# Cloning, Expression, and Production of Wild Type and Mutant Versions of HIV Reverse Transcriptase

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## Abstract

Reverse transcriptase (RT) is the essential enzyme that generates a complementary strand of DNA (cDNA) from RNA. Retrovirus RTs are one of the most commonly used enzymes in molecular biology and used in the diagnosis of COVID-19 disease. Among all known RTs, the reverse transcriptase from human immunodeficiency virus (HIV) is one of the most studied and well-known. Although HIV-RT has higher activity than other MLV and AMV RTs at temperatures above 50ºC, HIV-RT has relatively low fidelity. In the literature, it has been reported that some mutations in the HIV-RT confer increased fidelity. In this study, the RT encoding genes p66 and p51 of HIV were amplified by PCR, and the PCR products were cloned into the pJET1.2 cloning vector. Nucleotide sequence of plasmids carrying HIV-RT genes, were verified by the next-generation sequencing (NGS). To express the heterodimer proteins produced by p66 and p51 genes in the different hosts, the genes were cloned into the pET-28a(+) expression vector. Protein expression was confirmed by SDS-PAGE and coomassie brilliant blue staining. Amino acids in ß3-ß4 hairpin loops of the enzyme are important for the fidelity of HIV-RT. By examining this region K65R, V75I, D76V, and R78A mutations were inserted by site-directed mutagenesis techniques at cloned p66 gene on pET-28a(+). To test the enzyme activity, lysates of expressed protein subunits p66 and p51 were used as RT enzyme in cDNA reaction and qPCR for COVID-19 samples and, also in cDNA reaction and conventional PCR for ß-actin. In these reactions, combined lysates of p66 and p51 showed a good performance, which is comparable to the commercial enzyme. Single point mutations on p66 gene were by confirmed by NGS and, expression of mutant versions, activity assays and comparisons with wild type are still ongoing.
**Keywords**: Reverse transcriptase (RT), Cloning, Expression, Mutant Enzyme, RT Activity Test

## Introduction

The conversion of mRNA into cDNA (complementary DNA) is the essential step in studying the expression of the eukaryotic gene. The enzyme used first in this process, is RNA-dependent DNA polymerase, reverse transcriptase (RT). RT was discovered in 1970 by American scientists H.M.Temin and D.Baltimore, separately (1,2). Coupled with PCR, RTs have been widely used for the detection of RNAs, diagnosis of various cancers, and epidemic diseases, and cloning and sequencing (3). RTs from Molony murine leukemia virus (MLV), Avian myeloblastosis virus (AMV) and HIV-1, and a thermostable *Geobacillus stearothermophilus* group II intron have been extensively characterized. Today, these enzymes are readily available commercially(3).
The efficiency of RT is affected by RNA secondary or tertiary structure in qPCR. To overcome these shortcomings, the RTs' activity at temperatures above 50 ℃ is a great advantage. HIV RTs have high thermal stability compared to other RTs (4). HIV RT is a heterodimeric protein complex consisting of a small regulatory subunit p51 and a large catalytic subunit p66 (5). HIV RTs have low fidelity; however, they have the advantage of high thermostability and low RNase H activity (3,4).
In this study, we reported the cloning and expression of RT genes encoding the heterodimer p66 and p51 of HIV, respectively. A number of site directed mutations were performed on the catalytic subunit p66. Results suggest that expressed heterodimer proteins, p66 and p51, demonstrate a similar performance with the commercial enzyme MLV.

