

New Directions in Molecular Diagnostic Testing



An Educational Guide
for Clinical Laboratory
Professionals

Other Resources

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Further Reading:

Arnold, L.J., Hammond, P.W., Wiese, W.A., and N.C. Nelson. 1989. Novel assay formats involving acridinium-ester-labelled DNA probes. *Clinical Chemistry*. 35:1588-1594.

Chan, A.B. and J.D. Fox. 1999. NASBA and other transcription-based amplification methods for research and diagnostic microbiology. *Reviews in Med. Microbiol.* 10:185-196.

Ehrlich, G.S. and S.J. Greenberg. 1994. PCR-based diagnostics in infectious disease. Blackwell Scientific Publications.

Hill, C.S. 1996. Molecular diagnostics for infectious diseases. *Journal of Clinical Ligand Assay*. Vol.19, No.1

Kaul K, et al. 1994. Direct sputum analysis for tuberculosis by polymerase chain reaction vs conventional techniques in a public hospital. In: *Assessing Clinical Outcomes*. American Association for Clinical Chemistry.

McDonough, S.H., Giachetti C., Yang, Y., Kolk, D.P., Billyard, E., Mimms, L. 1998. High throughput assay for the simultaneous or separate detection of human immunodeficiency virus (HIV) and Hepatitis type C virus (HCV). 25:164-169.

Mullis, K.B., Ferre, F. and R.A. Gibbs (eds.). 1994. *The polymerase chain reaction*. Birhäuser, Boston.

Nelson, N.C. 1998. Molecular tools for building nucleic acid IVDs. In: *IVD Technology*. 4:48-54.

Nelson, N.C., Cheikh, A.B., Matsuda, E., and M. Becker. 1996. Simultaneous detection of multiple nucleic acid targets in a homogeneous format. *Biochemistry*. 35:8429-8438.

Shinnick, T.M. and V. Jonas. 1994. Detection of Mycobacterium tuberculosis by molecular methods. In: *Molecular approaches to the diagnosis of tuberculosis*. American Society for Microbiol. 30:517-530.

Wolcott, M.J. 1992. Advances in nucleic acid-based detection methods. *American Society for Microbiol.* 5:370-386.



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Introduction

Over the past decade, major advances have been made in molecular diagnostic testing for infectious diseases. Today, molecular diagnostic tests are playing an increasingly important role in the clinical microbiology laboratory because they can offer higher performance and shorter time-to-result than traditional tests.

This publication is designed to familiarize clinical microbiologists with the basics of molecular diagnostic technology, the types of tests available today, and the future direction of this exciting field.

Fundamentals of Molecular Biology

Molecular diagnostic assays are based on the detection of specific nucleic acid molecules contained in microorganisms. Identification of the presence of an organism's nucleic acid in a patient sample allows for the positive identification of that organism in the patient.

The two main types of nucleic acid are deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). DNA and RNA are very similar in chemical structure (*Table 1*) but have different functions in the cell.

Table 1

	DNA	RNA
Configuration	Double Strand	Single Strand
Sugar	Deoxyribose	Ribose
Purine Bases	Adenine	Adenine
	Guanine	Guanine
Pyrimidine Bases	Cytosine	Cytosine
	Thymine	Uracil

DNA is the information-storage molecule of all living cells, and is a major component of chromosomes. Typically, each cell contains only one or a few copies of DNA.

RNA serves as an intermediate molecule, converting the genetic information coded by DNA into the proteins that make up the cell. The three primary types of RNA are: messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA).

rRNA represents approximately 80% of the total cellular RNA. In microorganisms, there may be as many as 10,000 copies of rRNA per cell.

Molecular Diagnostics

Molecular diagnostics is the detection of nucleic acid sequences in cells to gain information that aids in the diagnosis of disease.

Molecular techniques are well suited to the identification of infectious organisms in human samples since each microorganism contains specific DNA or RNA sequences that are unique to that organism. By detecting these unique sequences, a specific identification of the microorganism can be obtained.

DNA Probes

The DNA probe is a basic component in most molecular diagnostic techniques.

The DNA probe is a labeled DNA strand that is synthesized in the laboratory. Its purpose is to hybridize with the target nucleic acid molecule to be identified.

A probe is composed of a strand of 30 to 40 nucleotides called an oligonucleotide. These oligonucleotides are labeled with a detector molecule. When hybridization occurs between the probe and the target organism's nucleic acid, the hybrid can be detected through the detector molecule (Figure 2).

