification that may be nearly achievable in a clean model system but is rarely achievable in practice with experimental or clinical samples.

Specificity may also be a significant problem with ethidium bromide-based detection methods. Often, PCR products from clinical or biological samples appear on the gel as a number of bands or as a smear. In these cases, it is difficult to determine whether the correct size band has been generated. To overcome this problem, two alternative gel-based methods have been used.

In one method, PCR products in an agarose gel are transferred to a membrane by standard blotting techniques and are subsequently detected with a labeled probe of specific sequence.[1] With proper hybridization conditions, this technique is sequence specific and will not detect nonspecific amplification products. However, in some instances, a significant amount of the PCR product is not full length, and the results may be ambiguous because the detected products will still appear as a smear. The sensitivity of blotting methods may vary, depending on the specific label and the detection method used. Radioactive labels or enzymatic labels coupled with chemiluminescent detection usually give the most sensitive results, typically, one to three orders of magnitude better than ethidium bromide staining. Blotting methods are also laborious and time consuming, and radioactive labels pose significant disposal problems.

An alternative to blotting-based methods is nested-primer amplification, followed by agarose gel electrophoresis and ethidium bromide staining. With this technique, specificity and sensitivity are enhanced by the amplification conditions while the detection method itself remains insensitive and nonspecific. With the nested-primer technique, a first round of amplification is performed in a normal way. A portion of the first-round amplification reaction is then used as the sample in a second round of amplification using primers that are internal, or nested, to the first set. Although the sensitivity of

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agarose gel detection is low, the overall sensitivity is high, because the target has been subjected to two rounds of amplification. The specificity of amplification is also enhanced because the primers used in the second round of amplification will only amplify the first-round amplicons. The disadvantages of this method are the lengthy and tedious amplification procedures, the cost of amplification reagents, the higher probability of amplicon contamination during the seeding of the second round of amplification, and the inability to utilize the dUTP/UNG procedure to prevent carryover contamination.[2]

## ADVANCED DETECTION METHODS

Advanced detection methods must provide significant advantages over traditional methods to justify the time, effort, and cost involved in converting to a new technology. Implementation of a new detection method into a laboratory will typically require significant investments in time to learn the new technology and to validate the performance of the new method against the currently used method. Moreover, advanced detection methods may require significant investment in capital equipment and the purchase of expensive consumables and reagents.

To overcome these drawbacks, advanced detection methods must exhibit superior sensitivity and specificity compared with traditional methods. Ten or fewer input target copies should be detectable after one round of amplification, and the detection should be sequence specific. Primer–dimers and other nonspecific amplification products should not be detected, and to ensure against carryover contamination, all detection methods should be capable of incorporating the dUTP/UNG decontamination procedure.[3]

Advanced detection methods should be easy to use, require little or no specialized training, and minimize tedious and laborious procedures. The detection method should require no purification of the PCR reaction products, require as little hands-on time as possible, and be amenable to automation with currently available equipment. Flexible throughput is desirable so that small or large batch sizes can be run without wasting reagents or consumables.

The total cost of an advanced detection method should be competitive with traditional detection methods, notwithstanding performance considerations. One-time costs are usually limited to capital equipment expenditures such as readers, shakers, and incubators—equipment that may already be available in many laboratories. Recurring costs include reagents, disposables, and labor. With advanced detection methods, however, cost savings may be realized from increased productivity, reduced hands-on time, higher sample throughput, fewer repeat amplifications because of ambiguous results, and reduced cost of amplification reagents if converting from nested-primer methods.

Universality is an important property of a detection method for most laboratories. A universal detection system uses common equipment, reagents (except sequence-specific components such as primers and probes), and a common procedure for detecting PCR products. This feature minimizes the equipment and number of different reagents that laboratories must purchase and store. A universal detection procedure makes detection of new analytes quick and facile. Another important characteristic of a universal detection system is that multiple targets can be detected in the same assay, a feature that is especially important to small-volume users.

Advanced detection methods can be developed in-house from numerous reports found in the literature. Advantages of using in-house methods are lower reagent costs and a higher level of technical knowledge about the assay that comes from hands-on development. Conversely, the laboratory may be

left with little or no technical expertise in the production, validation, or performance of the in-house assay if the individuals responsible for the development leave the laboratory or institution. Moreover, the reagents prepared for use in an in-house assay require additional in-house quality control and quality assurance programs on a routine and ongoing basis.

Commercially available detection methods offer several distinct advantages over in-house assays. Reagents in commercial kits have already been validated for their intended use, the laboratory does not need to develop a reagent quality control or quality assurance program, and the supply of reagents is usually reliable and consistent. The manufacturer often supplies defined protocols, and lot-to-lot or kit-to-kit variability is controlled, giving enhanced reproducibility. An additional convincing argument for using a commercially available detection method is the technical support that is available with the purchase of a commercial advanced detection system. Support should include detailed protocols, hands-on training, support literature, applications notes, and troubleshooting.

