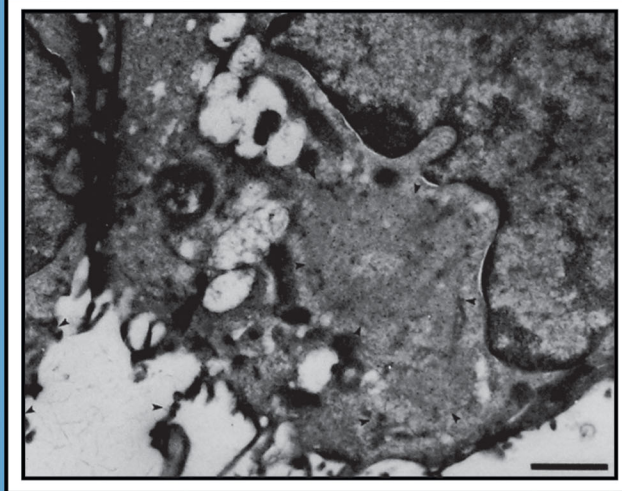
Immunological and Molecular Diagnosis of Infectious Disease



edited by

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Preface

This volume comprises the majority of the scientific contributions to the XIIIth International Convocation on Immunology, *Immunological and Molecular Diagnosis of Infectious Disease*, sponsored by The Ernest Witebsky Center for Immunology, State University of New York at Buffalo, held in Buffalo, June 1–5, 1996.

In recent years, new or previously unappreciated problems in the field of infectious diseases have emerged. In addition, new assay methods, particularly those involving DNA and RNA amplification and detection, have been developed for the rapid diagnosis of such diseases. While some of the infectious agents, such as *Helicobacter*, *Borrelia*, hantavirus and prions, have only recently come to prominence, others are previously encountered agents that are causing renewed concern after years of apparent control, such as *Mycobacterium tuberculosis* and hepatitis viruses.

Part I of these Proceedings treats methodologies of Immunological and Molecular Approaches, including DNA and RNA amplification methods, ELISA approaches, rapid diagnosis methods, biosensors, and flow cytometry. Part II deals with the more clinical aspects of Bacterial and Parasitic Infections comprising *M. tuberculosis*, *Borrelia burgdorferi*, *Streptococcus pyogenes*, Chlamydia, and schistosomes. Part III treats Viral Infections, e.g., hantaviruses, hepatitis C virus, cytomegalovirus, herpes viruses, and human immunodeficiency virus. Part IV groups Syndromes of Various Infectious Origins, including prions, chronic fatigue syndrome, and superantigens. All major topics discussed at the XIIIth Convocation are included in this volume, with one notable exception.

That exception is the presentation by Dr. Yi-Fu Zhou, of the Molecular and Cell Biology Laboratory, Cardiology Branch, NHLBI, NIH, Bethesda, Maryland, on "The Potential Role of Cytomegalovirus in Atherosclerosis and in Coronary Restenosis Following Angioplasty." To avoid duplication of publication, the text of Dr. Zhou's contribution could not be incorporated in this volume: the essence of the work by Dr. Zhou et al. was published shortly after his presentation in the New England Journal of Medicine (1). In the late 1980s and early 1990s epidemiological studies initiated in different medical schools in the United States (2) and Europe (2,3) indicated that a significantly high percentage of atherosclerosis and heart disease patients showed prior infection with cytomegalovirus (CMV), as contrasted with normal controls. In 1994, Speir et al. (also from the Cardiology Branch, NHLBI, NIH) proved the existence of a molecular link between CMV and the tumor suppressor protein p53 in coronary restenosis lesions (4). The 1996 paper (1) further strengthens the connection between CMV infection and arterial and heart disease.

While it is not feasible to treat all aspects of modern Immunological and Molecular Diagnosis of Infectious Disease in one volume, the 25 chapters of this work aim to provide highlights of the most recent developments in the clinical diagnosis of several major emerging and re-emerging infectious diseases. This volume points to the increasing reliance on these new technologies for what will soon become routine diagnostic procedures.

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ADDENDUM

Due to unforeseen circumstances the contribution by J.B. Myers and D. Amsterdam on "The Laboratory Diagnosis of Cytomegalovirus Infections" does not appear in this volume. However, it has been printed in Immunological Investigations, *26(3)*, 383 (1997), Marcel Dekker, Inc.

