Amplification of the *Gp41* gene for detection of mutations conferring resistance to HIV-1 fusion inhibitors on genotypic assay

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Abstract. Fusion inhibitors have potential for future use in HIV control programs in Indonesia, so the capacity to test resistance to such drugs needs to be developed. Resistance-detection with a genotypic assay began with amplification of the target gene, *gp41*. Based on the sequence of the two most common HIV subtypes in Indonesia, AE and B, a primer pair was designed. Plasma samples containing both subtypes were extracted to obtain HIV RNA. Using PCR, the primer pair was used to produce the amplification product, the identity of which was checked based on length under electrophoresis. Eleven plasma samples were included in this study. One-step PCR using the primer pair was able to amplify *gp41* from 54.5% of the samples, and an unspecific amplification product was seen in 1.1% of the samples. Amplification failed in 36.4% of the samples, which may be due to an inappropriate primer sequence. It was also found that the optimal annealing temperature for producing the single expected band was 57.2 °C. With one-step PCR, the designed primer pair amplified the HIV-1 *gp41* gene from subtypes AE and B. However, further research should be done to determine the conditions that will increase the sensitivity and specificity of the amplification process.

1. Introduction

Human Immunodeficiency Virus and Acquired Immunodeficiency Syndrome (HIV/AIDS) transmission has become a growing health problem in Indonesia. In 2013, the estimated number of HIV patients was 640,000, with prevalence rate of 0.5 [1]. Currently, most HIV infections are transmitted through heterosexual activity, usage of non-sterile injection needles by drug abusers, or passage from an HIV-positive mother to her fetus. HIV transmission has spread to 69.4% of regencies/cities throughout Indonesia, with the DKI Jakarta province reporting the highest number of infections [2]. A study conducted in Indonesia by Merati *et al.* [3] showed that among 108 patients, the most common HIV subtypes found in the population were AE (89.9%) and B (9.3%).

The Indonesian government has established guidelines for treating HIV-infected patients. Antiretroviral therapy (ART) should be started on patients with HIV/AIDS with CD4 concentrations of less than 350/mm3, those with clinical stage 3 or 4 AIDS regardless of CD4 levels, infected pregnant women, and patients with tuberculosis or hepatitis B coinfections. Currently, the ART regimen in Indonesia consists of nucleotide reverse transcriptase inhibitors (NRTIs), such as zidovudine, stavudine, and emtricitabine; non-nucleotide reverse transcriptase inhibitors (NNRTIs), such as efavirenz and nevirapine; and protease inhibitors (PIs), such as lopinavir and ritonavir. Prior to drug administration, resistance testing should be done with genotypic assays [4].

The National Working Group on HIV Drug Resistance (HIVDR) reports that inadequate supervision by health care providers, as well as low compliance and understanding on the part of patients receiving ART, has led to ineffective treatment in Indonesia [5]. This statement is supported by a study performed in Surabaya by Kotaki *et al.* [6], which showed that while drug resistance to reverse transcriptase inhibitors (RTIs) was only seen in 4.3% of patients who had not received treatment, it was observed in 37.7% of patients who had been receiving ART for two years [6,7]. Moreover, the high percentage of patients with drug-resistance mutations may lead to increased primary transmission of antiretroviral (ARV)-resistant HIV strains, which in turn will reduce the success of HIV control programs [8].

One strategy for overcoming the emergence of ARV-resistant mutants is the introduction of new classes of ARV drugs [9]. Examples of ARV drugs that have not yet been used in Indonesia are fusion inhibitors and integrase inhibitors [4], and these may be introduced into ARV regimens for national HIV-control programs. Australia and several European countries have approved fusion inhibitors for use in ART [10]. According to research in the Americas, the fusion inhibitor enfuvirtide was proven to provide both ARV and immunologic benefits in patients who had developed multidrug-resistant HIV-1 infections [11]. Considering the high probability that fusion inhibitors will be used in HIV treatments in Indonesia, it will be wise to develop the capacity to test resistance towards them. The Research Center for Virology and Cancer Pathobiology, Faculty of Medicine Universitas Indonesia, will do Sanger sequencing on the fusion domain of the envelope gene to test for fusion inhibitor resistance of HIV strains in Indonesia. In preparation for that, we amplified the *gp41* fusion gene as the first step toward a genotypic assay to detect mutations that confer resistance to fusion inhibitors.