ELISA-based detection methods offer the most promising alternative to gel-based detection methods because ELISA assays are relatively standardized, and ELISA techniques are familiar to most laboratory personnel throughout the world. In addition, a wide range of materials, equipment, and supplies are commercially available to support ELISA-based technology, and many laboratories are already thoroughly equipped to run ELISA-based assays. Other advanced detection methods based on alternative technologies, such as high-performance liquid chromatography (HPLC) or capillary electrophoresis, are currently in limited use or under development. In this chapter, a number of in-house and commercially available advanced detection systems will be discussed with emphasis placed on ELISA methods.

## ELISA DETECTION METHODS

### Oligonucleotide-based Detection Methods

Numerous new methods for detection of PCR products are found in the literature.[4–11] Two common detection methods use specific oligonucleotide sequences to either capture or detect the PCR amplicons.[12–14] In both formats, the PCR mixture contains one biotinylated primer and one unmodified primer. During amplification, the biotin-labeled primer is incorporated in the amplicon. In the oligonucleotide probe format (Fig. 1) the amplicon is denatured and hybridized to an enzyme-labeled oligonucleotide probe.[15] The reaction mixture is simultaneously captured onto a streptavidin-coated plate, excess labeled oligonucleotide is washed away, and the enzyme label generates a signal with an appropriate substrate.

In the oligonucleotide capture format (Fig. 2) the amplicon is denatured and hybridized to a sequence-specific capture oligonucleotide that is prebound to a solid phase. Sequences that are not captured are washed away, and captured sequences are detected through the biotin label with enzyme-labeled streptavidin.

Both of these formats are straightforward and relatively simple to design and construct in either a blot or microplate format. Both formats are limited in sensitivity by the fact that only a few enzyme labels are bound per amplicon. Moreover, optimal hybridization conditions may vary significantly among different oligonucleotide probes, because of sequence variations within the hybridization region. As a consequence, genotypically similar strains of an organism may be captured or detected with dissimilar efficiency because of minor sequence variation, thus making relational quantitation difficult.

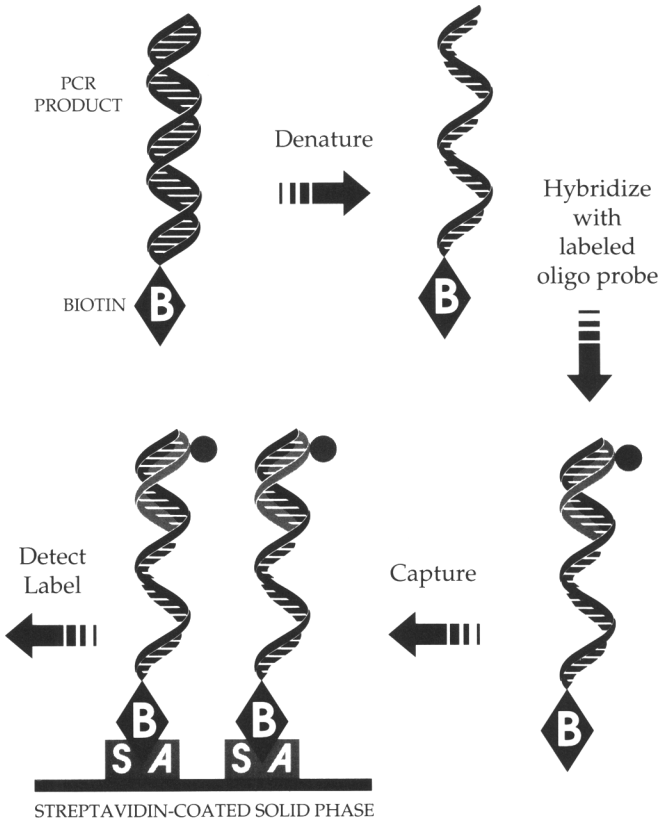


**FIGURE 1** Oligonucleotide probe format. After amplification, the PCR product is denatured and hybridized to a labeled oligonucleotide. Hybrids are captured onto a streptavidin-coated plate, and the label generates a detectable signal. This format is utilized in many detection assays including the QPCR System 5000.

## Immunological Detection Methods

An elegant ELISA method for the detection of PCR products has been described by Yolken that utilizes full-length RNA probes, solution hybridization, and a monoclonal antibody specific for RNA/DNA hybrids.[16,17] Biotinylated RNA probes complementary to the sequence of interest are transcribed from template DNA and labeled by the addition of biotinylated nucleotide triphosphate to the transcription reaction. PCR is performed with unmodified primers, and a portion of the PCR reaction is hybridized in solution with the biotinylated RNA probe. After hybridization, RNA/DNA hybrids are captured onto a microplate coated with anti-biotin antibodies. The captured hybrids are subsequently detected with a β-galactosidase conjugate of a monoclonal antibody specific for RNA/DNA hybrids, and the β-galactosidase label is detected with a substrate that produces a fluorescent signal.