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PART I

IMMUNOLOGICAL AND MOLECULAR APPROACHES

DNA AMPLIFICATION

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ABSTRACT

The polymerase chain reaction has become a mainstream tool for the molecular biologist. The sensitivity, efficiency, and speed of this method is unparalleled for the amplification and detection of exquisitely minute quantities of nucleic acids. Through repetitive cycles of heat denaturation of samples, followed by the base pairing of primers designed to identify one DNA sequence among the cellular heterogeneity, and finally synthesis of new DNA strands identical to the target, single molecules and individual genes can be detected and subsequently characterized. This method has revolutionized the study of gene organization, structure, and expression, not to mention offering newer, faster, and more economical means for the clinical detection infectious disease.

That PCR has been fruitful is undisputed; however, the method is not without shortcomings. Among the major limitations of this method are the absolute requirement for well-designed primers, the super sensitivity of this method to biological contaminants from any of a variety of sources, and subtle, though very important, inter- and intra-laboratory variations in technique.

UNDERSTANDING PCR

The Polymerase Chain Reaction[1] (PCR) has, without a doubt, revolutionized the entirety of molecular biology. Nearly every research project, application, and diagnostic methodology has implemented molecular cloning and identification at some level, and PCR has become, perhaps, the premier tool.

Recent refinements in PCR-related techniques have afforded an unprecedented level of sensitivity and resolution in most molecular assays. As a direct result of the sensitivity and resolving power of this technique, often only a few short hours span the beginning of the search for a new gene and the selection of the gene itself. The technique of PCR has obvious advantages, and the simple methodology has been adapted in order to develop assays for the identification of single copy gene sequences from complex genomes, the selection of

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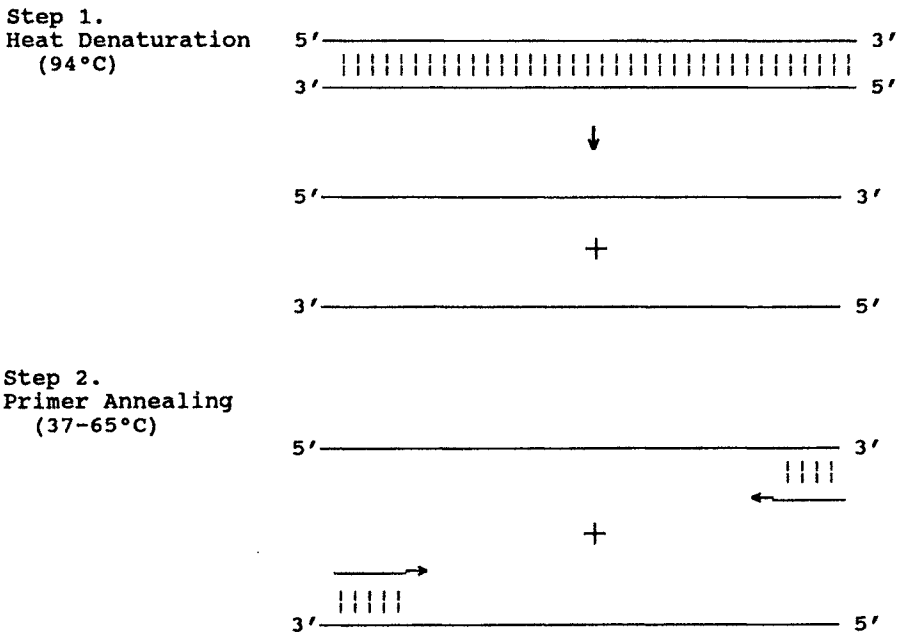
[1]The Polymerase Chain Reaction process is covered by patents issued to Hoffman-LaRoche, Inc. Use of the PCR process requires a license under their patents.

