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Introduction to tools for molecular diagnostics

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Introduction to tools for molecular diagnostics

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The correct and timely diagnosis of disease forms the cornerstone of modern medical practice. For decades, clinicians have relied on chemical and immunological assays to detect and characterize disease processes. However, with the advent of DNA-based molecular biological laboratory techniques and the sequencing of the human genome, nucleic acid testing (NAT) methods have come to the fore. Although NAT accounts for a small portion of the IVD market, it is the fastest-growing segment of the industry. The increasing emphasis on preventive healthcare, the continuing spread of infectious agents such as the human immunodeficiency virus (HIV), and the trend toward personalized medicine contribute to the expansion of NAT assays.

The TIGRIS DTS (Direct Tube Sampling) system by Gen-Probe Inc. (San Diego, CA) is a completely automated system for amplified nucleic acid tests.

Present and Future Applications

NAT focuses on identifying and characterizing RNA and DNA molecules. Such methods add an important weapon to the laboratorian's armamentarium of available tests. NAT provides highly sensitive and specific solutions for recognizing minute amounts of target analytes, often earlier in the disease state and with quantities lower than what previous test methods required.

The majority of NAT is used for detecting and managing infectious agents, including HIV, hepatitis B virus (HBV), hepatitis C virus (HCV), West Nile virus (WNV), Myobacterium tuberculosis, Neisseria gonorrhoeae, Chlamydia trachomatis, and human papilloma virus (HPV). The potential for expansion of NAT in the IVD industry is primarily due to the rapid growth in testing for infectious organisms and future testing for additional agents. For example, the clinical management of HIV patients relies on using protease inhibitors and other agents to suppress the replication of the virus in vivo. However, the emergence of viral strains that are resistant to such drugs has led to the need for periodic testing of the viral titer to ensure the continued effectiveness of the therapies.

In the public health arena, NAT methods are essential tools for identifying and containing emerging infectious agents. The sudden appearance of the severe acute respiratory syndrome (SARS) virus in Asia during the spring of 2002 affected travel worldwide and killed more than 800 people. The rapid development and implementation of NAT methods allowed labs to recognize the virus and devise containment procedures to stop the epidemic. A similar situation in the

United States involving WNV resulted in the development of NAT tests for screening donated blood.

In addition to infectious disease diagnosis and blood screening, growing areas of NAT research and development are genetic screening and pharmacogenetics. In clinical genetics, NAT provides information needed to counsel prospective parents on the risks of transferring inherited diseases to their children. With a simple oral swab or blood sample, NAT-based assays can quickly and accurately detect known gene mutations that are responsible for a variety of genetic disorders.

In the field of pharmacogenomics, NAT holds great promise for increasing the efficacy of drug therapies. Determining a patient's genotype in relation to the major drug-metabolizing enzyme systems, such as the cytochrome P450 isozymes, provides clinicians with the ability to tailor drug therapies to individual patients. Doing so could possibly prevent the lack of efficacy or adverse drug reactions caused by abnormally rapid or slow drug elimination kinetics.

NAT Technologies

Several different technologies are employed to perform NAT-based assays. At the forefront are the methods that amplify low-abundance DNA or RNA molecules to improve the sensitivity of detection. The polymerase chain reaction (PCR), a technology now licensed by Roche Molecular Diagnostics (Pleasanton, CA), was the first nucleic acid amplification technique developed for amplifying DNA molecules. This versatile method uses heat-tolerant DNA polymerase enzymes to produce double-stranded DNA molecules, or DNA amplicons. PCR utilizes carefully controlled heating and cooling cycles to facilitate the denaturation and replication of the target DNA molecules. This technique revolutionized the scientific research and medical fields by providing a means for isolating and analyzing low concentrations of DNA molecules. By coupling the DNA-based PCR technology with procedures to convert RNA into DNA, PCR can amplify DNA- and RNA-containing targets in diagnostic assays.

Another amplification protocol is transcription-mediated amplification (TMA) by **Gen-Probe Inc.** (San Diego). TMA is an isothermal NAT method that uses T7 RNA polymerase and reverse transcriptase in a single reaction to amplify either DNA or RNA analytes. The product of this amplification system is single-stranded RNA. Unlike PCR, TMA does not require complicated thermal cycling equipment. Converting RNA and DNA targets into RNA amplicons can be performed in a simple water bath or heat block.