This detection system is sensitive because multiple antibodies, and hence multiple enzyme labels, can bind to each captured hybrid. Specificity is also enhanced because the formation of RNA/DNA hybrids is favored at the hybridization temperature of 75°C. Because the RNA probes are complementary to the whole length of amplicon, small sequence variations that occur between different strains of the same species will have negligible effect on hybridization or detection efficiency.[18]

## SHARP Signal System

The SHARP Signal System (Digene Diagnostics, Inc.) is a capture ELISA assay that also utilizes an antibody to RNA/DNA hybrids (Fig. 3).[19] With the

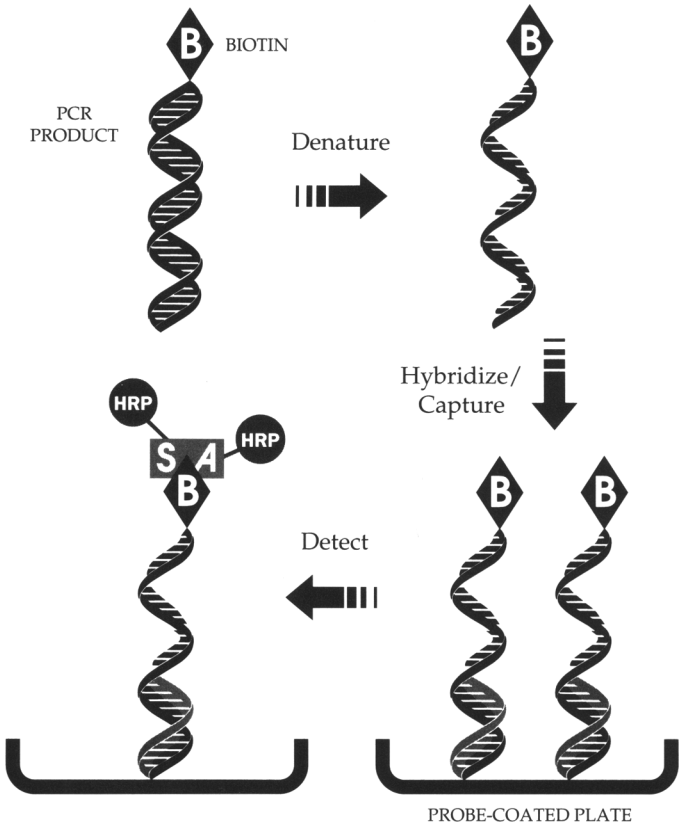


**FIGURE 2** Oligonucleotide capture format. After amplification, PCR products are denatured and captured by an oligonucleotide probe bound to a plate (solid phase). Captured nucleic acids are detected by a label incorporated into the PCR product. This format is utilized by the AMPLICOR, EnviroAmp, and HybriQuick assays.

SHARP Signal System, PCR is performed using one biotinylated and one unmodified primer. After amplification, a portion of the reaction mixture is denatured and then hybridized in solution to a complementary unlabeled RNA probe. The RNA/DNA hybrids thus formed are captured onto a streptavidin-coated microplate and detected with an alkaline phosphatase-conjugated antibody specific for RNA/DNA hybrids. After washing, signal is generated with a colorimetric substrate and read on a conventional microplate reader at 405 nm. The SHARP Signal assay procedure is summarized in Figure 4. In most applications, overall time to results is <4 hr, with hands-on time of ~1 hr. In one laboratory, the SHARP signal assay has been automated with standard ELISA robotic equipment and is currently being used for rapid PCR detection of human papillomavirus (HPV) in cervical specimens with excellent results.[20]

The SHARP Signal assay is quite sensitive because multiple antibodies, and hence multiple enzyme labels, can react with each captured hybrid. In a model system, the assay has been shown to be at least 100 times more sensitive than ethidium bromide staining of PCR products in agarose gels and can detect ~10 pg of biotinylated PCR product per well (Fig. 5). The detection assay is extremely flexible and easy to adapt to any target sequence because the RNA probes are unlabeled and can be easily produced by transcription of plasmid DNA containing a T7, SP6, or T3 RNA polymerase promoter. Alternatively, a promoter can be incorporated into a PCR primer, and after amplification with target DNA, the resulting PCR product can be used as the transcription template.[21,22]