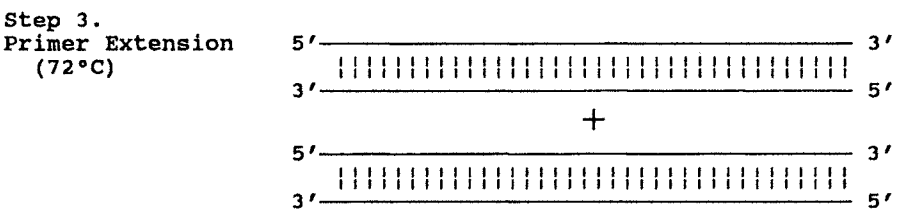
individual members of multigene families, identification and deliberate introduction of point mutations, deletions, amplifications, and gene rearrangements, and the identification of infectious agents by amplification of foreign genetic material from the blood of the host.

Comprehensive reviews of the subtleties of PCR have been published elsewhere (1,2,3), and a brief review of the method is presented here in order to convey the profound impact PCR continues to have on the daily routine of the most sophisticated and not-so-sophisticated laboratories around the world. The method of PCR is primer-mediated enzymatic amplification of specific genomic or cDNA sequences. Succinctly, PCR is possible because thermostable prokaryotic DNA polymerases are readily available, because single stranded DNA molecules (oligonucleotides) can be economically synthesized consisting of any combination of nucleotides desired by the investigator, because of instrumentation capable of efficient regulation of temperature, and because of DNA sequencing technology.

The polymerase chain reaction is divided into cycles, each of which is comprised of three discrete components, namely **denaturation** of both target sequences and primers, by which all of these molecules become single-stranded; **primer annealing**, by which the oligonucleotide primers involved base pair to a complementary sequence within the template (target) DNA; and **primer extension**, in which the polymerase activity of a thermostable enzyme (e.g. *Taq* polymerase) synthesizes DNA from the 3′-OH terminus contributed by each primer molecule which has base-paired to the template.

PCR: THE CYCLE





These three steps, as illustrated above, constitute "cycle 1" and the repetition of these steps using the products of the prior cycle as new template supports the geometric accumulation of those sequences. After only 30 cycles it is thus possible to achieve a multimillion fold amplification of template (2^{30} = 1,073,741,824; however the reaction is not 100% efficient).

The ability to perform PCR requires some knowledge of the sequence to be amplified or of the sequences which flank the region to be amplified. In the absence of direct nucleotide sequence information, the amino acid sequence of the corresponding peptide can be scrutinized in order to deduce the probable nucleotide sequence responsible for encoding that particular combination of amino acids. If this strategy is employed for primer design, a lack in the completeness of protein sequence data may not be an impediment because as few as 6 or 7 sequenced amino acids may be adequate for the design of an 18- or 21-mer oligonucleotide primer. Quite useful in this regard are protein domains containing large amounts or methionine and tryptophan, both of which are single codon amino acids. If such a domain has not been identified, eight additional amino acids have only two possible codons, a density of which may facilitate primer design. Thus, it may be possible to deduce an appropriate primer sequence to support PCR. Obviously, this is not a fool-proof method of cloning by PCR, however, because of the fair amount of degeneracy within the code itself (some amino acids have more than one possible codon).

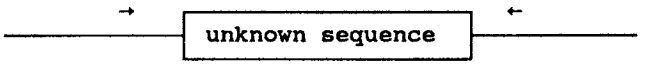
Fortunately, oligonucleotide primers are not required to anneal or match perfectly with the template. Were this an absolute requirement, the PCR process would be far less productive and far more frustrating indeed. Because of the extremely short nature of the oligonucleotide primers, the thermodynamic behavior of these molecules and their affinity for each other can be predicted with great accuracy. Often, temperature deviations of as little as one or two degrees mean the difference between successful amplification and the failure of that reaction. By lowering the temperature of the primer annealing component of each cycle (above) in the polymerase chain reaction, primers which are not perfectly base paired (matched) with their intended templates will be capable of base pairing among complementary nucleotides and at the same time will tolerate a mismatch at those locations where non-complementary nucleotides abut. The extent of base pairing is less critical than the location of mismatches; base pairing of the terminal 3′ dinucleotide is the only absolute requirement to support amplification.

QUANTIFICATION

Among the more popular applications of PCR technology is the method of transcript amplification for both sequencing and quantification purposes. Known as RNA PCR or RT-PCR, this method involves reverse transcriptase-mediated cDNA synthesis according to standard methodologies, using RNA purified according any of a variety of protocols (4), followed by PCR amplification of the newly synthesized cDNA. The mass of a PCR product so generated is directly proportional to the relative abundance of the transcript.

