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Automating nucleic acid amplification tests

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Automating nucleic acid amplification tests

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Although most nucleic acid amplification tests have generally had a history of excellent performance, they have not had widespread acceptance in clinical laboratories. Automation may help to make these tests more widely accepted.

Nucleic acid testing for infectious diseases has come into widespread use in many clinical microbiology laboratories in recent years. These tests have the potential to revolutionize infectious-disease diagnostics because they are usually more accurate than conventional tests and they can also identify organisms that are difficult or impossible to detect with culture tests.

Technologies

The three main types of nucleic acid testing technologies are nucleic acid probes, signal amplification, and nucleic acid amplification. Nucleic acid probe tests are the simplest of these technologies. These tests use a labeled nucleic acid probe to hybridize to target nucleic acid molecules. Gen-Probe Inc. (San Diego) developed the first FDA-approved nucleic acid probe test kits for infectious-disease testing in 1985.[1] These highly accurate tests are relatively simple to perform compared with the conventional culture and enzyme immunoassay (EIA) tests. The PACE 2 nucleic acid probe dual test for *Chlamydia trachomatis* (CT), and *Neisseria gonorrhoeae* (GC) is the most widely used nonculture test in the United States for these organisms.[2,3]

Signal-amplified tests are essentially nucleic acid probe tests that use multiple labels to amplify the signal of the assay. Examples of this type of test are bDNA (Bayer Diagnostics Inc.; Emeryville, CA) and the Hybrid Capture assay (Digene Corp.; Gaithersburg, MD).

Nucleic acid amplification tests are the most complex and sensitive of the nucleic acid testing technologies. They use enzymes to amplify the target nucleic acid and most are theoretically capable of detecting in a sample a single nucleic acid molecule. Most of the nucleic acid amplification tests use nucleic acid probes to identify the resulting amplified material.

Nucleic acid tests have become available in the last decade for use in clinical microbiology laboratories. Starting in 1993, FDA cleared for clinical diagnostic use several first-generation test kits that use various types of technologies to detect a variety of infectious organisms (see Table I).[4–8] Many other test kits to identify organisms such as the hepatitis C virus, hepatitis B virus, CMV, HTLV-I/II, and enterovirus are available for research use only.[9]

The primary advantage of nucleic acid amplification assays over DNA probe or signal amplification tests is that they often have significantly higher analytical sensitivity. This means that they can detect far smaller amounts of microorganisms than can be identified by nonamplified nucleic acid probe tests or other more-conventional tests.

Nucleic acid tests also provide greater flexibility than other technologies in the types of samples that can be analyzed. For example, DNA probe tests and some EIA tests can accurately detect CT and GC in endocervical or male urethral samples, but they are not as effective in urine samples. Only nucleic acid tests can detect these organisms in urine samples with adequate sensitivity. This enables the use of less-invasive procedures for collecting samples.

Nucleic acid amplification assays are

Nucleic Acid Amplification Technology	*Commercial Source*	*FDA-Cleared or -Approved Assays*
PCR	Roche Diagnostics	*C. trachomatis* *N. gonorrhoeae* HIV-1, *M. tuberculosis*
TMA	Gen-Probe Inc.	*M. tuberculosis* *C. trachomatis*
LCR	Abbott Laboratories	*C. trachomatis* *N. gonorrhoeae*
SDA	Becton Dickinson	*C. trachomatis* *N. gonorrhoeae*

Table I. FDA-approved nucleic acid amplification tests for infectious diseases.

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theoretically capable of detecting as little an amount as one organism in a sample. However, in practice, this sensitivity is rarely achieved. Sample inhibition can hinder the amplification reaction and result in false-negative results.[10,11] Sample inhibition occurs when factors in some samples inhibit the enzyme activities needed for the amplification reaction. The high sensitivity of nucleic acid amplification tests also makes the tests susceptible to contamination that can cause false-positive results. Sample inhibition as well as contamination problems are not typically observed with DNA probe or signal amplification tests.