2. Materials and Methods

The present research used a descriptive experimental design and was carried out over three months, from April through June 2015. The experiment was done at the Virology and Cancer Pathobiology Research Center for Health Services (VCPRC), Cipto Mangunkusumo Hospital. The type of data used was primary data acquired from prior experiments using plasma samples obtained from HIV-infected individuals. The data were analyzed to determine whether the designed primer was able to amplify the HIV-1 *gp41* gene.

The first step was to design a primer based on the sequence of the target region containing mutation locations that can confer resistance. HIV RNA was then extracted from the samples and converted into cDNA containing the target of amplification with two-step and one-step PCR, using the previously designed primer pairs. To check whether the amplification yielded the expected results, gel electrophoresis was done to determine the length of the amplification product. The primer performance was said to be good if there was only one bad with the expected length found in a sample. Optimization steps were then taken if the result was not as expected.

3. Results and Discussion

3.1 Results

A forward primer was designed with Primer Designer software targeting regions more than 100 bp before the first *gp41* mutation that may confer resistance. *Gp41* starts at base 7758, and according to the Stanford database, the first mutation that may confer resistance to fusion inhibitors is located at base 7866 [12]. The templates used for this primer design were the B and AE subtypes, and the primer had to fulfil the criteria listed in the methods [13]. The resulting primer was named 7579F. The reverse primer was BF_EnvR3, an existing primer in the VCPRC laboratory.

Table 1. Primer pair design

Primer	Sequence	Length	Tm
7579F	5'-TRC TAT TRA CAA GAG ATG GTG G-3'	22 bp	52 °C
BF_EnvR3	5'- YAR RTC TYG AGA TRC TGC TCC-3'	21 bp	61.6 °C

Initially, RevertAid enzymes were used to synthesize cDNA from DRHS 62, an existing RNA sample that is known to be positive. Primer BF_EnvR3 was used for the cDNA synthesis. The first-round PCR using the designed primer was then performed.
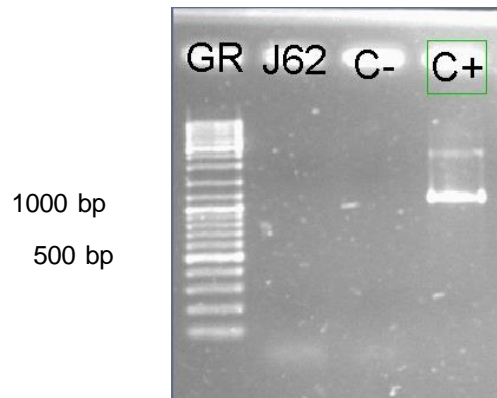

Figure 1. Two-step PCR of DRHS 62, using Platinum Taq HiFi enzymes, the template was made with RevertAid enzymes. J62: primers were 7579F and BF_EnvR3, template of DRHS 62 cDNA; C−: control negative using H_2O as template; C+: control positive using existing cDNA as template and AE_Pol4086F and AE_Pol5232R as primers. Expected band length is 1328 bp and is not seen.

Using a similar method, a different primer named AE_Env7633F was produced.