LIMITATIONS

While the promise of PCR has been fruitful indeed, the method does have limitations. A great many questions could certainly be answered, were there primers available to support analysis by PCR. A fundamental difficulty in many laboratories is the inability to perform PCR because the required primers have yet to be developed. This is especially true of research which involves the study of a "biological activity" of a yet-to-be characterized enzyme or other gene product. As stated above, the ability to perform PCR is dependent upon having some nucleic acid sequence information either about the sequence which is to be amplified, or about the sequences which flank the sequence to be amplified.



This sequence information can be either direct or indirect: direct sequences are derived by sequencing at least part of the target to be amplified, while indirect sequence information may be derived from another member of a multigene family, by characterization of the same gene in a different species (cloning by evolutionary relatedness), or by direct protein sequencing. The bottom line: no primers, no PCR.

That the polymerase chain reaction is too sensitive is a frequent complaint among investigators who routinely perform PCR. Given that a geometric amplification of template occurs, a lone carry-over molecule is enough to generate false signals and/or false positives. The gravity of such an occurrence is especially profound in a diagnostic setting for a number of obvious reasons. Contamination in the PCR environment has been traced to aerosol formation from micropipettors, tainted stock solutions of PCR reagents, contaminated thermal cyclers, lab coats and, in some cases, the epidermal layers of the investigator himself.

Lastly, the PCR technique requires empirical formulation of the exact conditions for amplification with each primer pair brought into the laboratory. Magnesium, a required divalent cation, is notorious for mediating the mispriming and amplification of sequences unrelated to the template and, by virtue of its ability to quantitatively bind dNTP's, exert

great influence on the efficiency of amplification in general. Moreover, the precise type of thermal cycler that is utilized may have a profound bearing on the efficiency and outcome of the reaction. Because of the way in which samples are heated and cooled, and the ramping time when moving among temperatures, enormous variation should be expected when comparing similar/identical protocols performed in different laboratory settings.

PCR has, without a doubt, revolutionized molecular biology, and all related disciplines. Experiments which in the past required days/weeks to perform can now be performed in a matter of hours. The ability to confirm a diagnosis by sensitive, specific means of DNA amplification affords the physician an opportunity to intervene more rapidly upon suspicion or clinical manifestation of infection or a disease state.

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BRANCHED DNA FOR QUANTIFICATION OF VIRAL LOAD

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ABSTRACT

This is a summary of a presentation made at the 13th International Convocation on Immunology. Nucleic acids in patient samples can be quantified directly using a solid phase nucleic acid hybridization assay based on branched DNA (bDNA) signal amplification technology. For example, HIV RNA is detected in a plasma sample by hybridization of multiple specific synthetic oligonucleotides to the target, 10 of which capture the target onto the surface of a microwell plate and 39 of which mediate hybridization of branched DNA molecules to the *pol* region of each HIV RNA molecule. Alkaline phosphatase-labeled probes bind to each arm of the branched DNA molecules. Detection is achieved by incubating the complex with a chemiluminescent substrate and measuring the light emission. The signal is directly proportional to the level of target nucleic acid, and the quantity of HIV RNA in a sample is determined by comparison with a 4-point standard curve. In order to ensure that different subtypes of HIV-1 were detected and quantified equally, in vitro RNA transcripts of the *pol* region of HIV subtypes A-F were purified and quantified by OD 260, phosphate analysis, and hyperchromicity. These characterized transcripts were then quantified using the bDNA assay. Comparisons were made using a ratio of signal per attomole for each transcript. Genetic subtypes A-F quantified within a factor of 1.5, indicating that the bDNA assay can be used to measure viral load in clinical samples regardless of genotype. Accuracy is important because several studies indicate that there may be a threshold level of virus which predicts progression of HIV disease. Detection of change in viral load is important in determining the efficacy of therapy. The bDNA assay for HCV RNA can be used to determine level of virus in HCV-infected individuals and assist in establishing prognosis prior to initiation of alpha-interferon therapy. Patients with lower levels of virus are more likely to have a sustained response to therapy. Patients who respond to treatment typically have a rapid decline in virus load within one to four weeks of the start of therapy. Many patients relapse when therapy is discontinued as evidenced by a rise in virus load to near pre-treatment levels. Sustained response is most often seen with patients who have lower pre-treatment levels of RNA.