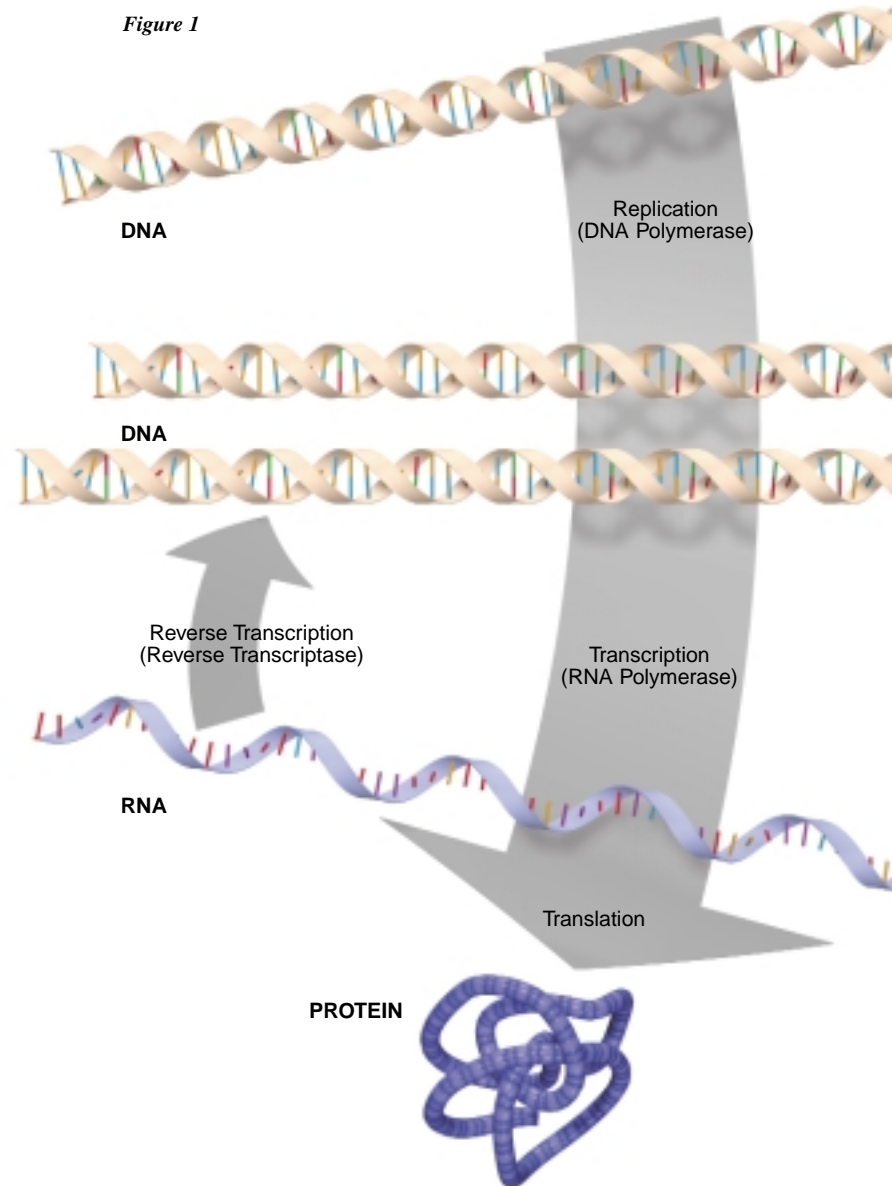
How Nucleic Acids Convey Genetic Information

The flow of genetic information typically goes from DNA to RNA to proteins through the processes of replication, transcription, and translation (Figure 1).

Replication: DNA can replicate to form new DNA during the process of cell division. This is achieved through the action of an enzyme called DNA polymerase.

