1-1-1996

Efficient long-PCR site-specific mutagenesis of a high GC template

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Recommended Citation

Chouljenko, V., Jayachandra, S., Rybachuk, G., & Kousoulas, K. (1996). Efficient long-PCR site-specific mutagenesis of a high GC template. *BioTechniques, 21* (3), 472-480. https://doi.org/10.2144/96213st05

Short Technical Reports

Efficient Long-PCR Site-Specific Mutagenesis of a High GC Template

BioTechniques 21:472-480 (September 1996)

ABSTRACT

A long PCR method was developed for the efficient site-specific mutagenesis of herpes simplex virus (HSV-1) DNA fragments with high GC content. In this protocol, a PCR product was partially extended first using a cloned DNA fragment. The final mutagenized fragment was produced after a second extension using another PCR product and final amplification using external primers. The sequential use of two extension reactions increased the predicted frequency of the engineered mutation in the final product to 100%. This method was used to generate a mutated glycoprotein K (gK) gene specified by HSV-1. A recombinant virus that carried the mutated gK gene caused extensive cell fusion of infected cells.

INTRODUCTION

One of the principal ways for polymerase chain reaction (PCR)-assisted mutagenesis is the "overlap extension" method, which requires that two different PCR DNA fragments overlap at the site to be mutagenized. This overlap is provided by a pair of mutagenic primers that produce the desired mutation (6,7). A variation of this "two-step PCR" theme is the "megaprimer" method, which utilizes one entire PCR fragment as a "megaprimer" and a distant primer (9).

We have developed a long-PCR site-directed mutagenesis method that results in nearly 100% mutagenesis of the final DNA products. This method utilizes long-PCR enzymes and conditions to efficiently amplify DNA fragments with above 50% GC content. We demonstrate this method by generating a new restriction endonuclease site and a missense mutation in the glycoprotein K (gK) gene specified by herpes simplex virus (HSV-1). A recombinant virus that carried the mutated gK gene caused extensive cell fusion of infected cells.

MATERIALS AND METHODS

Equipment and Reagents for PCR

All experiments were performed using the GeneAmp® PCR System 9600 (Perkin-Elmer, Norwalk, CT, USA). All PCR reagents were provided as part of the XL PCR Kit (Part No. N808-0182; Perkin-Elmer). Hot-Start PCR was performed with AmpliWax® PCR Gem 50 (Part No. N808-0150; Perkin-Elmer).

Restriction Enzymes, Bacterial Strains and Plasmid Vectors

Restriction enzymes were obtained from New England Biolabs (Beverly, MA, USA). Plasmid vector pUC19 and its host *E. coli* DH5α were obtained from Bethesda Research Laboratories (Gaithersburg, MD, USA).

Viruses and Cells

HSV-1(KOS) was originally obtained from Dr. P.A. Schaffer, Dana-Farber Cancer Institute, Boston, MA. Virus stocks were made and titered as described previously (1). Transfections were carried out on rabbit skin cells using the calcium phosphate technique (3,5).

Primers for Long PCR and Mutagenesis

Primers P1 (5′-GTT CAT AAC CCA CGA TCG CAG TTG-3′), P2 (5′-ATA CGC TGG CTG GGT TGG TCT TGG T-3′), mutagenic primer-MP3 (5′-TGG GGC GTA CTA CGT AAA TAC ATC-3′), mutagenic primer-MP4 (5′-CGA TGT ATT TAC GTA GTA CGC CCC ACC-3′), P5 (5′-GTT CGC GGT ACC CTG CAG CAA AG-3′) and P6 (5′-ACG GCC AAT TGG GAC CCA TGG GCG G-3′) were synthesized in our laboratory on an Applied Biosystems DNA Synthesizer Model 394 (Perkin-Elmer/Applied Biosystems Division [PE/ABI], Foster City, CA, USA) using phosphoramidite chemistry. The approximate location of primers P1, P2, MP3, MP4 and the gK gene on the viral genomic map is shown in Figure 1. The PCR fragment produced by the P1 and P2 primer pair was predicted to be 3340-bp-long with an average GC content of 64.3%. The DNA fragment amplified by P1/MP3 had a GC content of 66.1%, and the MP4/P2 PCR product had a GC content of 61.0%. Primers P5 and P6 bracket the point mutation and were used for detection of the engineered mutation in mutant viruses (Figure 1).

