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High throughput functional analysis of HIV-1 *env* genes without cloning

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Abstract

Functional human immunodeficiency virus type -1 *env* clones have been widely used for vaccine design, neutralization assays, and pathogenesis studies. However, obtaining bona fide functional *env* clones is a time consuming and labor intensive process. A new high throughput method has been developed to characterize HIV-1 *env* genes. Multiple *rev/env* gene cassettes were obtained from each of seven HIV-1 strains using single genome amplification (SGA) PCR. The CMV promoter was amplified separately by PCR. A promoter PCR (pPCR) method was developed to link both PCR products using an overlapping PCR method. Pseudovirions were generated by cotransfection of pPCR products and pSG3Δ*env* backbone into 293T cells. After infecting TZM-bl cells, 75 out of 87 (86%) of the *rev/env* gene cassettes were functional. Pseudoviruses generated with pPCR products or corresponding plasmid DNA showed similar sensitivity to six HIV-1 positive sera and three monoclonal antibodies, suggesting neutralization properties are not altered in pPCR pseudovirions. Furthermore, sufficient amounts of pseudovirions can be obtained for a large number of neutralization assays. The new pPCR method eliminates cloning, transformation, and plasmid DNA preparation steps in the generation of HIV-1 pseudovirions, this allows for quick analysis of multiple *env* genes from HIV-1 infected individuals.

Keywords

HIV-1; neutralization; envelope; pseudovirion; PCR

1. Introduction

Envelope genes of human immunodeficiency virus type 1 (HIV-1) have been extensively studied using pseudovirions by cotransfection of a backbone of an *env*-defective clone and an *env* expression plasmid (Helseth et al., 1990). This allows for the study of *env* genes from a large number of HIV-1 isolates (Derdeyn et al., 2004; Gao et al., 1996; Wei et al., 2003; Li et al., 2005; Li et al., 2006). Traditionally, *env* genes are amplified from either proviral DNA or viral RNA through RT-PCR and the products are then cloned into expression vectors for

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generation of pseudovirions. However, the *env* gene products amplified through bulk PCR may be affected by recombination or resampling during bulk PCR amplification (Fang et al., 1998; Liu et al., 1996). Since minor sequence changes can affect the biological functions of envelope glycoproteins (Cordonnier et al., 1989; Kalia et al., 2005; LaBranche et al., 1995; Morris et al., 1994; Shimizu et al., 1999; Shioda et al., 1994), it is important to avoid studying *env* genes containing artificial elements generated during PCR in vitro.

Non recombinant *env* genes can be obtained using limiting dilution or single genome amplification (SGA) methods (Liu et al., 1996; Palmer et al., 2005; Simmonds et al., 1990). Since only one amplifiable viral genome is amplified in each PCR reaction in either method, the products are not affected by recombination or resampling. However, because the PCR products are obtained through multiple reactions, numerous purifications, ligations, transformations, and plasmid DNA preparations are required to test the functionality of the *env* PCR products. As a result, this cloning step is time consuming, labor intensive, and costly.

The use of Env pseudotype viruses in a single round infection system has greatly improved the accuracy and simplicity of the evaluation of neutralization activity in vaccinated humans and experimental animals (Derdeyn et al., 2004; Gao et al., 1996; Helseth et al., 1990; Li et al., 2005; Wei et al., 2003). Given the extent of viral diversity that is seen among patients and even within a single individual, a large number of *env* clones are needed to understand neutralization profiles. For this reason, two panels of subtype B and C viruses have been proposed for use as standards for the evaluation of anti-HIV-1 neutralization activity in anti-HIV-1 sera (Li et al., 2005; Li et al., 2006). Analysis of a large number of Env pseudovirions from many different individuals using autologous and heterologous sera may lead to identification of signature sequences of critical neutralization epitopes and assist vaccine design. Therefore, a more efficient system is needed to generate a large number of Env pseudovirions. This study describes a promoter PCR (pPCR) method that can significantly decrease the labor, time, and cost needed to obtain a large numbers of Env pseudovirions by eliminating the cloning step.

