

PROTOTYPE DESIGN OF LYMPHOCYTE TCD4+ RESISTANT AGAINST HIV INFECTION GENERATED FROM PERIPHERAL BLOOD HAEMATOPOIETIC STEM CELL (PBMCs) By DELETION OF 32 bp CCR5 ENCODING GENE

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ABSTRACT

AIDS epidemic has spread to all parts of Indonesia and currently more than 150 countries reported the existence of HIV/AIDS from around the world. Additionally, HIV/AIDS treatment using ARV drugs also find obstacles that must be faced in terms of host, environment and the agent. The objective of this study was to generate lymphocytes TCD4+ that are resistant to HIV infection generate from PBMCs through by deletion of 32 bp CCR5 encoding gene. In principle, this study was done in three steps. First, isolation, culture and purification of lymphocyte TCD4+ from PBMC (Mather, 2008; Rantam, et.al., 2009). Second, lymphocyte TCD4+ characterization by PCR with primer F 5'CAAGTCGAGCGCCCCGCAAGGGG-3, R 5'GTCCGAGTGTGGCTGATCATCC-3 (Thomsen, et.al., 2002; Yuwono, 2006; Hall and Ziedonis 2007; Purwati, et.al., 2009). Third, designing of lymphocyte TCD4+ prototype which was resistant to HIV infection by deletion of 32 bp CCR5 full gene. **Results:** Twenty-four hours after culture, there were abundant cell growths. TCD4+ lymphocytes from isolated and cultured 10 ml PBMC were found to be 2 x 10⁷. Phenotype characterization of TCD4+ lymphocyte provided positive results, while the genotype showed similarities to that in corresponding gene bank of CCR5 variant A and variant B. Prototype of HIV resistant TCD4+ lymphocytes was made by nucleotide deletions in conserved areas, at position 554-576 bp, using restriction enzymes EcoRI checked using PCR and sequencing. In conclusion, prototype design of HIV resistant TCD4+ lymphocytes is obtained through the deletion of 32 bp CCR5 encoding full gene at GTCAGTATCAATTCTGGAA GAATTT CCAGACA using EcoRI enzyme.

Keywords: HIV/AIDS, resistant TCD4+ lymphocytes, mutant 32 bp CCR5, PBMCs, deletion

INTRODUCTION

AIDS epidemic has now spread to all parts of Indonesia. At the end of 2000, only 16 provinces have reported cases of AIDS, but in the end of 2003 it spread wider to 25 provinces, then in 2006 there were 32 provinces reporting AIDS cases. According

UNAID, more than 150 countries currently reported the existence of HIV/AIDS from around the world. Existing data indicate there is significant increasing number of AIDS cases. Based on those reports, research on preventing HIV infection is necessary (Depkes,2009; Widiyatna, 2009).

HIV is a spherical ss-RNA, with the outer layer or the envelope consists of glycoprotein gp120 attached to glycoprotein gp4. In the early events of infection, there is interaction between viral gp120 and CD4+ receptor found on the surface of target cells. Interaction of gp120 with CD4 leads to further binding with chemokine receptor that acts as a specific co-receptor CXCR4 or CCR5 or R5 and X4. CCR5 and CXCR4 performance is guided by controlling genes that determine whether it is vulnerable or resistant to HIV infection. Mutations in CCR5 encoding gene can serve as a protector or resistance to HIV infection. Thus, homozygous individuals are likely resistant to infection, whereas the heterozygotes are potentially vulnerable to HIV infection (Essex and Kanki, 1989; Anderson and Akkina, 2007). The aims of this study was to design the prototype of TCD4+ lymphocyte which is resistant against HIV infection generating from peripheral blood haematopoietic stem cell (PBMCs) by deleting of 32 bp CCR5 encoding gene, so we will be able to prevent the entry of HIV into the human body. This can be used as the basis for gene therapy approach, which is one of the promising alternatives to address the spread of HIV infections globally.

METHODS

In principle, this research was done in three steps. First, isolation, culture and purification of lymphocyte TCD4+ from PBMC (Mather, 2008; Rantam, et.al., 2009). Second, lymphocyte TCD4+ characterization

by PCR with primer F 5'CAAGTCGAGCGCCCCGCAAGGGG-3, R 5'GTCCGAGTGTGGCTGATCATCC-3 (Thomsen, et.al., 2002; Yuwono, 2006; Hall and Ziedonis 2007; Purwati, et.al., 2009). Third, designing the prototype of lymphocyte TCD4+ which is resistant to HIV infection by deletion of 32 bp of full gene CCR5.