Table 2. Alternate forward primer

Primer	Sequence	Length	Tm
AE_Env7633F	5'-GGA GGA RAT ATR ARG GAC AAT TGG AGA AGT G-3'	31 bp	59.1 °C

Both designed primers were then tested with first-round PCR using the pNL4-3 full genome plasmid as a template.
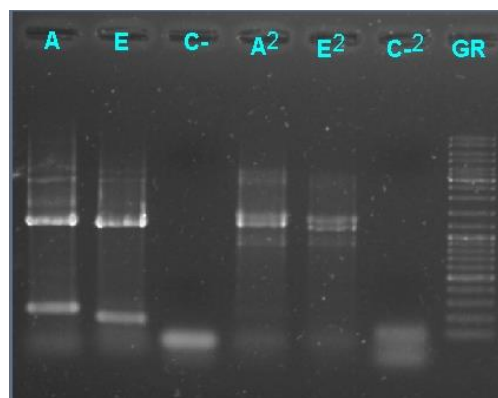

Figure 2. First- and Second-round PCR of *pNL4*-3. Platinum Taq HiFi enzymes were used. A: primers 7579F and BF_EnvR3 were used, first round, with expected band at 1328 bp; E: primers AE_Env7633F and BF_EnvR3 were used, first round, with expected band at 1274 bp; C−: control negative with H_2O as template; A^2: second round of A; E^2: second round of E; C^{-2}: second round of C−.

RNA was extracted from frozen plasma using the QIAmp Viral RNA Mini Kit, and cDNA was synthesized with RevertAid enzymes using BF_EnvR3 as the primer. First-round PCR was then done.
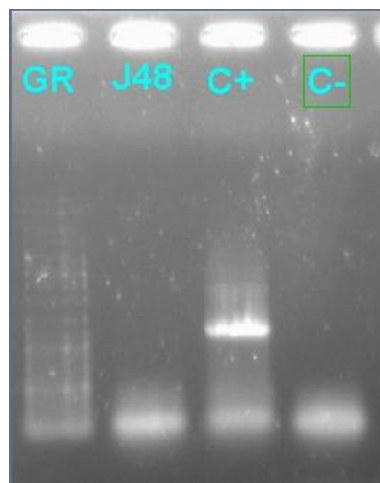

Figure 3. Two-step PCR of DRHS 48. DreamTaq enzyme was used. cDNA template was made from extracted RNA using RevertAid enzyme. J48: using primers AE_Env7633F and BF_EnvR3, with expected band at 1274 bp; C+: using same primer with J48, pNL-4-3 as template; C−: using control negative from DRHS 48 extraction as template.

Next, the RevertAid kit was checked using the RNA and primers provided in the kit.
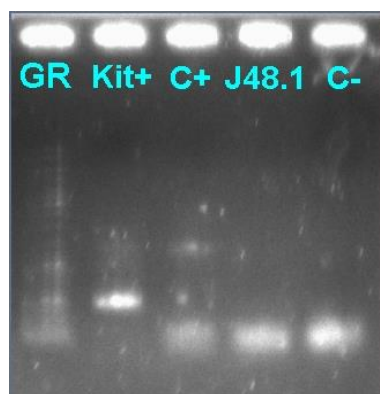

Figure 4. Repeating Two-step PCR of DRHS 48. Repeating the cDNA synthesis along with control positive from the RevertAid kit and first-round PCR with DreamTaq enzymes. Kit+: using GAPDH RNA (from the kit) as template and primers provided, with expected band at 496 bp; C+: control positive of primers AE_Env7633F and BF_EnvR3 using pNL-4-3 as template, with expected band at 1274 bp; J48.1: using AE_Env7633F and BF_EnvR3 as primers, newly synthesized cDNA from same RNA as Figure 6 as template; C−: using control negative from DRHS 48 extraction as template.