The Need for Automation

Although most nucleic acid amplification tests generally have had a history of excellent performance, widespread acceptance of many of these first-generation tests in the routine clinical laboratory has been limited. This has been attributed to higher reagent costs necessitated by these tests, increased labor requirements, specimen inhibition, and contamination problems.[10–14] These factors have contributed to slow market acceptance of many of the tests in an environment that is increasingly moving toward lower-cost, automated testing.

One exception to this lack of clinical laboratory acceptance has been in the case of the HIV viral-load tests.[8] The success of these tests has been driven by the clinical need for a quantitative test to monitor HIV drug treatment, coupled with the absence of practical alternative testing technologies.

In contrast to the HIV viral-load tests, nucleic acid amplification tests for CT and GC have had only limited acceptance, gaining only approximately 20% of the market share since the first FDA-approved polymerase chain reaction (PCR) test was introduced in 1993.[4] The limited acceptance of these tests is due in part to the availability of other tests such as the nonamplified nucleic acid probe tests and EIAs that are accurate, less expensive, and easier to run than the nucleic acid amplification tests.[2,3]

Some of the current first-generation tests have been partially automated in an attempt to reduce the labor requirements, thus enabling laboratory managers to increase overall laboratory efficiency. Most of these tests automate only the amplification and detection steps of the assay—not the labor-intensive sample-processing step.

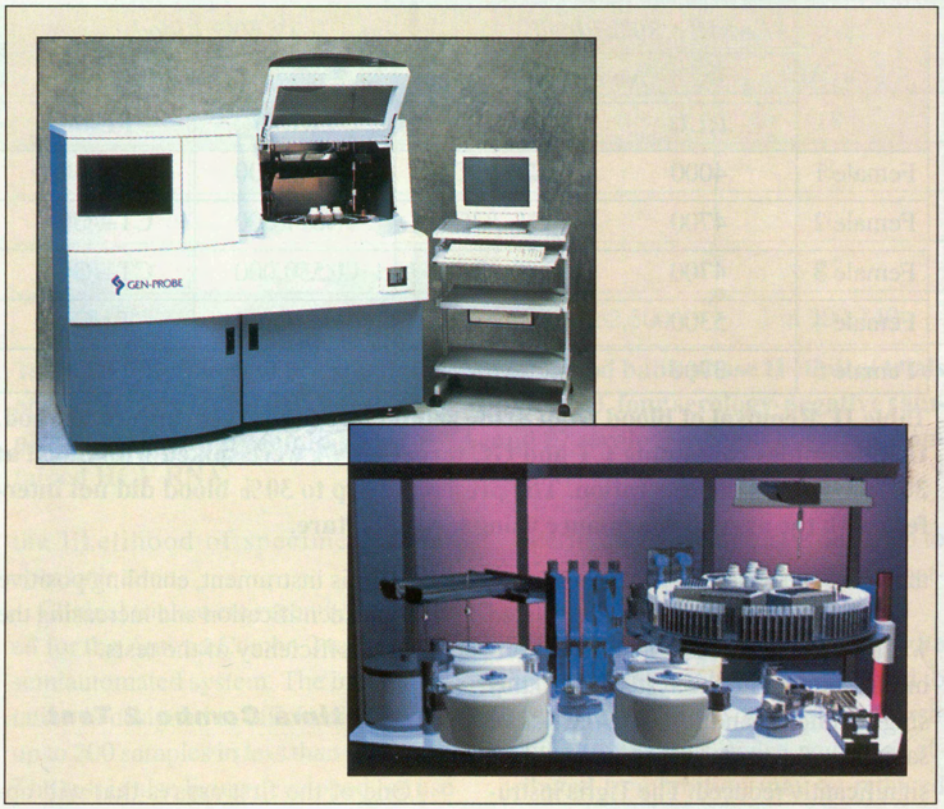

Figure 1. The Tigris instrument (top) fully automates all steps of the TMA tests. Bottom, model of the internal components of the Tigris.