2. Methods and Materials

2.1 Amplification of HIV-1 *env* genes

Seven plasma samples were collected from HIV-1 positive individuals enrolled in a study of current HIV-1 strains in Ndola, Zambia. The study was approved by the ethics committee of the Tropical Disease Research Centre, the Duke University Institutional Review Board, and the National Institutes of Health. Viral RNA was extracted from the plasma and eluted into 55 μ l of elution buffer using QiaAmp Viral RNA Mini kit (Qiagen; Valencia, CA). Reverse transcription was performed with 25 μ l of vRNA and 25 pmol primer OFM19 5'-GCACTCAAGGCAAGCTTTATTGAGGCTTA-3' in 100 μ l using Superscript III (Invitrogen; Carlsbad, CA). Single genome amplification (SGA) of the cDNA was performed to obtain the *rev/env* cassette and to avoid artificial recombination and resampling of the viral genomes (Palmer et al., 2005). The cDNA was diluted 1:3, 1:9, and 1:27 (15 reactions per dilution) to determine the dilution with a positive rate of 30% or less. One microliter of each diluted cDNA was used for the first round amplification with primers OFM19 and VIF1 5'-GGGTTTATTACAGGGACAGCAGAG-3' (Derdeyn et al., 2004). First round PCR was carried out with 1 unit of Platinum Taq Polymerase High Fidelity (Invitrogen; Carlsbad, CA), and 10 pmol of each primer in 20 μ l. The PCR thermocycling conditions were as follows: one cycle at 94°C for 2 min; 35 cycles of a denaturing step at 94°C for 15 sec, an annealing step at 55°C for 30 sec, an extension step at 68°C for 4 min; and one cycle of an additional extension at 68°C for 10 min. First round PCR products (2 μ l) were used for the second round PCR with primers env1Atopo 5'-CACCGGCTTAGGCATCTCCTATGGCAGGAAGAA-3' and envN 5'-CTGCCAATCAGGGAAGTAGCCTTGTGT-3'. The second round PCR was carried out in 50 μ l total volume with 2.5 units of Platinum Taq Polymerase High Fidelity and 15 pmol of

each primer. The PCR thermocycling conditions were as follows: one cycle at 94°C for 2 min; 45 cycles of a denaturing step at 94°C for 15 sec, an annealing step at 55°C for 30 sec, an extension step at 68°C for 4 min; and one cycle of an additional extension at 68°C for 10 min.

Similarly, HIV-1 *rev/env* cassettes were amplified from five functional *env* clones, CAP210.2.00.E8, DU422.1, ZM53.MPB12, ZM109.FPB4, and TRO.11, (Li et al., 2005; Li et al., 2006). Amplification was performed with 2 units of Platinum Taq Polymerase High Fidelity (Invitrogen; Carlsbad, CA), 5 pmol of each primer (*env1Atopo* and *envN*), and 100 pmol of each plasmid template in a 50 µl volume. The PCR thermocycling conditions were as follows: one cycle at 94°C for 2 min; 20 cycles of a denaturing step at 94°C for 30 sec, an annealing step at 55°C for 30 sec, an extension step at 68°C for 4 min; and one cycle of an additional extension at 68°C for 10 min. All PCR products were visualized on a 0.7% agarose gel and purified with the QiaQuick PCR Purification kit (Qiagen; Valencia, CA).

2.2. Amplification of CMV promoter

The CMV promoter was amplified from the pcDNA 3.1D/V5-His-TOPO cloning vector (Invitrogen; Carlsbad, CA). The PCR was carried out in a total volume of 50 µl with 2 units of Platinum Taq Polymerase High Fidelity, 100 pmol plasmid, and 10 pmol of each primer (CMVenv 5'-AGTAATCAATTACGGGGTCATTAGTTCAT-3' and CMVenv1A 5'-CATAGGAGATGCCTAAGCCGGTGGAGCTCTGCTTATATAGACCTC-3'). Each sample was subjected to one cycle at 94°C for 2 min; 30 cycles of a denaturing step at 94°C for 30 seconds, an annealing step at 55°C for 30 seconds, an extension step at 68°C for 4 minutes; and one cycle of an additional extension at 68°C for 10 min.

2.3 Promoter PCR (pPCR)

Overlapping PCR was performed using 100 ng of amplified HIV-1 *env*, 50 ng of CMV promoter, primers CMVenv and env1M 5'-TAGCCCTTCCAGTCCCCCTTTTCTTTTA-3' (20 pmol each) in a 50µl reaction with Platinum Taq Polymerase High Fidelity. The samples were subjected to one cycle at 94°C for 2 min; 20 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 4 minutes; and one cycle of an additional extension at 68°C for 10 min.