This type of study was an experimental laboratory (in vitro) study by deleting of 32 bp nucleotides (554-576 bp) full gene of CCR5 in lymphocytes TCD4+ of healthy individuals to become resistant to HIV infection. PBMCs were isolated from whole blood donors by using Ficoll-density gradient centrifugation according Hystopaque manufacturer's protocol. Purification of TCD4+ lymphocytes was performed using anti-CD4 magnetic beads. Then it was cultured in RPMI medium. To determine TCD4+ lymphocyte maturation we used a variety of methods, namely indirect immunofluorescence, flowcytometry, PCR and sequencing. Prototype design of TCD4+ lymphocytes which is resistant to HIV infection is principally conducted through 32 bp deletion of the gene encoding CCR5 nucleotide bases. In this study, we performed a full 32 bp deletion of the CCR5 by cutting conserved by cutting areas or areas that are not prone to mutations, GTCAGTATCAATTCTGGAAGAATTTCAGACA. By performing the deletion it was expected that HIV can not enter the cells due to its targeted mutation in CCR5. The deletion was done by using the restriction enzyme EcoRI (Promega)

RESULTS

Isolation, Culture and Purification of TCD4+Lymphocyte from PBMC

PBMCs were isolated from peripheral blood using Ficoll system in order to obtain buffy coat. The number of mononucleated cells contained in the buffy coat correlates with the amount of isolated peripheral blood.

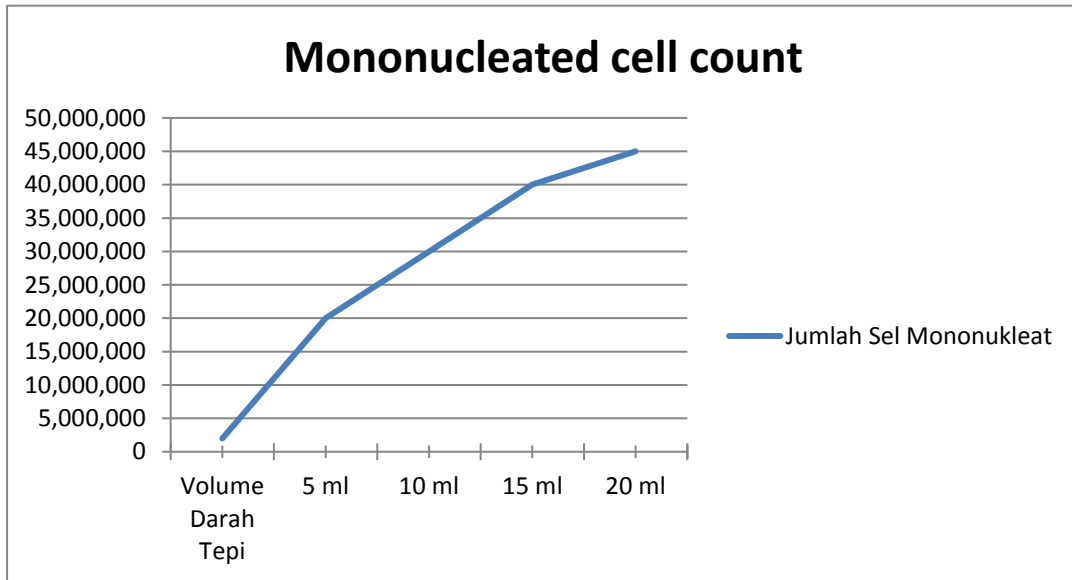


Figure 1. Mononucleated cell count contained in PBMCs

Purification of TCD4+ lymphocytes used magnetic beads containing anti-CD4+. Twenty-four hours after culture there were many cell growth. TCD4+ lymphocytes from

isolated and cultured 10 ml PBMC produced of 2×10^7 TCD4+ lymphocytes. The culture of TCD4+ lymphocyte culture can be seen in Figure 1.