However, sample processing is typically the most troublesome part of these tests. It has been called the Achilles' heel of first-generation nucleic acid amplification tests. Currently, sample-processing procedures are labor intensive, often requiring centrifugation and organic extraction steps. Also, these methods often do not adequately purify the target nucleic acid. They often leave inhibitory or interfering substances in the reaction mixture that can cause inhibition of the amplification reaction and result in false-negative results.[10, 11] The manual nature of current sample-processing techniques also can lead to specimen cross-contamination, which can cause false-positive results.[12–14]

Although some automated sample preparation systems are available for home-brew tests (e.g., the QIAamp kit from Qiagen Inc.; Valencia, CA), similar sample-preparation systems have not yet been integrated into FDA-approved kit tests.

Fully Automated Instrumentation

Full automation of assays and improved specimen-processing procedures can overcome many of the problems associated with first-generation tests. In particular, automating the sample preparation step can eliminate many contamination problems.

Gen-Probe Inc. is developing the first fully automated instrument for nucleic acid amplification testing. The company's Tigris instrument system has been designed to automate all the steps of the testing procedure, from sample processing through amplification and detection (see Figures 1a and 1b). The throughput goal of the instrument is up to 500 tests per 8-hour shift or 1000 tests per 12-hour shift. It takes about 3½ hours for the first result, and up to 125 sample results can be obtained each subsequent hour, depending on the requirements of

	30% Blood No RNA		30% Blood 5 fg CT/250 fg GC	
	RLU	Result	RLU	Result
Female 1	4000	CT–/GC–	1,483,000	CT+/GC+
Female 2	4700	CT–/GC–	1,671,000	CT+/GC+
Female 3	4700	CT–/GC–	1,530,000	CT+/GC+
Female 4	5300	CT–/GC–	1,847,000	CT+/GC+
Female 5	3700	CT–/GC–	1,553,000	CT+/GC+

Table II. Removal of blood from urine samples by the target capture method. Urine samples containing CT and GC target rRNA were spiked with blood at 30% (v/v) final concentration. The presence of up to 30% blood did not interfere with the assay performance using target capture.

the particular assay.

The only sample-handling step needed when using Tigris is to load the specimens directly into the system. With this single sample-handling step, the risk of sample cross-contamination should be significantly reduced. The Tigris instrument also automatically destroys amplicon (the amplified product) after testing is finished to help decrease the chance of carryover contamination.

Specimen bar code reading, automatic worklist creation from the input carousel, and bidirectional communication with a laboratory information system (LIS) will be an integral part of the Tigris instrument, enabling positive sample identification and increasing the overall efficiency of the tests.

Aptima Combo 2 Test

One of the first assays that will appear on the system is the Aptima Combo 2 test for CT and GC.[15,16] This is a second-generation assay using four core technologies—target capture specimen processing, transcription-mediated amplification (TMA), hybridization protection assay (HPA), and dual kinetic assay (DKA) detection technology.

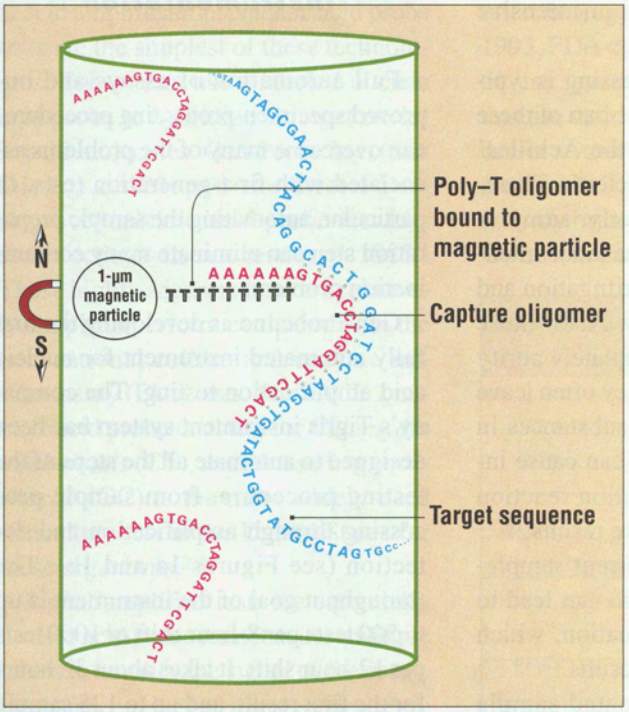

Figure 2. Diagram of the target capture specimen processing method for the Gen-Probe TMA assays.