Other nucleic acid amplification technologies used for detecting low-copy-level nucleic acid targets include strand displacement amplification (SDA) by **Becton, Dickinson and Co.** (Franklin Lakes, NJ), nucleic acid sequence-based amplification (NASBA) by **bioMerieux** (Marcy l'Etoile, France), and ligase chain reaction (LCR) by **Abbott Laboratories** (Abbott Park, IL).

Detection Methods

Various methods are used to detect and characterize the nucleic acid amplicons generated in amplified NAT assays. Early detection methods relied on hybridizing radioactively labeled DNA or RNA probes to amplicons separated in electrophoretic gels under denaturing conditions, with subsequent transfer of the nucleic acid to solid-membrane supports. Such blotting procedures are still being used with great effectiveness in the research setting. However, in the clinical diagnostics arena, they suffer from being laborious and of relatively low sensitivity compared with newer detection methodologies.

Of greater import for the clinical laboratory setting are chemiluminescent and fluorescent detection systems. Chemiluminescent probes are nucleic acid molecules modified with chemical labels that release light when treated with a cocktail of reagents that includes an oxidizing agent such as hydrogen peroxide. Chemiluminescent detection systems are highly efficient and provide

exquisite sensitivity. However, they are limited by the ability to detect simultaneously only two different targets in a single reaction.

Fluorescent-labeled probes provide greater multiplexing capabilities than chemiluminescent probes, since the chemical fluorophore labels used with the nucleic acid probes emit different wavelengths of light following stimulation by a light source. Sensitive detectors then differentiate the various probe signals and, based on the color of light emitted, the analytes present in the sample. This system also has the advantage of real-time detection, in which fluorescent probes hybridize to target amplicons as they are produced in the amplification reaction. This format reduces the time needed to perform the assay, which in turn increases the throughput of the lab performing the test. However, the price for this increased productivity is a loss of sensitivity in detecting nucleic acid targets, compared with chemiluminescent probe systems.

Certain NAT technologies achieve clinical utility by relying on enhancing the signal generated by the presence of a diagnostic target. Such technologies include the branched DNA (bDNA) method by **Bayer Diagnostics** (Tarrytown, NY) and the hybrid capture method by **Digene Corp.** (Gaithersburg, MD). Both of these methods rely on amplifying the signal generated after the targeted DNA or RNA hybridizes to a nucleic acid probe sequence. In the bDNA technology, a series of capture and linker oligonucleotides serve to join the target nucleic acid to the probe complex. This complex consists of a scaffold of DNA branches that contain binding regions for labeled probes. This configuration boosts the probe signal intensity by at least three orders of magnitude.

Digene's hybrid capture system is a signal amplification technology that uses antibodies specific for RNA-DNA hybrids to isolate nucleic acid targets in clinical samples. Secondary antibodies labeled with alkaline phosphatase subsequently bind to the hybrids, which are then detected with a chemiluminescent dioxetane substrate.

Automating NAT

Most NAT assays are complex procedures that require skilled technicians to master numerous techniques and specialized equipment. A shortage of qualified technicians coupled with stringent reimbursement policies by healthcare insurers have propelled the IVD industry to find more cost-effective ways to perform these tests.

One solution has been to automate NAT-based assays so that fewer and less-experienced operators are needed to conduct the increasing volume of tests. To meet this challenge, IVD manufacturers are racing to develop automated systems to perform NAT-based assays. The first fully automated NAT system to receive FDA approval is the TIGRIS DTS (Direct Tube Sampling) system by Gen-Probe, which is available to test for Neisseria gonorrhoeae and Chlamydia trachomatis. Without prior sample preparation, the TIGRIS system executes all aspects of NAT assays, including target capture, target amplification, amplicon detection, and reporting of results.

Other semiautomated platforms currently in development include the COBAS Amplicor analyzer by **Roche Diagnostics** (Indianapolis), and the GeneXpert system by **Cepheid** (Sunnyvale, CA). Both systems are intended for automating PCR-based NAT assays. The COBAS analyzer is a benchtop system that automates the amplification and detection steps of PCR-based assays. Sample preparation and capture of target analytes are performed using separate procedures prior to loading on the unit.

A small footprint for the support instrumentation makes Cepheid's GeneXpert system an attractive solution for point-of-care or low-volume settings. The system's disposable cartridge has a modular design that allows different assays to be run on the same instrument. By using a disposable-cartridge format, the system can accommodate a range of sample volumes. In addition to the processing chambers that store the reagents and a chamber where reactions take place, the cartridge has a fluidic valve that controls the flow of liquids from one chamber to

another, and a signal detection window. The GeneXpert system is being developed for group B streptococcus tests as well as other applications in biodefense and the life sciences.

