

# Amplification of Complete HIV-1 Envelope Genes from Purified CD4+ T Cell Populations

Yanmei Jiao, Juan Li, Wei Hua, Yanqing Gao, Tong Zhang, Hao Wu

## Abstract

Polymerase chain reaction (PCR) amplification of full-length HIV-1 envelope genes directly from uncultured clinical samples or from plasma of HIV patients using RT-PCR or from peripheral blood mononuclear cells (PBMC) DNA extracts of HIV patients is difficult. As HIV mainly infected CD4 positive cells, this paper first describes a sensitive method for the amplification of full-length HIV-1 envelope genes from purified CD4+ cells, and compares this method with other, less effective, procedures. This method were suitable for amplification of HIV-1 envelope genes directly from clinical samples especially from those of low viral load.

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**Key Words:** PCR; HIV-1; Envelope; gp160

## 1. Introduction

Cloning and subsequent expression of the full-length human immunodeficiency virus type 1 (HIV-1) envelope genes derived from uncultured material has become a prerequisite for the determination of their genotypic and phenotypic properties.<sup>1-3</sup> PCR methods for the amplification of full-length HIV-1 envelope genes from primary and continuous cell cultures have been previously described.<sup>1,2,4-7</sup> The amplification of full-length envelope genes from plasma of HIV patients using RT-PCR<sup>8-11</sup> or from peripheral blood mononuclear cells (PBMC) DNA extracts of HIV patients requires the use of nested primer polymerase chain reactions (PCR).<sup>12-16</sup> These methods have several disadvantages. Firstly, they require nested PCR, thus increasing the probability of introducing mutations due to the inherently

error-prone nature of polymerases. Secondly, full-length HIV-1 envelope genes are difficult to obtain from patient samples, even if nested PCR is used. Thirdly, the amplification full-length HIV-1 envelope genes from plasma samples by RT-PCR requires the isolation of RNA, thereby increasing the difficulty of the operation.

As the primary cell tropism of HIV-1 is CD4-positive T cells,<sup>17-21</sup> the probability of amplifying HIV-1 sequences from DNA isolated from purified CD4-positive cell is greatly increased. In this study, we successfully amplified complete HIV-1 envelope genes from DNA extracted from purified CD4+ T cells. This method proved to be more sensitive than amplification from both PBMC DNA and plasma RNA extracts.

## 2. Results and Discussion

### 2.1. Results

#### *Purification of CD4*

The purity of CD4+ cells was greater than 95%, as analyzed by flow cytometric analysis (Figure 1).

#### *Comparison of methods for the PCR amplification of full-length HIV-1 env genes*

The efficiency of amplifying full-length envelope genes using DNA samples from purified CD4+ cells, PBMC and RNA from plasma was assessed. DNA extracted from purified CD4+ cells from all three HIV-1-infected individuals were able to be PCR amplified using only the outer set of primers in one round of PCR (Figure 2). DNA extracted from the PBMC of three HIV-1-infected individuals were amplified by nested PCR (Figure 3). RNA extracted from the plasma of three HIV-1-infected individuals was amplified by the combination of RT-PCR and nested PCR (Figure 4).

### 2.2 Discussion

Cloning and subsequent expression of human immunodeficiency virus type 1 full-length envelope genes is often required for the determination of their genotypic and phenotypic properties.<sup>1-3</sup> Successful amplification of full-length HIV-1 envelope genes has been previously described using nested PCR from the DNA of patient PBMC<sup>12-16</sup> and by RT-PCR from plasma viral RNA.<sup>8-11</sup> Both of these methods needed nested PCR, which not only increased the probability of mutation, but also amplified full-length *env* hardly. As HIV mutates rapidly *in vivo*, it is important to identify the true *in vivo* envelope sequence to study HIV membrane properties.