METHOD

The branched DNA (bDNA) assay is a quantitative signal amplification method based on a series of specific hybridization reactions and chemiluminescent detection of hybridized probes in a microwell format. It involves binding of the target nucleic acid to the surface of a microwell plate by a series of probes that are complementary to capture probe on the plate, the other part of the probe being specific for the target. Another series of probes is added, designed to be complementary to the target and also bind synthetic branched DNA molecules. Each bDNA molecule has 15 arms, each arm able to hybridize to 3 alkaline phosphatase molecules. Thus, 45 alkaline phosphatase molecules can bind to each bDNA (Figure 1). In the HCV assay, 18 bDNA molecules are bound to each HCV RNA molecule in the 5' untranslated and core regions, and in the HIV assay 39 bDNA molecules are bound to each HIV RNA molecule in the *pol* region.

In order to ensure that different subtypes of HIV-1 were detected and quantified equally, *in vitro* RNA transcripts of the *pol* region of HIV subtypes A-F were purified and quantified by OD 260, phosphate analysis, and hyperchromicity. These characterized transcripts were then quantified using the bDNA assay. Comparisons were made using a ratio of signal per attomole for each transcript. Genetic subtypes A-F quantified within a factor of 1.5, indicating that the bDNA assay can be used to measure viral load in clinical samples regardless of genotype. As a result of a similar study of the HCV bDNA assay, the target probes were redesigned to ensure that each of the HCV genotypes is detected equally (1). The redesign increased the overall clinical sensitivity to over 95 percent (2). There was an overall percent coefficient of variation of 21-24% when over five hundred replicates of three different samples were tested over ten monthswith six lots of reagents by three different operators.

HEPATITIS

Hepatitis C virus (HCV), hepatitis B virus (HBV) can be quantified directly from serum or plasma using a solid phase nucleic acid hybridization assay based on branched DNA (bDNA) signal amplification technology. Assessment of serum alanine aminotransferase (ALT) and other liver enzyme measurements are markers of liver disease but not viral activity. Quantification of viral load has the advantage of providing information on viral kinetics and provides a greater knowledge of the disease process, as it yields a direct measurement of viremia.

HCV RNA baseline data obtained utilizing bDNA analysis of stored samples from interferon-treated patients has provided confirmation of the relationship between initial viral load and patient response to therapy. A sustained response was defined as normalization of serum ALT for at least 6 months after discontinuation of therapy. An analysis was performed on a series of patients from several worldwide studies of standard interferon therapy, where pre-treatment HCV RNA viral load was measured by bDNA. The results demonstrated that there was no significant difference in initial viral load between non-responders and responders who relapsed. The majority of long-term responders, however, had very low baseline viral load. An

increasing percentage of sustained response was noted in individuals with lower pre-treatment viral RNA levels. Quantification of HCV RNA by bDNA assay as a predictor of sustained response to administration of interferon in an HCV-infected population illustrates that in the entire population, 19 percent will have a sustained response to therapy, with this figure increasing as high as 49 percent as viral load decreases. Knowing the predicted response to interferon therapy may help set expectations for the patient. It is possible that those with high viral levels may benefit from higher or more frequent doses or longer term therapy.

Fluctuations in viral load are relatively insignificant in untreated patients with chronic HCV infection. Changes of threefold or 0.5 log or greater are rarely seen (3). In a subset of patients, however, RNA and ALT levels can fluctuate considerably, becoming stable only after a number of years (4). Fluctuations are also seen more frequently post-therapy. These observations have led to the theory that improved treatment efficacy may be achieved if treatment or re-treatment is initiated at a time point when a patient's viral load is at its lowest. This possibility is especially important for patients who have already received interferon therapy and have suffered relapse, as experience shows that a large proportion of these patients will relapse again. A small study was conducted in Japan and reported by Koga, Gastroenterology Seminars in 1993. Therapy-naïve individuals were grouped prior to initial treatment by their HCV RNA levels determined by bDNA, i. e. greater than 10 million genome equivalents/ml (n=10), 1 to 10 million (n=15) and less than 1 million (n=11). All participants responded to therapy but eventually relapsed. Viral levels were noted at the time of re-treatment, and, regardless of the individuals' pre-therapy levels, the only patients who experienced a sustained response were those who had viral loads of less than 1 million genome equivalents/ml at the start of re-treatment. Studies are now directed toward use of viral load in making decisions regarding the timing of initiation of treatment or re-treatment in hepatitis C-infected individuals.

