

Quantitation of HIV-1 by real-time PCR with a unique fluorogenic probe

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Abstract

Quantitation of HIV-1 specific RNA and DNA is pivotal to understanding the pathophysiology of HIV-1 diseases. A method has been developed for quantitation of HIV-1 DNA/RNA by real-time PCR using a unique fluorogenic primer-probe adduct known as scorpion. The probe hybridises to the extension of the adjoining primer intramolecularly, a process kinetically and thermodynamically more favourable than the conventional bimolecular probe-target hybridisation. Data presented in this paper indicate that the scorpion assay is extremely robust and is quite comparable to beacon-based assays. The scorpion assay is also comparable to quantitative competitive PCR (QC-PCR) assays but requires only a fraction of time and effort. Additionally, the dynamic range of the scorpion assay is several log-fold higher than the conventional end point PCR assays. As few as ten copies of vDNA can be detected in the presence of a large excess of exogenously added genomic DNA. Limiting dilution analysis indicates that the assay is capable of detecting a single copy of the viral template. Thus, the scorpion assay presents a specific and sensitive approach for quantitation of DNA/RNA templates by real-time PCR. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Upon infecting host cells the RNA genome of a retrovirus is reverse-transcribed, the complementary vDNA then integrates into the host chromosome and replicates along with the host

chromosome. In the instance of HIV-1, some of these integrated sequences endure productive replication propagating the life cycle of the virus. Thus, enumeration of proviral DNA in PBMC and other lymphoid tissues might provide an useful tool for prognostication, as well as for monitoring the efficacy of antiretroviral drugs. Furthermore, eradication of HIV-1 in infected individuals will require a complete understanding of the in vivo dynamics of viral replication during

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the entire course of infection and treatment. It will involve careful enumeration of both vRNA and vDNA in a variety of cell and tissue types.

Several methods have been described for vRNA/DNA quantification. The standard techniques are based upon PCR utilising either a competitor molecule (Ferre et al. 1994; Hockett et al. 1995) or a fluorescence tagged reporter (Heid et al. 1996; Tyagi and Kramer 1996). Whereas, the competitor assay is based on end-point PCR, the latter is a kinetic assay and measures fluorescence acquisition during PCR-amplification in real-time. The fluorescence is commonly introduced through hybridisation of a fluorescence tagged probe specific for the target sequence. The bimolecular reaction, however, imposes certain thermodynamic constraints on the kinetics of hybridisation.

Recently a novel real-time PCR assay was described (Whitcombe et al., 1999) in which the fluorescent-tagged probe was linked to one of the primers facilitating intramolecular hybridisation, which is thermodynamically more favourable than the bimolecular probe-target hybridisation. In this approach, the probe is located in the form of a hairpin loop at the 5'-end of the primer. The 5'-end of the probe is labelled with a fluorophore while the 3'-end is labelled with a quencher. The fluorophore and the quencher are brought in close proximity with the help of an irrelevant duplex stem structure so that there is no autofluorescence of the probe in the free state. The probe sequence is complementary to an internal region of the sequence extended by the adjacent primer. The polarity is such that the 5'-end of the probe is complementary to the 3'-end of the target sequence. Upon hybridisation of the probe to the target sequence, the hairpin stem is destabilised distancing the fluorophore from the quencher and allowing it to fluoresce freely. Thus, the extent of fluorescence is proportional to the number of the amplicons, which, in turn, is proportional to the number of the target molecules. In this report, we describe the application of this unique primer-probe (scorpion) to HIV-1 quantitation.

2. Materials and methods

2.1. Light cycler

Light Cycler (Wittwer et al., 1997) is a rapid microvolume thermal cycler with a built-in fluorimeter. Fluorescence-labelled PCR products are monitored on-line and real-time. Quantification is achieved with the help of an integrated software that calculates the copy number of the input templates with respect to a standard curve generated in parallel. Slopes of the fluorescence accumulation curves are calculated during the log-linear phase of amplification so that the quantitation is based on kinetic analyses and is free of ambiguities associated with end-point PCR.