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The current study compared the amplification of complete HIV-1 envelope genes from DNA of purified CD4<sup>+</sup> cells with those amplified from plasma using RT-PCR or from peripheral blood mononuclear cells DNA extracts using nested PCR. The current study shows that amplification of full-length envelope genes directly from DNA isolated from purified CD4<sup>+</sup> cells is more sensitive than from either PBMC or RT-PCR amplification from plasma, which required nested PCR. There are several reasons to explain why amplification of complete envelope genes from DNA of purified CD4<sup>+</sup> cells was more efficient than other methods tested. As HIV primarily infects CD4<sup>+</sup> T cells,<sup>17-22</sup> HIV DNA is likely to be present at high copy numbers in CD4<sup>+</sup> positive cells and, therefore, relatively easier to amplify. Moreover, amplified complete HIV-1 envelope genes from DNA samples of purified CD4<sup>+</sup> cells mean reducing the interference of unrelated sequences. Amplification of full-length *env* from the plasma required nested PCR for products to be visible. It is possible that contaminants in the RNA extraction procedure degraded the RNA template, decreasing the amount of HIV-specific sequence available for PCR. Likewise, the inefficient reverse transcription process is likely to contribute to the inefficiency of amplification of HIV sequences. In this study, we did not amplify the full-length HIV-1 envelope genes using nested PCR from the DNA of patient PBMC<sup>12-16</sup> and by RT-PCR from plasma viral RNA successfully. Maybe the PCR conditions should be optimized. However, amplification of full-length HIV-1 envelope genes from DNA extracted from purified CD4<sup>+</sup> cells as our experiment described was more efficient.

In conclusion, this paper described a method for the PCR amplification of full-length HIV-1 envelope genes from DNA extracted from purified CD4<sup>+</sup> cells. This method made amplify complete envelope genes easier compared with amplification it from PBMC and RT-PCR amplification it from plasma. And the method has not been through nested PCR, which reduces the possibility of mutation. This method is meaningful to analysis of clinical cases.

### 3. Experimental Section

#### 3.1. Study subjects

Three HIV-1-infected individuals were enrolled in our study (Table 1). The study protocol was approved by the Ethics Committee of our unit and written informed consent was obtained from each subject.

To determine if full-length *env* PCR products were successfully amplified and ligated to the pMD18-T cloning vector, we extracted plasmid DNA and used HindIII digestion (Figure 5) to verify predicted molecular sizes. The total size of the cloning vector (2700 bp) with full-length *env* insert (3084 bp) was 5784 bp. After restriction digestion of the plasmid DNA containing a single HindIII site, bands were identified on agarose gels that corresponded to the predicted molecular weight of cloned full-length *env*. To verify that cloned sequences were indeed of HIV-1 origin, plasmids were sequenced (Figure 6). Comparison of the sequences obtained by PCR to those in databanks confirmed

that they are of HIV-1 origin and can be classified as B/C subtypes.

#### 3.2. Purification of CD4<sup>+</sup> cells

PBMC were isolated from 20 mL of heparinized blood samples by Ficoll-Hypaque density gradient centrifugation. Approximately half of the isolated PBMC were used to obtain CD4<sup>+</sup> positive cells by positive selection using a CD4<sup>+</sup> cell isolation kit (Miltenyi Biotech, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. To determine the purity of CD4<sup>+</sup> positive cells in the preparation, surface staining for CD4 was performed using the conjugated monoclonal antibody, anti-CD4-APC (BD, San Jose, CA) and analyzed by flow cytometric analysis.

#### 3.3. Nucleic acid isolation

DNA was extracted from PBMC and purified CD4<sup>+</sup> cells using the Qiagen DNeasy kit according to the manufacturer's instructions (Qiagen, Germany). RNA was extracted from 200 µL of plasma using the Roche High Pure Viral RNA Kit (Roche Pharmaceuticals, Basel, Switzerland).

#### 3.4. PCR amplification

DNA extracted from PBMC and purified CD4<sup>+</sup>T cells were used as templates for the first round of PCR using outer primers previously described.<sup>6,7</sup> The primer names, sequences and corresponding nucleotide positions are: *env* O (outer sense primer 5'-TAGAGCCCTGGAAGCATCCAGGAAG-3', nt 5852-5876); *env* N (outer anti-sense primer 5'-TTGCTACTTGTGATTGCTCCATGT-3', nt 8912-8935). Sample DNA was used as the template in a total volume of 25 µL for the first round PCR containing 100 mM dNTPs, 0.6 U polymerase and 5 pmol each of primers *env* O and *env* N in 1xPCR reaction buffer. PCR was carried out using 30 cycles of 94 °C for 60 s, 55 °C for 60 s and 72 °C for 3 min with a final extension step of 72 °C for 10 min, after which the tubes were held at 4 °C. For DNA purified from CD4<sup>+</sup> cells, nested PCR was unnecessary as the first round PCR yielded a visible band; however, nested PCR was required for DNA purified from PBMC. Nested PCR was performed using 2 µL of the first round PCR product as the template in second round reactions described above and using primer *env* I (inner sense primer 5'-GATCAAGCTTTAGGCATCTCCTATGGCAGGAAGAA G-3', nt 5957-5982) with *env* M (inner anti-sense primer 5'-AGCTGGATCCGTCTCGAGATA CTGCTCCCACCC-3', nt 8881-8903). Amplimers were visualized by agarose gel electrophoresis of 5 µL of the reaction products in 1% (w/v) agarose gels followed by ethidium bromide staining, as previously described.<sup>22</sup> Full-length HIV-1 *env* was amplified directly from the viral RNA by limiting dilution nested RT-PCR. The reverse transcription and first round RT-PCR were performed using outer primers (*env* O and *env* N) in the Superscript one step RT-PCR for long templates kit (Invitrogen Life Technologies, Carlsbad, CA). Conditions for the one-step reverse transcription and first round PCR were as follows: 45 °C for 30 min, 94 °C for 2 min, 40 cycles of 94 °C for 15 s, 52 °C for 30 s, 68 °C for 3 min, and a final extension step at 72 °C for 10 min. The nested PCR was