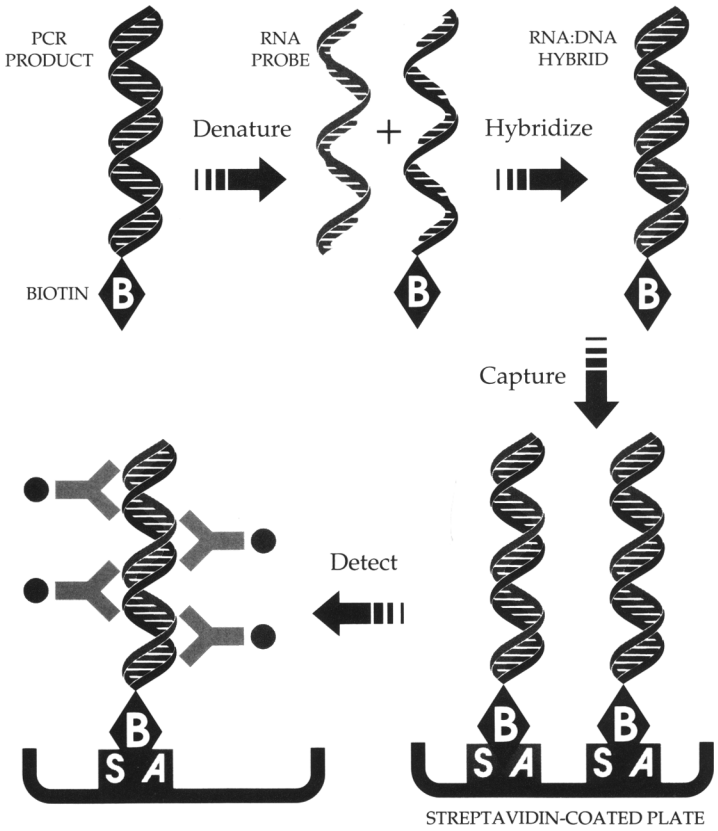


**FIGURE 3** The SHARP Signal System. PCR products are denatured and hybridized to unlabeled RNA probes. RNA/DNA hybrids are captured onto a streptavidin-coated plate and detected with an enzyme-conjugated antibody specific for RNA/DNA hybrids.

The performance of the SHARP Signal System has been evaluated in a model system with several target analytes.[23] In one study, plasmid DNA containing the 5′-noncoding region of hepatitis C virus (HCV) was serially diluted in buffer containing sheared herring sperm DNA. Aliquots containing 1 μg of sheared herring sperm DNA and 0, 10, $10^2$, $10^3$, $10^4$, and $10^5$ copies of the HCV sequence were PCR amplified for 35 cycles with primers directed against the HCV 5′ noncoding region (the sense primer was biotinylated on the 5′ end). A 5-μl aliquot of each amplification reaction was analyzed by ethidium bromide gel analysis and by the SHARP Signal System. The results are summarized in Figure 6. The data show that 10 input copies are detectable and that the signal generated is proportional to the number of input copies.

In a recent study using clinical specimens, the SHARP Signal System was compared with a nested-primer method for the detection of HCV in serum.[24] Specimens were processed by standard methods to preserve the RNA and to remove PCR inhibitors. cDNA copies of the RNA were prepared by a reverse transcriptase reaction using an antisense primer and the GeneAmp RNA PCR kit (Perkin-Elmer). One portion of the samples was then amplified with a single primer set containing one primer labeled with a 5′ biotin, and another portion of the samples was amplified using the nested primer method. The samples amplified with the single primer set were analyzed by the SHARP Signal System after one round of amplification. The samples amplified with the nested-primer technique were analyzed by ethidium bromide gel after the second round of amplification. Of the 99 clinical specimens tested, 47 were positive and 50 were negative by both methods. The remaining two samples were nested-primer positive, SHARP Signal System negative.