2.4 Single round infectivity assay

pPCR products were cotransfected with *env*-deficient HIV-1 backbone pSG3Δ*env* into 293T cells in a 24 well tissue culture plate using FuGENE6 transfection reagent (Roche Diagnostics; Indianapolis, IN) according to manufacturer instructions. Briefly, pPCR DNA (200 ng) or plasmid DNA (200 ng) and pSG3Δ*env* DNA (200 ng) were mixed with 1.2 µl of FuGENE6 (FuGENE:DNA ratio at 3 µl:1 µg) in a total volume of 20 µl with serum free DMEM, incubated for 30 minutes and added to 293T cells (70% confluence) seeded one day earlier at 5×10^4 per well. Transfected cells were maintained in DMEM with 10% FBS and Gentamicin. Forty-eight hours after transfection, supernatants were harvested. Equal volumes of pseudovirions were added to TZM-bl cells with DEAE (5 µg/ml) in a 96 well tissue culture plate (200 µl). Cultures were incubated for 48 hrs at 37°C with 5% CO₂.

2.5 Luciferase Assay

Supernatants (100 µl) from infected TZM-bl cells were removed and 100 µl of Bright-Glo Luciferase Assay substrate with buffer (Promega; Madison, WI) was added to the cells. Following a 2-minute incubation, 100 µl of cell lysates were added to a solid black 96 well plate. Luminescence was measured with a Wallac 1420 Multilabel Counter (PerkinElmer; Waltham, MA). The TCID₅₀ was determined as described previously (Li et al., 2005).

2.6 Neutralization Assay

Neutralization activity was measured as a reduction in luciferase activity after a single round infection of TZM-bl cells as previously described. (Li et al., 2005; Li et al., 2006; Montefiori, 2004). Equal amounts of pseudovirions (200 TCID₅₀) were used in each reaction.

Neutralization titers of pPCR pseudovirions were determined against six HIV-1 positive sera, one normal serum control, and three broadly neutralizing mAbs (4E10, 2F5, and 2G12).

2.7 Western Blot assay

Forty eight hours after cotransfection, the 293T cells were lysed with 250 µl of lysis buffer (50mM Tris-HCl, 150mM NaCl, 20mM EDTA, 1% Triton-X100, 0.1% SDS, pH 7.4). Cell lysates were mixed with denaturing sample buffer (24% Tris-HCl pH6.8, 20% SDS, 30% glycerol, 1.6% β-mercaptoethanol, 0.06% Bromophenol blue). Samples were boiled for 10 minutes and then loaded on a 4-15% gradient NuPAGE[®] SDS-PAGE gel (Invitrogen; Carlesbad, CA). Samples were normalized by the same amount of p24 protein. After the samples were transferred to a nitrocellulose membrane, the membrane was blocked (PBS containing 1% casein and 0.01% NaN₃) for 1 hr. The blot was then reacted with an HIV-1 infected patient serum (1:500) and mouse mAb 13D5 to the HIV-1 Env protein (1:3 000). Finally, the membrane was reacted with an IRDye800 conjugated affinity purified goat anti-human antibody (Rockland Immunochemicals; Gilbertsville, PA) and an Alexa-Fluor 680 conjugated goat anti-mouse antibody (Invitrogen; Carlesbad, CA). Fluorescence was detected with an Odyssey Infrared Imaging system (LiCor Biosciences; Lincoln, NE).

2.8 Sequence analysis

Sequence analysis of PCR products was performed by cycle-sequencing and dye terminator methods with an ABI 3100 genetic analyzer (Applied Biosystems; Foster City, CA). Individual sequence fragments for each *env* SGA were assembled and edited using the Sequencher program 4.7 (Gene Codes; Ann Arbor, MI). Deduced Env amino acid sequences were aligned using CLUSTAL W (Thompson et al., 1994).

2.9 Statistical analysis

Linear regression analysis was performed using SigmaPlot 8.0. (Systat Software, Inc; Point Richmond, CA). Luciferase data was analyzed using the two-tail Student T test with Excel (Microsoft Office 2004, Redmond, WA).

3. Results

To establish the promoter PCR (pPCR) method, the HIV-1 *env* gene (3 121 bp) and CMV promoter (655 bp) were amplified separately from known functional *env* clones TRO.11, CAP210.2.00.E8, DU422.1, ZM53.MPB12, and ZM109.FPB4 (Li et al., 2005; Li et al., 2006) The CMV promoter was added to the 5' end of the *env* PCR product by overlapping PCR (Fig. 1). The pPCR product was purified and cotransfected with an *env*-deficient proviral clone (pSG3Δ*env*) into 293T cells. The pseudovirions were harvested from the supernatants of the transfected cells 48 hrs after infection. An equal volume of supernatants containing pseudovirions generated with pPCR products or corresponding plasmid DNA were used to infect the TZM-bl cells. The luciferase activity of pPCR pseudovirions was on average 1.6 fold lower than that of their corresponding plasmid but significantly higher than that of the backbone control (Fig. 2). This suggests the pPCR method can be used to evaluate the biological functions of HIV-1 *env* genes by adding the CMV promoter at its 5' end without a cloning step.

