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New methods for the quantitation of microbial nucleic acids

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Abstract - Nucleic acid amplification reactions offer a great deal of potential for the accurate and sensitive detection/quantitation of microbial nucleic acids in human body fluids and have, in some cases, an edge over techniques using immunoassays. Successful detections include : nucleic acids from viruses such as HIV, rotaviruses, coxsackieviruses, papilloma viruses, pestiviruses as well as microorganisms in intestinal fluids.

The development of techniques for the amplification of nucleic acids from clinical samples represents a major advance in the ability to detect and characterize infecting viruses. The most widely used nucleic acid amplification technique is the polymerase chain reaction (ref. 1-3). Polymerase chain reactions and similar systems offer a number of important advantages in terms of viral diagnostics (Fig. 1). However, while these reactions have proven to be extremely valuable for the characterization of small numbers of samples, there are a number of technical problems which have limited the wide-scale application of this technique in diagnostic settings. Of particular importance is the occurrence of cross-contamination. The degree of containment available in clinical settings if often sub-optimal. Furthermore, as the methods are applied to additional body fluids, the problem of sample related inhibitors of enzyme-catalyzed amplification becomes more of concern to diagnostic microbiologists. For example, we have found that fecal samples can contain a material which inhibits the amplification of rotaviral RNA (ref. 4). This material, which is probably a heavy metal or other potent enzymic inhibitor, is resistent to phenol or guanidinium denaturation and co-precipitates with RNA in ethanol. While it can be removed by physical separation techniques such as CF-11 chromatography, the need to perform these reactions markedly increases the time involved in sample preparation and likewise increases the possibility of sample contamination.

Problems with Polymerase Chain Reaction

Cross-Contamination
- **Amplified Products**
- **Plasmids**
- **Sample DNA**

False Homology
- **Retroviruses**
- **Other DNA-Viruses**
- **Human Genome**

Interpretation of Results
- **Natural History**
- **Latency**
- **Infectivity**

Figure 1

There is also a need for a practical non-isotopic method for the quantitation of the amplification reactions. The rationale for the development of quantitative assays is related, not only to the need for the quantitation of microorganisms, but, more importantly, to the need for quality control procedures and inter-laboratory standarization. It is likely that no assay, no matter how sensitive, can be truly useful for clinical diagnostics until laboratories in different areas of the world can run the same test samples and get the same results in a consistent fashion.

There are many possible approaches to the quantitation of amplified nucleic acid hybridization assays, many of which involve labelled oligonucleotides of nucleotide bases (ref. 5). While a number of these approaches can be used for the assay quantitation, we believe that it would be advantageous to draw on the extensive experience available relating to immunoassays and to apply this experience to the detection of amplified nucleic acids. Our approach is based on the fact that monoclonal antibodies are developed which efficiently bind to DNA-RNA hybrids (ref. 6). These antibodies recognize repeating epitopes formed by the physical arrangements of the deoxy- and ribo-nucleotides. These antibodies are used with biotin-labelled probes in the format depicted in Fig. 2.

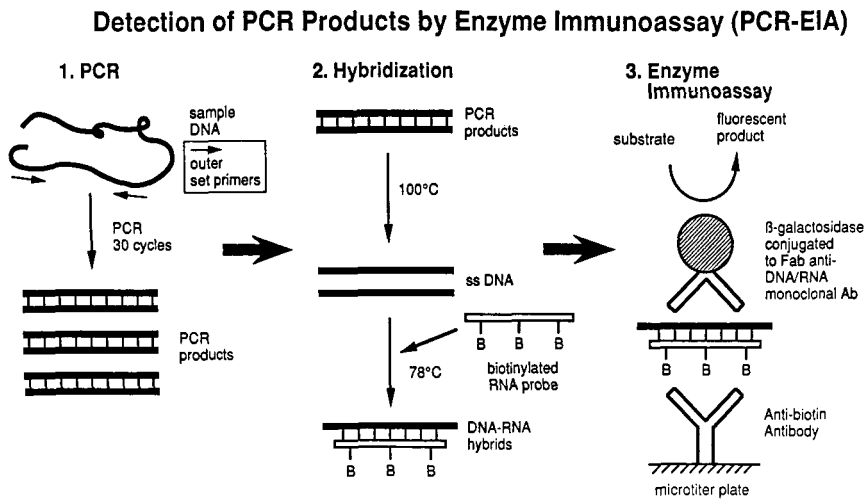

Figure 2

We had initially developed this system for the detection of nucleic acids from viruses such as Human Immunodeficiency Virus (HIV) (ref. 6), rotaviruses (ref. 7) and Coxsackieviruses (ref. 8) directly in clinical samples. The development of the polymerase chain reaction and similar methodologies has allowed us to adapt this method to the detection of amplified nucleic acids, with a resulting increase in sensitivity. It should be noted that we also use PCR amplification techniques to generate the biotin-labelled RNA probe, using sequences for the T7 promoter to generate a segment of double-stranded DNA which can serve as template for transcription in the presence of biotinylated nucleotides (ref. 9-10).