In the first step in the automated procedure, target capture technology is used to purify and concentrate rRNA target molecules.

The target capture procedure works by first lysing the microorganisms to release the target nucleic acid (see Figure 2). The target sequence is hybridized to an intermediate capture oligomer. This capture oligomer is then captured by poly-T oligomers bound to the surface of 1-µm magnetic particles. The particles are drawn to the side of the reaction tube by magnets and washed to purify and concentrate targets and remove extraneous material, including potentially interfering substances.

Target capture sample processing has been designed to effectively remove inhibitors and other interfering substances.[15,16] The target capture procedure reduces false-negative results by removing the inhibitors that may occur in the samples. Target capture simplifies sample processing and makes automation easier. This is due, in part, to the absence of centrifugation steps and other complex steps required in most of the current nucleic acid amplification tests.

Data in Table II demonstrate the efficiency of the target capture method in removing blood from urine samples containing CT and GC rRNA. First-generation amplification tests for CT and GC are inhibited by small amounts of blood. Concentrations of 6% blood or less in urine or swab specimens have been shown to inhibit all first-generation NAA assays.[19–21] Data show that up to 30% blood spiked into urine can be efficiently removed by target capture and does not interfere with the overall performance of the assay. Similar results have been obtained for other known inhibitory substances spiked into urine.[16]

Once the target capture step is complete, TMA is used to amplify the captured sequences on the surface of the magnetic particles. TMA is an RNA transcription amplification system that uses two enzymes to drive the reaction—RNA polymerase and reverse transcriptase (see Figure 3).

The reaction in the TMA process is isothermal; it is performed at a single temperature, unlike other amplification reactions, such as PCR, that require a thermal cycler instrument to rapidly cycle the temperature to drive the reaction. TMA amplifies RNA but it can also be modified to amplify DNA. RNA amplification product is produced, in contrast to many of the other nucleic acid amplification methods that produce DNA.

TMA uses two primers for each analyte in the reaction. One of the primers contains a promoter sequence for RNA

polymerase. In the first step of amplification, the promoter-primer hybridizes to the target rRNA at a defined site. Reverse transcriptase creates a DNA copy of the target rRNA by extension from the end of the promoter-primer. The RNA in the resulting RNA:DNA duplex is degraded by the RNAse H activities of the reverse transcriptase. A second primer then binds to the DNA copy. A new strand of DNA is synthesized from the end of the primer by reverse transcriptase creating a double-stranded DNA molecule. RNA polymerase recognizes the promoter sequence in the DNA template and initiates transcription. Each of the newly synthesized RNA amplicons reenters the TMA process and serves as a template for a new round of replication leading to an exponential expansion of the RNA amplicon. Since each of the DNA templates can make 100–1000 copies of RNA amplicon, this expansion can result in the production of up to 10 billion amplicons in 15–30 minutes.

After the CT and GC targets are coamplified using TMA in a single reaction well, they are simultaneously identified with DKA detection. This technology is based on the HPA technology used in nearly all of the Gen-Probe nucleic acid test assays.[17] DKA uses two different acridinium ester molecules attached to two different nucleic acid probes.[18] One of the acridinium ester molecules has fast light-off kinetics, and the other acridinium molecule has slower kinetics. By targeting two different target sequences, assays can be developed to simultaneously detect two different organisms and two results can be obtained simultaneously in the same reaction well, using one specimen in one reaction (see Figure 4). No reflex testing is necessary to identify the individual organisms.

