Amplification and non-isotopic detection of specific DNA sequences in a single microtitre well

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Summary

We report the development of a convenient and reliable polymerase chain reaction (PCR)-based microassay for the amplification and detection of specific DNA sequences with potential applications in the diagnostics field. The major features of our system are: (a) the complete system is carried out entirely in the same microtitre well; (b) the PCR is performed in two instead of the traditional three temperatures, thus reducing the time for 35 cycles to under 2 h; (c) the probe is already immobilized onto the solid phase, allowing direct hybridization of the PCR products; (d) one of the two primers is already biotinylated at the 5' end, thus detecting one of the two actual specific products, and (e) the whole process is designed to an enzyme-linked immunosorbent assay (ELISA)-like system for easy use and takes only 3 h, rendering the system particularly suitable for a busy clinical laboratory and automation. The method was successfully applied for the detection of human immunodeficiency virus type 1 (HIV-1) from patient lymphocyte samples.

Key words: PCR, HIV-1, molecular diagnosis

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Introduction

The polymerase chain reaction (PCR) has had a great impact in molecular biology since the demonstration of the technique as a very sensitive *in vitro* amplification method of specific target DNA sequences[1–4]. Although the potential application of PCR as a molecular diagnostic tool has already been demonstrated with the detection of specific nucleotide sequences of various pathogens, genetic disorders and allelic variations, two basic problems continue to hinder the routine use of the method: (a) the actual amplification remains relatively time-consuming, and (b) classical detection methods of the PCR products using gel electrophoresis and either ethidium bromide staining or autoradiography are unsatisfactory.

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We have developed a simple and reliable PCR-based diagnostic microsystem which overcomes some of the major inherent drawbacks of PCR, making the method more accessible to non-experts and amenable to automation as it applies colorimetric detection procedures. The system was successfully evaluated for the detection of human immunodeficiency virus type 1 (HIV-1) from lymphocyte DNA material of HIV-1 seropositive patients.

Materials and methods

Selection of primers

A GC-rich as well as specific and conserved DNA region of HIV-1 was selected to allow annealing and enzymatic extension from the primer end to occur at a single temperature, thus reducing the PCR cycle to two instead of three temperatures. The oligonucleotide primers HP1 (5'-GAAGGAGCCACCCCACAAG-3') and Bio-HP2 (5'-biotin-GGCTGCTTGATGTCCC-CCC-3') were selected which span HIV-1 *gag* gene sequences in positions 1317–1379 of pHXB2. These

primers were synthesized by the phosphoramidite method. The biotinylation of the HP2 primer was done by inserting first an amino group at the 5' end[5] and then coupling with biotin by the mixed anhydride method[6].

Design and immobilization of the probe to the solid phase

The probe (HP3) used in the study (5'-CTCTCTT-TAAACACCATGCTAAACACAGC-3') comprised essentially of two regions, a 'spacer' consisting of the first five bases with a primary amino group at the 5' end and a second region with the bases complementary to a specific region of the PCR product. As control probe (HPC) was used a complementary oligonucleotide to HPV DNA consisting of the sequence: (5'-CTCGTGGAACTTCACTTTTGTTAGCCTG) which also had an amino group at the 5' end.

The probe was immobilized directly onto Falcon flexible 96 U-bottomed microtitre wells (Becton Dickinson, USA) or onto wells pre-coated with 100 µl of 5% bovine serum albumin (BSA), using carbodiimide chemistry[7,8] with the following modifications: in each well, 50 µl of 1 µM solution of the probe in 0.5 mg ml^{-1} p-nitrophenol (p-NPh) were added followed by the addition of 50 µl of 0.5 mg ml^{-1} 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC). The plates were incubated at 37° C for 2 h and excess reagents were washed with 0.1% Tween 20 in 1.5 M NaCl (wash buffer). The coated plates were stored dried at –20° C until use. They were stable for at least 6 months.

Simultaneous amplification and detection assay

Either plasmid DNA (pHXB2) or DNA isolated from lymphocytes of HIV infected patients was used as target DNA. To each microtitre well containing directly immobilized probe were added 10 µl of sample DNA, 40 µl of amplification reaction mixture and 1.5–2.0 U of DNA polymerase (Minotech, Greece). The amplification reaction mixture consisted of 0.25 mM of each dNTP, 0.5 µM of HP1 and Bio-HP2 in a buffer of 10 mM Tris-HCl, pH 8.6 containing 50 mM potassium chloride, 3 mM magnesium chloride, 0.01% gelatin and 0.02% BSA. The plate was then placed in the microtitre block of a thermal cycler (LEP, UK).