2.2. Scorpions and primers

Schematics of the free and hybridised scorpion are shown in Fig. 1A and B, respectively. Fig. 1C shows the nucleotide sequence of the HIV-1 amplicon including the scorpion probe. The scorpion primers are usually set close to each other so that the amplicon is quite short, 100–200 bp. Two primers (underlined) were designed from the RT region of the pol gene of HIV-1 (GenBank accession # K03455) to yield an amplicon of 104 bp. A probe sequence (bold italics) was designed so that it was complementary to the PCR-extension of the forward primer and started after 11 nucleotides from the beginning of the extension (bold letters). Each end of the probe was linked to a complementary GC containing hexanucleotide that forms a duplex stem at room temperature. The 5' end of the stem was covalently linked to a fluorophore, 6-carboxyfluorescein (FAM) while the 3' end was successively linked to a quencher methyl red (MR) and a chemical blocker hexaethylene glycol (HEG). Due to fluorescence resonance energy transfer to MR, FAM remains quenched at room temperature (Fig. 1A) while HEG prevents the probe from being copied by the reverse primer during the extension step of PCR. The probe sequence orientation was such that its 5'-end was complementary to the 3'-end of the

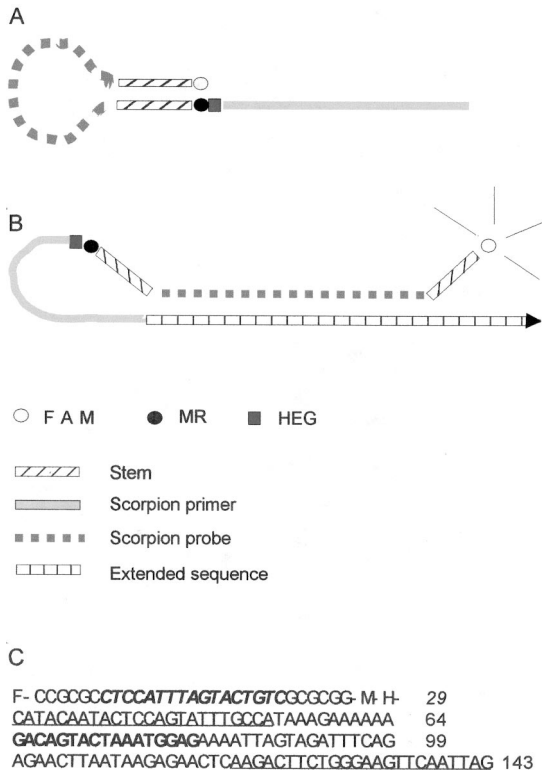


Fig. 1. (Continued)

extended target sequence. During the annealing step of the PCR cycle, concurrent with the primer annealing, the probe sequence loops around and intramolecularly hybridises to the adjoining strand of the amplicon generated in the previous round, and fluoresce. In the subsequent step, the probe is displaced from the template following extension of the reverse primer and returns to the unquenched form. Thus at each annealing step the fluorescent signal increases proportional to the increase in the number of amplicons.

The scorpion sequence was synthesised by Osweil (University of Southampton, UK). The beacon probe and the corresponding primers were from the HIV-1 gag gene as described before (Vet et al., 1999). The beacon was synthesised by Stratagene (San Diego, CA). Both the scorpion and the beacon were purified by double HPLC to remove any unbound fluorophore. The primers for QC-PCR were from the HIV-1 pol gene as described before (Hockett et al., 1999).

2.3. Template DNA

SG3 plasmid (Ghosh et al., 1993) containing the entire HIV-1 sequence was from George Shaw (UAB, Birmingham, AL). The U1 cell line (Folks et al., 1987) was from Tom Folks (CDC, Atlanta, GA). Plasmid and cellular DNA were purified using Qiagen columns (Qiagen, Valencia, CA). DNA mass was determined by an extremely sensitive (detection limit < 50 pg) fluorometric assay using PicoGreen (Molecular Probes, Eugene, OR). Input target copies were calculated based on the size of the plasmid or the genome and the corresponding DNA mass. The copy numbers were verified by limiting dilution assay (LDA, Taswell, 1981).