The Aptima Combo 2 test also uses a new sample transport system that incorporates a penetrable cap. The system extracts a specimen from a closed tube, which removes a major time-and-labor issue for operators and will help reduce the likelihood of specimen cross-contamination.

Testing Agency	*No. Donations Tested*	*TMA-Positive, Sero-Negative*	
		HIV-I	*HCV*
ARC	5,510,000	2	18
ABC	1,990,000	2	8
AIBC	350,000	0	1
Total	7,850,000	4	27
Yield:		1 in 1,962,500	1 in 290,740

Table III. Summary of interim data from the blood bank phase II clinical trials of the Procleix HIV-1/HCV test. As of March 2000, four serology-negative samples were shown to contain HIV-1 RNA, and 27 serology-negative samples contained HCV RNA.

Clinical trials were recently completed for the Aptima Combo 2 test using a semiautomated system. The instrumentation is designed to efficiently process up to 200 samples in less than six hours. The test is also being developed for use in the fully automated Tigris system format. The processing steps of the Aptima technology on the Tigris instrument are shown in Figure 5.

The schematic diagram shows the main subsystems of the instrument and their functions. Steps A and B are the only manual steps.

A. Multitube unit (MTU) reaction tubes are loaded into the MTU input.
B. Samples are placed in the sample carousel.
C. Samples and target capture reagent are transferred to the MTU tubes and mixed.
D. Target capture incubation, capture sequences hybridize to target molecules.
E. Target molecule hybrids are captured on beads.
F. Target capture magnetic particle wash removes specimen matrix.
G. Amplification reagent addition, mix, oil addition.
H. Enzyme reagent addition, amplification incubation.
I. HPA and DKA hybridization and selection steps.
J. Read in luminometer.
K. Add deactivator and aspirate waste.

Performance of the test is designed to be equivalent on both the semiautomated and Tigris platforms.

The Aptima Combo 2 test on the Tigris instrument has been designed to address many of the drawbacks of first-generation assays. False negatives attributed to inhibition may be reduced or eliminated using target capture technology. The Aptima Combo 2 test on the Tigris requires no manual intervention after the samples are loaded into the machine so labor requirements are reduced, which should decrease the overall cost to run the assays. Automation may also decrease the likelihood of operator error that can lead to contamination.

Blood Bank Test for HIV-1 and HCV

Despite improvements in HIV and HCV serological tests in recent years, instances of viral transmission via transfusion still occur because of donations that take place while a donor is in the preseroconversion window phase, is infected with immunovariant viruses, or is a nonseroconverting chronic carrier. Direct, sensitive detection of viral nucleic acid could substantially decrease the incidence of transfusion-induced infections.

Gen-Probe Inc., in partnership with Chiron Corp. (Emeryville, CA), has developed a multiplex test for the simultaneous detection of HIV-1 and HCV RNA in donated blood samples. This assay was developed under a contract