The amplification step consisted of: (a) five cycles of 90° C for 1 min and 70° C for 1 min, (b) 30 cycles of 90° C for 0.5 min and 70° C for 1 min; the last cycle having an extension time of 3 min at 70° C. The plate, still in the thermal cycler block, was heat-treated for 5 min at 90° C and 30 min at 42° C for the specific PCR product to be hybridized on the immobilized probe. The plate was then removed from the thermal cycler and was processed for detection of the hybridized products. The excess reagents were washed off with wash buffer followed by incubation with 100 µl well^{-1} streptavidin-peroxidase conjugate (40 ng ml^{-1}) at 37° C for 20 min. After three washings the biotinylated product was revealed by adding 100 µl well^{-1} substrate (0.25 mM 3,3',5,5'tetramethylbenzidine, 0.03% v/v hydrogen peroxide, pH 5.2). The reaction was stopped after 15 min with 50 µl well^{-1} 2 M sulphuric acid. The absorbance was measured at 450 nm (Multiscan, Flow, Finland).

Sample preparation

Total DNA was isolated from patient lymphocytes[9]. Briefly, lymphocytes were isolated from heparinized blood by Ficoll-Paque (Pharmacia) centrifugation at 500 g. The cells (5×10^6) were suspended and lysed in 10 mM Tris-HCl pH 8.3, containing 1 mM ethylene diamine tetracetic acid (EDTA), 0.5% Triton X-100, 0.001% sodium dodecyl sulphate (SDS) and 300 µg proteinase K ml^{-1} and digested with proteinase K at 55° C for 1 h. The lysates were stored at –80° C until used.

Results

Probe immobilization

Simultaneous DNA amplification and detection necessitates an *in vitro* hybridization system in which the oligonucleotide probe is stably immobilized onto the solid phase. Our approach was to couple the probe covalently to plain polyvinyl microtitre wells by applying carbodiimide chemistry. Assessing the hybridization efficiency of the immobilized probe by using biotinylated complementary oligonucleotide (Table 1), the best results were obtained by coupling the probe directly to the plastic surface via an amino group introduced at the 5' end. The optimum probe concentration for maximum coupling was 50 pmol well^{-1}. The specificity of the system was confirmed using a nucleotide probe for the human papilloma virus. Repeated

Table 1. Comparison of probe immobilization efficiency

Immobilization conditions of probe	*Absorbance (450 nm)*	
	Specific DNA template	*Negative control*
Amino-HP3 p-NPh, EDAC, plain wells	0.97	0.11
Amino-HP3 p-NPh, EDAC BSA-coated wells	0.45	0.08
HP3 p-NPh, EDAC plain wells	0.4	0.11
Amino-HPC p-NPh, EDAC plain wells	0.17	0.09

p-NPh, p-nitrophenol; EDAC, 1 ethyl-3-(3-dimethylaminopropyl)-carbodiimide.

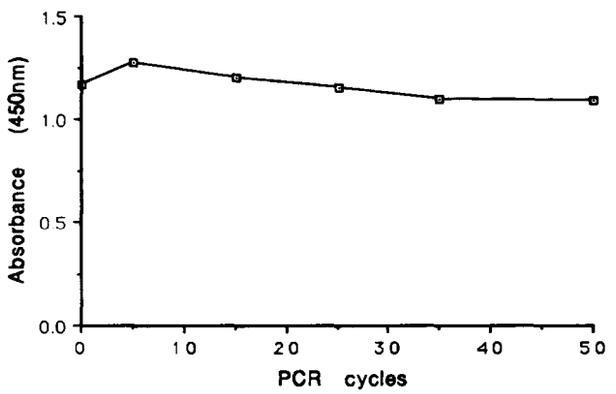

Figure 1. Stability of the immobilized probe HP3 to thermal cycling. Fifty microlitres of the amplification reaction buffer was added to each well containing the immobilized probe and subjected to thermal cycles at 90° C for 0.5 min and 70° C for 1 min each cycle. The hybridization efficiency of the probe in each case was evaluated by adding 1 μg well^{-1} biotinylated complementary oligonucleotide to the probe. The hybridized molecules were revealed with the streptavidin-peroxidase system as described in the Materials and methods section.

thermal cycling up to 50 cycles (Figure 1) had no apparent adverse effects on the stability of the immobilized probe and the hybridization. The system was not suitable for temperatures exceeding 95° C as distortion of the plastic interfered with the assay.