The new pPCR method was then tested with a patient plasma sample (05ZM373). Viral RNA was extracted and reverse transcribed into cDNA. A total of 48 PCR reactions were performed

and five SGAs were obtained. The CMV promoter was added to all *env* PCR products at the 5' end by pPCR (Fig. 3A). After purification, the pPCR products were cotransfected with pSG3Δ*env* backbone clone into 293T cells. Supernatants (20 μl) from transfected cells were used to infect TZM-bl cells. Luciferase activity was determined 48 hrs post infection. Four pPCR pseudovirions yielded positive responses. Three had slightly higher infectivity than the pPCR TRO.11 control, while 05ZM373.2 was less infectious than the TRO.11 control (Fig. 3B). 05ZM373.1 did not yield infectious pseudovirions and the luciferase activity was similar to the background level of pSG3Δ*env* and CMV promoter controls.

Western blot analysis was then performed to determine if the low or no infectivity of 05ZM373 pPCR pseudovirions was due to the low level of Env protein expression. The transfected 293T cells were lysed and equal amounts of cell lysates (normalized by p24 content) were separated by SDS-PAGE. Env proteins were detected for four pPCR pseudovirus. However, a lower level of Env protein was detected for 05ZM373.2 (Fig. 4). No Env protein was detected in 05ZM373.1. The infectivity of pPCR pseudovirions was in good agreement with the level of Env protein expression (Figs. 3B and 4). The precursor and cleaved Gag proteins were detected at comparable levels among all samples. Although the Env expression level of pPCR transfection was much lower than that of plasmid transfection (Fig. 4), the infectivity of the pseudovirions derived from pPCR products was not significantly effected (less than two fold) compared to plasmid-derived pseudovirions (Fig. 2). This result suggests over-expression of Env protein by plasmids is not required for the generation of infectious viruses with pPCR products.

To investigate if variable levels of infectivity and Env protein expression were determined by Env amino acid sequences, all five 05ZM373 SGAs were sequenced. Open reading frames (ORFs) were intact in the four *env* genes that were positive in the luciferase assay. A premature stop codon (position 149) was found in the nonfunctional *env* gene (05ZM373.1), thus explaining the lack of Env protein expression and infectivity of 05ZM373.1 pseudovirions. It was not clear why protein expression and infectivity were lower for 05ZM373.2. To determine the reproducibility and fidelity of pPCR products, the same *env* gene was amplified by the pPCR method in five independent reactions. The pPCR products were sequenced and all five were identical, suggesting the pPCR method does not introduce detectable differences in sequence (data not shown).

In addition to evaluating the functionality of Env proteins from HIV-1 infected individuals, pseudovirions are also widely used for neutralization assays. To determine the sensitivity of 05ZM373 pPCR pseudovirions to neutralizing antibodies, their neutralization profiles were determined for six HIV-1 positive sera, a normal control serum, and three monoclonal antibodies (2G12, 2F5, and 4E10). Although four pPCR pseudovirions were from the same patient, three showed similar neutralization sensitivity to all sera and mAbs but 05ZM373.2 was slightly more resistant to all sera and 4E10 (Table 1).

To confirm these results, viral RNA was obtained from six additional patient plasma samples (05ZM375, 05ZM379, 05ZM381, 05ZM394, 05ZM401, 05ZM413). Between five and twenty-one SGAs were obtained from each subject (Table 2). The CMV promoter was successfully added to most of *env* PCR products (33%–100%) at the 5' end by pPCR. The functional *env* rate was 86% (73% – 100%) among 87 pPCR products from different HIV-1 strains. Phylogenetic analysis of all SGA sequences showed that they were subtype C viruses (data not shown). These results indicate the pPCR method is more efficient than the traditional cloning method to test functionality of multiple HIV-1 *env* genes from the same infected individual.

Dose dependent luciferase activity was investigated by cotransfection of variable amounts of *env* pPCR products with the same amount of pSG3Δ*env* (400 ng) into 293T cells in a 24 well

plate. The pPCR DNA was serially diluted 1:2 (800 ng - 12.5 ng). Luciferase activity decreased proportionally with decreasing pPCR DNA amounts. Linear regression analysis showed a good correlation ($R^2 = 0.9958$) between DNA concentrations and luciferase activities among the tested concentrations (Fig. 5). Since 200 ng of pPCR DNA yielded only slightly less luciferase activity than 400 ng ($p = 0.192$) and significantly higher luciferase activity than 50 ng of pPCR DNA ($p < 0.002$), 200 ng of DNA was routinely used for initial screening of functional *env* genes.