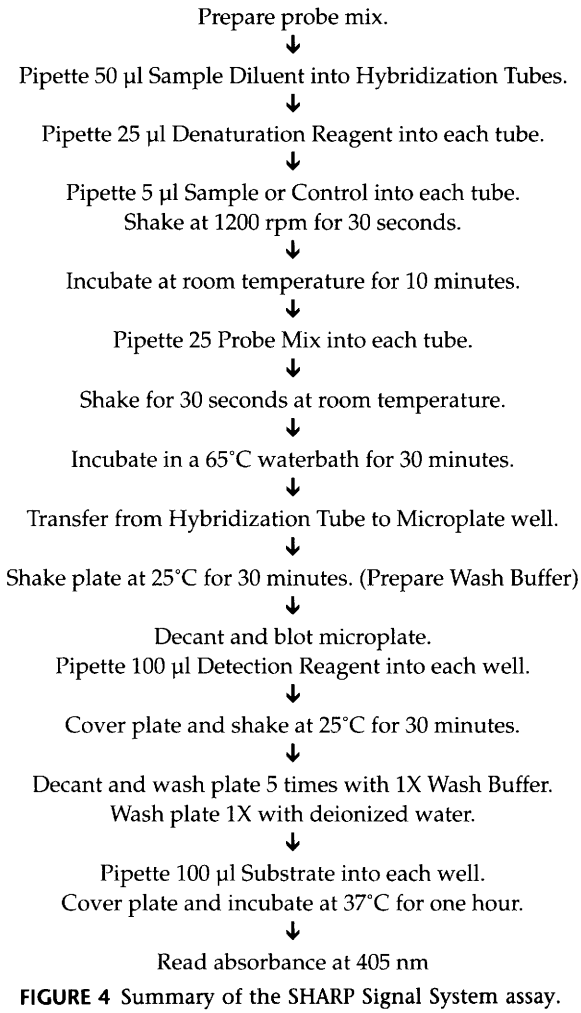


**FIGURE 4** Summary of the SHARP Signal System assay.

Overall, the SHARP Signal System exhibited 98% agreement with the nested-primer method.

The SHARP Signal System assay for PCR products provides sufficient reagents to perform 192 tests in an 8-well strip, 96-well microplate format. Minimal reagent preparation is necessary. A control probe and positive and negative assay controls are provided so that the user can verify assay performance. RNA probes, primers, and detection controls for the amplification and detection of specific infectious disease targets such as human immunodeficiency virus (HIV), HCV, hepatitis B virus (HBV), HPV, *Mycobacterium tuberculosis* (Mtb), and cytomegalovirus (CMV) are currently available, and additional targets are under development. The methodology of the SHARP Signal System allows for maximum flexibility because the same reagents, except for the RNA probe, are used for all assays and the detection procedure is universal for all targets. Thus, in a single assay on a single capture plate, a user can test for several analytes simultaneously.

Use of unlabeled RNA probes may be superior to conventional DNA probes for several reasons. (1) RNA/DNA hybrids are more stable than DNA/DNA hybrid structures, thus allowing the use of higher temperatures to increase the stringency of probe/target association and to favor probe/target hybridization over reannealing of the target strands.[18] (2) A posthybridization RNase digestion increases the specificity of detection and reduces background by removing free or nonspecifically bound RNA probe. (3) Strand-specific

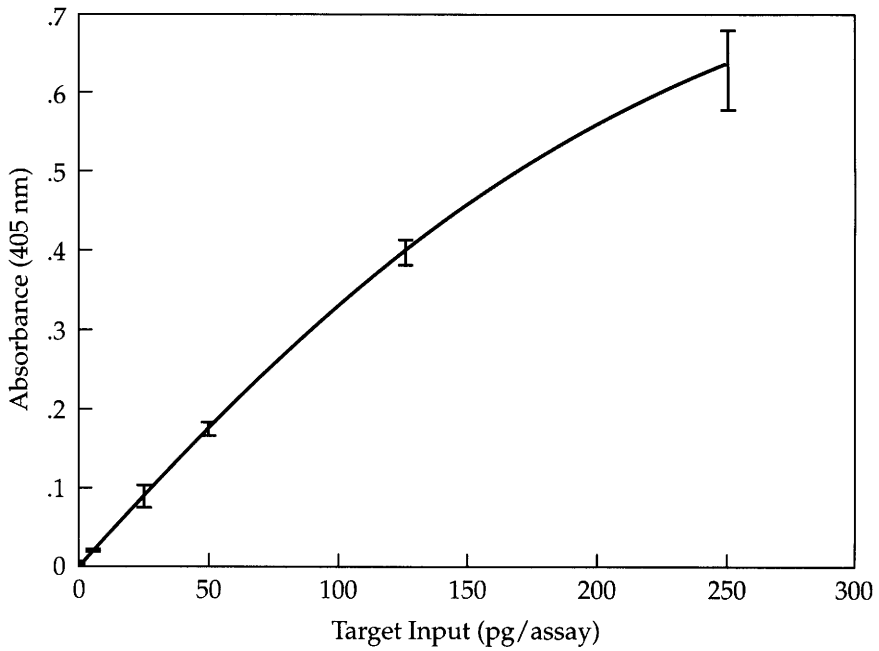


**FIGURE 5** Sensitivity of the SHARP Signal System with 95% confidence intervals. PCR product from the L1 region of HPV-16 was quantitated, diluted, and tested in the SHARP Signal System.

single-stranded probes are more sensitive and efficient in detecting target sequences because of a lack of competition from probe reassociation.