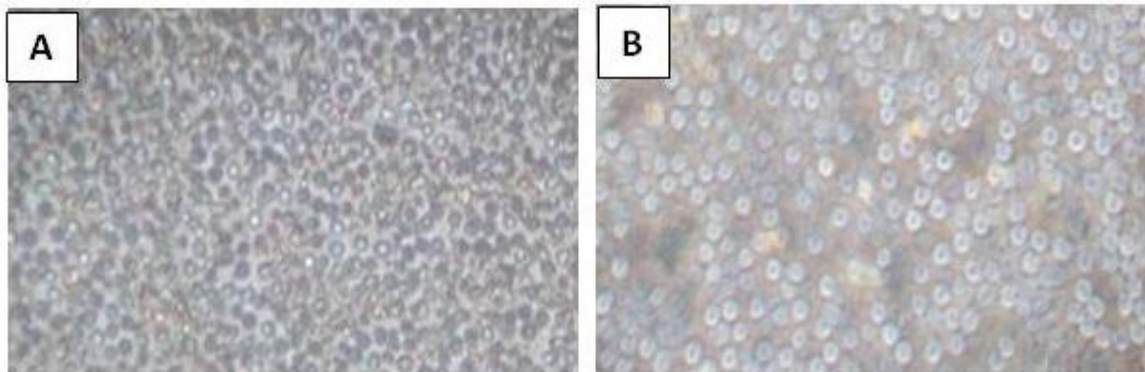


Figure 2. TCD4+ lymphocyte culture. A. 4 days after culture TCD4+ lymphocytes is growing, seen under an inverted microscope in magnification 40x. B. Mature lymphocytes are then identified by CD4+, observed under the inverted microscope 40x.

Characterization of TCD4+ lymphocytes aims to identify whether isolated and purified TCD4+ lymphocytes have expressed CD4+ receptor. Characterization was done phenotypically and genotypically. Phenotypes

were examined using immunofluorescence and flowcytometry. Genotype characterization was examined using PCR and sequencing.

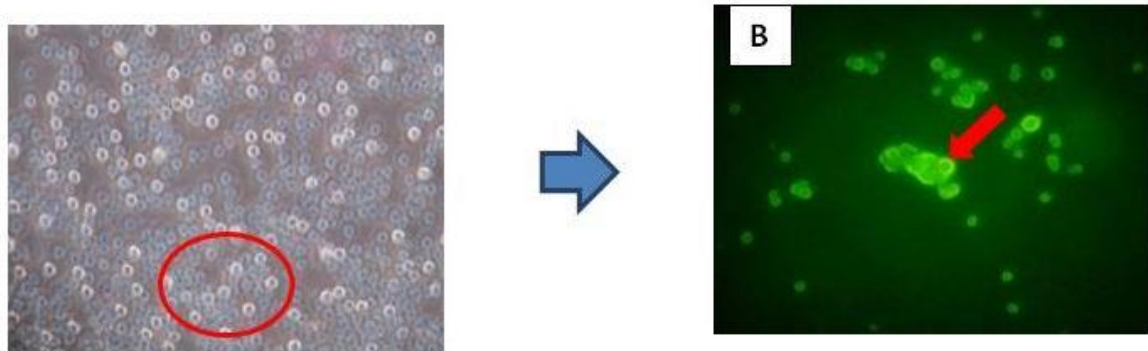


Figure 3. TCD4+ expression analysis. A. Mature TCD4+ lymphocytes ready for characterization. B. Cells showing green fluorescence are expressing CD4+ cells. Observed under a fluorescence microscope, 40x magnification.

Based on the examination using flowcytometry TCD4+ lymphocyte counts compared with the number of haematopoietic

stem cells are characterized by expression of CD45+

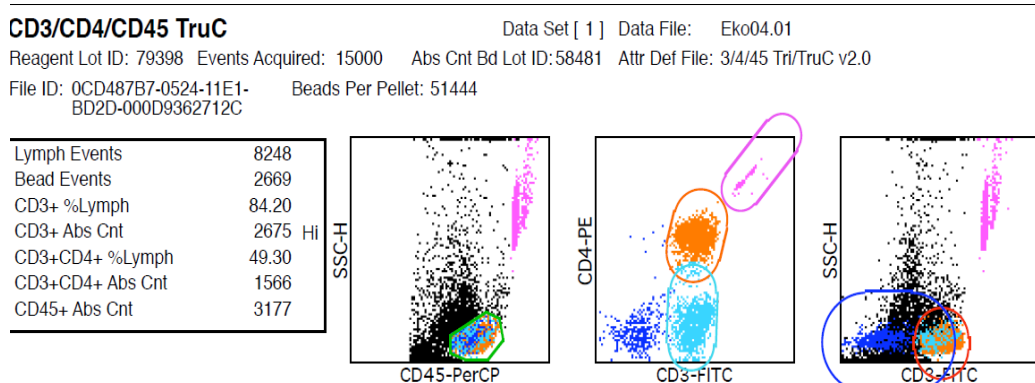


Figure 4. Analysis of the expression of CD4 + based on flowcytometry.