At the end of therapy, HCV RNA is often undetectable in the serum, even though many of these patients relapse. Determining whether a patient has truly cleared virus can be problematic due to residual viral replication in the liver. If a follow-up liver biopsy is performed to determine improvement in histology, examination of the liver biopsy material for HCV RNA could be a way of establishing a true patient response. A modification of the bDNA assay can be used to quantify HCV RNA in liver tissue, requiring only simple RNA extraction from liver biopsy samples, followed by the standard microplate assay (5). Since pure nucleic acid is not required for the bDNA procedure, the extraction procedure was optimized for RNA recovery, yielding results that reflect the concentration of the virus in the liver of the patient. In contrast, reverse transcription-PCR (RT-PCR) has been found to be inhibited by heme, present in high concentration in liver tissue, which necessitates purification of RNA and results in loss of quantitative recovery.

HIV

Using bDNA, individuals classified as long-term survivors have been shown to have lower a viral load than those who progress more rapidly (6). In a study using the bDNA assay

to measure HIV RNA in plasma of individuals enrolled in MACS, a large cohort study, it was shown that those with high levels of HIV RNA progressed to AIDS more rapidly than those who maintained low levels of RNA (7).

Many HIV-infected individuals have high CD4 levels and high viral load (8). It may be useful to stratify patients by viral load rather than CD4 level for entry into clinical trials. The bDNA assay is currently being used in many laboratories to monitor changes in levels of HIV RNA for several antiviral treatment trials. Two such studies led to new insight into HIV pathogenesis, indicating that HIV replication in vivo is continuous and highly productive (9, 10). It was suggested that because of the rapid turnover of HIV in plasma, protocols for monitoring the antiviral activity of new compounds should be modified to focus on the first few days following drug initiation. New recommendations on the use of HIV RNA quantification include two baseline measurements 2 to 4 weeks apart, a measurement 3 to 4 weeks after initiating a new antiviral therapy, and routine measurements every 3 to 4 months when CD4 counts are done (11). A decrease in HIV RNA of at least 0.5 log is considered incative of antiviral activity.

NEW DEVELOPMENTS

It is often noted that the bDNA assay is not as sensitive as target amplification methods. However, the achievable analytical sensitivity is as high as PCR. The bDNA procedure is similar to an enzyme immunoassay, and the sensitivity is limited by background. In order to reduce non-specific hybridizations, two new bases, iso-cytosine (iso-C) and iso-guanosine (iso-G), were synthesized and utilized in the development of new probes (12). These bases form strong hybridizations with each other, but, as they are not naturally occurring bases, they do not hybridize with natural guanosine and cytosine. In this manner, nonspecific hybridization between the bDNA amplifier and the capture probes has been reduced by substituting some cytosine and guanosine bases with iso-C and iso-G. This method has been used together with modifications in probe design and multi-site preamplification molecules in a bDNA assay for HIV RNA, increasing the sensitivity by more than two orders of magnitude.

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NASBA TECHNOLOGY: ISOTHERMAL RNA AMPLIFICATION IN QUALITATIVE AND QUANTITATIVE DIAGNOSTICS

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ABSTRACT

Nucleic acid amplification technologies allow for the development of highly sensitive and specific diagnostic assays. The capacity to amplify and detect analyte targets, which may be present in a clinical sample as a single copy, is characteristic of many of these amplification technologies. NASBA is an isothermal method of nucleic acid amplification with such capability, and is particularly well suited for the amplification of RNA analytes. NASBA utilizes the coordinated activities of three enzymes (AMV-RT, RNase H, T7 RNA polymerase), and two oligonucleotide primers which are specific for the analyte target. The amplification process is part of a total system which includes a versatile nucleic acid isolation procedure, and powerful detection methodology. In this report, the development of NASBA technology for the detection of human Retrovirus RNA will be discussed. Specifically, a qualitative NASBA assay for the RNA of HTLV I, and a quantitative NASBA assay for HIV-1 will be described.