DNA amplification using two temperatures

Considerable time would be saved in the amplification process if the necessary temperatures could be reduced to two from the traditional three without compromising efficiency. This was achieved by selecting compatible primers to HIV DNA with high GC : AT ratio (Td = 62° C for HP1, and Td = 64° C for Bio-HP2) and lower melting temperature (Tm = 76.7° C for product) which allowed annealing and enzymatic extension to take place simultaneously at 70° C. Comparison of amplification efficiency between the two-temperature and three-temperature PCR (Figure 2) showed that at low starting DNA concentrations (<100 fg well^{-1}) the two systems gave similar results, whereas at higher concentrations the two-temperature system clearly gave more efficient amplification.

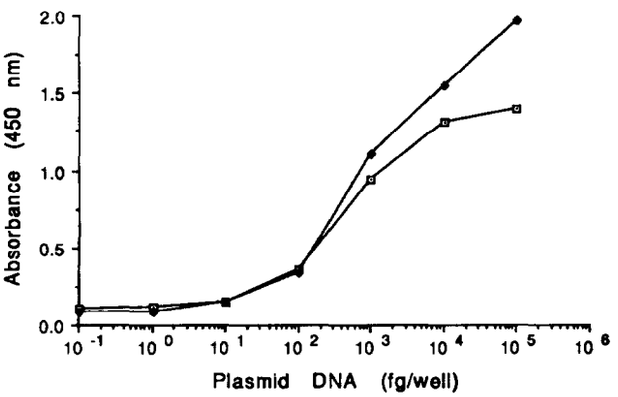

Figure 2. Comparison of sensitivity with two- (—◆—) and three-temperature (—⊡—) amplification using HXB2 plasmid DNA as template. The two-temperature amplification protocol is as described in Materials and methods. In the three-temperature protocol, each cycle consisted of 90° C for 1 min, 60° C for 0.5 min and 72° C for 0.5 min in the first five cycles followed by the reduction of denaturing time to 0.5 min in the next 30 cycles. Product hybridization and detection was performed as described in Materials and methods.

Simultaneous DNA amplification and hybridization

The efficiency of simultaneous amplification of the target DNA and hybridization of the resultant products to the immobilized probe was compared with that using the two processes separately. The results (Figure 3) showed no important differences in the reactivity of the two systems.

Amplification and detection of HIV-1 DNA from patient lymphocytes

Total DNA samples from lysed lymphocytes taken from 12 HIV-1 infected patients and three HIV-1 serologically negative subjects were used to assess the potential diagnostic application of the system described. The samples were amplified using two temperatures and the products were hybridized *in situ*. Detection of the hybridized products was achieved with streptavidin-peroxidase which reacted with biotin

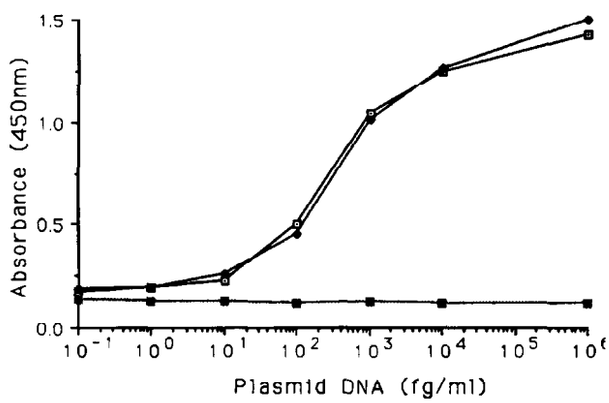

Figure 3. Comparison of sensitivity in detecting amplified DNA using *in situ* hybridization with hybridization after product transfer using HXB2 plasmid as template. Direct hybridization (—⊡—) of product DNA was performed as described in Materials and methods using amino-HP3 as specific probe or amino-HPC as negative control probe (—■—). In the indirect approach (—◆—), amplification was performed in Eppendorf tubes followed by a final denaturation at 90° C for 3 min. Fifty microlitres of amplified product was transferred to amino-HP3 probe-coated wells and hybridized at 42° C for 30 min. After washing, the bound biotinylated products were detected using streptavidin-horseradish peroxidase as described in Materials and methods.