The flexibility of the system is further increased because the assay uses easily produced unlabeled RNA probes allowing users to develop their own probe/primer set as outlined by the protocol shown in Figure 7. A PCR product containing the target sequence of interest can be directly cloned into a plasmid containing a T7 RNA polymerase promoter.[25] After plasmid DNA

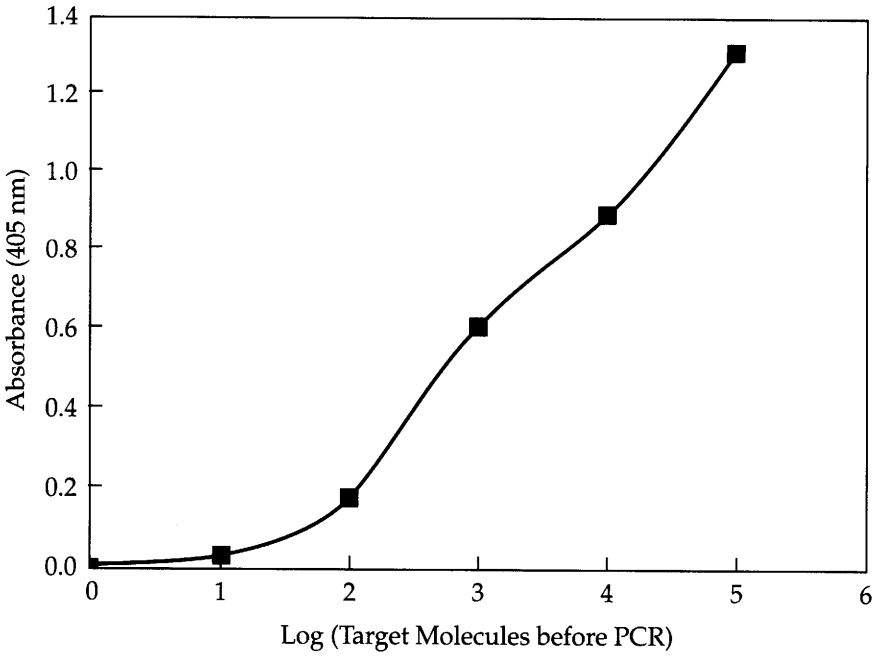


**FIGURE 6** SHARP Signal System. Detection of amplified plasmid DNA containing the HCV 5′-noncoding region.

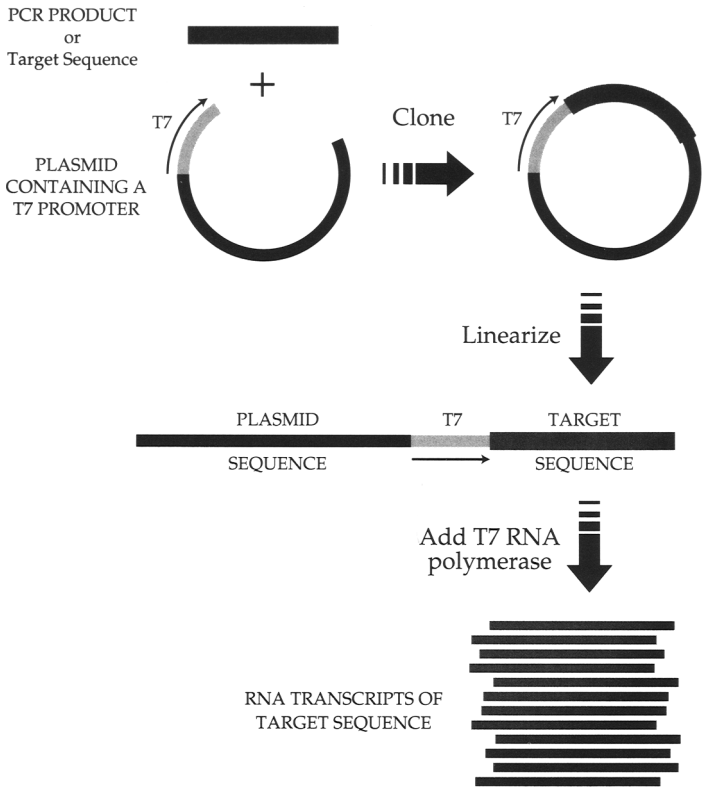


**FIGURE 7** Preparation of RNA probes from PCR products or other DNA fragments. PCR products or other DNA fragments are cloned into a plasmid containing a T7 RNA polymerase promoter. After propagation and purification of the plasmid DNA, the DNA is linearized and RNA transcripts are produced.

has been purified, RNA is easily (and inexpensively) transcribed from the plasmid DNA producing a high yield. The RNA is purified by a simple lithium chloride precipitation and is then ready to use. Biotinylated and unmodified primers may be ordered from commercial suppliers; however, the strand of amplification product containing the biotinylated primer must be the opposite sense of the RNA probe or detection will not occur. Alternatively, a custom RNA probe and primer set for specific targets of interest can be ordered commercially.

For even greater flexibility, standard cloning techniques can be used to prepare much longer RNA probes. For instance, the HBV probe (Digene Diagnostics, Inc.) is a genomic, full-length RNA probe of ~3200 bases. By choosing the appropriate primer sets, many different genes can be amplified and then detected with the same probe in the same assay. The "extra" RNA that is not complementary to the much shorter PCR product does not interfere with the assay.