New samples, DRHS 09 and DRHS 32, were next extracted for the RNA. After that, the new method of one-step PCR using SuperScript III enzymes was applied.
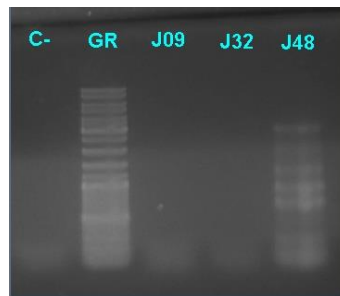
Figure 5. One-step PCR of New Samples. SuperScriptIII enzyme was used to amplify newly extracted samples using AE_Env7633F and BF_EnvR3 as primers, with expected band at 1274 bp. J09: DRHS 09 as template; J32: DRHS 32 as template; J48: DRHS 48 as template; C-: using control negative from extraction process as template.

To check whether the extraction was successful, the existing primer pairs for the *p24* region were used in one-step PCR.
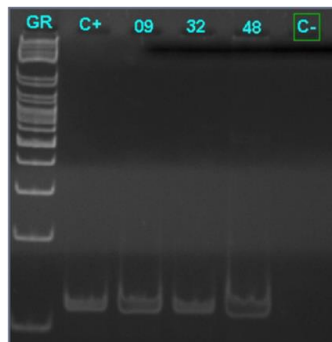

Figure 6. One-Step PCR of *p24* Gene. SuperScriptIII enzyme was used with primer pair HI857F and HI967C targeting the p24 region, with expected band at 110 bp. C+: existing RNA as control positive; 09: DRHS 09 RNA as template; 32: DRHS 32 RNA as template; 48: DRHS 48 RNA as template; C−: control negative from extraction process as template.

To find the optimal conditions for PCR using the above primers, the thermal cycler with gradient feature was used to test various annealing temperatures.
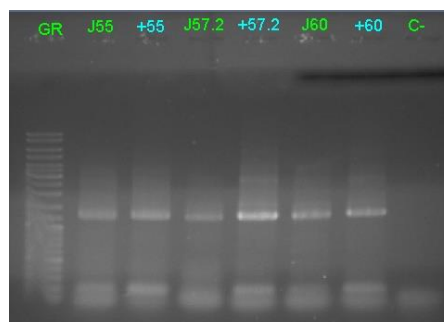

Figure 7. One-step PCR With Varying Annealing Temperatures. SuperScriptIII enzyme was used with primer pair AE_Env7633F and BF_EnvR3, expected band at 1274 bp. Three temperatures for annealing were used: 55 °C, 57.2 °C, and 60 °C. J: using DRHS 09 RNA as template; +: using existing RNA as template; C−: using control negative from extraction process as template.

RNA was extracted from the frozen plasma samples DRSS 20, DRSS 22, DRSS 24, DRSS 45, DRSS 57, DRHS 07, DRSS 64, and DRSS 66.
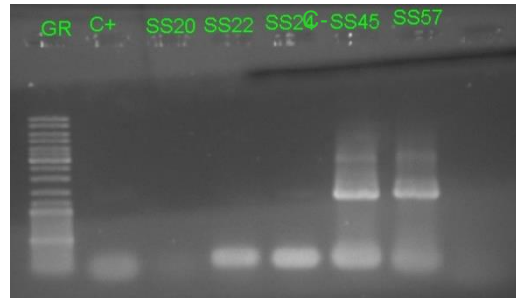

Figure 8. One-step PCR of Five New Samples. SuperScriptIII enzyme was used with primer pair AE_Env7633F and BF_EnvR3, expected band at 1274 bp. C+: using existing RNA as template; SS20: DRSS 20 as template; SS22: DRSS 22 as template; SS24: DRSS 24 as template; SS45: DRSS 45 as template; SS57: DRSS 57 as template; C−: using control negative from extraction process as template.
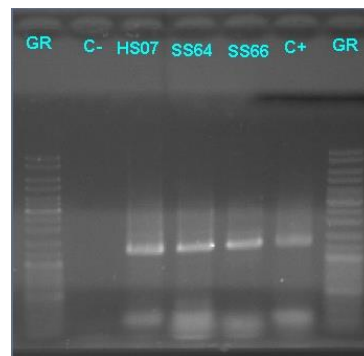

Figure 9. One-step PCR of Three New Samples. SuperScriptIII enzyme was used with primer pair AE_Env7633F and BF_EnvR3, expected band at 1274 bp. C+: using DRHS 09 RNA as template; HS07: DRHS 07 as template; SS64: DRSS 64 as template; SS66: DRSS 66 as template; C−: using control negative from extraction process as template.