2.4. PCR-amplification

For the scorpion assay, PCR amplifications

Fig. 1. (a) Schematic of the HIV-1 scorpion showing the stem-loop structure. (b) Schematic of the scorpion after hybridisation of the probe sequence to the amplicon. During the annealing step of PCR three things happen concurrently: (1) the free scorpions, denatured in the earlier step, return to their closed configuration and remain quenched; (2) the primers, including the scorpion primers, anneal to their respective targets; and (3) the amplified strands extended by the scorpion primers undergo intramolecular hybridisation, the probe portion of the scorpion fold around and hybridise to the complementary portion of the extended sequence. This results in the release of fluorescence proportional to the number of the amplicons as the quencher (MR) is now removed from the vicinity of the fluorophore (FAM). The intramolecular hybridisation step is extremely rapid and distinguishes the scorpion assay from the bimolecular hybridisation-based assays. (c) Nucleotide sequence of the entire amplicon including the probe sequence. The 5'-end starts with the fluorescent dye (FAM), followed, respectively, by six nucleotides (nt 1–6) forming one arm of the stem, seventeen nucleotides (nt 7–23) of the probe sequence that hybridises to nt 2748–2764 of the HXB2CG sequence of HIV-1 (GenBank Accession # K03455) and then another six nucleotides (24–29) complementary to nt 1–6, forming the other arm of the stem. The probe sequence is followed by the quencher methyl red (MR) and the chemical blocker hexaethyleneglycol (HEG) to prevent Taq polymerase from copying 5' to the primer sequence that follows it. The forward PCR primer (nt 30–53) corresponds to nt 2713–2736 of HXB2CG. The reverse PCR primer (nt 120–143) corresponds to nt 2803–2827 of HXB2CG.

were carried out in 10 μ l containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 6 mM MgCl₂, 0.25 mM each of the four dNTPs, 0.5 μ M each of the scorpion and the reverse primer, 1 μ g of BSA, 0.5 unit of Taq DNA Polymerase (Roche Biochemicals) and template DNA. After initial heating at 95°C for 1 min, each of the subsequent 45 cycles consisted of denaturation at 95°C for 0 s, annealing at 59°C for 5 s and extension at 72°C for 30 s. Fluorescence was monitored at each cycle during the annealing step. For the beacon assay, PCR amplification was carried out essentially the same way as above but in the presence of the primers and the beacon as described by Vet et al. (1999). PCR amplification for QC–PCR assay was carried out exactly as described by Hockett et al. (1999).

2.5. Principle of real-time PCR

Real-time PCR, as opposed to end-point PCR, depends on continuous monitoring of amplification using a fluorescence-labelled reporter. The built-in fluorimeter records the fluorescence acquisition (Rn) at each cycle so that at any given cycle the amount of fluorescence registered is proportional to the amount of the PCR product generated through that cycle, providing the basis for real-time homogeneous PCR. However, as the PCR amplification becomes less and less exponential with progressive cycling, the fluorescence accumulation curves often reach the same plateau level with widely disparate initial target concentrations typical for end point PCR. The threshold cycle (C_T), on the other hand, remains proportional to the initial target concentration, increasing as the concentration of the latter decreases. The threshold cycle (C_T) is defined as the cycle at which the fluorescence acquired during amplification is significantly higher than the baseline (background) level. Extrapolation of the log-linear phase of the fluorescence accumulation curve to the baseline provides the C_T value, which is a unique signature of the target concentration. The integrated software calculates the C_T value corresponding to each PCR reaction automatically. A standard

curve is drawn using known input target copies (X-axis) versus the corresponding C_T values (Y-axis) using the least squares fit method. The correlation coefficient (r^2) should be as close to 1.0 as possible. The copy number in a test sample is then deduced from its C_T value plugged into the linear regression equation of the standard curve.

