Downloaded from genome.cshlp.org on June 23, 2026 . Published by Cold Spring Harbor Laboratory Press

Basic methods for the detection of PCR products.

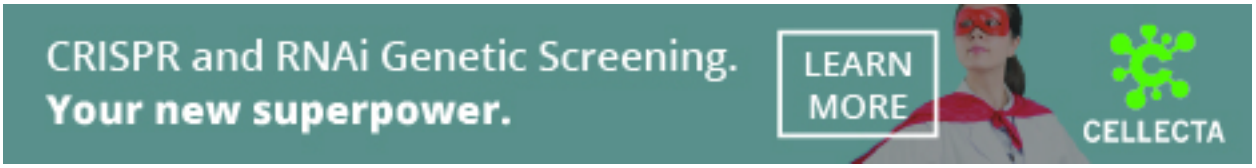
F J Jenkins

Genome Res. 1994 3: S77-S82

References This article cites 9 articles, 3 of which can be accessed free at:
http://genome.cshlp.org/content/3/5/S77.full.html#ref-list-1

License

Email Alerting Service Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or **click here.**



To subscribe to *Genome Research* go to:
https://genome.cshlp.org/subscriptions

Copyright © Cold Spring Harbor Laboratory Press

Downloaded from genome.cshlp.org on June 23, 2026 . Published by Cold Spring Harbor Laboratory Press

Basic Methods for the Detection of PCR Products

Frank J. Jenkins

Department of Microbiology and Immunology and the Neuroscience Program, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4799

PCR technology is quickly becoming an essential component of every laboratory involved in molecular biology experimentation. Equipment and supplies for PCR experiments are expensive, and a significant investiture of personnel time and laboratory space is required to incorporate them into the laboratory's repertoire of techniques. One of the many choices an investigator must make when using PCR is how to detect the amplified product. Detection methods range from procedures that are relatively insensitive and nonspecific to those that are very sensitive and highly specific.

Whereas some PCR reactions yield a single amplified product at levels that are readily detectable by many different procedures, other reactions produce several DNA bands consisting of the expected amplified product along with bands representing nonspecific amplification. Optimization of the PCR conditions can often reduce, but not entirely eliminate, the nonspecific products in these reactions. In addition, in some reactions containing both the specific and nonspecific products, the amount of specific product may be quite small and not easily detectable. The ideal method for detection should allow for an accurate determination of the desired product regardless of the number of nonspecific bands produced. In this section, I will describe the basic methods for detecting PCR products, focusing on the strength and weakness of each method.

DIRECT VISUALIZATION USING ETHIDIUM BROMIDE

For the molecular biologist, one of the easiest and most common methods employed for detecting PCR products is the use of ethidium bromide/gel electrophoresis.[1] Ethidium bromide is a fluorescent dye that intercalates between the stacked bases of DNA causing the DNA to fluoresce when exposed to UV light at 260 nm. Ethidium bromide can be either added to an agarose gel solution prior to pouring the gel or it can used as a solution to stain the gel following electrophoresis. DNA products are visualized and can be photographed under UV light. Identification of the PCR product is based on the appearance of a DNA band of the expected length. Sizing of the DNA bands is achieved by running the PCR products next to DNA markers. The majority of PCR products are between 100 and 500 bp in length; therefore, 2% agarose gels are generally used for electrophoresis. Although many laboratories use standard electrophoresis grade agarose, agaroses specially designed for the separation of small DNA fragments are available (e.g., NuSieve and MetaPhor from FMC Bioproducts, Rockland, ME). The major disadvantage to the use of ethidium bromide as a sole method for PCR product detection is that the dye can only detect bands that contain ~5 ng or more of DNA.[2] For some experiments this may be a more than adequate level of detection, whereas for others it is too insensitive and therefore not appropriate. A second disadvantage of using ethidium bromide is that all of the DNA products (both specific and nonspecific) produced during the PCR reactions will be stained. In cases where the nonspecific bands are in excess of the specific product and/or are very close to the same size as the expected DNA product, the use of ethidium bromide to detect the PCR products can lead to faulty interpretations of the results.