For samples with negative PCR results, one-step PCR targeting the *p24* region was done.

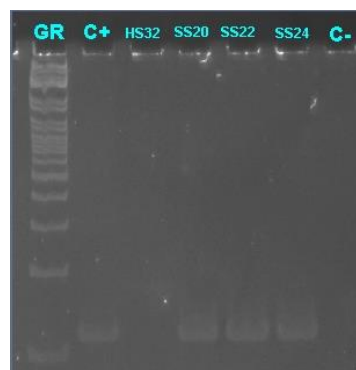
Figure 10. One-step PCR to Check for the Presence of RNA. SuperScriptIII enzyme was used with primer pair HI857F and HI967C, expected band at 110 bp. C+: using DRHS 09 RNA as template; HS32: using DRHS 32 as template; SS20: using DRSS 20 RNA as template; SS22: using DRSS 22 RNA as template; SS24: using DRSS 24 RNA as template; C−: using control negative from extraction process as template.

Table 3. Summary of research results

Sample	Viral Load (copies/ml)	Subtype	Resulting band	RNA
DRHS 07	23,999	B	Expected	
DRHS 09	17,296	CRF01_AE	Expected	
DRHS 32	515,966	CRF01_AE	None	V
DRHS 48	1,369,136	CRF01_AE	Multiband	V
DRHS 62 (RNA)	10,000,000	CRF01_AE	None	Not checked
DRSS 20	560,643	CRF01_AE	None	V
DRSS 22	477,907	CRF01_AE	None	V
DRSS 24	1,628,913	CRF01_AE	None	V
DRSS 45	7,813,116	CRF01_AE	Expected	
DRSS 57	1,891,104	CRF01_AE	Expected	
DRSS 64	508,164	CRF01_AE	Expected	
DRSS 66	133,526	CRF01_AE	Expected	

3.2 Discussion

Initially, this research used two-step PCR so that the researchers could store the samples in the form of cDNA, which is more stable than RNA, and to make it easier to optimize the PCR conditions [14]. As seen in Figure 4, the primers and PCR conditions failed to yield the expected bands using a template known to be positive (DRHS 62 RNA). The probable causes for this were degraded RNA, primers that did not work well on PCR, failed cDNA synthesis, or incompatible reagent. New primers were therefore designed using cDNA synthesis with BF_EnvR3, and the same reverse primer was used for amplification. To check whether the primers were successful, a full genome plasmid of subtype B, pNL-4-3, was used as the template for PCR to exclude the probability of degraded RNA or failed cDNA synthesis [15]. As shown in Figure 5, the expected bands were observed for both the old set of primers and the new set, indicating that the primers should work well if they are compatible with the sample sequence.

A new plasma sample, DRHS 48, was then extracted to ensure that the RNA was not degraded. However, as seen in Figure 6, the expected band was still not observed. To test the reagent, the process was repeated, this time using the control positive provided within the kit. Figure 7 shows that the expected band for the control positive were observed, meaning that the reagents worked well. Therefore, the causal possibilities were incompatibility between reagents and HIV amplification, incompatibility between primer and sample sequence, and absence of extracted RNA.