3. Results

Initial studies focused on the quantitation of HIV-1 specific DNA (vDNA). The SG3 plasmid (Ghosh et al., 1993) carrying a full-complement of HIV-1 sequence and the U1 cell line (Folks et al., 1987) carrying proviral HIV-1 were used as the source of vDNA template. Preliminary experiments were directed toward optimising the various parameters of PCR including cycling conditions (time and temperature) and Mg concentration. Conditions were chosen such that the C_T values were the lowest possible and the fluorescence acquisition curves were robust and parallel to each other at various template concentrations. In general, the cycling time was much shorter (less than 30 s per cycle) and the Mg concentration was somewhat higher (4–8 mM Mg) than those in the conventional PCR.

3.1. Quantitation of HIV-1 DNA

Fig. 2A shows a typical experiment with various copy numbers of the SG3 plasmid DNA and the pol scorpion as described in Section 2. Although with decreasing template concentrations the start of the fluorescent curves were displaced towards the higher cycle numbers, the maximum fluorescence achieved overlapped at various template concentrations analogous to that observed in end-point PCR. However, the log-log plot of the threshold cycles (C_T) against the corresponding target concentrations (Fig. 2B) shows > 99% correlation ($r^2 = 0.9989$) providing the basis for quantitative real-time PCR. Thus, kinetic assays overcome the problem of anomalous end points observed in standard PCR.

3.2. Sensitivity, dynamic range and assay reproducibility

The sensitivity of the scorpion assay was tested by limiting dilution assay (Fig. 3A). The copy number of SG3 was first calculated based on DNA fluorometry using PicoGreen. DNA was then diluted to 0.3, 0.6 and 1.2 copies per assay tube. Ten tubes at each concentration were assayed for the presence of SG3 sequence using the pol scorpion. The percent of negative tubes were plotted against the input copy number in a semilog plot. According to Poisson's distribution at a limiting dilution of one copy, 37% of the tubes are expected to be negative (Taswell, 1981). Extrapolation of the 37% negative point on the Y-axis intersected at about 0.95 copy on the X-axis, in excellent agreement with fluorometric determination of the copy number, implying the scorpion assay is both sensitive and robust (that is no appreciable false negatives or positives). Next, the dynamic range and reproducibility of the assay were investigated. Fig. 3B shows the assay is quite linear ($r^2 = 0.9983$) over seven logs of target concentration, from 10 to 10^8 (the highest concentration tested) input copies of the SG3 plasmid DNA. The data represent the aggregate of three

independent assays with triplicate determinations at each concentration ($n = 3 \times 3$). The huge linear dynamic range is another feature that separates real-time PCR from end-point based QC-PCR, which is applicable only over a narrow range of template/competitor concentration.

Table 1 shows the inter- and intra-assay variability associated with the assay. The coefficient of variation (CV) was calculated for the triplicate determinations in each of the three assays (intra-assay variability). The CV% varied up to 45% (average 24%) within the three experiments. Calculation of CV% amongst the three independent assays (inter-assay variability) revealed a smaller range of variation, 5–20% (average 12%), indicating the assays were quite reproducible.

3.3. Comparison with the Beacon and QC-PCR assays

Another type of real-time PCR assay involves the molecular beacon (Tyagi and Kramer, 1996). This approach utilises a pair of primers as in conventional PCR and an oligonucleotide probe with a stem-loop structure that fluoresces only upon hybridisation with one of the amplicon strands. A well-characterised beacon from the gag