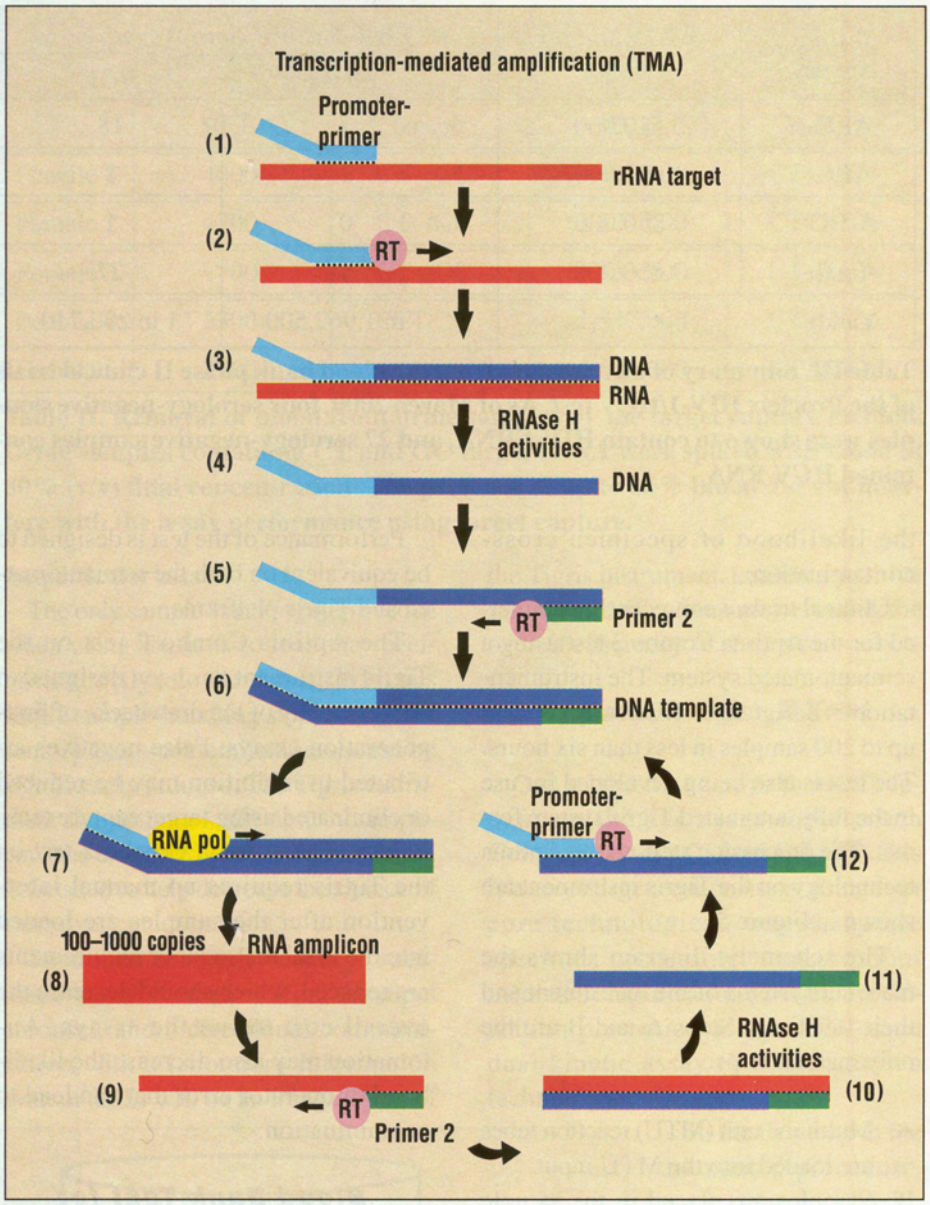

Figure 3. Diagram of the TMA reaction.

with the National Heart, Lung, and Blood Institute for the purpose of decreasing the risk of HIV or HCV transmission via transfusion. The Chiron Procleix HIV-1/HCV assay uses the same four technologies as the Aptima Combo 2 tests. The test was developed and manufactured by Gen-Probe Inc., and is distributed to the blood-screening market worldwide by Chiron Blood Testing, a division of Chiron Corp. The test is currently approved and used to screen the blood supply in several countries outside the United States. It is in phase III clinical trials at several blood bank sites in the United States using the Chiron Procleix system incorporating an automated pipetting station. Automation of the test on the Tigris instrument is in development.

Analytical sensitivity studies have shown that the assay can reliably detect less than 100 copies per mL of HIV-1 or HCV RNA.[22] These same studies have shown that the test can detect all known subtypes of both viruses, including Group O and Group N strains, with similar sensitivity.

The American Red Cross (ARC), America's Blood Centers (ABC), and Association of Independent Blood Centers (AIBC) have been conducting phase II clinical trials with the assay. In the United States, samples from individual donations are pooled into lots of 16 donations each, and tested with the Procleix HIV-1/HCV assay, in addition to all existing serological tests required. Interim data have shown that out of a total of 7,850,000 whole-blood donations collected between April 1999 and March 2000, four HIV and 27 HCV serology-negative samples were identified with the test.[23–24] (see Table III). This represents samples that could have been transfused and potentially infected as many as 93 people with a virus if the assay had not been implemented (up to three people may be transfused with products from a single blood donation).