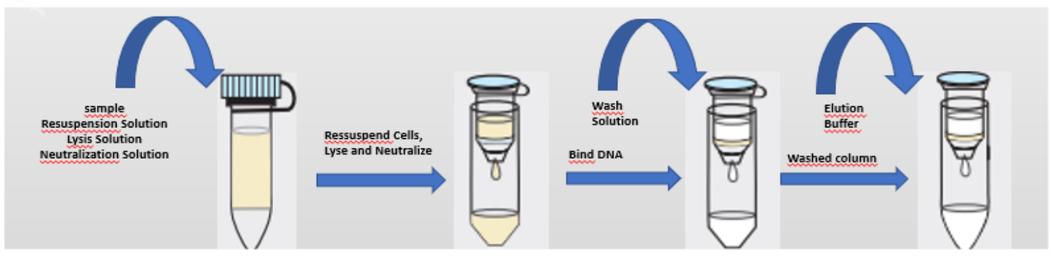
## Materials and Methods

### Reagents and plasmids

In the study, pHIV-pTp66p51 vector was used for amplfying genes encoding heterodimer p66 and p51. Escherichia coli DH10ß and Escherichia coli BL21(DE3) were used as the host for recombinant proteins. pJET1.2 and pET28a were utilized for cloning genes encoding HIV RTs. PCR enzymes and restriction enzymes were purchased from Thermo Scientific and New England Biolabs (NEB), respectively. Oligonuclotides were synthesized by Sentebiolab Biotech. Long PCR enzyme was used for site-directed mutagenesis assay. Bacterial strains were grown at 37℃ on Luria Bertani (LB) medium with the appropriate antibiotics required for plasmid selections (e.g., Ampisilin at 100mg/ml, kanamycin at 100mg/ml).

### Construction of bacterial vectors expressing HIV-RT

•*Isolation of target genes by Miniprep*




- *Amplification of target genes by PCR*


- *Cloning of HIV-RTs*


### Expression of HIV-RTs

1. When the absorbance of the culture at 600 nm was 0.1 (OD600= 0.1), it was inoculated again in a 1 L beaker into 100 mL of LB with kanamycin and incubated at 37°C with shaking until $OD_{600}$ reaches 0.6-0.9.
2. When OD600= 0.6-0.9, it was induced with IPTG at a final concentration of 0.5 mM and incubated for hours at 37°C.
3. The culture was spinned at 5000 rpm for 5 minutes. The supernatant was discarded and the pellet was taken.
4. The pellet was dissolved in 1ml Lysis buffer, lysozyme was added, incubated for 1 hour at 37 °C.

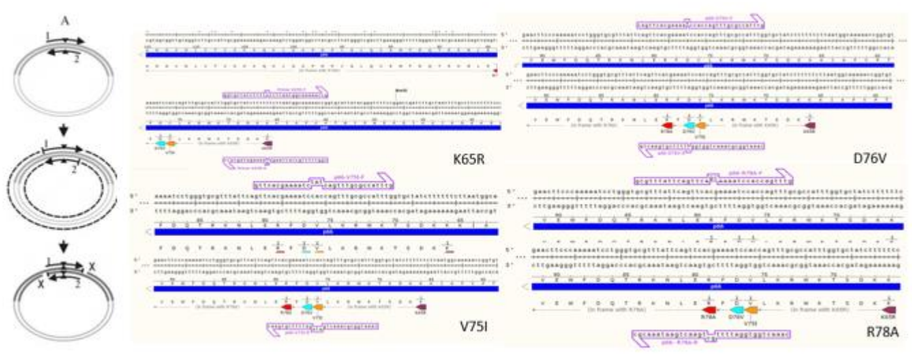
The enzyme-treated lysate was precipitated at 5000 rpm for 5 minutes, 20 µl of the supernatant was taken, 20 µl of SDS Page 2X loading dye was added, and it was kept at 100 °C for 5 minutes, then loaded onto the gel

### Generating site- directed mutant RT library

*1. Determination of mutation points*

Studies in the literature to increase the accuracy of the RT enzyme were examined. In line with literature, it was decided to make K65R, V75I, D76D and R78A mutations.

*2. Design of mutation primers and mutagenesis*




Using primers for mutagenesis, long PCR was optimized to insert mutations into p66 gene by amplfying the whole vector containing p66 gene. Amplified vector was treated with DpnI to digest original plasmid without mutations. Long PCR product was transformed into *Escherichia coli* DH10B cells. Vector sequences with mutant p66 were confirmed by NGS.

### Reverse Transcription PCR Assays

A two-step RT-PCRassay was used to determine the efficiency of the reverse transcription reaction at different temperatures. Reverse transcription reactions (20 µL) were conducted using buffer, RNase inhibitor, 100 µM oligo(dT), Beta-actin mRNA, and 1ul of HIV- RTs lysates. The cDNA synthesis reactions were conducted at temperatures (50°C) for 60 min and then stopped when the mixtures were heated at 85 °C for 5 min.