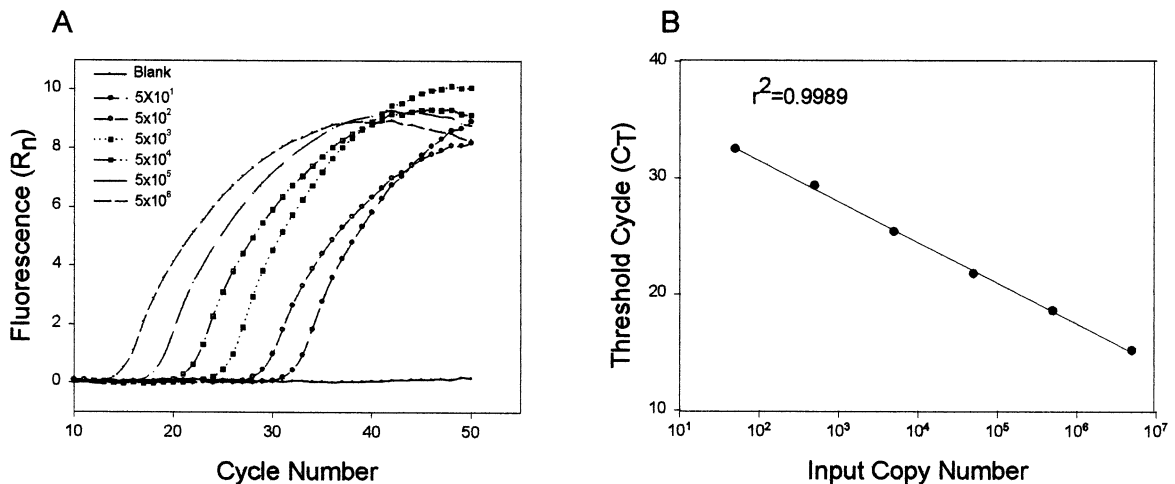


Fig. 2. Scorpion assay at various SG3 plasmid DNA copy number. Each data point represents the mean value of triplicate determinations. (a) Fluorescence acquisition (R_n) curves at various input copy numbers of the SG3 plasmid. Fluorescence data at baseline were plotted throughout the PCR cycles at each input copy (shown in the insert). (b) Linear relationship between threshold cycles (C_T) and input SG3 copy numbers. Linear regression ($r^2 = 0.9989$) results in an equation of $y = -3.5x + 38.5$.

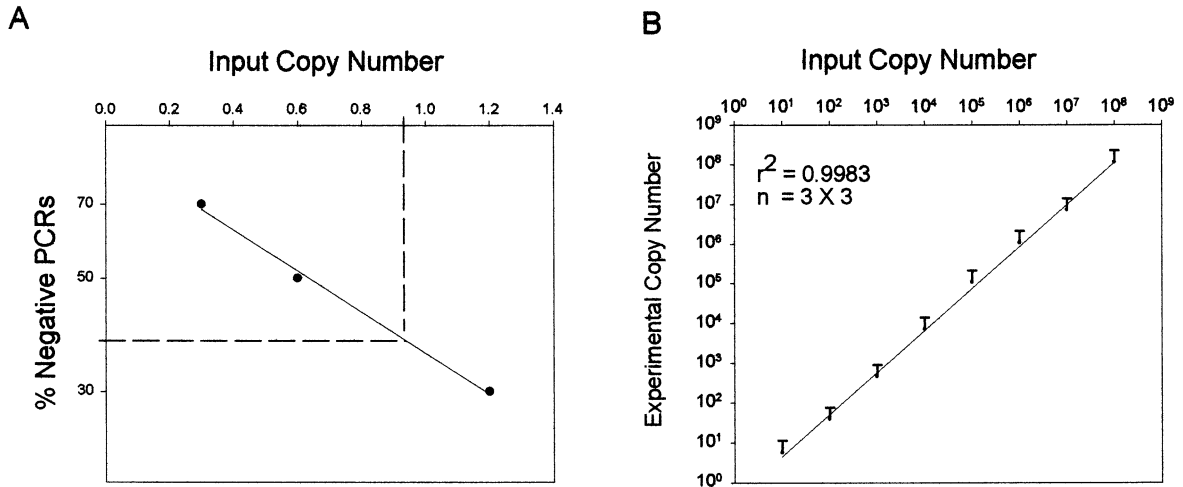


Fig. 3. (a) Limiting dilution assay (LDA) of SG3 plasmid DNA. Ten PCR-amplifications were performed at each of the three dilutions of the SG3 at an input copy number of 0.3, 0.6 and 1.2, respectively. Percent assays failing to show any amplification were plotted against the respective input copy numbers. A dashed line was drawn parallel to the X-axis from the Y-axis at 37% negative to intercept the limit dilution plot. The point of intersection was then extended parallel to the Y-axis to intercept the X-axis at ~ 0.95 copy indicating the Scorpion assay is sensitive to one molecule. (b) Linear dynamic range of the Scorpion assay. Experimentally determined copy numbers were plotted against the input copy numbers of SG3 in a log-log scale. Each data point represents a mean value with the standard error for triplicate determinations of three successive assays ($n = 3 \times 3$). The mean values and CV% are shown in Table 1.