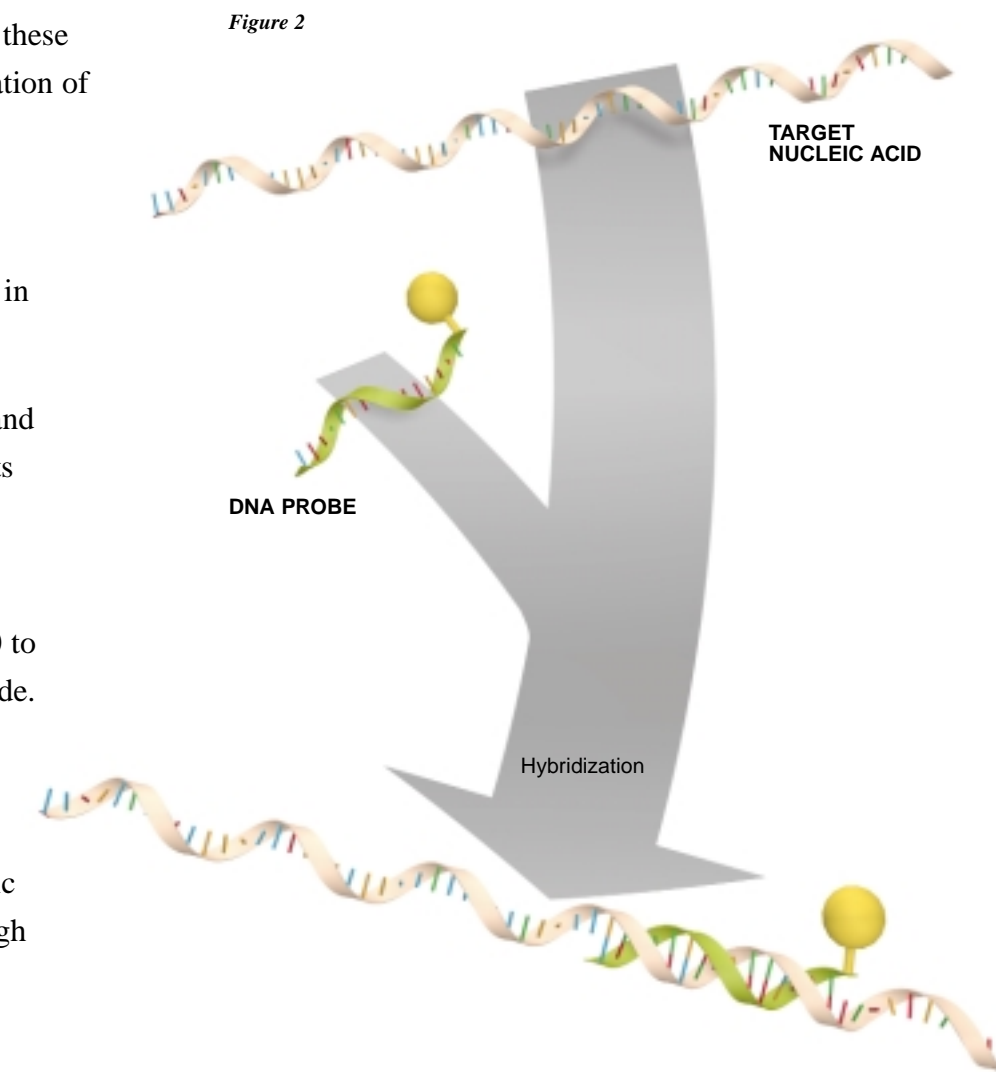
Transcription: The transcription of RNA from DNA occurs through the action of the enzyme RNA polymerase. RNA is then translated into proteins.

Reverse Transcription: In certain viruses called retroviruses (such as HIV-1), the transcription process is reversed. RNA can serve as a template to produce new DNA in a process called reverse transcription. This is mediated by the reverse transcriptase enzyme.



DNA Probe Assays

The three primary types of DNA probe assays used for detecting infectious organisms are culture identification, direct probe, and nucleic acid amplification assays.



Culture Identification Assay: In culture identification assays, the organism is first grown in culture, after which its nucleic acid is detected by DNA probes. The molecular culture identification tests most widely used in the clinical laboratory are the ACCUProbe® line of tests from Gen-Probe. These tests detect clinically relevant bacteria, mycobacteria, and fungi.

Direct Probe Assay: In direct probe assays, the DNA probe is introduced into the processed patient specimen. If the suspected infectious organism is present, the probe will detect the organism's nucleic acids directly in the specimen.

Examples of direct probe assays are the GEN-PROBE® PACE® 2 tests for the detection of *C. trachomatis* and *N. gonorrhoeae* from the same sample. Molecular techniques make it relatively easy to detect multiple organisms in a single sample, while nonmolecular methods such as enzyme immunoassays (EIA) usually cannot accurately detect more than one organism at a time.

The superiority of molecular techniques is due in part to the very high specificity of the probes for the target nucleic acid and lack of cross-reaction with other targets. The PACE 2 assay has high clinical sensitivity due to the targeting of rRNA, which is present in thousands of copies in each cell. This is in contrast to DNA, which is present in only a few copies per cell.

Direct probe assays such as the PACE 2 assay can detect certain types of organisms directly in samples with high sensitivity because of the relatively large number of organisms in a typical sample. However, other organisms such as viruses exist in too few numbers to be directly detected with DNA probes. Nucleic acid amplification technology is needed with these samples to amplify the target nucleic acid from the microorganisms.

Nucleic Acid Amplification Assay: Nucleic acid amplification techniques enzymatically multiply a specific nucleic acid sequence exponentially, resulting in the production of billions of copies of the sequence in a short period of time. The amplification product, or amplicon, is easily detected by DNA probes or other methods to definitively identify the organism present in the sample.

The Nucleic Acid Amplification Process

A nucleic acid amplification assay is typically a three-stage process: sample preparation, amplification, and detection.

Sample Preparation

The first step is to extract the target nucleic acid from the organisms. Extraction may be performed using physical means such as sonication, or chemical reagents such as detergent. After extraction, the target molecule must be stabilized against degradation by nucleases. Substances that can inhibit or interfere with the amplification reaction must also be eliminated.