The Procleix assay incorporates an internal control to monitor for inhibition of the amplification reaction. The phase II studies have demonstrated an initial internal control failure rate of less than 0.8%, and a 0% failure rate upon repeat testing.[24] This again provides evidence that the target capture method is able to virtually eliminate inhibition in the assay.

These studies show that this assay has a significant sensitivity advantage over the existing serological tests for the detection of virus in donated blood samples. Preliminary data indicate that HIV-1 can be detected an average of 16.3 days before the presence of an antibody, and 7.5 days before P-24 antigen can be detected.[24] HCV can be detected an average of 32.8 days before antibody can be identified.

Implementation of the assay in routine blood bank testing will achieve the FDA and NIH goal of using the latest technology to continue to improve the safety of the U.S. blood supply. The high volume of donated samples processed through blood bank laboratories necessitates the use of efficient, automated testing. The ability of this assay to detect both organisms simultaneously, together with its eventual implementation on the fully automated Tigris system, will allow blood bank laboratories to efficiently use the more sensitive and specific TMA test with donated blood samples.

A triplex TMA assay for simultaneous detection of HIV-1, HCV, and HBV is currently being developed for use on the Chiron Procleix system and

positive amplification reaction. These safeguards minimize the risk of contamination in the Tigris instrument.

Conclusion

Nucleic acid amplification testing has the potential to revolutionize not only infectious-disease testing, but also many other areas of clinical testing in the fields of genetics, cancer, and other chronic human diseases. Although many new FDA-approved tests are becoming available, clinical laboratories have difficulty implementing routine nucleic acid amplification testing because of the high labor requirements, contamination, and inhibition problems of the first-generation tests. Larger laboratories are particularly hindered by the relatively high labor requirements and low throughput of many of the new test systems.

New automated instruments such as the Tigris instrument will alleviate these concerns by decreasing the labor requirements and thus the overall costs of this type of testing. Many laboratories will be able to take advantage of the higher throughput afforded by the Tigris instrument. Full automation will also reduce the risk of human error and contribute to increased accuracy of results. Second-generation automated technologies will make nucleic acid amplification testing a reality for any clinical laboratory and lead to improvements in healthcare through more-rapid and more-accurate diagnostic tests.

References

1. CH Hill, "Molecular Diagnostics for Infectious Diseases," *Journal of Clinical Ligand Assay* 19, no. 1 (1996): 43–52.

2. PC Iwen et al., "Evaluation of Nucleic Acid Based Test (PACE 2C) for Simultaneous Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in Endocervical Specimens," *Journal of Clinical Microbiology* 33, no. 10 (1995): 2587–2591.

3. R Warren et al., "Comparative Evaluation of Detection Assays for *Chlamydia trachomatis*," *Journal of Clinical Microbiology* 31, no. 6 (1993): 1663–1666.

4. JE Bauwens et al., "Diagnosis of *Chlamydia trachomatis* Urethritis in Men by Polymerase Chain Reaction Assay of First-Catch Urine," *Journal of Clinical Microbiology* 31, no. 11 (1993): 3013–3016.

5. A Miettinen et al., "Comparison of Enzyme Immunoassays Antigen Detection, Nucleic Acid Hybridization and PCR Assay in the Diagnosis of *Chlamydia trachomatis* Infection," *European Journal of Clinical Microbiological Infectious Disease* 14 (1995): 543–549.

6. V Jonas et al., "Detection and Identification of *Mycobacterium tuberculosis* Directly from Sputum Sediments by Amplification of rRNA," *Journal of Clinical Microbiology* 32 (1993): 2410– 2416.