gene of HIV-1 (Vet et al., 1999) was compared with the scorpion (Fig. 4A) using SG3 template as described in Fig. 3. Linear regression analysis showed an excellent correlation ($r^2 = 0.9988$) between the two assays. The 'gold standard' for quantitative PCR, however, is QC-PCR (Ferre et al., 1994; Hockett et al., 1995) that utilises an internal competitor to compensate for the tube-to-tube variation observed in end-point PCR. Therefore, we compared QC-PCR, using a pair of primers and a competitor (Hockett et al., 1999) from the same region as the scorpion target sequence, with the scorpion-PCR. The experiment was carried out as before but utilised genomic DNA from the U1 cell line carrying integrated HIV-1 DNA as the target. Fig. 4B shows that there is an excellent correlation between the two assays ($r^2 = 0.9901$), similar to that observed with the beacon probe, implying quantitation by real-time PCR based on C_T is free from the vagaries encountered in routine end-point PCR.

3.4. Effect of exogenous DNA

The experiments described above validate the performance characteristics of the scorpion assay for quantitative studies. However, these assays were carried out either with plasmid DNA (SG3), or with DNA from a cell line (U1 cell). HIV-1 patients treated with antiretroviral drugs may contain only a few copies of the viral RNA/DNA sequence in their blood/PBMC/LN samples demanding an extremely sensitive detection method. We tested this scenario by adding an excess of genomic DNA (500 ng DNA derived from normal PBMC, $\sim 100\,000$ cell equivalent) to a wide range of HIV-1 template DNA (10; 100; 1000; and 10 000 HIV-1 copy equivalent of U1 DNA). Linear regression analysis (Fig. 5) showed that the slopes of the two lines, with and without exogenous DNA, are comparable indicating the scorpion assay is both specific and sensitive for extending to clinical assays. In the event, a higher level of genomic DNA is needed for the assay (e.g. in heavily

suppressed patients), it might be necessary to generate the external standard curve with a comparable level of exogenous DNA in order to compensate for the differential effect of the latter. It should be noted that for most accurate quantitation, the slopes of the fluorescence acquisition curves of the test samples should be parallel to those of the external standards (i.e. the curves should be parallel to one another despite varying crossing points), and the reactions should be optimised accordingly.

4. Discussion

A real-time quantitative PCR assay was developed based on a unique fluorogenic primer-probe, named scorpion (Figs. 1 and 2). The scorpion assay is sensitive to a single target molecule as determined by the limiting dilution assay (Fig. 3A), and has a huge dynamic range of linear amplification to accommodate virtually any number of target molecules that can be solubilised in the reaction mixture (Fig. 3B). The scorpion assay compares quite favourably with the beacon-based assay and the quantitative competitive PCR (QC-PCR) assay (Fig. 4A and B, respectively).

However, the scorpion assay, like the beacon assay, has many advantages over the QC-PCR assay. For instance, unlike in the QC-PCR, there is no need for post-PCR manipulation in real-time (homogeneous) assays, minimising the danger of

contamination arising from PCR derived amplicons, the most common source of contamination in PCR assays. QC-PCR normally requires multiple assays for a single target and downstream processing is extremely time-consuming limiting its use in time sensitive cases. Finally, QC-PCR is subject to formation of heterodimers between the target and the competitor strands further complicating the quantitative analysis (Freeman et al., 1999). Additionally, scorpions have both kinetic and thermodynamic advantage over molecular beacon and TaqMan probes in that the hybridisation in scorpions is unimolecular (intramolecular) as opposed to bimolecular hybridisation with the latter. Unimolecular hybridisation is independent of the concentration of the reactant (zero-order reaction) and is extremely rapid compared to the bimolecular hybridisation, which is dependent on the concentration of the reactants (first-order reaction).

