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

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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-

odology has been improved by amplifying the wild-type template together with two competitors in a single reaction tube, followed by evaluation of the PCR products by laser-induced fluorescence (4). In a similar approach, we coamplified, in a single reaction tube, four competitors and wild-type template in order to allow accurate quantitation after separation of PCR products on ethidium bromide-stained gels.

MATERIALS AND METHODS

Construction of Competitor Templates Containing a Nucleotide Deletion or Insertion

Synthesis of the competitor templates and oligonucleotides used are depicted in Figure 1. The primers chosen for this study were those most frequently used for a conserved *env* region, SK68 and SK69 (7). Wild-type proviral HIV-1 DNA [10^5 copies of pBH10 (6) containing HIV-1 III B sequences] was amplified with primers SK68 DelI/SK69, resulting in a 122-bp deletion mutant (wild-type: 142 bp). The 122-bp deletion fragment was reamplified with primers SK68 DelII/SK69 to obtain a 105-bp fragment. Lengthened competitors were constructed by amplifying wild-type DNA with primers X-env (containing a randomly chosen 21-base nucleotide sequence combined with sequence env) and SK69. This product was then amplified with SK68-X, containing the SK68 sequence combined with sequence X, and primer SK69. The resulting 163-bp fragment was amplified with primers Y-X/SK69 and subsequently SK68-Y/SK69 to obtain a 187-bp fragment.

The four different templates were individually re-amplified with restriction site-bearing primers NcoI-SK68 and PstI-SK69 and digested with the two respective enzymes. Cleaved products were ligated in *Nco*I/*Pst*I sites of plasmid pGEM®-5Zf(+) (Promega, Madison, WI, USA). After transformation into XL1-Blue MRF′ bacteria (Stratagene, La Jolla, CA, USA), the competitor plasmids were prepared with the QIAGEN® Plasmid Kit (Qiagen, Chatsworth, CA, USA) following the supplier's instructions. To determine the exact copy number of the respective competitor plasmids precisely, both measurement of optical density at 260 nm and comparison of the fluorescence intensity of the competitor plasmids on 1% agarose gels with other plasmids of known concentrations (including wild-type plasmid pBH10) were performed. Furthermore, the copy numbers determined as described above were verified in a conventional quantitative competitive PCR assay (5) using known concentrations of wild-type pBH10 as a standard. When testing an estimated 10 000 copies of pBH10 in five individual PCR runs, the actual mean copy numbers obtained were 10 397 for pInsII, 9905 for pInsI, 8285 for pDelI and 8574 for pDelII. The concentrations of the different competitor plasmids were then adjusted accordingly (so as to contain 10 000 copies each).

PCR

Each PCR was carried out in 50 µL of a solution containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton® X-100, 200 µM