## OTHER ELISA DETECTION METHODS

### AMPLICOR

The AMPLICOR PCR assay (Roche Diagnostic Systems) is a complete sample preparation, amplification, and detection system for specific analytes. PCR amplification is performed with one biotinylated and one unmodified primer. As in the oligonucleotide capture format (Fig. 2), the PCR product is denatured and hybridized to a specific capture probe that has been prebound to the microplate. After capture, the biotin label on the capture DNA strand is

detected with a streptavidin–horseradish peroxidase conjugate following a standard immunoassay procedure. Results are obtained in 4–5 hr.[26,27] The AMPLICOR system contains all of the reagents necessary for sample collection, preparation, PCR amplification, and detection. The assay utilizes the PCR System 9600 thermal cycler and incorporates the dUTP/UNG procedure for control of carryover contamination. An FDA-approved version of the AMPLICOR PCR assay is currently available for detection of *Chlamydia trachomatis*. Because the AMPLICOR assay is a complete package designed to detect a single target, the assay is convenient but lacks the flexibility of other detection systems. Also, the cost of the AMPLICOR system may not be feasible for a research laboratory.

## GEN-ETI-K DEIA

DNA enzyme immunoassay (DEIA) (GEN–ETI-K DEIA, Sorin Biomedica) is also an antibody-based detection system (Fig. 8).[28] Before detection, a biotin-labeled, target-specific capture probe is bound to a streptavidin-coated plate with an overnight incubation. Prepared capture plates may then be stored dry for 1 month. PCR amplification is performed with unmodified primers. After amplification, the PCR products are denatured by heating to 100°C, and a portion of the denatured PCR reaction is hybridized to the immobilized capture probe. Following hybridization and wash steps, a mouse monoclonal antibody specific for double-stranded DNA is added to the wells and reacts with the captured sequences that have formed double-stranded DNA complexes with the capture probe. After a second washing, an enzyme-

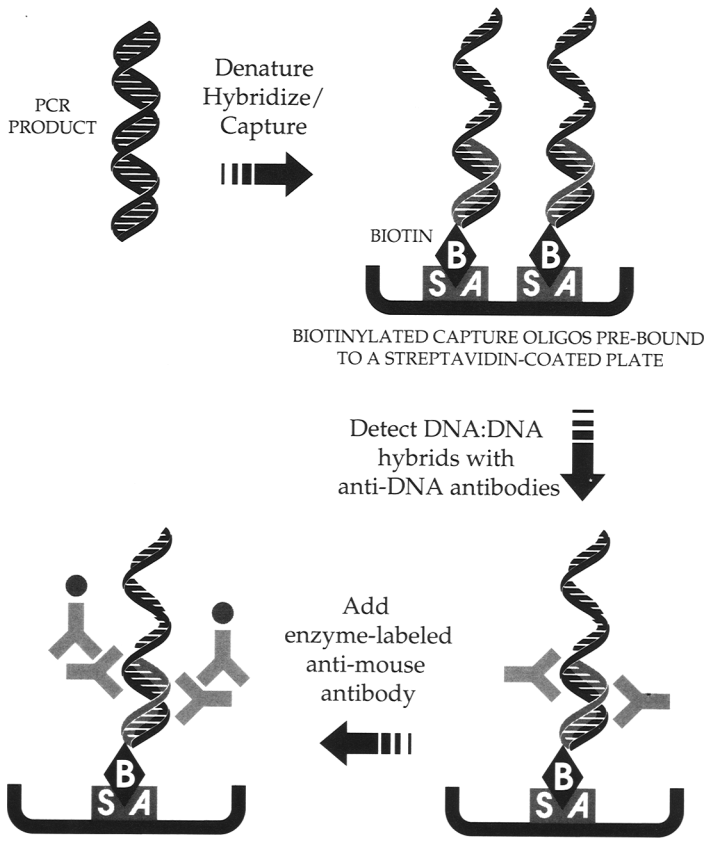


**FIGURE 8** DEIA. PCR products are denatured and hybridized with a solid-phase capture probe. A mouse monoclonal antibody specific for DNA/DNA hybrids is added, followed by an enzyme-labeled anti-mouse antibody.

labeled anti-mouse antibody is added followed by a third washing and colorimetric detection. Drawbacks of this method include lack of flexibility because once the capture probe is bound to the plate, only a single analyte can be detected. In addition, multiple antibody additions and wash steps make the detection procedure more labor intensive than other methods.