Nucleic Acid Amplification

Nucleic acid amplification techniques enzymatically amplify the nucleic acid in a test tube to a very high level so that DNA probes can be used to identify the resulting copies of nucleic acid, or amplicon.

Today, there are many different types of nucleic acid amplification technologies, including Polymerase Chain Reaction (PCR), Ligase Chain Reaction (LCR), Transcription-Mediated Amplification (TMA), Nucleic Acid Sequence-Based Amplification (NASBA), and

Strand Displacement Amplification (SDA). There are currently several FDA-cleared nucleic acid amplification diagnostic kits in wide-spread use in clinical laboratories. The most commonly used kits utilize either TMA, PCR, or LCR amplification technologies (*Table 2*).

Table 2

	TMA	PCR	LCR
Target	DNA or RNA	DNA	DNA
Enzymes	RNA Polymerase Reverse Transcriptase	DNA Polymerase	DNA Polymerase DNA Ligase
Thermal Conditions	Isothermal	Thermal Cycling	Thermal Cycling
Amplicon Product	RNA	DNA	DNA
Special Equipment Needed	Luminometer	Thermal Cycler, Microtiter Plate Reader/Washer	Thermal Cycler LCx Instrument
Company	Gen-Probe Incorporated	Roche Diagnostics	Abbott Laboratories

PCR: Developed in 1985, PCR was the first nucleic acid amplification method. PCR uses the DNA polymerase enzyme to replicate target DNA molecules in the organism to be identified (*Figure 3*). RNA can also be targeted but requires the addition of a reverse transcriptase step to generate a DNA target. PCR uses a thermal cycler instrument to alternately heat and cool the reaction mixture.

DNA primers hybridize to the target molecules and initiate polymerization of new DNA molecules. Each cycle results in a doubling of the target nucleic acid molecule and leads to the production of billions of copies of the DNA sequence. The reaction is usually completed in about 90 minutes.

Figure 3

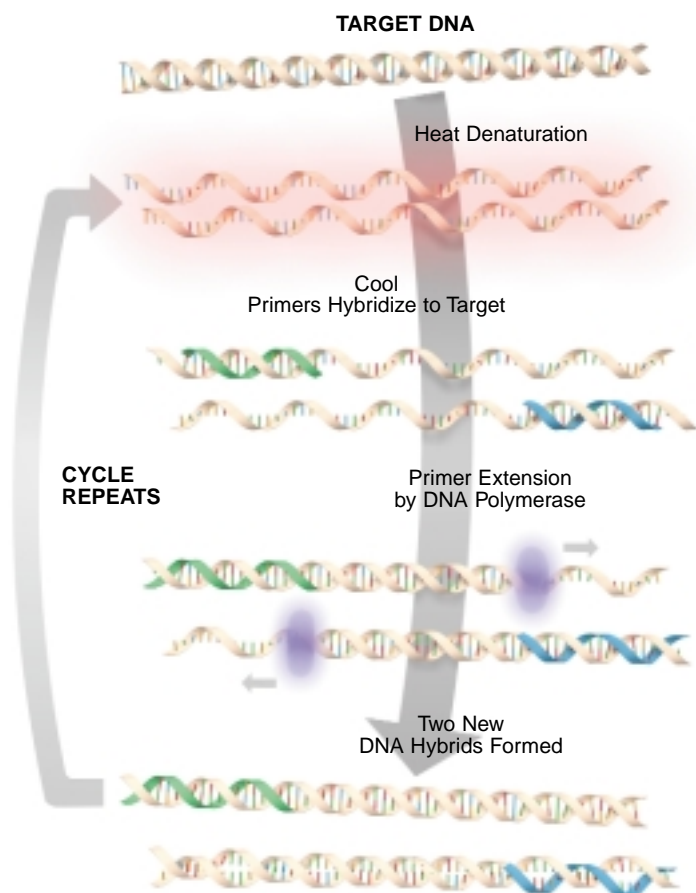
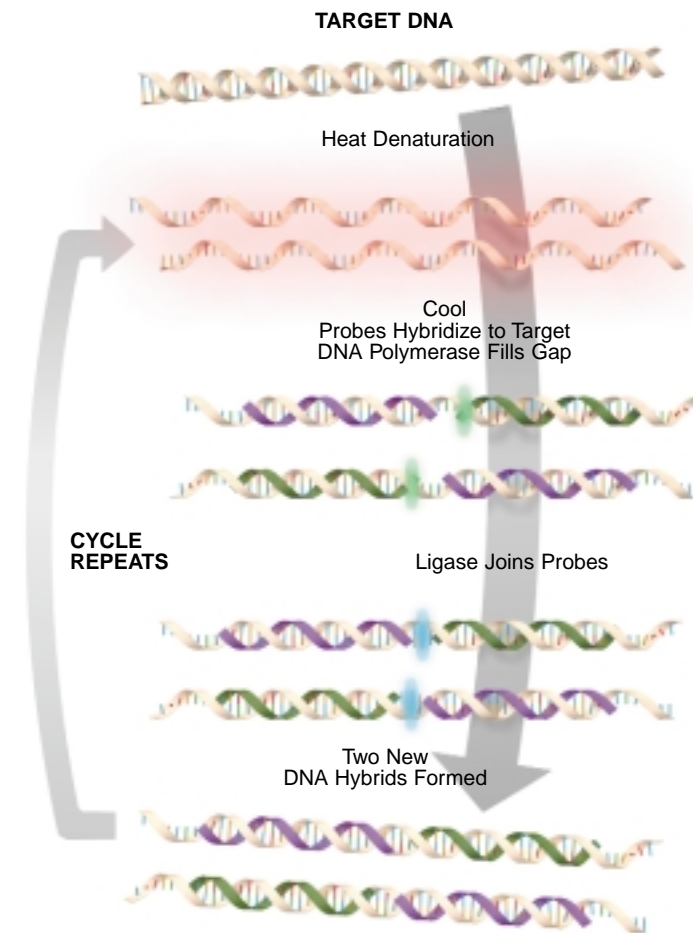