INCORPORATION OF RADIOACTIVE AND NONRADIOACTIVE LABELS

A procedure for direct visualization of amplified PCR products that is more sensitive than ethidium bromide staining involves the incorporation of radioactive or nonradioactive labels directly into the amplified products.[3] Incorporation of specific labels is achieved by decreasing the amount of one or more cold (nonlabeled) deoxynucleotide triphosphates (dNTPs) and adding a corresponding labeled dNTP.[4] Radiolabeled dNTPs can be either ^{32}P or ^{35}S,

3:S77–S82©1994 by Cold Spring Harbor Laboratory ISSN 1054-9805/94 $1.00

Downloaded from genome.cshlp.org on June 23, 2026 . Published by Cold Spring Harbor Laboratory Press

with ^{32}P more commonly employed. Nonradioactive labels include biotin and digoxigenin. Detection of PCR products labeled with radioisotopes is achieved by autoradiography of either dried gels[5] or membranes to which the DNA products have been transferred.[6] The detection of PCR products labeled with biotin or digoxigenin requires the transfer of the DNA products to a membrane. Immobilized DNA bands labeled with biotin can be visualized by treatment of the membrane with a streptavidin–enzyme conjugate [such as horseradish peroxidase (HRP)] and a chromogen substrate appropriate for the particular enzyme (such as tetramethylbenzidine for HRP). DNA bands labeled with digoxigenin can be visualized with a digoxigenin-specific antibody conjugated to an enzyme like HRP (digoxigenin labeled dNTPs and anti-digoxigenin antibodies conjugated to HRP are available from Boehringer Mannheim Corporation, Indianapolis, IN). The sensitivity of these methods is greater than ethidium bromide staining in that they can detect DNA bands with a concentration of <1 ng. The major disadvantage of these methods, shared with ethidium bromide, is that all PCR products, both specific and nonspecific, are visualized. Because the sensitivity of detection is increased by direct labeling of the PCR products, the number of nonspecific bands seen is often even greater when compared with ethidium bromide staining. For most applications, an increased detection of nonspecific products is not acceptable, and, therefore, direct labeling of PCR products is not commonly employed.

DETECTION OF SPECIFIC PCR PRODUCTS USING A DNA PROBE

PCR is used to amplify specific regions of a given strand of DNA. In many instances, PCR is used to answer questions regarding the presence or absence of different DNAs in various tissues or samples (e.g., the presence of a particular virus). For these procedures, the detection of specific PCR products is extremely important. The presence of a DNA band in an agarose gel whose length corresponds to the length of the expected PCR product does not provide positive identification. Positive identification of a specific PCR product requires the use of a DNA probe (15–21 nucleotides in length) that hybridizes to a region of the DNA located internally between the two PCR primers. DNA bands produced by either nonspecific amplification of DNA or by multimer formation of the PCR primers would not hybridize to the internal DNA probe and, therefore, would not be detected. Hybridization with the DNA probes only occurs with those DNA bands that contain the specific sequence of the probe. Thus, in instances where ethidium bromide staining or direct labeling of PCR products demonstrates the presence of several DNA bands, the use of a DNA probe would only visualize the specific amplified product. The DNA probes can be either radiolabeled or labeled with nonradioactive markers such as biotin or digoxigenin. In addition to providing specificity of detection, the use of labeled DNA probes provides increased sensitivity over ethidium bromide staining as well. For these reasons, the use of DNA probes represents the most accurate and widely used method for the detection of PCR products.