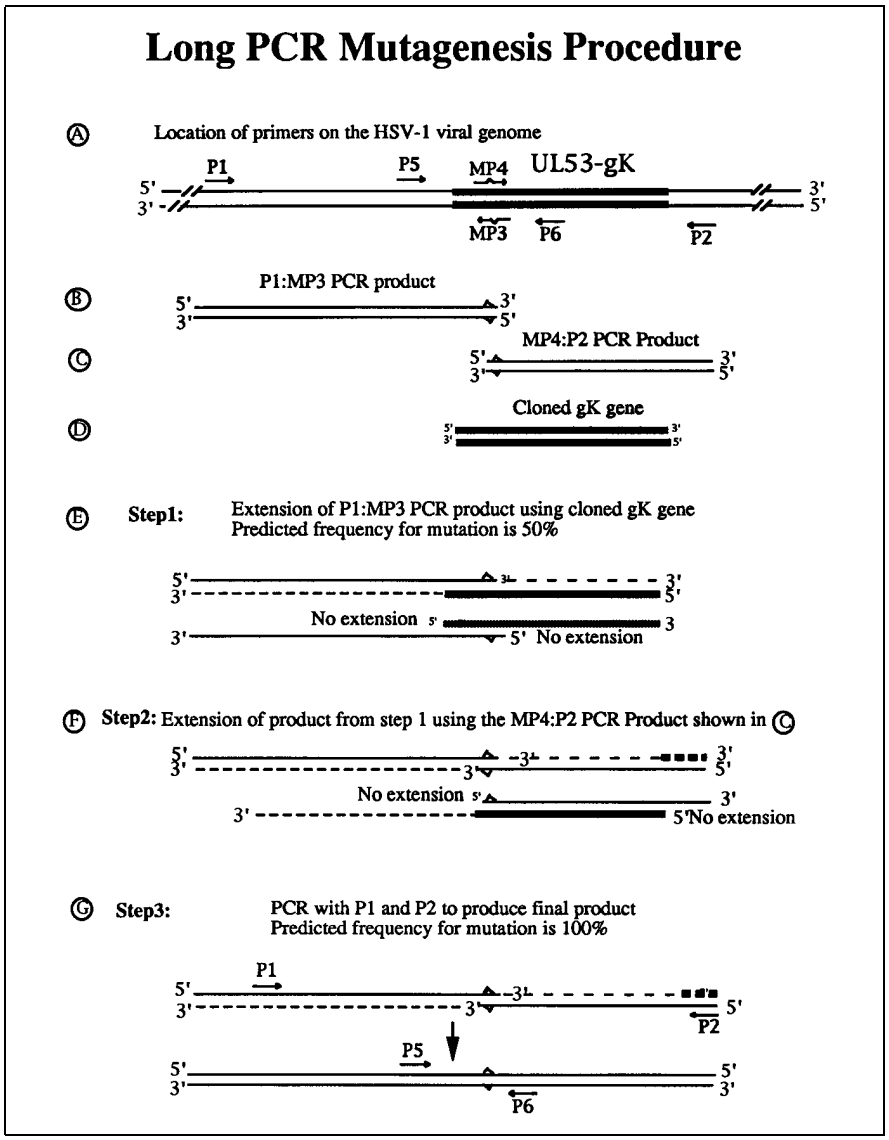

Figure 1. Schematic diagram of the mutagenesis procedure. (A) The HSV-1 genome showing the location of all the primers and the UL53 (gK) gene. The location of the engineered mutation is denoted (^). (B and C) The P1/MP3 PCR product (B) and the MP4/P2 PCR product (C). (D) The cloned DNA gK fragment used for the first extension reaction. (E) The first extension reaction (---) of the P1/MP3 PCR product using the cloned gK DNA fragment. (F) The extension of the PCR product from step E using the PCR DNA fragment represented in step C. (G) The final PCR product using primers P1 and P2. This DNA fragment contains the desired mutation (^).