### Quantitative Real-Time PCR Assay

In qPCR reactions, HIV-RT lysates, which were expressed in our laboratory were used as RT enzymes. "Senteligo™ SARS-CoV-2 (COVID-19) Multiplex qPCR Detection Kit" protocol was used for reaction conditions.

## Results

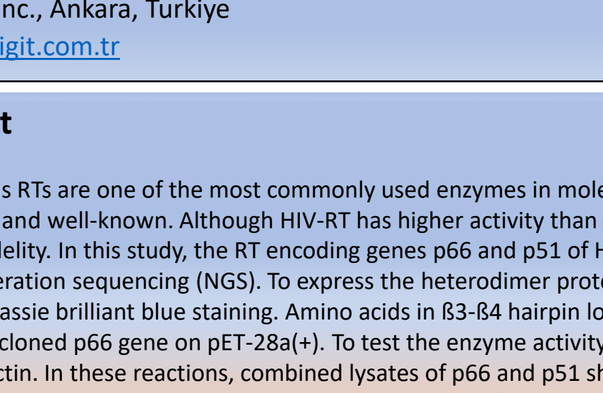


Fig1. Genes encoding heterodimer p66 and p51 were amplified by PCR.

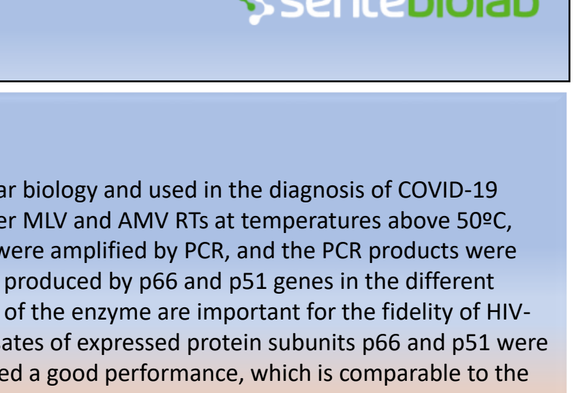


Fig 2. Colony PCR results showing confirmation of p51 and p66 inserts in pJET1.2 cloning vector.

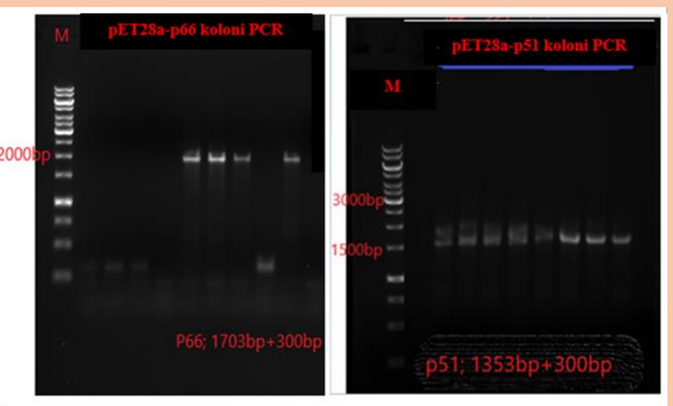


Fig 3. Colony PCR results show confirmation of p51 and p66 inserts in pET28a expression vector.

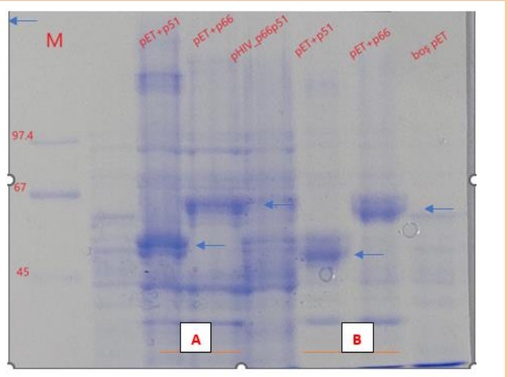


Fig4. SDS-PAGE analysis of the expression of HIV-RTs, p51 and p66.
The gel was stained with coomassie blue. A: induced with 0.5mM IPTG, B: induced with 1mM IPTG.