Figure 4

LCR: LCR is a probe-based amplification system. LCR differs from PCR because it amplifies the probe molecule rather than producing amplicon through polymerization of nucleotides. Two probes are used per each DNA strand and are ligated together to form a single probe. LCR uses both a DNA polymerase enzyme and a DNA ligase enzyme to drive the reaction (Figure 4).



Like PCR, LCR requires a thermal cycler to drive the reaction and each cycle results in a doubling of the target nucleic acid molecule. The LCR reaction is also completed in about 90 minutes.

TMA: TMA is an RNA transcription amplification system using two enzymes to drive the reaction: RNA polymerase and reverse transcriptase (Figure 5). TMA can be used to target both RNA and DNA.

TMA has several other differences in comparison to PCR and LCR:

- TMA is isothermal. A water bath or heat block is used instead of a thermal cycler.
- TMA produces RNA amplicon rather than DNA amplicon. Since RNA is more labile in the laboratory environment than DNA, this helps reduce the possibility of carry-over contamination.
- TMA produces 100-1000 copies per cycle in contrast to PCR and LCR that produce only two copies per cycle. This results in a 10 billion fold increase of copies within about 15-30 minutes.

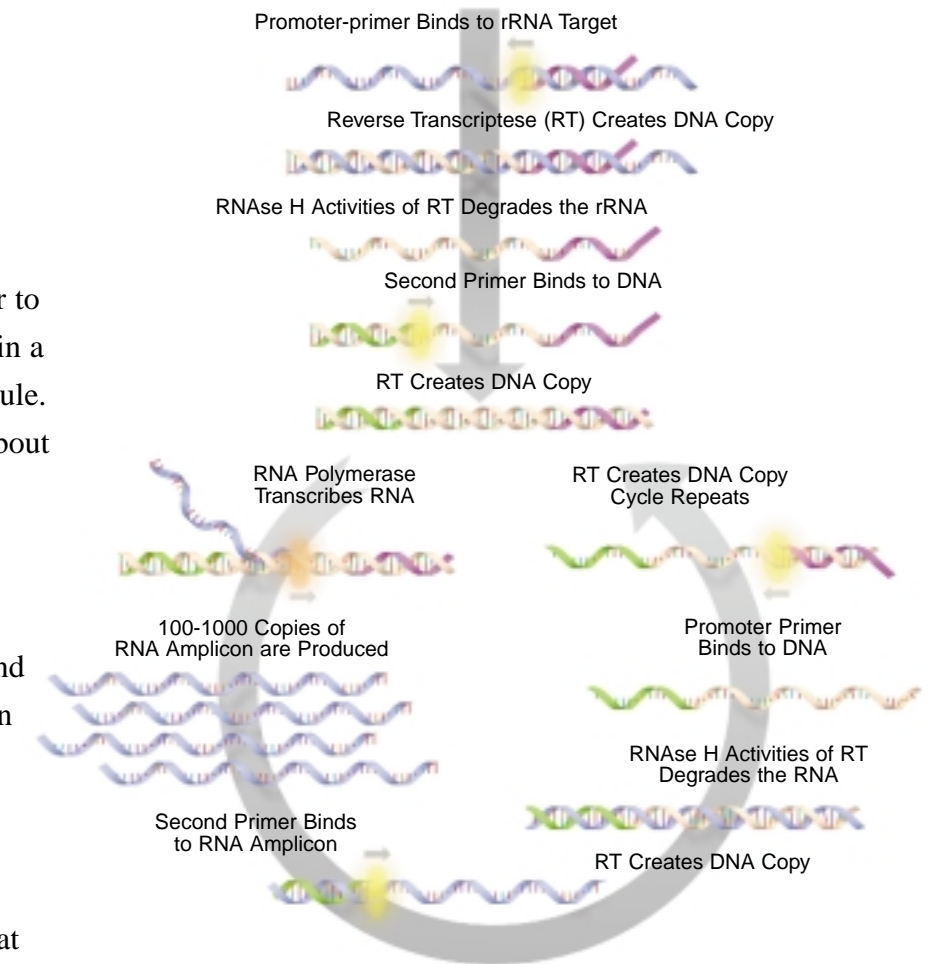


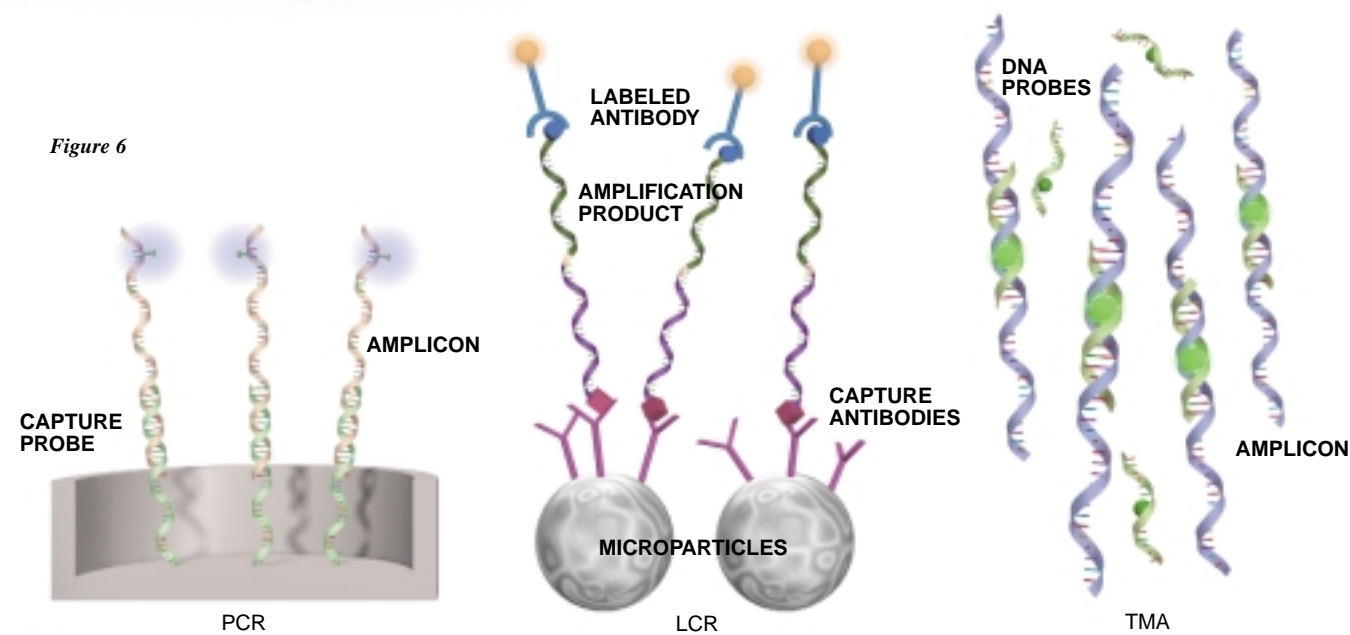
Figure 5

Detection of the Amplification Product

After the amplification reaction, a detection step must be performed to detect the amplification product created. Each of the commercial amplification methods uses a different detection system (Figure 6).

PCR Detection System: The Roche PCR assays use a biotin-avidin horseradish peroxidase system in a microtiter plate format. The biotin-labeled amplicon is captured by specific capture probes bound to the bottom of the microtiter plate wells. Avidin-horseradish

Amplification Tests in the Clinical Microbiology Laboratory



peroxidase is added and followed by a washing step. Substrate is added and the absorbance of the reaction is measured in a photometer.

LCR Detection System: The Abbott LCR assay uses an antibody-antigen reaction to detect the ligated probes. Each of the two probes is labeled with a different hapten molecule. Probes are captured by antibodies bound to magnetic particles. After washing, a second antibody with a fluorescent label is introduced and will bind only to the ligated probes bound to the particles. A substrate is added and fluorescent signal is measured in a fluorimeter.

TMA Detection System: The GEN-PROBE TMA assays use the Hybridization Protection Assay (HPA) format, which has been used successfully for many years in the GEN-PROBE direct probe and culture identification assays.

The HPA technique uses a specific DNA probe labeled with an acridinium ester detector molecule that can emit a chemiluminescent signal.