We have applied this method to the detection of a number of different viral nucleic acids in biological fluids. Using a model system consisting of the replication of Human Immunodeficiency Virus (HIV) in cultivated lymphocytes, we found that we could utilize primers directed at sequences in the gag gene to detect as few as 2 infected cells with a low co-efficient of variation. We then applied this system to the detection of HIV DNA in lymphocytes. We found that this system could be applied to the detection of HIV in lymphocytes obtained from virtually all adults with symptomatic or asymptomatic HIV infection. We have also applied this system to the detection of HIV nucleic acids in the lymphocytes of infants born to HIV infected mothers. In such cases, standard serological assays are not reliable due to the fact that the transplacental transmission of HIV antibodies can occur in the absence of active infection. Clinical correlations between the detection of HIV and clinical status are being performed as part of ongoing studies. Similar assays have been devised for a number of microorganisms including rotaviruses, papilloma viruses, Pestiviruses, and Chlamydia trachomatis (ref. 11).

The above assay format provides for an objective method for the quantitation of nucleic acid amplification reactions. However, it does not address the question of sample interference or contamination. When faced with that problem in the development of immunoassays in the past, we and others found that solid phase formats were useful for the specific retention of target and the removal of interfering substances (ref. 12). We have thus developed similar formats for nucleic acid amplification reactions. For each target, we identify one genomic region which is used for binding to the solid phase and another which is used for amplification (Fig. 3). The target is specifically bound to the solid phase; contaminating materials are removed by washing prior to the performance of the amplification reactions. These washing steps serve to remove both materials which would interfere with the amplification and yield false negative results as well as contaminating target fragments generated by previous amplification reactions that would yield false positive results.

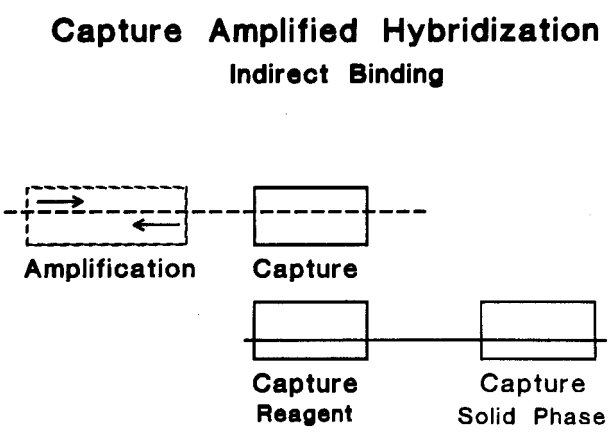

Figure 3

We have developed some preliminary data with the HIV system. It has been found by numerous investigators that whole blood contains heme-iron and other potent inhibitors of DNA amplification reactions. However, we have found that target nucleic acids can be bound to the solid phase and the inhibitory activity can be removed by washing prior to the amplification reactions. Preliminary studies indicate that the inhibitory activity found in blood and in fecal samples can be effectively removed by this method. The availability of a simple method for the removal of interfering substances present in body fluid samples would be an important step in the development of practical systems for the detection of agents in a wide range of body fluids.

We have also found that the binding and washing steps effectively remove contaminating fragments of DNA amplified in previous reactions. Since these fragments do not contain regions homologous to the solid phase probe, they do not bind to the solid phase. They are thus removed by washing prior to the amplification reactions and do not result in the generation of measurable signal. On the other hand, standard amplification protocols result in the re-amplification of these fragments and the generation of what are essentially false-positive reactions. It should be noted that this system will not prevent false-positive results from contamination with actual virus; however, it is likely that amplified fragments will be present in the laboratory environment in higher concentrations and will thus serve as the principal source of cross-contamination.

In conclusion, nucleic acid amplification reactions offer a great deal of potential for the accurate and sensitive detection of nucleic acids in human body fluids. The development of practical methods for the accurate quantitation of these reactions will allow for their application to the diagnosis of a wide range of human diseases.

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