Analysis of TCD4+ lymphocytes by flowcytometry obtained results as shown in the image above that the number of haematopetic stem cell in this case CD45+ was 21,366 cells/0.5 ml of culture

results (top) and 3177/0.1 ml of culture results (below). While the analysis CD45+ is based on SSC-H (Side Scattered) complexity shown by the dotted orange and blue colors. The CD3+ with CD4+ showed that CD3+ showed

complexity in blue and CD4+ in orange colors. Quantitatively there were of 9115 CD4+ cells (top) and 3166 cells (bottom). It shows there were high TCD4+ lymphocyte counts from isolation and culture derived from PBMCs.

Characterization of TCD4+ lymphocytes by PCR

Genotype characterization was carried out by PCR and sequencing. Characterization by one step PCR is to obtain CCR5 encoding gene expression, where CCR5 gene encoding was used as the basis for making TCD4+ lymphocytes prototypes that are resistant to HIV infection by 32 bp deletion of genes encoding nucleotide bases CCR5. We obtained PCR results with the PCR product of 200 bp as shown in the image below :

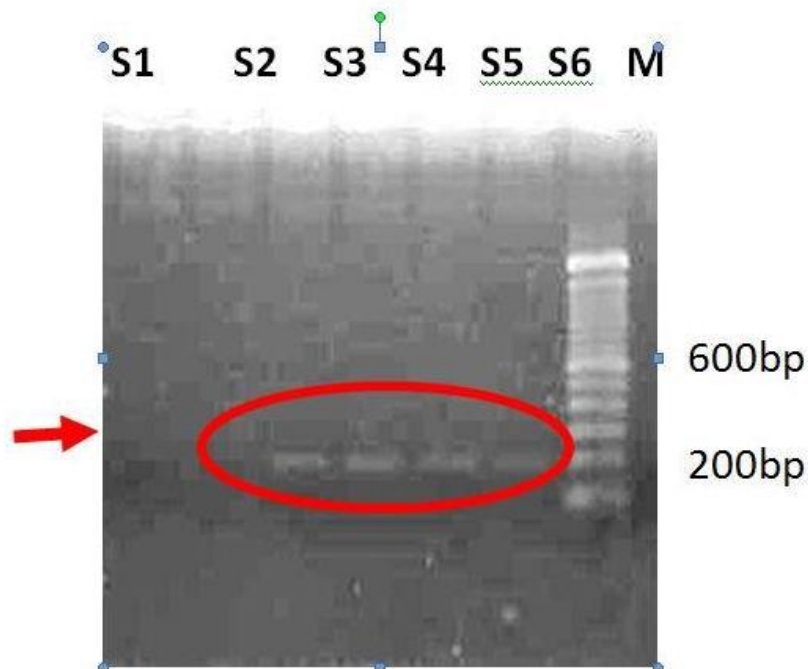


Figure 5. Analysis of the gene encoding CCR5 by PCR visualized by electrophoresis on 2% agarose and stained with ethidium bromide showing the absence of cDNA strand in lanes 1-2, whereas lanes 3-6 shows strands of cDNA of 200 bp, M is the marker.