The probe hybridizes with the RNA amplicon produced in the TMA reaction. Separation of hybridized from unhybridized probes occurs in the solution phase using a reaction that selectively destroys the acridinium ester on the unhybridized probe.

The acridinium ester on the hybridized probes is protected within the double helix, and will chemiluminesce when exposed to detection reagents. This is a homogeneous reaction, and no wash step or solid substrate is used in the assay. Because both TMA and HPA detection occur in a single test tube, there is no transfer of reagents out of the reaction tube. This minimizes the risk of carryover contamination.

The main advantages of amplification assays for the detection of microorganisms are the higher sensitivity and faster time-to-result compared with many current microbiology techniques. Amplification assays are theoretically capable of detecting as little as one organism in a sample. Among the organisms for which amplification tests are currently available are:

Chlamydia trachomatis

Amplification tests for the detection of *C. trachomatis* have been shown to have a higher sensitivity than chlamydial culture and immunoassays. These tests can also be used effectively with noninvasive specimens such as urine, allowing for expanded screening of certain high-risk populations such as adolescents.

Mycobacterium tuberculosis

Nucleic acid amplification tests can specifically detect *M. tuberculosis* directly in respiratory samples. These tests are similar to culture in sensitivity but can detect *M. tuberculosis* within

a few hours. By contrast, culture may take six to eight weeks for results. Use of amplification tests together with culture and smear can help the laboratorian quickly differentiate between *M. tuberculosis* and other mycobacteria. This can result in a much faster diagnosis of *M. tuberculosis*, allowing for quicker isolation and treatment of the patient, and more efficient contact tracing. Amplification assays for *M. tuberculosis* have been shown to benefit the clinical outcome of the patient in a cost-effective manner.

HIV

Nucleic acid amplification viral load tests that quantitatively measure HIV in patient's plasma have become routine and indispensable for monitoring the drug therapy of HIV-infected patients. Numerous studies have demonstrated that viral load HIV tests can improve patient health in a cost-effective manner.

The Future of Amplification Testing

Other Organisms

Amplification methods can also be used for the vast number of bacteria and viruses that are difficult or impossible to culture on synthetic media, such as *Mycobacterium leprae*. These methods have also been used to identify new microorganisms that cannot be grown in culture, such as the hepatitis C virus.

Limitations of Amplification Tests

Although amplification assays are sensitive and rapid in comparison to culture, there are several limitations with the first-generation amplification assays that have limited their acceptance in clinical microbiology laboratories.

One of the most significant issues has been the threat of carryover contamination, which can lead to false positives. In addition, it is difficult or impossible to verify positive amplification results in the clinical lab.

Another concern with first-generation assays is inhibition. All amplification assays are susceptible to substances in certain samples

that can inhibit the enzymes that drive the amplification reactions. Inhibition can result in false-negative results and reduce the clinical sensitivity of the assays. Proper sample preparation should eliminate most of the potential inhibitors found in clinical specimens.

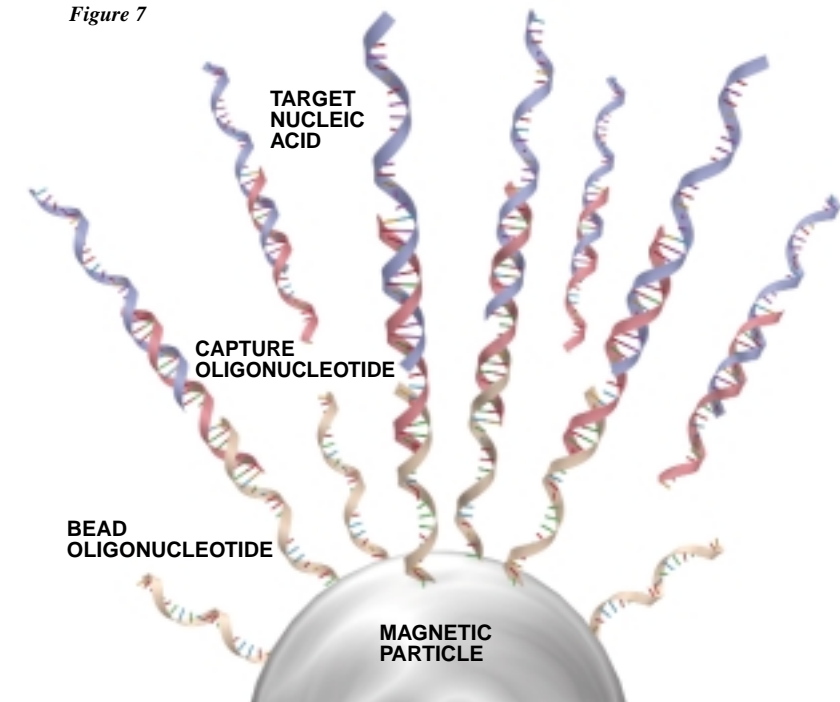
Other drawbacks to first-generation amplification tests involve increased labor requirements, high cost, and low assay throughput. These factors have contributed to slow acceptance of many of these tests in clinical laboratories, which are increasingly moving toward low-cost, automated testing.