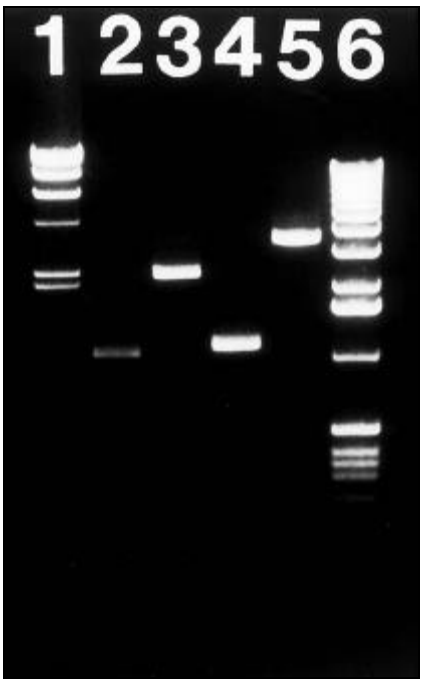

Figure 2. Agarose gel electrophoresis of the different dsDNA fragments amplified by PCR. Lane 1: λ phage DNA digested with *Hin*dIII (marker); lane 2: cloned gK 1020-bp DNA fragment; lane 3: P1/MP3 2257-bp PCR product; lane 4: MP4/P2 1108-bp PCR product; lane 5: P1/P2 3340-bp PCR product; lane 6: Molecular mass marker (1-kbp ladder).

Extension of the P1/MP3 PCR Product Using the Cloned gK DNA Fragment

The PCR-derived DNA fragment (500 ng) was mixed with the cloned DNA fragment (125 ng), denatured and re-annealed. The total volume of DNA mixture was brought up to 55.6 µL, and the extension reaction mixture (100 µL) consisted of 55.6 µL DNA solution, 4.4 µL $Mg(OAc)_2$, 30.0 µL 3.3× XL buffer, 8.0 µL of 2.5 mM stock dNTPs, 2.0 µL r*Tth* Pol. XL. The thermal profile was 3 min at 95°C; 1 min at 94°C and 15 min at 72°C repeated 7 times.

Final Overlap-Extension

The P1/MP3 PCR extended product (50 ng) was gel-purified using the Prep-A-Gene® purification method as detailed by the manufacturer (Bio-Rad, Hercules, CA, USA) and was used for the next overlap-extension with the MP4/P2 PCR product (50 ng). The extension reaction mixture (100 µL) was as described above. The thermal profile was 3 min at 95°C; 1 min at 94°C and 15 min at 72°C repeated 3 times.

Final PCR Conditions

The primer pair P1/P2 was used to amplify the 3340-bp DNA fragment. One microliter undiluted and 1 µL from a 1:10 dilution of the final overlap-extension reaction were used in the final PCR. PCR parameters were 3 min at 95°C; 30 min at 94°C and 8 min at 68°C repeated 30 times and final extension for 15 min at 72°C. Hot-start conditions were applied according to the manufacturer (PE/ABI), and the composition of lower and upper reaction mixtures were as follows: lower reaction mixture (40 µL total) was: 12.4 µL water, 12.0 µL 3.3× XL Buffer, 8.0 µL of 2.5 mM stock dNTPs, 1.6 µL of 20 µM stock Primer P1, 1.6 µL of 20 µM stock Primer P2, 4.4 µL of 25 mM stock $Mg(OAc)_2$; upper reaction mixture (60 µL) was: 38 µL water, 18 µL 3.3× XL Buffer, 1 µL of 7 µg/mL stock viral DNA, 2 µL of 2 U/mL stock r*Tth* DNA Pol. XL.

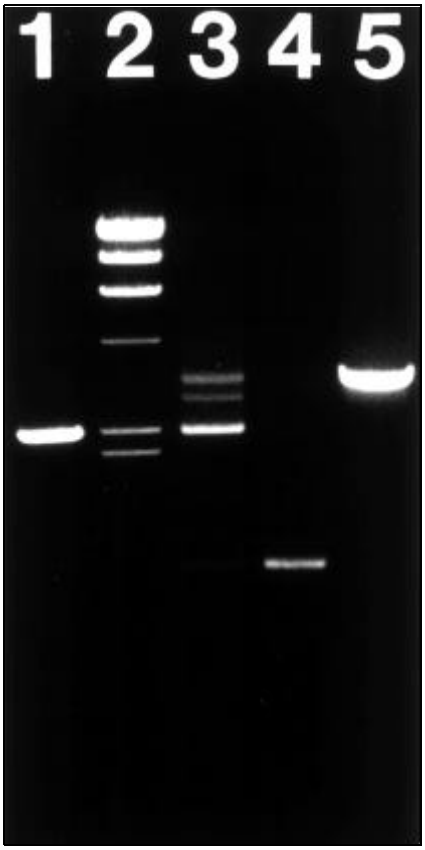

Figure 3. Agarose gel electrophoresis of dsDNA fragments after the first extension. Lane 1: P1/MP3 2257-bp PCR product; lane 2: λ phage DNA digested with *Hin*dIII (marker); lane 3: P1/MP3 PCR product after extension with the cloned gK DNA fragment; lane 4: cloned gK DNA fragment before extension (1020 bp); lane 5: P1/P2 3340-bp PCR product.