Sequencing was performed to characterize TCD4+ lymphocytes genotypically, in order to obtain the nucleotide basal composition of the gene encoding CCR5 expressed by

TCD4+ lymphocytes. Sequencing analysis of the research results obtained as in the image below:

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ATGGATTATCAAGTGTCAAGTCCAATCTATGACATCAATTATTATACATCGGAGCCCTG
CCAAAAAATCAATGTGAAGCAAATCGCAGCCCGCCTCCTGCCTCCGCTCTACTCACTGG
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TGTTTCATCTTTGGTTTTGTGGGCAACATGCTGGTCATCCTCATCCTGATAAACTGCAAAA
 GGCTGAAGAGCATGACTGACATCTACCTGCTCAACCTGGCCATCTCTGACCTGTTTTTCC
 TTCTTACTGTCCCCTTCTGGGCTCACTATGCTGCCGCCAGTGGGACTTTGGAAATACAA
 TGTGTCAACTCTTGACAGGGCTCTATTTTATAGGCTTCTTCTCTGGAATCTTCTTCATCAT
 CCTCCTGACAATCGATAGGTACCTGGCTGTCGTCCATGCTGTGTTTGCTTTAAAAGCCAG
 GACGGTCACCTTTGGGGTGGTGACAAGTGTGATCACTTGGGTGGTGGCTGTGTTTGCGT
 CTCTCCCAGGAATCATCTTTACCAGATCTCAAAAAGAAGGTCTTCATTACACCTGCAGC
 TCTCATTTTTCCATACAGTCAGTATCAATTCTGGAAGAATTTCCAGACATTAAGATAGTC
 ATCTTGGGGCTGGTCCTGCCGCTGCTTGTGTCATGGTCATCTGCTACTCGGGAATCCTAAAA
 ACTCTGCTTCGGTGTGCGAAATGAGAAGAAGAGGCACAGGGCTGTGAGGCTTATCTTCAC
 CATCATGATTGTTTATTTTCTCTTCTGGGCTCCCTACAACATTGTCCTTCTCCTGAACACC
 TTCCAGGAATTCTTTGGCCTGAATAATTGCAGTAGCTCTAACAGGTTGGACCAAGCTAT
 GCAGGTGACAGAGACTCTTGGGATGACGCACTGCTGCATCAACCCCATCATCTATGCCT
 TTGTCGGGGAGAAGTTCAGAACTACCTCTTAGTCTTCTTCCAAAAGCACATTGCCAAA
 CGTTCTGCAAATGCTGTTCTATTTTCCAGCAAGAGGCTCCCGAGCGAGCAAGCTCAGT
 TTACACCCGATCCACTGGGGAGCAGGAAATATCTGTGGGCTTGTAG

Figure 6. Sequencing analysis of CCR5 full gene encoding.

Then we performed basal nucleotide deletion in conserved area, namely at position 554-576 bp (the image above is shown with red nucleotide bases), and then performed a deletion by using restriction enzymes EcoRI.

Deletion results was checked by PCR in order to verify whether the composition of the nucleotide deletion had occurred. Visualization of deletions results are shown in Figure 7:



Figure 7. PCR analysis of the full gene and 32 bp deletion of the CCR5 encoding gene. A. Visualisasi by electrophoresis. B. CCR5 full gene encoding with PCR showed cDNA strand of 1059 bp, whereas the deletion of 32 bp in the CCR5 encoding gene by PCR showed a cDNA strand with bp lower than the full gene. M is the marker.

Based on the PCR results, the deletion of the CCR5 encoding encoding has occurred, which subsequently was checked by sequencing examination, revealing that the nucleotide base had already lost as many as 32 bp in the area in question in accordance with the method.

DISCUSSION

The role of CCR5 in the pathogenesis of HIV entry into target cells has been a concern of experts both in basic science and in infectious tropical disease, in relations to HIV/AIDS epidemic in the world. This study was designing a prototype of TCD4+ lymphocyte resistant against HIV infection generated from peripheral blood haematopoietic stem cells (PBMCs) by deletion of 32 bp encoding the CCR5 gene, to prevent the entry of HIV to TCD4+.

In this research, a few steps had been made to create prototype design of TCD4+ lymphocytes resistant to HIV infection. First, isolation, culture and purification of TCD4+ lymphocytes. Second, characterization of TCD4+ lymphocyte using immunofluorescent, flowcytometry, PCR and sequencing. Third, we created a prototype design of TCD4+ lymphocytes resistant to HIV infection by deleting 32 bp of CCR5 encoding gene.

If someone is found to have natural mutations in CCR5 encoding gene, then the person becomes resistant to HIV infection and thus a useful gene therapy for the prevention of HIV entry into target cells is associated with a mutation in CCR5 presenting as 32 bp deletion of CCR5 encoding gene. CCR5, instead of CXCR4, was chosen because of the consideration that CXCR4 has many biological functions in the body, so when mutations in CXCR4 was made, many body's physiological systems will be disrupted (Liu et al, 1996; Chakraborty and Jones, 1999; Rizzardi and Pantaleo, 1999; Hammer, 2005 ; CDC, 2009 ; Hutter, et al, 2009).