One of the criticisms against quantitation by real-time PCR as opposed to by QC-PCR is that the former assay is based upon external standards and does not address the subtle differences in the samples owing to variations in extraction efficiency and/or varying levels of inhibitors present therein. However, it is possible to circumvent these problems by normalising the target measurements to measurements of another internal sequence using a second scorpion. The second scorpion can be run in a parallel reaction, or be duplexed in the same reaction (von Ahsen et al.,

Table 1
Intra- and inter- assay variability

Input copy	Assay 1 ^a		Assay 2 ^a		Assay 3 ^a		Assay 1–3	
	Mean copy	CV%	Mean copy	CV%	Mean copy	CV%	Mean copy	CV%
10	9	34.12	11	31.01	10	37.02	10	13.51
100	85	1.66	63	45.68	61	21.19	70	19.27
1000	977	35.77	968	0.079	1062	12.03	1002	5.17
10 000	9196	21.58	10 767	9.93	10 844	19.23	10 269	9.06
100 000	120 567	7.62	95 580	13.88	102 233	8.80	106 127	12.19
1 000 000	942 033	16.50	1 068 400	13.15	1 002 533	4.52	1 004 322	6.29
10 000 000	9 792 667	19.70	9 855 333	14.07	12 343 333	9.56	10 663 778	13.64
100 000 000	99 860 000	10.44	100 513 333	11.53	79 980 000	3.17	93 451 111	12.49

^a average of three replicates.

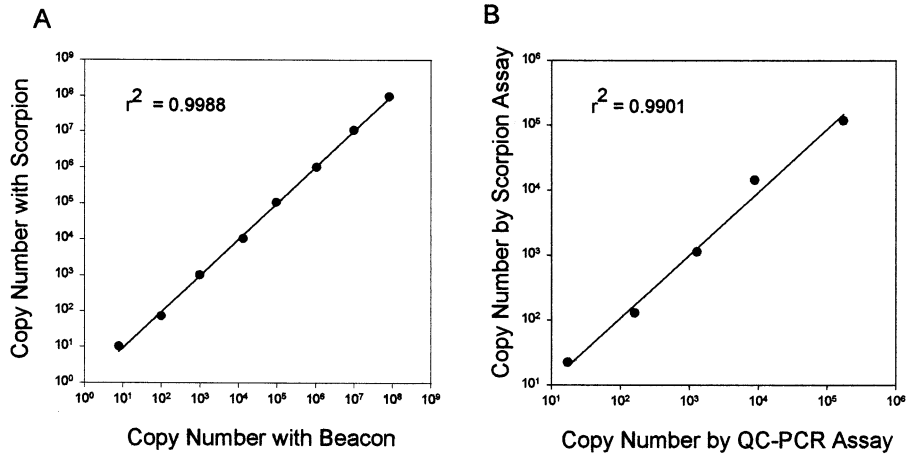


Fig. 4. Comparison of the Scorpion assay with bonafide quantitative PCR assays. Each data point represents the mean value of triplicate determinations. (a) Comparison of Scorpion with Beacon. SG3 plasmid was assayed at various copy numbers using either the Scorpion or a Beacon, and the experimentally determined copy numbers were plotted against each other. (b) Comparison of the Scorpion assay with the quantitative competitive-PCR (QC-PCR) assay. Various copy numbers of HIV-1 from U1 cells were assayed by the Scorpion and the QC-PCR methods, and the experimentally determined copy numbers were plotted against each other as in above.