performed using inner primers (env I and env M) under the following conditions: 94 °C for 2 min, 40 cycles of 94 °C for 15 s, 55 °C for 30 s, 68 °C for 3 min, and a final extension at 72 °C for 10 min.

### 3.5. PCR product identification

The PCR product was purified using the Qiagen DNeasy kit (Germany) and ligated to the pMD18-T vector (TaKaRa, Japan) according to the manufacturer's instructions. To confirm that we had successfully amplified the full-length *env* and ligated it to the pMD18-T vector, we extracted plasmid DNA from bacterial colonies and performed restriction digestion with HindIII (TaKaRa, Japan) to confirm fragment sizes (pMD18-T vector, 2700 bp and full-length *env*, 3084 bp). The cloned sequences were also analyzed by dye-deoxy chain termination sequencing.

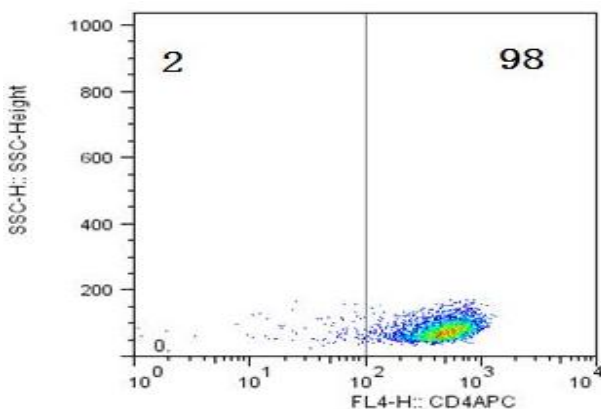
## 4. Conclusion

This paper described a method for the PCR amplification of full-length HIV-1 envelope genes from DNA extracted from purified CD4<sup>+</sup> cells. This method made amplify complete envelope genes easier compared with amplification it from DNA extracted of PBMC and RT-PCR amplification it from plasma. And the method has not been through nested PCR, which reduces the possibility of mutation.

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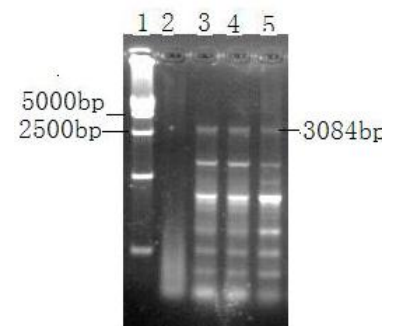
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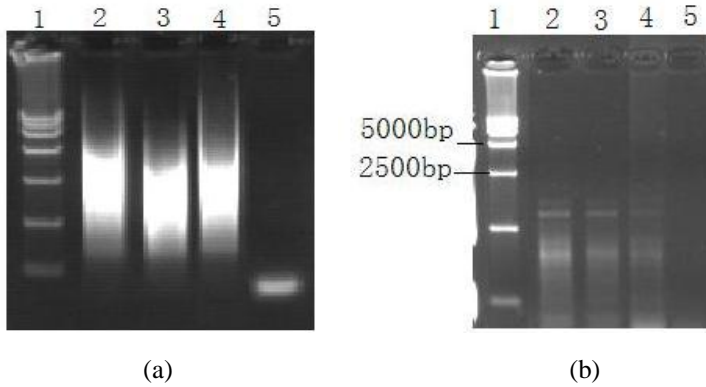