Gen-Probe is currently developing amplification tests that will address many of the issues associated with the first-generation nucleic acid amplification tests. Emerging technologies include:

Target Capture

Newly developed Target Capture sample preparation partially purifies the target nucleic acid before the amplification step (Figure 7). Magnetic particles (approximately one micron in diameter), with synthetic oligonucleotides bound to the surface are used to capture the target nucleic acid. These bead oligonucleotides hybridize to a capture oligonucleotide which contains a sequence specific for the target nucleic acid. The target nucleic acid is captured on to the bead, the magnetic particles are drawn to the side of the test tube by a magnet, and wash steps remove substances that can potentially interfere with the assay. This virtually eliminates the inhibition that can occur with some samples and allows the use of different types of samples that are difficult to amplify with current first-generation amplification tests.

Figure 7



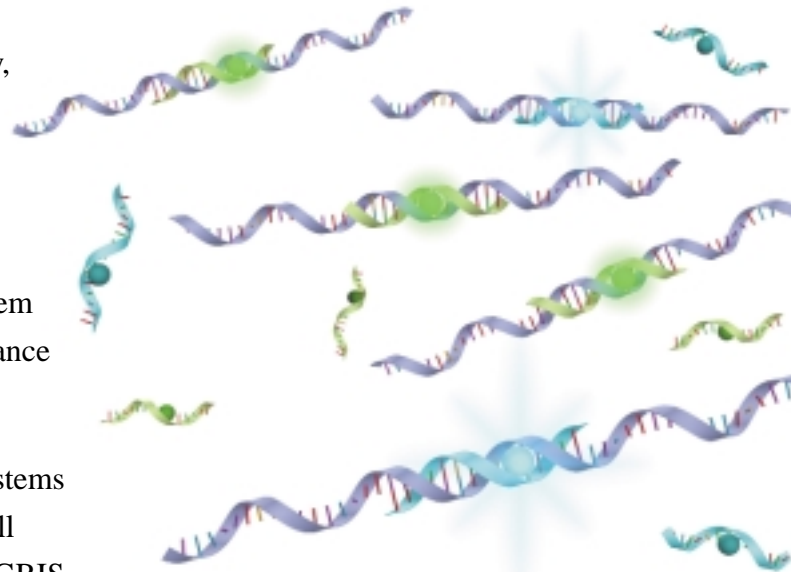
Dual Kinetic Assay (DKA)

The Dual Kinetic Assay is a further advancement of the HPA technology. DKA allows simultaneous detection of amplicon from two independent amplification reactions (multiplex amplification). DKA uses two different acridinium ester detection molecules attached to two different DNA probes specific for the amplified products (Figure 8). The acridinium ester molecules have different chemiluminescent kinetic properties that

can be differentiated by the luminometer instrument. Assays using DKA have been developed that can simultaneously amplify, detect, and differentiate between two different organisms in the sample. Target capture, together with TMA and DKA, constitutes the GEN-PROBE® APTIMA™* assay system. This second-generation system will have improved accuracy and performance over first-generation tests.

TIGRIS™*: Automated instrumentation systems are being developed to meet the needs of all clinical laboratories. The GEN-PROBE TIGRIS system is a fully automated instrument that will completely automate GEN-PROBE APTIMA assays. The TIGRIS instrument performs full automation of sample processing, amplification, and detection, and can process up to 500 specimens during an eight-hour shift, or 1000 specimens in a 12-hour shift. This will be the first totally automated instrument for nucleic acid amplification tests and will increase sample throughput and decrease labor costs.

Figure 8



The automated APTIMA assays will allow all clinical laboratories to use nucleic acid amplification technology to improve the accuracy, time-to-result, and cost-effectiveness of clinical microbiology diagnostic testing.

As a result, molecular diagnostics will play an increasingly important role in the clinical laboratory and will help clinical laboratories respond to patient and clinician demands for faster, more accurate results.

* TIGRIS instrument system and APTIMA assays are currently under development.

Summary

Clinical laboratory professionals who seek timely and accurate results are increasingly turning to the emerging technologies of molecular diagnostic testing. These technologies offer positive identification of an organism's nucleic acid in the cell, for a higher degree of diagnostic accuracy. In addition, their shorter time-to-result permits more rapid treatment of the patient. Research and development in molecular testing are moving ahead at a rapid pace, resulting in tests for an ever-broadening range of diseases, and making this one of the most promising areas of the medical sciences field.