Deletion of 32 bp in CCR5 resulted in the dysfunction of the receptor. Thus, HIV cannot enter into the target cells. Two copies of this allele is shown to provide a powerful protective effect against HIV infection. This allele was found in 5-14 % of the population in Europe, but it is rare in populations in Africa and Asia. Some research suggests that individuals with one copy of the allele is able to inhibit the progression toward AIDS in the period of approximately two years. Deletion of 32 bp of CCR5 would alter CCR5 protein on the outer surface of the TCD4+ cell. It gives a great influence on the progression of HIV infection. 32 bp nucleotide deletion was found in 2 of the 25 people exposed to the

HIV virus and 2 are resistant to HIV. After analyzing the sequence as compared with their parents (Wild Type/WT) it was found that there was 32 bp deletion at nucleotide in position 794-825 bp from 1055 bp of the parents. With the change of the CCR5 encoding gene, the amino acid sequence produced in the area of the mutation also changed (Samson et al, 1996; Pardis, et al, 2005).

The research was carried out with isolation using Ficoll hystopaque gradient 1,007 that aimed to precipitate erythrocytes, and the culture of TCD4+ lymphocytes from PBMC of healthy people. It was hoped that we would obtain good TCD4+ lymphocytes. The purification of TCD4+ lymphocytes was performed using magnetic beads deluted with PBS. Subsequently, TCD4+ lymphocyte culture was done using RPMI medium. The maturity of TCD4+ lymphocytes started to appear at passages 4 and culminated in passage 7 and then the number declined again (Rantam, et.al., 2009; Purwati, et.al., 2010)

The kinetic of cell growth was measured in the format of culture cycle standard starting with a slow phase (lag), logarithmic phase, stationary phase, decline phase and then died. These phases are influenced by microenvironment, and may further affect cell multiplication because Go, S, G1, G2 cycles are inhibited. Empirically, when the cells were isolated and cultured, cell viability depends on the ability to attach on the base of the petridish or plate or flash. Therefore, cell growth required fast adaptation in the new microenvironment. This is the answer why

late initial phase results in slow mitosis process in cell proliferation (Blau et al 1985).

Characterization of TCD4+ lymphocytes could be based on phenotype or genotype. Phenotype characterization is based on the differentiation cluster, where CD4 that can be checked with the direct and indirect immunofluorescent examination and examination with Fluorescence Activated Cell Shorting (FACS). To determine the maturation of these cells, we require an indicator. In this regard the indicator is CD4+. From the results of flowcytometry, we obtained average number of TCD4+ lymphocytes as high as 50-60 % of the total CD45 + or hematopoietic stem cell. This is due to the possibility of differentiation of hematopoietic stem cell to CD3/NK cell (Jackson, et al, 1999; Rantam, et.al., 2009). Genotype characterization can use PCR and sequencing. DNA results of CCR5 in this study was shown in agarose gel of DNA bands parallel to the standard marker of 180 bp. In this study, using BLAST (Basic Local Alignment Tool) program we found high levels of suitability (Query Sequence) as much as 80-100 % with the Genbank database where the composition of the nucleotide sequences was in accordance to the results of CCR5 variant A and variant B.

TCD4+ lymphocytes prototype design started with a 32 bp deletion of the CCR5 full gene . CCR5 full gene was obtained by PCR and sequencing. PCR was performed using primers for forward : 5' - CCCAGTCACGACGTTGTAAAACG - 3', and reverse : 5' -

TAATACGACTCACTATAGGG - 3'. Then, deletions was made in conserved area in 554-576 bp. Results revealed two bands with different positions where the higher band at position 1059 was CCR5 full gene while the lower is CCR5 with deletion of 32 bp, resulting in functional loss of CCR5, so HIV can not enter into the target cells.

CONCLUSION

The prototype design of HIV-resistant TCD4+ lymphocyte can be made by deleting 32 bp of CCR5 full gene encoding in GTCAGTATCAATTCTGGAA GAATTT CCAGACA using EcoRI enzyme.

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