For the next step of the research, the method was changed from two-step to one-step PCR using SuperScript III enzymes. New samples were extracted and one-step PCR was done; however, as seen in Figure 8, the expected band was still not observed for samples DRHS 09 and DRHS 32, while there were unspecific bands for DRHS 48. The absence of the expected band could mean either absence of RNA, suboptimal PCR conditions, or unsuitable primers for the sample. One-step PCR using an established set of primers targeting the p24 region was done to check for the presence of RNA. Figure 9 shows the presence of the expected band, indicating that the extraction was successful. To determine optimal conditions for the PCR process, a thermal cycler with gradient feature was used to vary the annealing temperature between 55 °C, 57.2 °C, and 60 °C. This time, the expected bands were observed in all conditions, with the most clear and specific band at the annealing temperature of 57.2 °C (Figure 10). Since the subtype of the DRHS 09 sample was CRF01_AE, the primer set was suitable to amplify subtype AE. The previous one-step PCR used the same method and sample, so the negative result may have been due to different enzyme lots or technical mistakes by the researchers. From this point on, 57.2 °C was the annealing temperature used for all PCR processes involving the primer pair of AE_Env7633F and BF_EnvR3.

The results showed that the primer was compatible with the extracted samples from the patients using a one-step process, and it is possible that two-step PCR is unsuitable for amplifying HIV genes. This could be due to the basic differences between one-step and two-step PCR. In one-step PCR, less pipetting and handling is involved, which reduces pipetting errors and contamination. The secondary structure is also less affected by the one-step method. One-step PCR allows more efficient priming and performs better than two-step PCR with lower RNA copy numbers. Moreover, when using multiple samples targeting only one region, one-step PCR is more efficient than the two-step method [13].

As optimal PCR conditions were finally determined, more samples were extracted for RNA and amplified using the same method. Only one sample with subtype-B HIV was used due to limited availability. The results are shown in Figure 11 and Figure 12. Among all of the samples, three did not yield the expected band: DRSS 20, DRSS 22, DRSS 24 (Figure 11). This means the primer set also works for HIV-1 subtype B. The negative results may be due to an unsuitable primer for the sample, absence of RNA in the sample, or degraded RNA. To test for the presence of RNA, one-step PCR targeting the p24 region was done. Figure 13 shows the expected band, therefore RNA was present. In conclusion, the primers used were not suitable for these three samples. DRHS 32 showed no band with this round of PCR, while on previous attempts; the expected band was observed (Figure 9). This indicates that on this attempt, the RNA might have been degraded or there were technical errors by the researchers.

In conclusion, the primer pair of AE_Env7633F and BF_EnvR3 was able to produce the expected amplification results, which included the *GP41* protein. Among 11 extracted samples, the primer pair with one-step PCR was able to amplify the *gp41* gene in 54.5% of the samples (n=6), one of which was the B subtype. In 1.1% of the samples, PCR showed multiband profiles, and in 36.4% of the samples, PCR failed to deliver amplification products. The negative results may have been caused by incompatibility with the nucleotide sequence of the sample. The multiband results from DRHS 48 could not be investigated further because the sample was finished due to previous amplifications. These results do not correlate with the viral load of the sample. There were several limitations to this research, including time constraints as well as budget and resource limitations. As a result, only 11 samples were used, and optimization steps were not done on every sample. The results of this experiment have low reproducibility since repetition could not be performed, and the findings may not be a good representation of the primer performance due to the small sample size.

4. Conclusion
The primer pair of AE_Env7633F and BF_EnvR3 can be used for amplification of the gp41 region containing mutations that confer resistance to fusion-inhibitor drugs. One-step PCR and an annealing temperature of 57.2 °C are optimal for amplification of the gp41 gene using this primer pair. Under optimal conditions, this pair shows good performance in producing amplification results from both the subtype B and AE templates