**Figure 1 (left).** The purity of CD4<sup>+</sup> cells was greater than 95% by flow cytometric analysis.

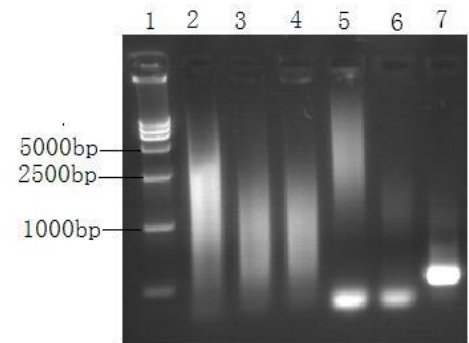
**Figure 2 (right).** The PCR products from DNA extracted from purified CD4<sup>+</sup> cells of three HIV-1-infected individuals. Lane 1, DNA marker; 2, negative control; 3, patient 1; 4, patient 2; 5, patient 3.



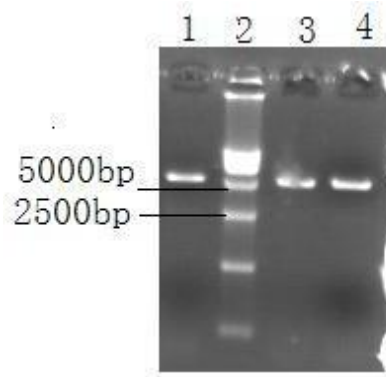
**Figure 3.** The PCR products from DNA samples extracted from PBMC of three HIV-1-infected individuals. (a) First round PCR products. Lane 1, DNA marker; 2, first round PCR product for patient 1; 3, first round of patient 2; 4, first round of patient 3; 5, negative control. (b) Second round PCR products. Lane 1, DNA marker; 2, second round of patient 1; 3, second round of patient 2; 4, second round of patient 3; 5, negative control.



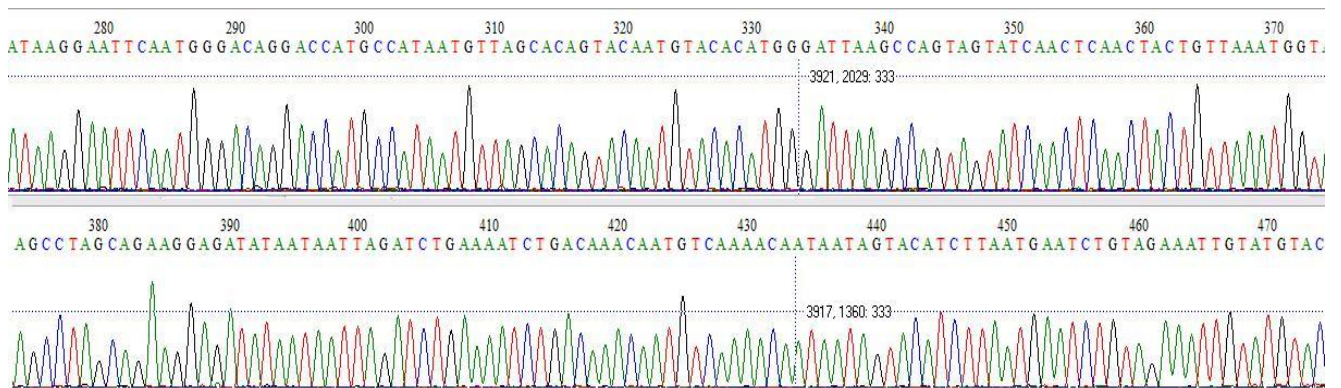
**Figure 4.** RT-PCR and nested PCR amplification products of RNA samples extracted from plasma from three HIV-1-infected individuals. Lane 1, DNA marker; 2, first round of patient 1; 3, first round of patient 2; 4, first round of patient 3; 5, second round of patient 1; 6, second round of patient 2; 7, second round of patient 3.



**Figure 5.** (right) Agarose gel electrophoresis of the HindIII restriction digestion products. Lanes 1, 3, and 4 contain the plasmid DNA enzyme digestion products. Lane 2 DNA marker.



**Figure 6.** Sequences obtained from plasmid DNA containing full-length HIV-1 env PCR products.



**Table 1.** Characteristics of subjects in this study.

Patient	Gender	Virus load (Copies/mL)	CD4 counts (Cells/ $\mu$ L)
1	Male	96000	89
2	Female	32700	271
3	Male	8234	540