Microtiter format gene quantification by covalent capture of competitive PCR products: application to HIV-1 detection

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ABSTRACT

We have developed a simple gene quantification system using the competitive polymerase chain reaction (CPCR) followed by microtiter format analysis. CPCR is carried out using a mutant competitor with the same size as the target DNA product, and a minimal base exchange to insure the same amplification kinetics. One primer is aminated at the 5' end to produce PCR products that are captured onto carboxylated wells of microtiter plates through peptide bond formation. The non-aminated DNA strands are stripped off from the wells by alkali washing, and the remaining aminated strands are hybridized with either a digoxigenin-labeled wild type-specific oligonucleotide probe or a competitor-specific probe. To standardize the hybridization conditions of the probes, a DNA construct containing wild type and mutant competitor sequences in tandem is captured at different concentrations, hybridized with the probes, and used to generate a standard curve. Bound probes are detected by anti-digoxigenin antibody conjugated with peroxidase and chromogen. Optical densities are recorded with a conventional microtiter plate reader and converted to concentrations according to the standard curves. The ratios of wild type DNA to mutant competitor are used to determine the initial amounts of wild type DNA in the samples. This method was used successfully to quantify human immunodeficiency virus type 1 (HIV-1) *env* gene in human lymphocytes. It only requires a thermal cycler and a conventional microtiter plate reader, and can be readily done on a large scale. Potential applications include detection of other pathogens, diagnosis of genetic disorders and studies of gene expression.

INTRODUCTION

The polymerase chain reaction (PCR) is a powerful tool to detect trace amounts of nucleic acids. PCR has been used in the diagnosis of infectious and genetic diseases (1,2). However, gene quantification by PCR is not straightforward for several reasons. First, the absolute quantity of PCR products does not necessarily bear a close relationship to the abundance of the initial target DNA because the amplified DNA increases exponentially with

cycle number and reaches a plateau (3). Second, the kinetics of each amplification reaction depend upon not only the initial amount of target DNA but also the length of the products, the priming sequences, potential inhibitors in the test samples and variations in the concentrations of reaction components.

The only established method to overcome the inconsistency between the amount of input target and output product DNA is the use of a competitor nucleic acid, which shares the same primer regions with the wild type target and is mixed with sample DNA prior to amplification (3). A competitor differs from the target DNA of interest by having either a small intron or deletion, or a mutated internal restriction enzyme site. Nominal and competitor PCR products are usually distinguished by agarose gel electrophoresis and quantified by radioactivity, chemiluminescence or ethidium bromide staining.

We have developed a simple, non-radioactive and microtiter format analysis method to detect nominal and competitor PCR products specifically, and have applied the method to a nucleotide sequence from the *env* gene of HIV-1. The method does not require that the target competitor DNA differs in length or in internal restriction sites. The method can be carried out with a thermal cycler and a conventional ELISA system, and is adaptable to a large scale clinical laboratory.

MATERIALS AND METHODS

RNA extraction and reverse transcription

Total RNA was isolated from CEM cells infected with HIV-1 (4) using RNA STAT-60 (Tel Test B). It was reverse transcribed with SuperScript II reverse transcriptase (Life Technologies) and a HIV-1 *env* gene specific primer 88-272, as described (5).

Construction of competitor DNA and standard DNA

A short fragment of the HIV-1 *env* gene was amplified with two gene specific primers, 88-79 and 88-272, as reported earlier (5). The amplification mixture contained 100 μ l of 20 mM Tris-HCl (pH 8.2), 10 mM KCl, 2 mM MgCl₂, 6 mM (NH₄)₂SO₄, 0.1% TritonX-100, 10 ng/ μ l BSA, 200 μ M dNTPs, 2.5 u Pfu DNA polymerase (Stratagene). The primers were at a concentration of 1 μ M. The amplification included 30 cycles of 95°C for 30 s, 42°C for 30 s and 72°C for 1 min followed by final extension for 7 min. PCR products were subcloned into pBluescript II SK+ (Stratagene), and termed pENVwt. In order to generate a mutant

Figure 1. Wild type (pENVwt), mutated competitor (pENVcomp), and tandemly aligned genes (pENVwc). Each was subcloned into pBluescript II plasmid. pENVcomp was added to the samples before PCR. pENVwc was used to amplify the standard construct. The priming regions (88-79, 88-272, T7 and T3 rev) and probe detection regions (HE3 and HE5) are boxed. The T7 and T3 Rev priming regions came from the pBluescript II plasmid.

env gene fragment, pENVwt was reamplified either with 88-79 and HE1 primer (5'-CGC CAT TTG ACC ATT CAT TTG TAC ATG GTC) or with 88-272 and HE2 primer (5'-AAT GGT CAA ATG GCG TTA CAC ATG GAA TTA G). Each product was purified to remove free primers with Magic PCR preps (Promega) and the mixture was reamplified with 88-79 and 88-272. The resulting mutant gene fragments were subcloned into the same vector and termed pENVcomp.