Use of Radioactive Probes

The most common method for radiolabeling oligonucleotide probes involves the end-labeling of the oligonucleotide with [γ-^{32}P]ATP by T4 polynucleotide kinase. This procedure is fast, easy, and very efficient producing labeled DNA probes with high specific activities. Potential disadvantages include the required use of radioactive materials and, thus, the associated problems of radioactive handling and disposal, and the relatively short life span of the radioactive probe. The short half-life of ^{32}P (14.7 days) requires that the DNA probes be used soon after labeling; therefore, the radioactive probes must

Downloaded from genome.cshlp.org on June 23, 2026 . Published by Cold Spring Harbor Laboratory Press

TABLE 1 End-labeling of Oligonucleotide with [γ-^{32}P]ATP

Mix the following reagents in order in an Eppendorf tube:	
1. 10× T4 polynucleotide kinase buffer	(1 µl)
2. 0.1–0.5 µg oligonucleotide	(1–5 µl)
3. Sterile distilled water (bring volume to 8 µl)	(2–6 µl)
4. 75 µCi of [γ-^{32}P]ATP	(1 µl)
5. T4 polynucleotide kinase	(1 µl)

Incubate at 37°C for 30–60 min.
Terminate reaction by addition of 10 µl of 0.5 M EDTA.

generally be made every 10–14 days. A protocol for a typical end-labeling reaction is shown in Table 1. On completion of the labeling reaction the radiolabeled probes do not need to be separated from unincorporated label and can be used directly in hybridization reactions.

Use of Nonradioactive Probes

A variety of different nonradioactive labels are available for labeling DNA probes, including biotin, digoxigenin, HRP, and fluorescein.[7] The major advantage to the use of these labels is that they do not have a short half-life, unlike radioactive labels, and can last for weeks or even months. In addition, the problems associated with handling and disposal of radioactivity do not exist. Some labels, such as fluorescein, have limited sensitivity[8] whereas others, such as biotin and digoxigenin, have compared favorably with radioactive labels.[9]

A more recent nonradioactive labeling procedure that has been developed utilizes chemiluminescence. This procedure combines nonradioactive labeling with autoradiography in that the addition of a specific substrate to the labeled probe results in the emission of light, which can be visualized by exposure to X-ray film. Chemiluminescence substrates are available for both HRP and alkaline phosphatase-conjugated antibodies. An advantage of this detection method over conventional nonradioactive labeling procedures is an increase in sensitivity of the PCR product and a decrease in the amount of time required for detection.

SPECIFICITY OF DETECTION USING OLIGONUCLEOTIDE PROBES

The specificity of detection associated with labeled DNA probes is the result of our ability to control the stringency of hybridization conditions between the DNA probe and membrane-bound PCR products. Stringency conditions during hybridization are determined by the DNA sequence of the individual DNA probe. Generally, stringent conditions can be defined as hybridizations that occur at a temperature 5°C below the melting temperature (T_m) of the probe. The T_m of the oligonucleotide probe shorter than 20 bp is calculated with the following formula: $T_m = (4\times GC) + (2\times AT)$, where GC is the number of guanosine and cytosine residues and AT is the number of adenosine and thymidine residues in the sequence of the DNA probe. A typical protocol for the transfer of DNA bands to Nytran membrane and hybridization with an internal oligonucleotide probe is described below.

TRANSFER OF PCR PRODUCTS FROM AN AGAROSE GEL TO NYTRAN MEMBRANE

Reagents

1. Denaturation solution
   0.5 M NaOH
   1.5 M NaCl

Downloaded from genome.cshlp.org on June 23, 2026 . Published by Cold Spring Harbor Laboratory Press

2. Neutralization solution
   1.0 M Tris-Cl (pH 7.4)
   1.5 M NaCl
3. 20× SSPE
   3.0 M NaCl
   0.2 M $NaH_2PO_4 \cdot H_2O$
   0.02 M EDTA
   pH 7.4
4. Whatman filter paper
5. Nytran membrane

Procedures

1. Following electrophoresis, soak the agarose gel in denaturation solution (enough to cover the gel slightly) for 30 min at room temperature. If you require a picture of the DNA bands, the gel should be stained with ethidium bromide and photographed prior to soaking the gel in the denaturation solution.
2. Transfer gel to neutralization solution (enough to cover the gel) for 15 min at room temperature.
3. Transfer DNA to Nytran membrane (0.45 μ pore size) using 20× SSPE. Allow transfer to run overnight.
4. Cross-link DNA to membrane by exposure to UV light (e.g., 120,000 μjoules in a Promega Stratolinker).
5. Bake membrane at 80°C for 1 hr in a vacuum oven. Store membrane at 4°C.