Large amounts of infectious virus stocks are required to determine neutralization characteristics for each pseudovirion with multiple HIV-1 positive sera and neutralizing mAbs. To determine if sufficient amounts of pseudovirus stocks can be generated using the pPCR method, four different transfection conditions were tested with different numbers of cells (Table 3). Transfection in T25 or T75 flasks yielded slightly higher luciferase activity than in 24 well or 6 well plates. However, the TCID₅₀ titers were much higher in T25 or T75 flasks than in 24 well or 6 well plates. Since the medium volume in T75 was more than other cell culture containers used in this study, the total TCID₅₀ was as high as 2 million. Therefore, many neutralization assays can be performed from one transfection with the pPCR product in a T75 flask.

To determine if pPCR pseudovirions have altered neutralization characteristics, pPCR products were obtained from five functional molecular clones and neutralization profiles were compared between pseudovirions derived from pPCR DNA transfections and corresponding HIV-1 *env* plasmid counterparts. Pseudovirions were generated with five pPCR-plasmid pairs and their neutralization profiles were determined with six HIV-1 positive sera and a normal control serum in addition to three monoclonal antibodies (2G12, 2F5, and 4E10). The neutralization titers in each pPCR-plasmid pair were comparable for both sera and monoclonal antibodies (Table 4). These results indicate pseudovirions derived from the pPCR products or traditional plasmids have similar neutralization characteristics.

4. Discussion

The newly developed promoter PCR (pPCR) method can be used for high throughput functional analysis of HIV-1 *env* genes by directly adding a cytomegalovirus (CMV) immediate enhancer/promoter to the 5' end of the HIV-1 *rev/env* gene PCR products. Since the steps of ligation, transformation, and plasmid DNA preparation are eliminated in the pPCR method, a large number of quasispecies HIV-1 *env* genes from a single individual can be quickly analyzed (Table 5). Coupled with the SGA method to obtain HIV-1 *env* genes directly from patient samples, pPCR also avoids analysis of artificial *env* genes generated either through recombination between different viral genomes or resampling of viral genomes during bulk PCR amplification (Fang et al., 1998; Liu et al., 1996). The high success rate of incorporated CMV promoter from a single PCR reaction greatly reduces time required to prepare DNA for transfection compared to traditional cloning methods (Table 5). Similar infectivity and neutralization profiles of pseudovirions generated with pPCR products and matched cloned plasmids indicate this method is reliable for determining neutralization characteristics of interested HIV-1 *env* genes. This new method can substantially reduce time and resources needed for characterization of HIV-1 *env* gene functions.

A virus stock containing as high as two million TCID₅₀ can be obtained by transfecting 4 μ g of pPCR DNA together with the pSG3 Δ *env* backbone clone into 293T cells in a T75 flask. Since 200 TCID₅₀ are normally used for one reaction in a standard neutralization assay in 96 well plates (Li et al., 2005), pseudovirions from one transfection can be used for many neutralization assays. In addition to function studies of multiple *env* genes from the same individual, the Env pseudovirions can be used for detailed analysis of neutralization profiles

using a relatively large panel of mAbs and anti-HIV-1 sera. The new method may also be used to determine neutralization escape mutants among wild-type viruses by analyzing a large number of quasispecies viral populations from longitudinally collected samples.

Recently, it has been reported that the biological phenotype of polioviruses is determined not only by the sequences present in the viral genome, but also by quasispecies diversity of the virus population (Vignuzzi et al., 2006). Studies of multiple functional HIV-1 *env* genes individually and as a quasispecies viral population may shed new light on the understanding of biological functions, viral fitness, neutralization, and neutralization escape. Furthermore, the pPCR method can also be used to quickly characterize other proteins without cloning.

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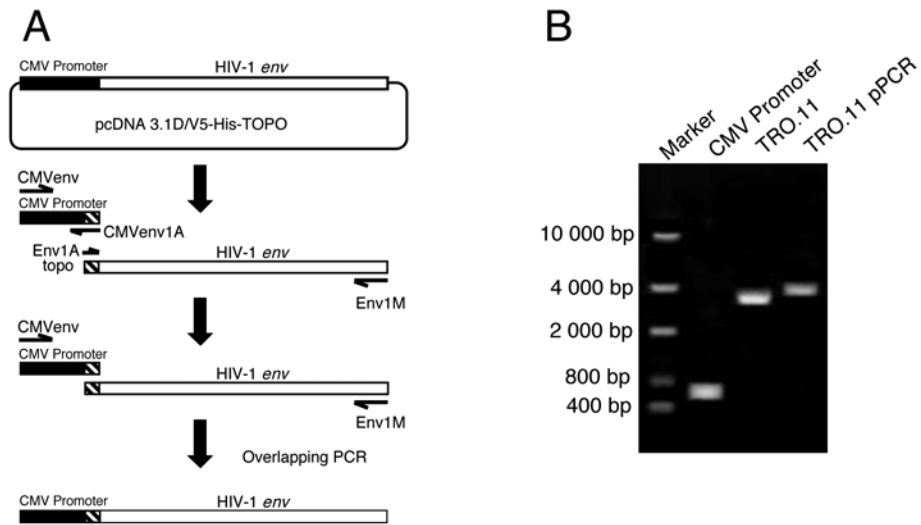