## OTHER DETECTION METHODS

### Electrochemiluminescence

The QPCR System 5000 (Perkin-Elmer) applies a recently developed detection technology to PCR product detection.[29–31] Electrochemiluminescence is the ability of a substance to emit light when stimulated by an electric field. With this detection method, PCR amplification is performed with one biotinylated and one unmodified primer. After amplification, the PCR products are hybridized to oligonucleotide probes containing the electrochemiluminescent label, Tris (2,2′-bipyridine)-ruthenium(II) chelate (TBR). After hybridization, the reaction mixture is mixed with streptavidin-coated magnetic beads that bind the biotin-labeled hybrids. The magnetic beads are then loaded into the QPCR instrument, which contains an electrochemiluminescent detection chamber. The magnetic beads are bound magnetically to an electrode and are washed to remove unbound materials and unhybridized TBR-labeled probe. Subsequently, the electrode supplies an electric field to the bound material, and TBR-labeled probe that has hybridized to capture PCR product is stimulated by the electric field and produces light. The emitted light intensity is measured by a photomultiplier tube and converted to a signal output. The magnetic beads are then released from the electrode, and the electrode is washed and cleaned in preparation for the next sample.

The QPCR system is highly automated and can process 50 samples in ~1 hr but requires 2.5 hr to process 96 samples.[32] Because the signal is light output, the linear dynamic range is greater than most colorimetric-based detection systems. However, the QPCR system requires a significant investment in capital equipment, and although TBR-labeled phosphoramidite is available, TBR-labeled primers are not yet routinely available from commercial suppliers.

### Reverse Dot Blots: HybriQuick and EnviroAmp

The HybriQuick (MicroProbe) and EnviroAmp (Perkin-Elmer) detection systems employ the same basic reverse dot blot detection technology,[33,34] but utilize different formats for processing and detection. In a reverse dot blot, a target-specific capture oligonucleotide is bound to a solid phase, usually a filter or membrane. PCR amplification is performed with at least one biotinylated primer, and after amplification, the PCR product is denatured and hybridized to the capture probe. After washing, enzyme-labeled avidin or streptavidin is used to detect the biotin label on the captured PCR product. A colorimetric reaction then produces a colored spot on the filter or membrane.

The EnviroAmp assay utilizes filter strips that are prebound with capture probe specific for *Legionella*. All processing, including hybridization, washing, and detection, is performed manually. The HybriQuick system utilizes a probe analysis card (PAC) that contains HIV-specific capture probes prebound to immobilized beads. After denaturation, the sample is simply spotted onto the appropriate site on the PAC. The MicroProbe Affirm processor then processes the cards through the remaining assay steps and color intensity is read by visual inspection. The Affirm processor can process six samples in 38 min.

Both of these methods allow only a limited sample throughput and provide only qualitative results. The manual methodology required by the EnviroAmp system is tedious and time consuming while the HybriQuick

method requires a capital equipment investment in the Affirm processor and the use of expensive PACs. Neither method allows flexibility in assay design because there is no provision for allowing the user to bind alternate capture probes to the solid phase.

## HPLC

HPLC can be used to separate DNA fragments by size-exclusion (SE) or ion-exchange (IE) chromatography. IE chromatography is used more commonly to detect PCR products because of the high resolution obtained with this approach. HPLC detection of PCR products is a simple technique that requires few reagents or manipulations. After amplification, a portion of the reaction mix is loaded onto the column. With an anion-exchange column and gradient elution, DNA fragments are separated according to size and are eluted from smallest to largest. A specific PCR product is detected by the appearance of a peak corresponding to the expected product size. The technique is similar to gel electrophoresis, except that the resolution and sensitivity of the HPLC separation are much greater than gel methods. Quantitative measurement of the amounts of PCR product is also performed easily and accurately by the detector integrator; therefore, HPLC can be an especially useful technique for optimization of PCR conditions. An additional advantage of HPLC detection is that purified PCR products can be recovered for future manipulation such as cloning or sequencing. Because a typical HPLC separation takes from 10 to 60 min, sample throughput with HPLC analysis is low. However, for analysis of only a few samples per batch, HPLC might be an appropriate method. Because HPLC systems are quite expensive, HPLC detection is probably only appropriate for those laboratories that already have an HPLC system in place.

## CONCLUSIONS

Traditional gel-based methods for detection of PCR products lack sensitivity and specificity or require tedious blotting techniques to achieve adequate results. To overcome these drawbacks, a wide range of techniques and formats have been developed that are significant improvements over traditional gel-based assays. Advanced detection assays developed in-house and commercially available assays are now being used that provide rapid, sensitive, and specific detection of PCR products in standard ELISA formats. Before choosing a new detection method, laboratories should examine their current and expected future needs and expectations with respect to assay format, per-sample cost, assay flexibility, sample throughput, capital investment, and technical support. A final note of advice: PCR detection assays are only as good as the PCR amplification. A sensitive detection assay may not compensate for an extremely poor amplification. Therefore, investment in PCR optimization is well worth the effort, regardless of the detection system used.

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