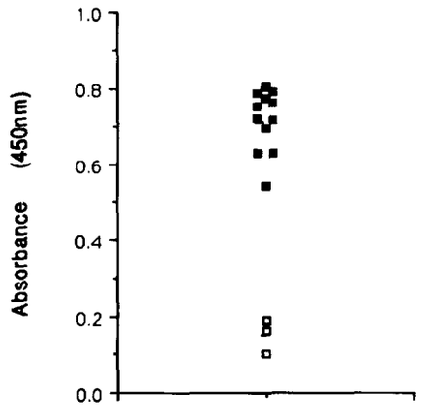

Figure 4. Simultaneous amplification and detection of HIV-1 DNA from patient lymphocytes. Samples from 12 seropositive patients (■) and three healthy donors (□) were amplified in polyvinyl microtitre wells using two temperatures followed by *in situ* hybridization of the products. Detection of the hybridized products was achieved with streptavidin-peroxidase as described in Materials and methods.

at the 5' end of the product. The results summarized in Figure 4 showed a clear discrimination in reactivity between the HIV-1 positive and the control samples.

Discussion

PCR is recognized as an extremely efficient technique for DNA amplification. Although the method is widely applied in research labs, the complicated multiple-step procedures currently used often lead to problems of carryover of amplified product, resulting in false-positive reactions. Such drawbacks hinder the routine use of the technique as a molecular diagnostic tool in clinical labs[10]. There is also a need for a simplified, non-isotopic hybridization and detection system. To address these problems we developed a convenient enzyme-linked immunosorbent assay (ELISA)-like assay system which combines amplification with non-radioactive detection in the same microtitre well.

An essential step towards combining amplification and detection was the stable immobilization of the probe onto the microwell surface for the hybridization of the products. Several immobilization methods have been described including passive adsorption[11], UV irradiation[12], covalent coupling on carboxylated surfaces[13], with base-modified DNA molecules[14], using polylysine[15] or BSA[16] as linkers and to surface grafted with spacer arms terminating in secondary amino groups[17]. These methods have been adapted for polystyrene rather than polyvinyl surfaces, the amplified products therefore require transferring for the hybridization and detection step, as temperature changes are not tolerated during amplification. Our method of chemically coupling the probe via a primary amino group at the 5' end allows the immobilization of the probe directly onto a polyvinyl surface. Polyvinyl microtitre plates are normally preferred for use in PCR because of their thin walls and flexibility, which allows better contact and heat transfer at the thermal cycler block. Our results from repeated thermal cycles indicated satisfactory stability and hybridization capability. A limiting factor of the system is that the denaturation temperature cannot exceed 95° because higher temperatures can lead to distortions of the plastic.

Amplification with the traditional PCR takes place with thermal cycles of three temperatures. Annealing and enzymatic extension may require similar temperatures under certain conditions. We investigated this aspect because of the practical applications in time saving during the amplification step. It was achieved with the use of GC-rich primers with dissociation temperatures at 62° and 64°, and with selected working temperature for annealing-extension at 70°. At low concentrations of target DNA, the amplification efficiency was similar to that with three temperatures, whereas at higher concentrations the two-temperature amplification was more effective. This may be explained by the fact that the enzymatic efficiency of *Taq* polymerase is time-dependent. An added advantage of the new approach was also that the high annealing temperature eliminated the problem of generation of non-specific products which is a phenomenon often associated with low annealing temperatures.

The use of biotinylated primers during PCR amplification was originally described for the capture of product-probe hybrids[18]. Our biotinylated primer was applied for direct detection of the amplification product after capture of the product by hybridization with the immobilized probe, as described previously[19]. The detection assay was simple, sensitive and convenient particularly with clinical samples. Combination of amplification and detection in the same microtitre well was as efficient as when the two steps were performed in different wells. The advantage of this approach is the avoidance of the need to carry over the product after amplification.

In conclusion, the assay we have described is a microtitre-based system which allows amplification and detection of amplified DNA sequences in the same well. It utilized a novel amplification with two-temperature thermal cycles which considerably reduces non-specific reactions and saves time and a simple nonradioactive detection of the product. Application of the system with genomic DNA successfully discriminated DNA samples between HIV-1 seropositive patients and seronegative subjects. The assay is convenient and particularly suitable for busy clinical labs and ELISA-type automation systems.

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