The cloned mutant *env* gene fragment was isolated from pENVcomp and inserted into pENVwt, to generate a standard plasmid containing tandemly aligned wild type and mutant *env* genes (termed pENVwc, Figure 1).

Amplification of HIV-1 *env* gene with competitor DNA

The reverse transcribed HIV *env* gene was mixed with known amounts of mutant competitor plasmid DNA, the insert fragment of which was released from vector with *PvuII* restriction enzyme digestion. The mixture was subjected to amplification in 100 μ l of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 200 μ M dNTPs, 2.5 u Taq DNA polymerase (Boehringer Mannheim) and 88-272 and 5' end aminated 88-79 primers at a concentration of 1 μ M. The amplification consisted of 30 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 1 min followed by final extension for 7 min. Free primers were removed with Magic PCR Preps columns and eluted with 1 mM EDTA (pH 8.0).

Amplification of standard DNA

Tandemly aligned *env* gene and its mutant were amplified from pENVwc using T3 Rev primer (5'-AAT TAA CCC TCA CTA AAG) and aminated T7 primer (5'-primary amine-AAT ACG ACT CAC TAT AG). The amplification consists of 30 cycles of 95°C for 30 s, 41°C for 30 s and 72°C for 1 min followed by final extension of 7 min. Free primers were removed as described above. The concentration of the product DNA was determined by direct visualization on agarose gel with ethidium bromide, compared to known DNA standards. This was used to standardize each probe hybridization.

Figure 2. Schematic diagram of microtiter format gene quantification by covalent capture of PCR products.

Covalent capture of amplified DNA

20 μ l of 8 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Pierce), 5 mM N-hydroxysulfosuccinamide (Pierce) were placed in each well of 96 well microtiter plates with carboxylated surfaces (Costar). The concentrations of both were chosen to optimize DNA capture onto the plate (6). Immediately, 20 μ l aliquots of PCR products diluted in ME buffer (50 mM 2-[N-morpholino] ethanesulfonic acid and 1 mM EDTA, pH 5.5) were added to each of two wells and were incubated at 37°C for 2 h. The plates were washed once with PBS and blocked with 1% BSA in PBS for 1 h. After washing three times with PBS, 100 μ l of 0.1N NaOH was placed and left for 10 min. Plates were then washed once with 0.1 $\times$ SSC, and twice with HW buffer (3 $\times$ SSC, 0.1% N-lauroylsarcosine).

Sequence-specific oligonucleotide hybridization

Two oligonucleotides HE3 (5'-CAT TGT ACT GTG CTG AC) and HE5 (5'-ACG CCA TTT GAC CAT TC) were synthesized to detect the wild type *env* gene and the mutant competitor, respectively. They were labeled with a single molecule of digoxigenin-ddUTP (Boehringer Mannheim) using terminal transferase. 5 pmol/ml of labeled oligonucleotide in HW buffer

Figure 3. Standard curves of HE3 and HE5 probe binding to standard constructs. These were used to convert optical density values to target DNA concentrations.

was placed into the well and the plates were incubated at 65°C for 20 min, and then at 42°C for 90 min. After washing three times with HW buffer and once with blocking buffer [0.5% Genius blocking reagent (Boehringer Mannheim) in 100 mM Tris-HCl (pH7.5), 800 mM NaCl], they were incubated with 100 μ l of 150 mu/ml peroxidase conjugated anti-digoxigenin antibody (Boehringer Mannheim) in blocking buffer for 30 min. The wells were washed once with blocking buffer, twice with 100 mM Tris-HCl (pH7.5), 800 mM NaCl, and incubated with 100 μ l of tetramethylbenzidine and peroxidase (Kirkegaard & Perry Laboratories). The reaction was stopped with 1M o-phosphate at the appropriate time point. Optical densities were measured at 450 nm with a microplate reader (Molecular Devices) and were transformed to concentrations using the Deltasoft software program (Biometallics). Figure 2 diagrams the entire procedure.

RESULTS

Generation of standard curve using a standard construct

One to 1000 fmol of the amplified standard constructs were covalently captured onto wells of a microtiter plate in duplicate. After removal of non-targeted strands with 0.1 N NaOH, one set of wells was incubated with digoxigenin-labeled HE3 and the other was incubated with labeled HE5. Hybridized probes were detected with anti-digoxigenin antibody conjugated with peroxidase and chromogen. As shown in Figure 3, both probes showed a similar hybridization efficiency. A set of standard curves were included in each experiment.