Figure 1. Generation of promoter PCR (pPCR) amplicons. (A) Schematic presentation of the pPCR procedure. The CMV promoter and the HIV-1 *rev/env* cassette were amplified separately. The CMV promoter was then added to the HIV-1 *rev/env* gene at the 5' end by overlapping PCR. (B) PCR and pPCR amplification of the CMV promoter and the *env* gene from a functional *env* control clone TRO.11 visualized on a 0.7% agarose gel.

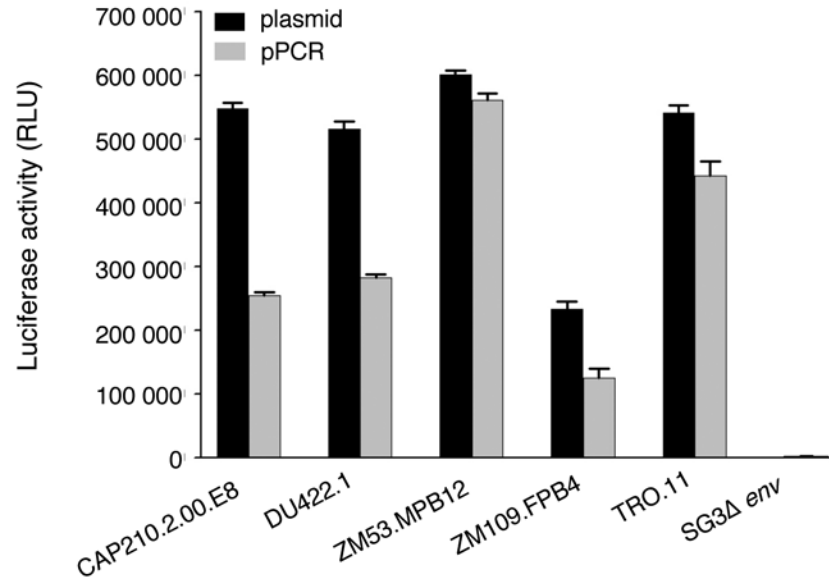


Figure 2. Infectivity of pseudovirions derived from pPCR DNA or corresponding functional *env* clones. The pPCR product (1000 ng) or corresponding *env* plasmid (1000 ng) was cotransfected with pSG3Δ*env* into 293T cells in a T25 flask. Supernatants were harvested 48 hrs after transfection and were used to infect TZM-bl cells. The luciferase activity was measured two days after infection. The data is shown as the mean \pm standard error ($n = 24$ independent assays).