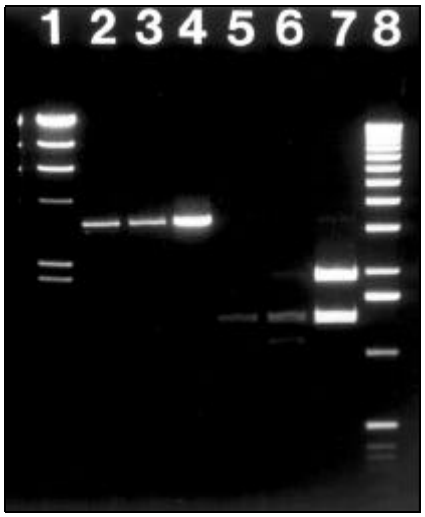

Figure 4. Agarose gel electrophoresis of mutagenized fragments restricted with *Sna*BI. Lane 1: λ phage DNA digested with *Hin*dIII (marker); lanes 2 and 3: P1/P2 mutagenized PCR products from fully and partially extended DNA species shown in Figure 3, lane 3; lane 4: P1/P2 unmutagenized PCR products; lanes 5–7: DNA fragments shown on lanes 2–4, respectively, digested with *Sna*BI; lane 8: molecular mass marker (1-kbp DNA ladder).

Preparation of Viral DNA and Marker-Transfer Experiments

Viral DNAs were prepared as described (10). For marker-transfer studies, Vero cells were co-transfected with DNA fragments produced by PCR and intact wild-type HSV-1(KOS) viral DNA (1,2). For PCR analysis, viral DNA mini-preparations were prepared from plaque-purified virus stocks as described previously (1). Viral DNAs from each of the viral plaques were subjected to PCR analysis using internal gK primers P5 and P6, and the subsequent PCR products were digested with restriction endonuclease *Sna*BI.

RESULTS

Long-PCR of the HSV-1 gK Gene Region

The gK gene is approximately 63% GC-rich; however, it contains short segments (50–150 bp) with over 80% GC content (11). We were able to efficiently

Figure 5. Agarose gel electrophoresis of viral PCR products restricted with *Sna*BI. P5/P6 PCR product from a putative mutagenized syncytial mutant virus (lane 1), laboratory virus strain KOS (lane 3) and laboratory syncytial strain MP (lane 5). These three PCR DNA fragments were digested with *Sna*BI (lanes 2, 4 and 6, respectively). λ phage DNA digested with *Hin*dIII (lane 7).

amplify up to 7 kbp of DNA sequence spanning the gK gene with an average GC content of over 67%. PCR of a 7-kbp DNA fragment produced approximately 0.3 µg per 100 µL reaction in comparison with over 2 µg for templates of up to 4.5 kbp (data not shown).

PCR Mutagenesis

We designed a pair of mutagenesis primers, MP3 and MP4, that created a new restriction endonuclease site (*Sna*BI and an Ala to Val change at position 40 of the gK primary sequence. Herpes simplex viruses that have Val at position 40 are known to cause extensive cell fusion of infected cells in tissue culture (4,8).

The mutagenesis procedure is outlined in Figure 1 and is composed of three main steps. In step 1, the cloned gK gene is used to extend the P1:MP3 PCR product. Fifty percent of the extension reaction products are predicted to contain the engineered mutation. Similarly, in step 2, a second extension is predicted to raise the frequency of the mutation to 100%. The relevant PCR products are shown in Figure 2. P1/P2 produced a 3340-bp DNA fragment, primer pair P1/MP3 produced a 2257-bp DNA fragment and primer pair MP4/P2 produced a 1108-bp DNA fragment. A 1020-bp *Hin*dIII/*Hin*dIII DNA fragment containing the entire gK gene sequence was excised from plasmid vector pUC19 and gel-purified.