CPCR of HIV *env* gene and competitor mixtures at known ratios

One attomol of wild type DNA (pENVwt) was mixed with increasing amounts of competitor DNA (pENVcomp) ranging from 1.95 zeptomol to 128 attomol. They were subjected to CPCR and 3/50 of the products were captured onto two wells. The ratios of wild type DNA products to competitor DNA products were determined by probe hybridization followed by calculation using the standard curves. The ratios in the products bore a consistent relation to the ratios in the initial mixtures. (Figure 4).

Figure 4. Demonstration of a linear relationship of wild type to competitor DNA ratios in the PCR products compared to the initial DNA. One attomol of wild type DNA was mixed with a competitor at ratios ranging from 1.95 zeptomol to 128 attomol, and then was amplified by CPCR.

Figure 5. Determination of HIV-1 *env* DNA content in the samples. The point of equivalence where the competitor added would give 1:1 ratio of wild type to competitor was calculated. The amount of competitor represents the amount of wild type DNA in the initial sample.

Quantification of HIV *env* genes in HIV infected CEM cells

Total RNA was extracted from 3×10^5 of HIV-1 infected CEM cells and converted to *env* cDNA using an *env* gene-specific primer (88-272). 1/10 of reverse transcribed cDNA was mixed with 1 attomol, 100 zeptomol or 10 zeptomol of competitor DNA. Each mixture was amplified by 30 cycles and 1/6 of the products was used for analysis. The amounts of competitor DNA added and the ratios of wild type products to mutated products were plotted in a log scale graph. The initial amount of HIV-1 *env* cDNA was determined by calculating the amount of competitor which would give a 1:1 ratio (Figure 5). To determine the reproducibility of the method, a cDNA sample was amplified three separate times, and then the PCR products were assayed by ELISA. The obtained data were 3.4×10^2 , 3.8×10^2 and 3.2×10^2 femtomol, respectively.

DISCUSSION

CPCR is a method to precisely determine the amount of target DNA sequences in a test specimen (7). Quantification depends upon the relative amounts of PCR products derived from wild type and competitor DNA. Therefore, the amplification kinetics of competitor and wild type DNA must be the same. In previous methods, competitors with different lengths have been preferred to competitors with mutated internal restriction enzyme sites. This is probably because complete enzymatic digestion of the CPCR products is difficult to monitor but is absolutely necessary for quantification. In the method reported here, we used a competitor of the same length as a wild type DNA with a minimal change (17 bases, the same GC content), and without any need for enzymatic cleavage.

Wild type and competitor products were quantified by hybridization in microtiter plates. Unlike previous CPCR methods, electrophoresis was not required. Only a simple ELISA reader and immunoassay analysis software are requisite, and the method is fully compatible with the equipment present in clinical chemistry laboratories.

Probe hybridization-based quantification of PCR products can eliminate false positive results derived from non-specific amplification. However, potential flaws can come from differences in hybridization or labeling efficiency of the probes. The standard construct we used has tandemly arranged wild type DNA and competitor DNA sequences. Since the standard curves are generated from the results of hybridization of each probe with the standard construct, labeling or hybridization efficiency does not affect the results.

It is essential for accurate and quantitative analyses to generate conditions where the oligonucleotide probes can hybridize to all potential target sequences in the PCR products. We used 5'-aminated primers to produce PCR products, one strand of which has a primary amine at the 5' terminus. This permitted the covalent capture of the aminated DNA-strand to carboxyl groups on derivatized ELISA plates. Since peptide bonds are resistant to alkali, only the aminated strands remained on the plates after alkali washing. The alkali wash was absolutely necessary to permit the probes to hybridize to their target sequences without competition from the other DNA strands. No probe binding was detected without alkali treatment.

Experiments measuring HIV-1 *env* cDNA content in HIV-1 infected CEM cells showed that the assay is reproducible among different amplification reactions and using different standard curves. The assay was no longer quantitative when the amount of input DNA was less than 1 zeptomol, although PCR products were detectable (data not shown). This is probably because the amplification of such a few DNA molecules is subject to variation. Thus, we estimate that the sensitivity of the technique is 1 zeptomol.

Recently Piatak and colleagues (3) reported that HIV RNA levels in plasma specimens from affected patients reflect viral replication and correlate with disease progression. They implied that quantification of plasma RNA may be useful in assessing prognosis, and the efficacy of antiretroviral therapy. Accordingly, it is reasonable to follow the HIV viral load in plasma of every infected patient periodically. The method described here can be readily used with RNA competitors, and is adaptable to clinical laboratories to facilitate such clinical studies. Other potential applications of the method include the detection of additional

pathogens, diagnosis of genetic disorders, and studies of gene expression.

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