2000). In the latter instance, a different fluorochrome must be used and both targets must amplify optimally under the same cycling condition in a dual-color read out system. For DNA quantitation, the second target sequence could be any stretch of the genome with a single copy and for RNA, this could be a house-keeping gene such as beta-actin or GAPDH. The ratio of the two measurements will serve to correct for the differences in extraction efficiency and in the case of RNA, for RT efficiency, as well. The final numbers can be expressed in terms of units of genomic DNA or total RNA measured spectrophotometrically, or more accurately, fluorometrically using a DNA or RNA specific dye (e.g. PicoGreen or RiboGreen). It should be noted, however, that many of the PCR-related problems (e.g. 'plateauing' effect, effect of inhibitors, etc.) encountered in end-point PCR are less of a concern in real-time PCR as the measurements in the latter instance are carried out in early cycles of PCR when the DNA amplification is most robust and exponential. Thus, the issue of disproportionate amplification, which is a constant problem in end-point PCR and must be dealt with (e.g. QC-PCR), is mostly academic in real-time PCR.

The *pol* scorpion was tested successfully against several other HIV-1 templates including NL-4.3, YU-2 and Cdcy, as well as several clinical specimens. The *pol* scorpion was also, as expected, effective for RT-PCR. Two other scorpions targeting the *env* and *gag* genes of HIV-1 and SHIV performed equally well (data not shown). Additionally, we have established a scorpion assay for the 2-LTR circles of HIV-1, a putative marker for latent viral reservoir and ongoing viral replication (Sharkey et al., 2000; data not shown). It should be pointed out here that no single scorpion (or beacon, or any other primer/probe sequence) is likely to amplify all the clinical HIV-1 isolates because of the rapid sequence evolution. Therefore, whenever possible scorpions should be designed on the conserved regions of HIV-1, and in the instance of a negative reaction, a second amplification should be attempted with a different scorpion targeting another region of the genome. Sequence variation may also lead to differential amplification or probing of clinical isolates so that for a rigorous analysis, it would be wise to carry out two independent scorpion-based amplifications at all instances.

Recent literature suggests that real-time PCR is the choice over the competitor-based PCR for

quantitation of DNA and RNA. A flurry of reports were published of late utilising the FRET (fluorescence resonance energy transfer), TaqMan, or the beacon technique for quantitation/detection of viral sequences (Nitsche et al., 1999; Espy et al., 2000), for gene rearrangements (Emig et al., 1999; Pfitzner et al., 2000) and for single nucleotide polymorphism, SNP (Dianzani et al., 1999; Germer et al., 2000). Quantitation of retroviruses including HTLV-1 (Nagai et al., 1998; Miley et al., 2000), SIV (Suryanarayana et al., 1998) and HIV-1 (Lewin et al., 1999) were also reported using either the TaqMan or the beacon technique. The scorpion approach described above provides another option with distinct advantages over the existing methods.

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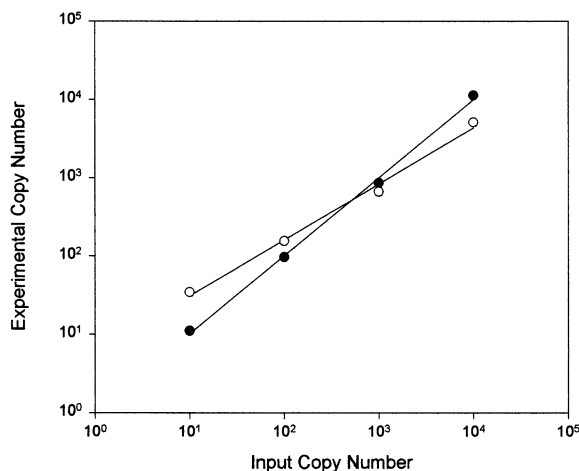


Fig. 5. Effect of exogenous DNA on the sensitivity of the scorpion assay. The scorpion assay was carried out with 10; 100; 1000 and 10 000 copy equivalent of HIV1 template from U1 cell DNA in the presence of 500 ng of genomic DNA derived from normal PBMC. Open circles, 500 ng exogenous DNA; closed circles, no exogenous DNA. Linear regression analysis indicates that there is a small intercept in the presence of exogenous DNA. The data for test samples can be corrected, if desired, by running the standard curve in the presence of exogenous DNA.

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