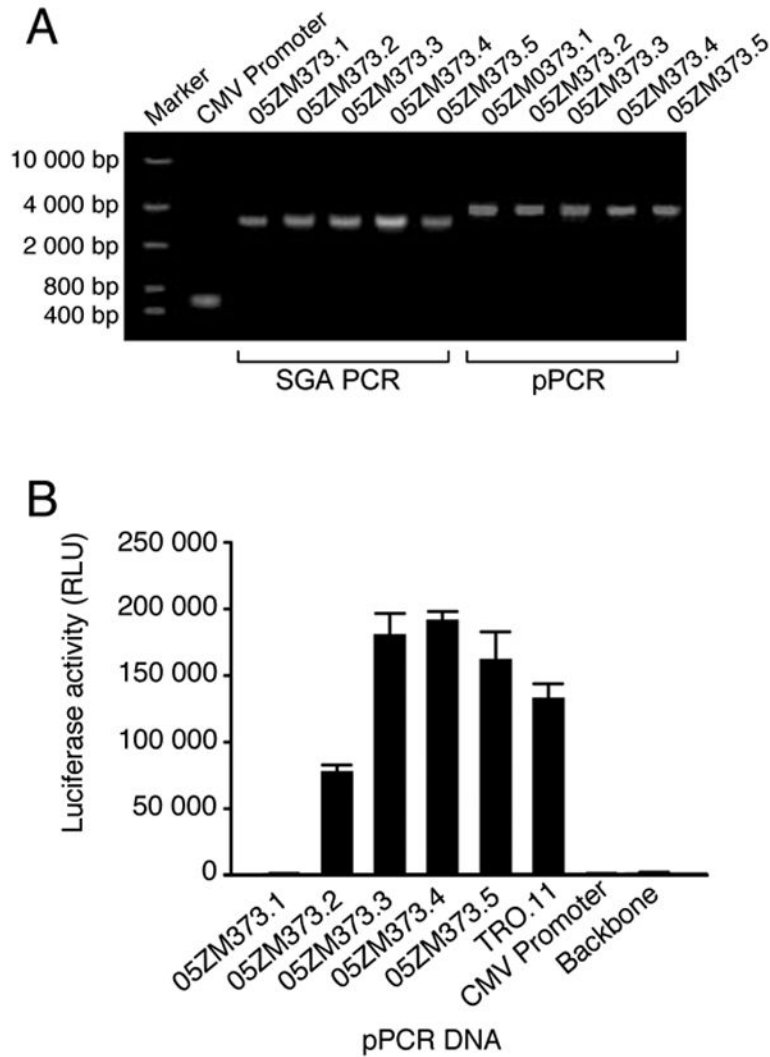


Figure 3.

Infectivity of pseudovirions derived from pPCR from patient 05ZM373. (A) PCR and pPCR amplification of *env* genes from patient 05ZM373 plasma. Five HIV-1 *rev/env* cassettes were obtained using the single genome amplification (SGA) method. The CMV promoter was amplified and then added to all five *env* genes at the 5' end by pPCR. (B) The pPCR DNA was cotransfected with pSG3 Δenv into 293T cells. Pseudovirions were harvested 48 hrs after transfection and were used to infect TZM-bl cells. The luciferase activity was measured two days after infection. The data is shown as the mean \pm standard error ($n = 4$ independent assays).

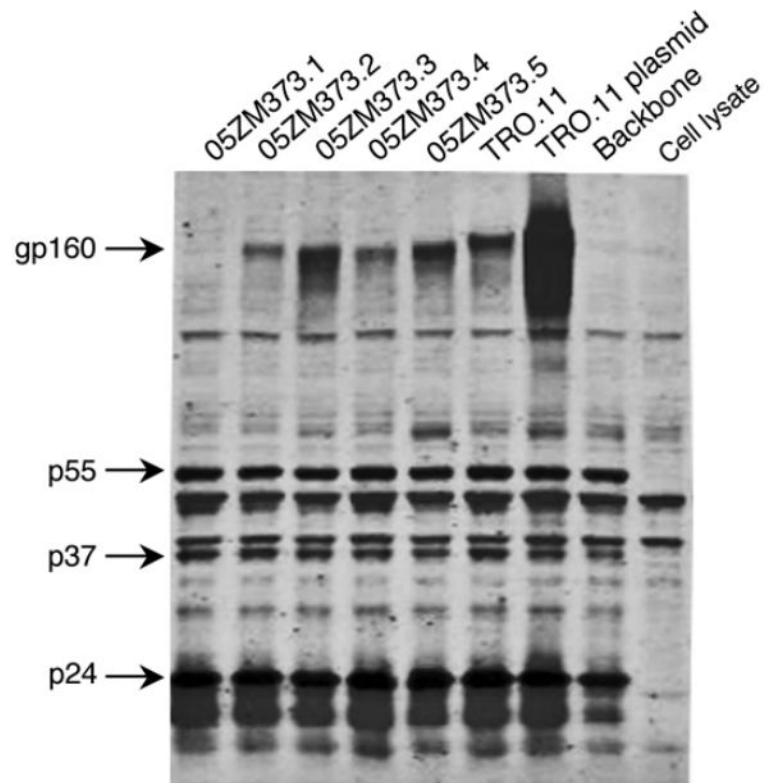


Figure 4.

Western blot analysis of HIV-1 protein expression. The 293T cells transfected with pPCR products and pSG3 Δenv were lysed 48 hrs after transfection. The viral proteins were separated on a 4–12% gradient reducing gel, transferred to nitrocellulose, and were reacted with an HIV-1 infected patient serum and a mouse mAb 13D5 to the Env protein. Viral proteins were visualized with secondary antibodies IRDye800 conjugated goat anti-human and Alexa-Fluor680 goat anti-mouse using a LiCor Odyssey Infrared Imaging system.