7. DV Ferrero et al., "Performance of the Gen-Probe Amplified *Chlamydia trachomatis* Assay in Detecting *Chlamydia trachomatis* in Endocervical and Urine Specimens from Women and Urethral and Urine Specimens from Men Attending

Sexually Transmitted Disease and Family Planning Clinics," *Journal of Clinical Microbiology* 35, no. 11 (1998): 3230–3233.

8. J Mulder et al., "Rapid and Simple PCR Assay for Quantitation of Human Immunodeficiency Virus Type 1 rRNA," *Plasma: Application to Acute Retroviral Infection, Journal of Clinical Microbiology* 32 (1994): 292–300.

9. PR Murray et al., *Manual of Clinical Microbiology*, 7th ed. (Washington, DC: American Society of Microbiology, 1999).

10. JB Mahony, "Role of Confirmatory PCRs in Determining Performance of Chlamydia Amplicor PCR with Endocervical Specimens from Women with a Low Prevalence of Infection," *Journal of Clinical Microbiology* 32, no. 10 (1994): 2490–2493.

11. J Mahony et al., "Urine Specimens from Pregnant and NonPregnant Women Inhibitory to Amplification of *Chlamydia trachomatis* Nucleic Acid by PCR, Ligase Chain Reaction, and Transcription Mediated Amplification: Identification of Urinary Substances Associated With Inhibition and Removal of Inhibitory Activity," *Journal of Clinical Microbiology* 36, no. 11 (1998): 3122–3126.

12. DJ Farrell, "Evaluation of Amplicor *Neisseria gonorrhoeae* PCR Using cppB Nested PCR and 16S rRNA PCR," *Journal of Clinical Microbiology* 37, no. 2 (1999): 386–390.

13. AM Gronowski et al., "Reproducibility Problems with the Abbott Laboratories LCx Assay for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*," *Journal of Clinical Microbiology* 38, no. 6 (1999): 957–959.

14. EM Peterson et al., "Reproducibility Problems with the Amplicor PCR *Chlamydia trachomatis* Test," *Journal of Clinical Microbiology* 35, no. 4 (1997): 957–959.

15. J Shaw et al., "Performance of Aptima Combo 2 Test with High Target Level Samples and Closely Related *Neisseria* Organisms," (paper presented at the American Society for Microbiology, 100th General Meeting, Los Angeles, May 2000).

16. J Carlson et al., "Target Capture as Sample Processing for Transcripted-Mediated Amplification (TMA) and Subsequent Detection and Differentiation of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in a Single Tube," *Clinical Chemistry*, 44 (1998): A5.

17. NC Nelson et al., "Detection of Acridinium Esters by Chemiluminescence," in *Nonisotopic Probing, Blotting, and Sequencing*, ed. LJ Kricka (San Diego: Academic Press, 1995): 391–428.

18. NC Nelson et al., "Simultaneous Detection of Multiple Nucleic Acid Targets in a Homogeneous Format," *Biochemistry* 35 (1996): 8429– 8438.

19. *Chlamydia trachomatis* assay package insert, Abbott Laboratories, Diagnostics, August 1996.

20. Cobas Amplicor CT/NG Test for *Chlamydia trachomatis* package insert, Roche Diagnostics, 1999.

21. Gen-Probe Amplified *Chlamydia trachomatis* assay package insert, Gen-Probe Inc., 1999.

22. L Mimms et al., "Preclinical Performance of the TMA HIV-1/HCV Assay and HIV-1 and HCV Discriminatory Assays," in *Proceedings of the International Society of Blood Transfusion (ISBT) Congress* (Lancaster, Lancashire, UK: ISBT, 2000).

23. SL Stramer et al., "U.S. Experience with Nucleic Acid Testing (NAT) for HIV-1 and HCV" (Gaithersburg, MD: American Red Cross).

24. L Mimms, "TMA Assays for Blood Screening: HIV-1/HCV Assay, HIV-1/HCV/HBV Triplex, HIV-1/HCV/HBV Discriminatory Assays," (paper presented at the Transfusion 2001 Conference, Cambridge, UK, April 2000).