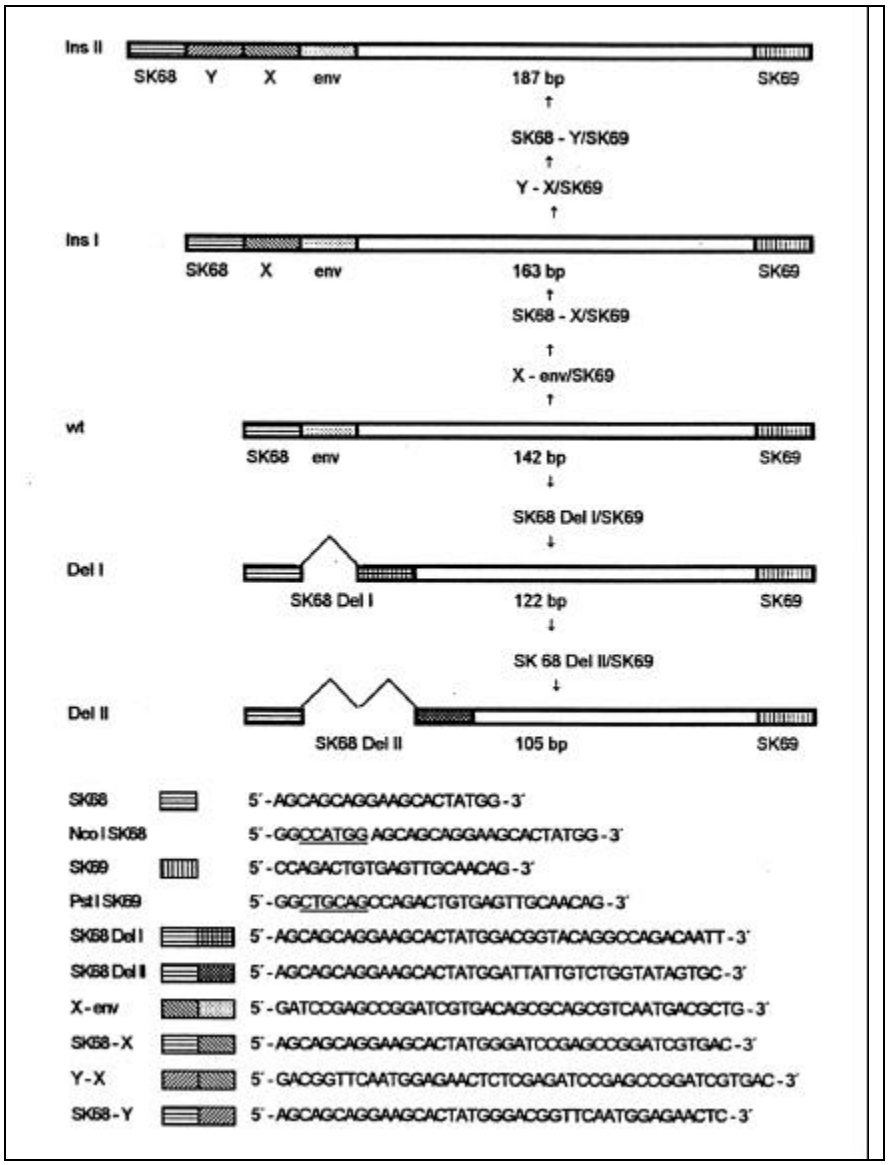

Figure 1. Scheme for the construction of competitive templates and sequences of the used oligonucleotides. Wild-type proviral DNA was amplified with the oligonucleotides depicted in the diagram, resulting in fragments lengthened to 163 and 187 bp and fragments deleted to 122 and 105 bp.

of each dNTP, 1 U DynaZyme (Finnzymes Oy, Espoo, Finland), 250 ng each of SK68 and SK69, the sample to be determined and the respective competitive templates. The samples were overlaid with mineral oil and amplified 40 cycles: step 1, 1 min at 94°C; step 2, 1 min at 60°C; step 3, 1 min at 75°C (extended 1 s per cycle); and final incubation at 75°C for 1 min.

Sample Analysis

Eight microliters of the PCR product were run on a 3.5% NuSieve® GTG® Agarose gel (FMC BioProducts, Rockland, ME, USA) stained with ethidium bromide and scanned with a video densitometer (Hirschmann GmbH, Taufkirchen, Germany). Peak areas of the 187-, 163-, 122- and 105-bp competitor fragments and 142-bp wild-type fragment were analyzed in each lane, and the copy number of the sample was determined using a single linear regression plot (5). To correct for the different lengths of the competitive templates used, the fluorescence of each competitive template was multiplied with a specific correction factor (i.e., 142/187, 142/163, 142/122 and 142/105).

RESULTS AND DISCUSSION

To determine the optimal molar ratios of the four different competitor plasmids to be used in quantitative multiple competitive PCR (QMC-PCR), different molar ratios of each competitor were amplified by PCR and separated on an agarose gel. We compared a constant copy number of 3000 copies for all four templates (Figure 2A, lane 3) with different molar ratios in which the copy number of the templates used was in inverse relationship to the length of the fragment, beginning with 3000 copies for the longest 187-bp fragment. Concentrations of competitor templates analyzed were 3000, 6000, 9000 and 12 000 copies (lane 4); 3000, 6000, 12 000 and 24 000 copies (lane 5); 3000, 9000, 27 000 and 81 000 copies (lane 6); and 3000, 12 000, 48 000 and