INTRODUCTION

The capacity to detect and quantitate specific RNA analytes is clinically relevant for several reasons. For example, the capacity to detect RNA is critical in determining the efficacy of an antimicrobial therapy. In the case of bacteria, RNA serves as a marker for viable

organisms. Since the DNA of an infecting bacteria may persist despite the fact that the organism is no longer viable, DNA is a less useful analyte for this purpose. Therefore, the effectiveness of a therapy can be determined through the post-treatment screening for bacterial RNA. In cases of viral infection, RNA serves as a marker for the intracellular replication of the virus. RNA is also the genetic material of several pathogenic viruses (e.g., Retroviruses, Enteroviruses, etc.). Thus, antiviral therapies can also be evaluated with assays that target viral RNA. Further, the expression of specific cellular transcripts is indicative of particular disease states. This has recently been clearly demonstrated for several forms of cancer (10). It is also possible to conduct genetic screening using RNA targets. An additional benefit of targeting expressed cellular RNA transcripts for genetic or disease diagnoses is the fact that these analytes are typically present in multiple copies, whereas their DNA counterparts are usually present as two copies.

NASBA is an isothermal method of nucleic acid amplification which is most useful in the amplification of RNA analytes since reverse transcription is directly incorporated into the amplification pathway. The process involves three enzymes and two oligonucleotide primers (11). Briefly, the initial event is the annealing of the first primer (P1; antisense) to the RNA analyte. The 3' half of the primer is complimentary to the analyte; the 5' half encodes the T7 RNA polymerase promoter. After primer annealing, avian myeloblastosis virus reverse transcriptase (AMV-RT) extends the primer, producing a cDNA copy of the RNA analyte. The RNA portion of the hybrid is destroyed via the action of RNase H, allowing the second primer (P2; sense) to anneal to the newly synthesized cDNA. The DNA dependent DNA polymerase activity of AMV-RT is now engaged, resulting in the extension of P2, creating a double stranded cDNA copy of the original RNA analyte. Importantly, this cDNA encodes a T7 RNA polymerase promoter at one end. This then serves as a substrate for T7 RNA polymerase, which associates with the promoter and begins to transcribe large amounts of antisense RNA corresponding to the original RNA target. This antisense RNA transcription product can then serve as a template for additional

amplification in the process, however the primers will anneal in reverse order (P2 followed by P1). It is this cyclic phase of the process which is responsible for the enormous level of amplification.

Since the entire NASBA amplification process is isothermal, there is no need for temperature cycling. The typical level of amplification is a factor of 10^9. The amplified product is single stranded RNA, and can therefore be readily subjected to different probe hybridization analyses without the need for a denaturing step. Although the NASBA amplification process can be applied to double stranded DNA analytes, two heat denaturing steps are required. In the absence of these heating steps, the process is completely specific for single stranded RNA targets. Thus, background DNA will not be coamplified.

It is the appropriateness of NASBA for RNA which has led to its extensive development as a retroviral diagnostic assay. A quantitative form of the NASBA assay for HIV-1 has been available for some time (14). More recently, we have developed a qualitative assay for HTLV I RNA. Both of these NASBA assays utilize the same nucleic acid isolation procedure (2). However, different detection technologies are used for the different configurations of these assays. In this report, a description of the component technologies comprising the total NASBA system will be presented. The strategy utilized in the quantitative HIV-1 assay, as well as performance data for this assay, will be provided. Finally, the qualitative NASBA assay for HTLV I RNA will be described.

MATERIALS AND METHODS

Nucleic Acid Isolation. The procedure for nucleic acid isolation is the same for both qualitative and quantitative forms of the NASBA assay. The procedure makes use of the guanidine isothiocyanate (GuSCN)-acidified silica method of Boom et al. (2). Briefly, one volume of clinical specimen (whole blood, plasma, serum, sputum, cells, tissue homogenate, CSF, etc.) is added to 9 volumes of lysis buffer (5.25 M GuSCN, 50 mM Tris, pH 7.2, 20 mM EDTA, 1.3% Triton X-100). This