HYBRIDIZATION WITH AN INTERNAL OLIGONUCLEOTIDE PROBE

Reagents

1. Hybridization solution (100 ml)
   25 ml—20× SSPE
   5 ml—100× Denhardt's solution
   1 ml—10% SDS
   69 ml—deionized water
2. 100× Denhardt's solution
   2% Ficoll 400
   2% polyvinyl-pyrrolidone (soluble)
   2% bovine serum albumin
3. First wash buffer
   2× SSPE
   0.1% SDS
4. Second wash buffer
   5× SSPE
   0.1% SDS

Procedures

1. Wet the membrane with deionized water and place in a sealable bag with 10 ml of hybridization solution. Submerge sealed bag in water bath at a temperature of 5°C below the T_m of the oligonucleotide probe.
2. Hybridize for a minimum of 1 hr.
3. Add radiolabeled oligonucleotide probe (0.1–0.2 μg/10 ml of buffer) and hybridize for 3–20 hr.
4. Remove membrane from bag and wash with first wash buffer (no. 3 above) for 30 min at room temperature. Repeat.

Downloaded from genome.cshlp.org on June 23, 2026 . Published by Cold Spring Harbor Laboratory Press

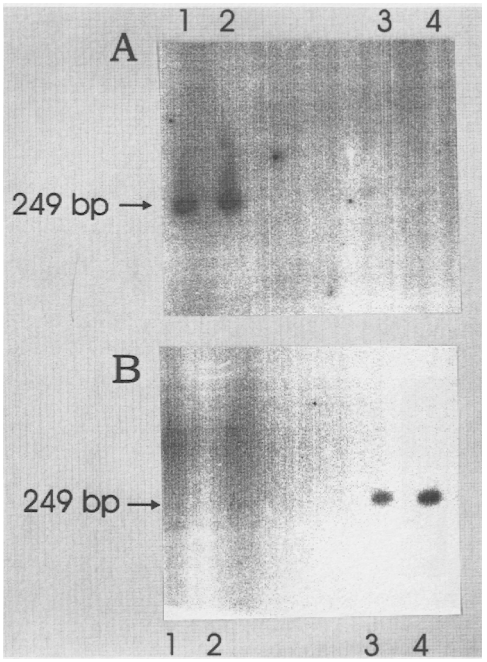


FIGURE 1 Phosphorimage of a Southern Blot. DNAs from HHV-6 strain Ull02 (lanes *1,2*) and strain Z29 (lanes *3,4*) were analyzed by PCR using HHV-6-specific primers as described in the text. PCR analysis was performed on either 0.1 μg (lanes *1,3*) or 0.01 μg (lanes *2,4*) of each DNA. Aliquots of each PCR reaction were separated on duplicate 2% agarose gels, transferred to Nytran membranes, and hybridized under stringent conditions with ^{32}P-labeled oligonucleotide probes specific for HHV-6 variant A (*A*) or HHV-6 variant B (*B*) DNA. Following hybridization, specific bands were visualized by scanning on a PhosphorImager (Molecular Dynamics, CA).

5. Wash membrane in second wash buffer (no. 4) for 30 min at hybridization temperature. Repeat.
6. Air-dry the membrane and expose for autoradiography.

USE OF ALLELE-SPECIFIC PROBES TO DIFFERENTIATE CLOSELY RELATED SEQUENCES

Stringent hybridization conditions not only allow for the detection of specific DNA products but also permit the PCR products of closely related sequences to be distinguished from each other by using specific probes that can recognize as little as 1-bp difference between sequences. Figure 1 demonstrates this type of specificity. In this experiment, PCR analysis was used to distinguish between two different groups of human herpesvirus type 6 (HHV-6) designated as HHV-6 variant A and HHV-6 variant B.[10] DNAs from HHV-6 strain Ull02 (a member of the variant A group) and HHV-6 strain Z29 (a member of the variant B group) were analyzed by PCR using a primer set that recognizes both virus groups[11] and the amplified products were separated on a 2% agarose gel. An ethidium bromide stain of the agarose gel showed a 259-bp product from both Ull02 and Z29 DNAs (data not shown). The amplified products from duplicate gels were transferred to a Nytran membrane and hybridized under stringent conditions (5°C below the T_m of the probes) to either a variant A (Fig. 1A) or variant B (Fig. 1B) oligonucleotide probe, which differed in their nucleotide sequence by only 1 base (Table 2).[12] As seen in Figure 1, under stringent conditions, each probe hybridized only to the corresponding variant group A or group B DNA. Thus, the use of specific DNA probes allows us to distinguish PCR products differing by as little as 1 base in the DNA sequence.