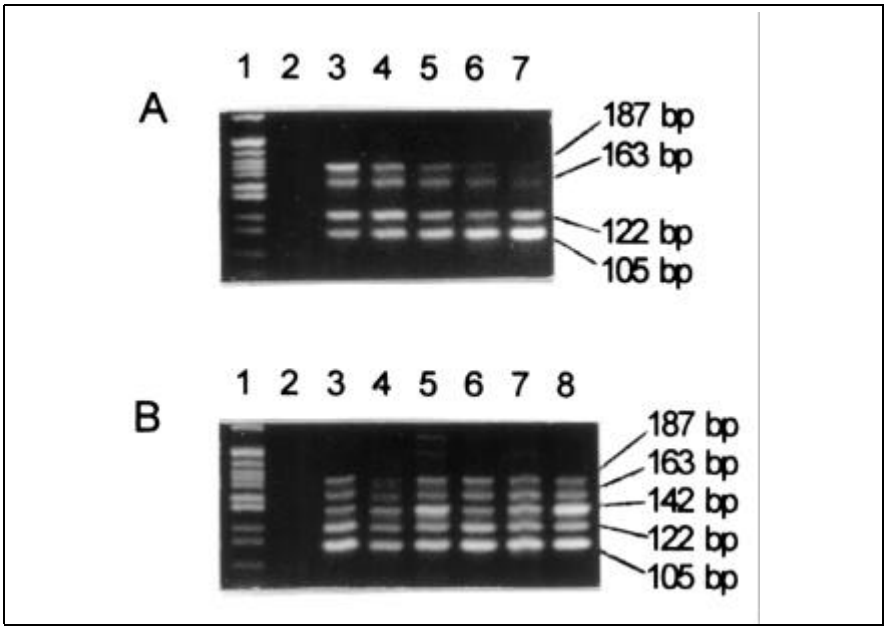

Figure 2. (A) Ethidum bromide-stained gel of amplified products using different molar ratios of the 4 different competitor templates. Lane 1, pBR322 digest; lane 2, negative control (buffer); lane 3, equal molar ratios (3000 copies per template); lane 4, copy numbers increasing by 3000 copies/template (3000-12 000); lanes 5–7, molar ratios increased by factors of 2 (3000–24 000) and 3 (3000–81 000) and 4 (3000–192 000), respectively. From lanes 4–7, the number of templates analyzed was in inverse relationship to the length of the fragment (starting with 3000 copies for the longest fragment). (B) Typical QMC-PCR experiments. Lane 1, pBR322 digest; lane 2, negative control; lanes 3–5, copy numbers of 500, 1000 and 2000 wild-type pBH10 determined within a range of 300–2400 competitive templates; lanes 6–8, copy numbers of 5000, 10 000, 20 000 wild-type pBH10 determined within a range of 3000–24 000 competitive templates. Length of wild-type fragment: 142 bp.

Table 1. Inter-Assay Variability of QMC-PCR

Copies of pBH10	Experiment No. 1	2	3	4	5	6	7	8	($s_{\bar{x}}$)*
500	553	512	587	554	602	581	563	**568**	566 (29)
1000	1018	1204	1119	1073	911	**1186**	914	1046	1059 (111)
2000	2011	1923	1885	2218	1948	2075	2204	**2018**	2035 (124)
5000	4114	5401	4918	4688	5729	5607	6102	**6179**	5342 (719)
10 000	8743	11 179	11 608	9664	9698	**10 996**	10 341	8170	10 050 (1207)
20 000	18 753	20 622	20 009	20 502	19 686	**19 422**	20 561	20 974	20 066 (742)

Results represent number of copies found in 8 different QMC-PCR experiments. Experiments of bold data are depicted in Figure 2B.
*$s_{\bar{x}}$ is standard error of the mean.

192 000 copies (lane 7). Up to an increase in template concentration by a molar ratio of 3 (i.e., up to lane 6), all the expected bands were detectable. The ratios of the different percentages, checked with a video densitometer, coincided well with the ratios of the competitor templates introduced.

The reproducibility of the QMC-PCR was tested by adding 500, 1000, 2000, 5000, 10 000 and 20 000 copy numbers of wild-type pBH10 to reaction tubes containing either 300, 600 1200 and 2400 or 3000, 6000, 12 000 and 24 000 competitor templates. Each experiment was performed eight times and yielded highly reproducible results. The data are shown in Table 1, and typical experiments (bolded in the table) are depicted in Figure 2B. Occasionally bands other than the intended products (possibly heteroduplexes) were observed (Figure 2B, lanes 5, 7 and 8), but these bands did not affect the accuracy of quantitation. To demonstrate the accuracy of the analytical method used, the PCR runs for which the data are bolded in Table 1 were evaluated five times with the video densitometer. This yielded standard deviations (*s*) of 24, 32, 86, 179, 327 and 337 for the values 568, 1186, 2018, 6179, 10 996 and 19 422, respectively.

Performance of the QMC-PCR was not hampered by adding backgrounds of lysates ranging from 10^4 to 10^5 uninfected cells (data not shown), and the method is also suitable for analysis of HIV-1-infected peripheral blood mononuclear cells (PBMC). When 2, 4, 6 or 8 µL lysate of HIV-1-infected PBMC were analyzed, 4410, 10 373, 13 991 and 15 798 copies, respectively, of HIV-1 DNA were detected. These results corresponded well to the copy numbers to be expected when analyzing increasing amounts of the same lysate.

The value of this method lies in the field of routine diagnosis, especially where the approximate copy numbers to be expected are known. Even when the approximate range is not known, this assay is nevertheless advantageous compared to standard competitive PCR, as each reaction tube offers a range extending almost to a factor of 30. When the copy number of the sample falls within the range chosen, a single sample assay provides four points for construction of a regression curve. The reduced amount of sample and labor required for QMC-PCR should make it an interesting and useful methodology for widespread use.

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