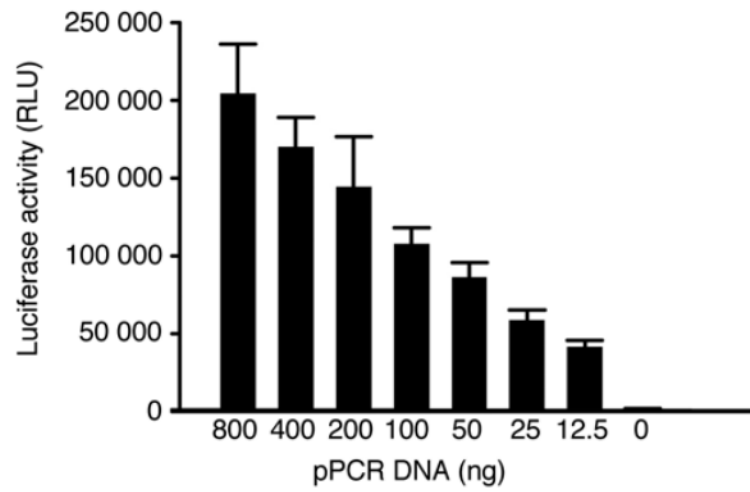


Figure 5. Dose dependent infectivity of pPCR pseudovirions. The pPCR products were 1:2 serially diluted (800 ng – 12.5 ng) and cotransfected with pSG3 Δenv into 293T cells. The infectivity was determined by measuring luciferase activity. The data is shown as the mean \pm standard error (n = 6 independent assays).

Table 1
Comparison of neutralization sensitivity of pseudovirions to HIV-1 positive sera and mAbs

Pseudovirus	ID ₅₀ in TZM-bl cells									
	Reciprocal serum dilution					Normal Sera				
	BB8	BB12	BB28	BB55	BB70	BB106	2G12	2F5	4E10	μg/ml
05ZM373.2	34	375	215	276	56	74	>50	>50	>50	>50
05ZM373.3	43	>540	320	406	68	124	>50	>50	40	>50
05ZM373.4	160	>540	374	>540	93	184	>50	>50	26	>50
05ZM373.5	48	>540	430	490	204	150	>50	>50	29	>50

Table 2
Summary of functional analysis of pPCR pseudovirions

Virus	No. of SGA	No. of pPCR	No. of functional <i>env</i> genes (%)
05ZM373	5	5	4 (80)
05ZM375	21	21	18 (86)
05ZM379	16	14	12 (86)
05ZM381	16	12	11 (91)
05ZM394	15	5	5 (100)
05ZM401	19	15	11 (73)
05ZM413	18	15	14 (93)
Total	110	87	75 (86)

Table 3

Comparison of TCID₅₀ titers of pseudovirions harvested from different cell cultures.

	pPCR DNA	Luciferase Activity (RLU)	TCID ₅₀ /ml	Volume	Total TCID ₅₀
24 well	200 ng	296 610	13 975	0.5 ml	6 988
6 well	200 ng	262 924	10 687	2 ml	21 374
T25	1 000 ng	460 090	69 877	5 ml	349 385
T75	4 000 ng	423 603	91 376	24 ml	2 193 024

Table 4 Comparison of neutralization titers of pseudovirions generated from HIV-1 *env* plasmids and pPCR products

Pseudovirus	ID ₅₀ in TZM-bl cells											
	Reciprocal serum dilution					Normal Sera					µg/ml	
	BB8	BB12	BB28	BB55	BB70	BB106	Normal Sera			2G12	2F5	4E10
CAP210.2.00.E8	<20	24	52	46	292	<20	<20			>50	>50	<2
	<20	<20	25	<20	147	<20	<20			>50	>50	<2
DU422.1	35	320	93	67	279	74	<20			>50	>50	<2
	30	249	79	37	197	44	<20			>50	>50	<2
ZM53.MPB12	<20	<20	390	<20	<20	22	<20			>50	>50	6
	<20	<20	279	<20	<20	<20	<20			>50	>50	11
ZM109.FPB4	103	49	44	46	282	<20	<20			>50	>50	3
	69	38	31	40	168	<20	<20			>50	>50	2
TRO.11	>540	>540	40	208	104	90	<20			<2	>50	<2
	293	402	21	152	41	77	<20			<2	>50	<2

Table 5

Comparison of different methods used for functional analysis of HIV-1 *env* quasispecies populations from one infected individual

	Bulk PCR	SGA^a	pPCR
PCR reaction	one	multiple	multiple
Ligation	single	multiple	no
Transformation	single	multiple	no
Plasmid DNA preparation	multiple	multiple	no
Recombination	yes	no	no
Resampling	yes	no	no
Time needed for transfection	days	weeks	hours

^a single genome amplification