The primers that carried the desired mutations were MP3 and MP4 (Figure 1). The cloned gK DNA fragment (Figure 1D) was used to extend the 2257-bp DNA fragment generated by primer pair P1/MP3 (Figure 1B). The results of this experiment are shown in Figure 3. The P1/MP3 PCR-extended product produced two slower migrating DNA species. The largest DNA fragment was tentatively identified as representing the fully extended 3146-bp product. The second DNA species was assumed to represent a partially extended product. Each of these two DNA species was gel-purified and used separately for the next overlap extension with the MP4/P2 PCR product (Figure 1F). Final amplification was performed with primer pair P1/P2 (Figure 1G). PCR-amplified DNA fragments of full length were produced by both the putative fully extended DNA species (Figure 4, lane 2) and by the partially extended DNA species (Figure 4, lane 3). Final PCR products were tested for the presence of a new *Sna*BI restriction endonuclease site by digestion with *Sna*BI. The unmutagenized P1/P2 PCR product (Figure 4, lane 4) contains a single *Sna*BI site, which upon digestion, produces two DNA fragments (Figure 4, lane 7). As expected, mutagenized PCR products (Figure 4, lanes 2 and 3) contained an additional *Sna*BI site producing three DNA fragments of the expected apparent molecular mass (Figure 4, lanes 5 and 6). A small amount of either unmutagenized or partially cleaved PCR product was also detected, as evidenced by the DNA species (Figure 4, lanes 5 and 6) that co-migrated with a similar DNA fragment visible in the unmutagenized sample (Figure 4, lane 7).

Marker Transfer of the *Syn*1 Mutation (Ala to Val Change) and the *Sna*BI Restriction Site into the KOS Genome

Full-length mutagenized DNA fragments were purified from agarose gels and were co-transfected with intact HSV-1(KOS) viral DNA into rabbit skin cells. Individual viral plaques were collected, virus was plaque-purified three times under agarose overlay and viral DNA stocks were prepared as described previously (1,2). Viral DNAs were subjected to PCR using primers P5 and P6, which were designed to bracket the site of the mutation (Figure 1A). P5 is located 377 bases 5′ to the engineered *Sna*BI site, and P6 is located 1030 bases 3′ to the *Sna*BI site. Therefore, digestion by *Sna*BI of the P5/P6 product is expected to produce two DNA species of approximately 377 and 1030 bp. PCR fragments obtained from purified viral DNAs were restricted with *Sna*BI. Viral isolate SJ101, which caused extensive cell fusion, contained the engineered *Sna*BI restriction site as seen by the predicted restriction profile (Figure 5, lane 2). Control viral strains HSV-1(KOS) (Figure 5, lane 1) and HSV-1(MP) (Figure 5, lane 6) did not contain this *Sna*BI restriction site.

DISCUSSION

Mis-incorporation of nucleotides by the error-prone *Taq* DNA polymerase can prematurely terminate DNA synthesis during PCR amplification and result in lower PCR yields and inability to amplify relatively long DNA fragments of greater than 2 kbp. To increase the amplification efficiency of HSV-1 DNA fragments with an average GC content of 67%, and to overcome the possibility of generating random mutations within the amplified DNA sequence, we exclusively used the Long PCR Kit manufactured by Perkin-Elmer. This PCR kit utilizes a mixture of r*Tth* DNA Polymerase, XL and Vent® DNA Polymerases, which is known to correct mis-incorporated nucleotides and long-PCR conditions that result in efficient amplification of DNA templates.

A major advantage of the site-directed mutagenesis method presented here over the splice overlap and megaprimer methods is that the sequential use of two extension steps and use of the cloned gK DNA fragment increases the predicted frequency of the engineered mutation in the final product to nearly 100%. This is because DNA polymerases can extend only in the 5′ to 3′ direction (Figure 1). The overlap of the cloned DNA gK fragment with the first PCR product is only 106 bases, yet it results in an efficient extension of approximately 3000 bases. It is possible that much longer primers of approximately 100–150 nucleotides may render a single-step overlap-extension protocol feasible. However, long primers require substantial purification, and they are expensive to synthesize. The visualization and gel purification of the initially extended PCR using the cloned gK gene provide an intermediate quality-control checkpoint, which, in our opinion, outweighs the additional labor associated with this purification. We have found that this is an efficient and reproducible method to mutagenize long DNA fragments of herpes simplex viruses with above 50% GC content.