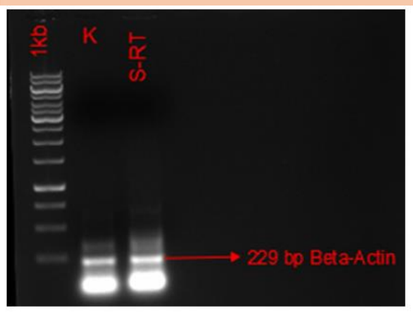


Fig 5. Beta-actin gene was amplified by Reverse Transcription PCR.
K: Commercial RT enzyme, MLV, and S-RT: HIV-RT lysates produced in our laboratory.

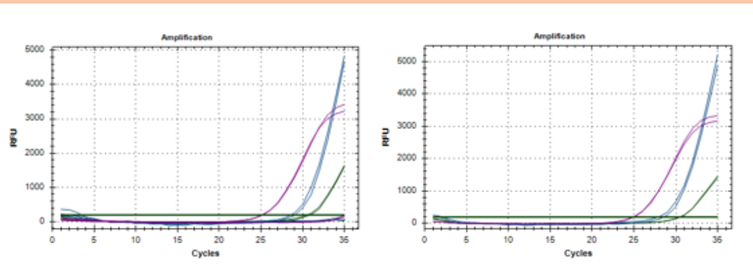


Fig 6. qPCR was performed to amplify SARS-CoV-2 target genes Commercial RT enzyme, MLV, and HIV-RT lysates produced in our laboratory were used as RT in these reactions.

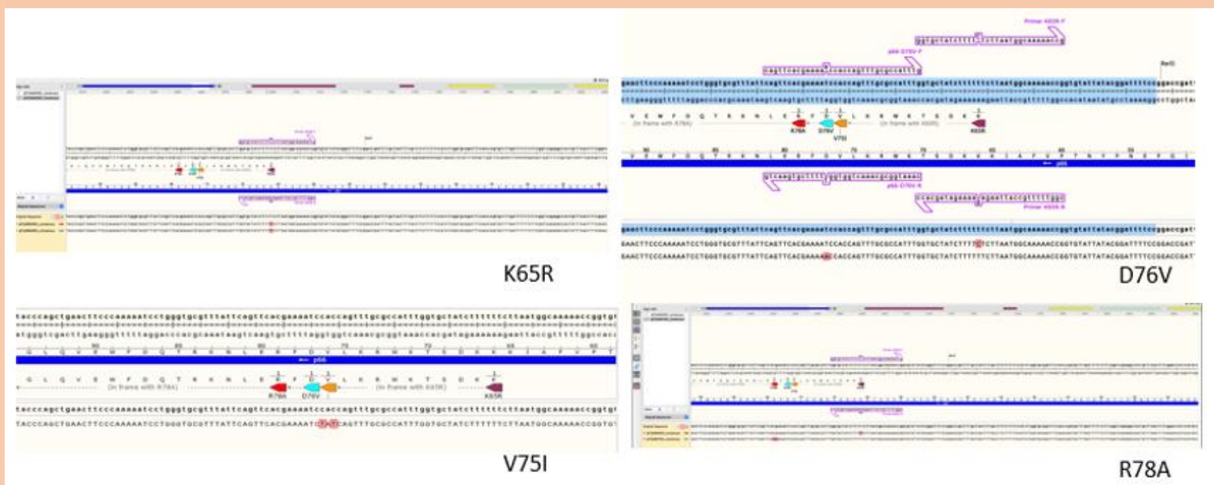


Fig 7. NGS analysis of site-directed mutations on pETp66

## Conclusion

Results demonstrated that cDNA synthesis and RT-qPCR amplifications can be performed by using combined lysates of p66 and p51 and our lysates showed a good performance, which is comparable to the commercial enzyme. Single point mutations K65R, V75I, D76V, and R78A on p66 gene were confirmed by NGS. Expression of mutant versions on pET28a, activity assays in qPCR and comparisons with wild type RT are still ongoing.

### References

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