TABLE 2 Variant A and B Probe Sequences

Variant A probe: ATTCCAAG<u>T</u>TTTTATGA
Variant B probe: ATTCCAAG<u>C</u>TTTTATGA

Downloaded from genome.cshlp.org on June 23, 2026 . Published by Cold Spring Harbor Laboratory Press

The combination of the sensitivity of the PCR reaction, along with the sensitivity and specificity of product detection by the use of labeled DNA probes, explains why PCR technology has become so valuable in the fields of molecular biology and clinical microbiology.

ACKNOWLEDGMENTS

I thank Dr. Phillip Pellet (CDC, Atlanta, GA) and Dr. Niza Frenkel (Tel Aviv University, Tel Aviv, Israel) for the Z29 and Ull02 DNAs, respectively.

The opinions or assertions contained herein are the private views of the author and should not be construed as official or necessarily reflecting the views of the Uniformed Services University of the Health Sciences or Department of Defense. There is no objection to its presentation and/or publication.

REFERENCES

1. Sambrook, J., E.F. Fritsch, and T. Maniatis, eds. 1989. In *Molecular cloning: A laboratory manual*, 2nd ed., p. 615. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
2. Sharp, P.A., B. Sugden, and J. Sambrook. 1973. Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose. *Biochemistry* **12:** 3055–3059.
3. Pang, S., Y. Koyanagi, S. Miles, C. Wiley et al. 1990. High levels of unintegrated HIV-1 DNA in brain tissue of AIDS dementia patients. *Nature* **343:** 85–89.
4. Hayashi, K. 1991. PCR-SSCP: A simple and sensitive method for detection of mutations in the genomic DNA. *PCR Methods Applic.* **1:** 34–38.
5. Jenkins, F.J. and M.K. Howett. 1984. Characterization of mRNAs that map in the BglII N fragment of the herpes simplex virus type 2 genome. *J. Virol.* **52:** 99–107.
6. Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98:** 503–510.
7. Levenson, C. and C. Chang. 1990. Nonisotopically labeled probes and primers. In *PCR protocols: A guide to methods and applications* (ed. M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White), pp. 99–112. Academic Press, San Diego, CA.
8. Landegren, U., R. Kaiser, and L. Hood. 1990. Oligonucleotide ligation assay. In *PCR protocols: A guide to methods and applications* (ed. M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White), pp. 92–98. Academic Press, San Diego, CA.
9. Whetsell, A.J., J.B. Drew, G. Milman, R. Hoff, E.A. Dragon, K. Adler, J. Hui, P. Otto, P. Gupta, H. Farzadegan, and S.M. Wolinsky. 1992. Comparison of three nonradioisotopic polymerase chain reaction-based methods for detection of human immunodeficiency virus type 1. *J. Clin. Microbiol.* **30:** 845–853.
10. Ablashi, D., H. Agut, Z. Berneman et al. 1993. Human herpesvirus-6 strain groups: A nomenclature. *Arch Virol.* **129:** 363–366.
11. Collandre, H., J.-T. Aubin, H. Agut, J.-M. Bechet, and L. Montagnier. 1991. Detection of HHV-6 by the polymerase chain reaction. *J. Virol. Methods* **31:** 171–180.
12. Dewhurst, S., K. McIntyre, K. Schnabel, and C.B. Hall. 1993. Human herpesvirus 6 (HHV-6) variant B accounts for the majority of symptomatic primary HHV-6 infections in a population of U.S. infants. *J. Clin. Microbiol.* **31:** 416–418.