REFERENCES

1. **Baghian, A., L. Huang, S. Newman, S. Jayachendra and K.G. Kousoulas.** 1993. Truncation of the carboxy-terminal 28 amino acids of glycoprotein B specified by herpes simplex virus type 1 mutant amb1511-7 causes extensive cell fusion. J. Virol. *67*:2396-2401.
2. **Cavalcoli, J.D., A. Baghian, F.L. Homa and K.G. Kousoulas.** 1993. Resolution of genotypic and phenotypic properties of herpes simplex virus type 1 temperature sensitive mutant (KOS) tsZ47: evidence for allelic complementation in the UL28 gene. Virology *197*:23-34.
3. **Chen, C. and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. *7*:2745-2752.
4. **DebRoy, C.** 1990. Nucleotide sequence of the herpes simplex virus type-2 syn gene that causes cell fusion. Gene *88*:275-277.
5. **Graham, F.L. and A.J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology *52*:456-457.

6. **Ho, S.N., H.D. Hunt, R.M. Horton, J.K. Pullen and L.R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene *77*:51-59.
7. **Horton, R.M., S.N. Ho, J.K. Pullen, H.D. Hunt, Z. Cai and L.R. Pease.** 1993. Gene splicing by overlap extension. Methods Enzymol. *217*:270-279.
8. **Pogue-Geile, K.L., G.T. Lee, S.K. Shapira and P.G. Spear.** 1984. Fine mapping of mutations in the fusion-inducing MP strain of herpes simplex virus type 1. Virology *136*:100-109.
9. **Sarkar, G. and S.S. Sommer.** 1990. The "megaprimer" method of site-directed mutagenesis. BioTechniques *8*:404-407.
10. **Walboomers, J.M. and J. Ter Schagget.** 1976. A new method for the isolation of herpes simplex virus type 2 DNA. Virology *74*:256-258.
11. **Zhang, X. and K.G. Kousoulas.** 1993. Direct sequencing of PCR-amplified high GC DNA. BioTechniques *14*:376-377.

This publication is identified as #120 of the VMP Gene Probes and Expression Systems Laboratory "GeneLab". This work was supported by Public Health Service Grant No. A127886 to K.G.K. G.R. was supported by the Howard Hughes Medical Institute through an undergraduate science enhancement grant to Louisiana State University. V.C. is a senior postdoctoral fellow and visiting scientist from the Institute of Molecular Biology and Genetics, Kiev, Ukraine. Address correspondence to Konstantin G. Kousoulas, Department of Veterinary Microbiology & Parasitology, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803-8416, USA. Internet: vtgusk@lsuvax.sncc.lsu.edu

Received 30 June 1995; accepted 18 March 1996.

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Quantitative Multiple Competitive PCR of HIV-1 DNA in a Single Reaction Tube

BioTechniques 21:480-484 (September 1996)

ABSTRACT

A quantitative multiple competitive PCR (QMC-PCR) for determination of DNA copy numbers is described. Four competitive DNA templates for the env *region of HIV-1 were constructed with sizes longer (187 and 163 bp) or shorter (122 and 105 bp) than the 142 bp of the wild-type PCR product. Varying amounts of each of these competitors are introduced together with the sample into a single reaction tube. Since competitors and wild-type fragments share the same primer recognition sequence (SK68/SK69), amplification occurs according to the rate of the introduced copy numbers. The PCR products are run on an agarose gel, and the copy number of the sample is determined by analyzing the bands with a video densitometer and calculating the equivalence point in a linear regression plot.*

INTRODUCTION

Over the past years, polymerase chain reaction (PCR) technology has been widely used for highly sensitive molecular diagnosis of infectious agents, including human immunodeficiency virus type 1 (HIV-1). For quantitation of specific nucleic acid templates, competitive PCR seems to be the most promising approach. This method overcomes the variabilities of PCR by co-amplification of internal competitive standard DNA and specific template in the same reaction tube and thus allows more accurate quantitation. Although a great variety of competitive PCR protocols are in use (1–3,5,8), they all involve mixing varying amounts of competitor, usually different in size from the wild-type product, with a constant amount of the sample to be determined. This requires at least four to five different PCRs to obtain an accurate determination of copy number in a regression plot. Recently this meth-