References
[1] UNAIDS 2013 *Epidemilogical Fact Sheet on HIV/AIDS*. Available from http://www.unaids.org/sites/default/files/epidocuments/IDN.pdf.
[2] Direktorat Jenderal Pengendalian Penyakit dan Penyehatan Lingkungan 2013 *Laporan Situasi Perkembangan HIV&AIDS Di Indonesia Tahun 2013*. (Jakarta: Kementrian Kesehatan RI).
[3] Merati T P, Ryan C E, Spelmen T, Wirawan D N, Bakta I M, Otto B, *et al.* 2012 CRF01_AE dominates the HIV-1 epidemic in Indonesia. *Sexual. Health*. **9** 414- 21. https://doi.org/10.1071/SH11121.
[4] Direktorat Jenderal Pengendalian Penyakit dan Penyehatan Lingkungan 2011 *Pedoman Nasional Tatalaksana Klinis Infeksi HIV dan Terapi Antiretroviral pada Orang Dewasa*. (Jakarta: Kementrian Kesehatan RI).

[5] National Working Group on HIVDR n.d. *Monitoring HIV Drug Resistance in Indonesia*. Available from http://ino.searo.who.int/LinkFiles/Other_Content_WHD11-Seminar_Presentation-2-dr_Miko.pdf.

[6] Kotaki T, Khairunisa S Q, Witaningrum A M, Muhammad M Q Y, Sukartiningrum S D, Diansyah M N, *et al.* 2015 HIV-1 transmitted drug resistance mutations among antiretroviral therapy-Naïve individuals in Surabaya, Indonesia. *AIDS Res. Ther.* **12** 1-7 https://doi.org/10.1186/s12981-015-0046-y.

[7] Khairunisa S Q, Kotaki T, Witaningrum A M, Yunifiar M, M Q, Sukartiningrum S D, Nasronudin and Kameoka M 2015 Appearance of drug resistance-associated mutations in human immunodeficiency virus type 1 protease and reverse transcriptase derived from drug-treated Indonesian patients. *AIDS Res. Human. Retroviruses*. **31** 255–59. https://doi.org/10.1089/aid.2014.0221.

[8] Vercauteren J, Wensing A M J, van de Vijver D A M C, Albert J, Balotta C, Hamouda O, *et al.* 2009 Transmission of drug-resistant HIV-1 is stabilizing in Europe. The *J. Infect. Dis*. **200** 1503–8. https://doi.org/10.1086/644505.

[9] Matthews T, Salgo M, Greenberg M, Chung J, DeMasi R and Bolognesi D 2004 Enfuvirtide: the first therapy to inhibit the entry of HIV-1 into host CD4 lymphocytes. *Nature. Rev. Drug. Discovery*. **3** 215–25. https://doi.org/10.1038/nrd1331.

[10] Greenberg M L 2004 Resistance to enfuvirtide, the first HIV fusion inhibitor. *J. Antimicrob. Chemother.* **54** 333-40. https://doi.org/10.1093/jac/dkh330.

[11] Lalezari J P, Henry K, O'Hearn M, Montaner J S G, Piliero P J, Trottier B, *et al.* 2003 Enfuvirtide, an HIV-1 fusion inhibitor, for drug-resistant HIV infection in north and south America. *N. Engl. J. Med*. **348** 2175–85. https://doi.org/10.1056/NEJMoa035026.

[12] Stanford University 2008 *Fusion Inhibitor Resistance Mutations*. Available from: http://hivdb.stanford.edu/DR/FIResiNote.html.

[13] Kamel A and Abd-Elsalam 2003 Bioinformatic tools and guideline for PCR primer design. *African. J. Biotechnol*. **2** 91–5.

[14] Wacker M J and Godard M P 2005 Analysis of one-step and two-step real-time RT-PCR using superscript III. *J. Biomol. Tech*. **16** 266–71.

[15] Lu W, Salzwedel K, Wang D, Chakravarty S, Freed E O, Wild C T and Li F 2011 A single polymorphism in HIV-1 subtype C SP1 is sufficient to confer natural resistance to the maturation inhibitor bevirimat. *Antimicrob. Agents. Chemother*. **55** 3324–29. https://doi.org/10.1128/AAC.01435-10.