Point-of-Care Challenges

The ultimate goal for many users of NAT assays is to have point-of-care diagnostic tests, in which a patient sample is analyzed directly in a physician's office or at the hospital bedside. The challenges associated with this goal are substantial and, with the current technology, not possible for NAT. Many of the advantages offered by the high throughput, automated sample-to-result platforms described above could apply to point-of-care unit-dose tests. However, their throughput requirements, instrumentation size, regulatory requirements, and cost hinder their use in point-of-care applications.

Developing point-of-care NAT systems will require assays and devices that are fully integrated and operator-free from sample to result. The systems will need to be rapid, as well as compact enough to fit in a doctor's office, and portable for bedside testing or environmental applications. The cartridges used in the devices will need to be closed to reduce contamination and allow for easy disposal. In addition, point-of-care NAT will require different assays that can be run on a single instrument. This might be achieved by having either a number of individual assay cartridges each performing a different test, or a single multiplexed assay cartridge running multiple tests.

IVD manufacturers have suggested various solutions to meet these requirements. The major current trend in the industry toward developing point-of-care NAT systems is in the area of microfluidic devices and related biomicroelectromechanical systems (bioMEMS).

Beyond Mere Miniaturization

Microfluidic devices are characterized by having very small channels or chambers in which analytes from clinical samples are processed, chemically manipulated, and detected. The technology needed for such devices has progressed rapidly during the past few years. Such progress is reflected by the large volume of literature that has been published in this area.

When assessing new developments and devices, IVD manufacturers should consider carefully how traditional test-tube-scale assay systems and research-based devices can be converted into commercially viable products. Changing the scale at which assays are conducted can create unanticipated problems with the behavior of sample materials and assay reagents, as well as the performance of the underlying assay chemistry. Microfluidic device development is not merely an exercise in miniaturizing existing technology. It is a complex process whereby existing assay chemistries and hardware platforms are codeveloped into new integrated systems.

Efforts to develop bioMEMS devices for clinical diagnostics have led to commercializing several related, but different, technologies used in massively high-throughput genomic screening assays. Such technologies, the so-called lab-on-a-chip platforms, include the LabChip products by Caliper Life Sciences (Hopkinton, MA) and the GeneChip microarray system by **Affymetrix** (Santa Clara, CA). These platforms offer the advantages of performing assays in microfluidic environments. The advantages are characterized by reduced reagent usage, shorter assay times, and, in some cases, the ability to perform reactions that are impossible in the macro world.

The bioMEMS-based platforms are different in that the disposable cartridges need to handle tens of milliliters of processed sample (the volume after completing sample preparation), as opposed to the single microliter, nanoliter, or even picoliter volumes used in existing lab-on-a-chip devices. Designing and building bioMEMS devices that can accommodate such larger sample volumes present significant engineering challenges. Such systems contain a network of individual building blocks. The ability to design, test, and fabricate successfully these building blocks (i.e., the fluidic

valves, channels, pumps, and mixers that can process larger sample volumes) will be crucial to developing bioMEMS devices.

One important design element for bioMEMS platforms is that they must include totally automated sample preparation of raw patient samples. This type of sample preparation has been lacking in lab-on-a-chip devices, such that target purification, extraction, capture, and volume reduction (from milliliter sample volumes to microliter processing volumes) usually occur “off chip.” Choosing whether to perform such sample preparation onboard a chip is a balance between technology and economics. If an IVD manufacturer decides to include sample processing in the supporting instrument, then the instrument must remain compact and ergonomically amenable to point-of-care settings. Even the most technologically advanced or economically viable chip will not be successful in a point-of-care setting if it requires an instrument the size of a washing machine to run it.

As these technical challenges are being overcome, devices with simple chip-to-world interfaces that are disposable, reliable, cost-effective, and contain the internal controls required for regulatory approval are being developed. As technology brings NAT diagnostics closer to patients, the potential for patient-specific diagnostics becomes more realistic.

Conclusion

The miniaturizing of NAT technology will likely mature in the coming decades, and as a result, the role of the NAT diagnostic test in patient care will increase greatly in prominence. Achieving the current miniaturization goals of design engineers will allow future implementation of rapid, inexpensive point-of-care tests performed at the bedside. Such tests will be complemented by fully automated, high-throughput, multiplexed methods performed in central labs. A vast menu of tests for infectious organisms will then be available for such platforms, along with genomic DNA testing for individualized pharmacotherapy. All of these developments in concert will serve to improve healthcare and medical knowledge in the 21st century.

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