Cloning and Expression of Serotype-2 Simian Betaretrovirus Reverse Transcriptase Gene Isolated from Indonesian Cynomolgus Monkey in *Escherichia coli*

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In this study, we isolated simian betaretrovirus serotype-2 (SRV-2) reverse transcriptase (RT) gene from infected Indonesian cynomolgus monkey (*Macaca fascicularis*). The gene was then cloned in *Escherichia coli* expression system. The SRV-2 RT gene is located between nucleotides 3284-4925 in the polyprotein (*Pol*) region encodes 547 amino acids. Analysis of expression using SDS-PAGE and western blot techniques showed a specific band of 64.9 kDa, indicating SRV-2 RT recombinant enzyme. Purification of SRV-2 RT recombinant enzyme produced 312 µg mL^{-1} protein with 7.1 U µL^{-1} enzyme activities. Application of this recombinant enzyme in reverse transcription-PCR (RT-PCR) of β-globin and β-actin genes produced DNA fragments of 206 and 350 bp, indicating amplification of β-globin and β-actin genes, respectively. Therefore, the expressed SRV-2 RT enzyme was proven to be functional, although the activity was low.

Key words: *Escherichia coli* expression system, recombinant enzyme, reverse transcriptase, SRV-2

Pada penelitian ini, dilakukan isolasi gen *reverse transcriptase* (RT) simian betaretrovirus serotipe-2 (SRV-2) yang menginfeksi monyet ekor panjang (*Macaca fascicularis*) asal Indonesia. Gen tersebut selanjutnya diklonakan ke dalam sistem ekspresi *Escherichia coli*. Gen RT SRV-2 berada pada posisi nukleotida 3284-4925 di daerah *polyprotein* (*Pol*) yang mengkodekan 547 asam amino. Analisis hasil ekspresi menggunakan teknik SDS-PAGE dan western blot menunjukkan adanya pita protein spesifik berukuran 64,9 kDa yang diperkirakan sebagai enzim rekombinan RT SRV-2. Pemurnian enzim rekombinan RT SRV-2 menghasilkan 312 µg mL^{-1} protein yang memiliki aktivitas enzim sebesar 7,1 U µL^{-1}. Aplikasi enzim rekombinan RT SRV-2 pada teknik *reverse transcription*-PCR (RT-PCR) terhadap target gen β-globin dan β-aktin menghasilkan pita DNA berukuran 206 dan 350 pb. Dengan demikian, enzim rekombinan RT SRV-2 terbukti berfungsi memiliki aktivitas enzim walaupun aktivitasnya masih rendah.

Kata kunci: enzim rekombinan, reverse transcriptase, sistem ekspresi *Escherichia coli* , SRV-2

Reverse transcription is an essential and critical step in the life cycle of all retroviruses replication (Telesnitsky and Goff 1997; Sarafianos *et al.* 2009). Reverse transcriptase (RT) is a multifunctional enzyme that catalyzes the formation of double-stranded deoxyribonucleic acid (DNA) from single-stranded ribonucleic acid (RNA) viral genomes (Telesnitsky and Goff 1997; Hizi and Herschhorn 2008). This enzyme is responsible for genome replication of viruses possessing RNA- and DNA polymerase and RNase H activities (Hizi and Herschhorn 2008).   RT has become an important enzyme in molecular biology, genetics and medicine for the synthesis of complementary DNA (cDNA) from messenger RNA (mRNA) (Telesnitsky and Goff

1997; Herschhorn and Hizi 2010). The application of this enzyme combining with PCR amplification technique (RT-PCR) has expanded our knowledge of numerous cellular regulatory mechanisms and has become a gold standard to facilitate the coding region of any gene of interest. The development of biotechnology has been relying on the availability of this efficient RTs (Herschhorn and Hizi 2010). Until nowadays, the reverse transcriptases of human immunodeficiency virus type-1 (HIV-1) and Moloney murine leukemia virus (MMLV) have been extensively characterized. The RT of HIV-1, the virus causing acquired immunodeficiency syndrome (AIDS), is a prime target for the development of antiretroviral drug therapy of HIV-1/AIDS and the most studied enzyme in biomedical research (Kohlstaedt *et al.*1992). Meanwhile, the MMLV-RT is the most widely used

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enzyme for cDNA synthesis and RNA amplification due to its robust catalytic activity and high fidelity (Kotewitcz *et al.*1988; Taube *et al.* 1998).

Simian betaretroviruses (SRVs) are the causative agents of simian acquired immunodeficiency syndrome (SAIDS) in Asian macaques with varying severity (Marx *et al.* 1984; Gardner *et al.* 1988; Lerche 2010). Regarding potentially active infection and immune abnormality affected by this virus, SRVs is a pathogenic agent that should be eliminated in the *Macaca* breeding colony (Marx *et al.* 1984; Morton *et al.* 2008). This SRVs infection poses some problems for breeders providing a population of SRV-free macaques, since macaques are frequently used as animal model in biomedical researches (Lerche and Osborn 2003). While SRV is an important pathogen in Asian macaques and causes a potential confounding variable in biomedical research, SRV also provides a valuable viral model to compare with other retrovirals for a better understanding of aspects of retroviral-host interactions, infection mechanism, retroviral structure, antiretroviral, and vaccine development (Montiel *et al,* 2010; Lerche and Osborn 2003). Recently, SRV-2 have been isolated and characterized from wild Indonesian *Macaca fascicularis* of which the sequence analysis of envelope region showed 96% homology to SRV-2 (Iskandriati *et al.* 2010). The SRVs' genome contains three major genes, 5'-*gag-pol-env*-3'. Each gene plays a role in the production of viral proteins (Marracci *et al.* 1995; Marracci *et al.* 1999). The RT sequence is located within the *pol* coding domain of this retrovirus. The gene encoding RT can be isolated and cloned to produce RT recombinant enzyme.

In this study, we report the expression of SRV-2 RT gene isolated from Indonesian *M. fascicularis* in *Escherichia coli*, its purification, and its enzymatic activity measurement of the gene product.

MATERIALS AND METHODS

Isolation of SRV-2 RT Gene Using PCR Technique. DNA was extracted from PBMCs (peripheral blood mononuclear cells) of Indonesian *M. fascicularis* infected SRV-2 (archived sample of PSSP LPPM IPB) using QIAmp Blood DNA Minikit (Qiagen, Hilden, Germany). The SRV-2 RT gene was amplified using specific primers SRV-2: RT3284U (5'CACCCCTGTTTGGGTTGATC-3') and SRV-2 RT4925R (5'-CTAGTGATTACCTTGAGATAAAGG TCC-3'). The forward primer was designed with the addition of CACC nucleotides that would facilitate the

cloning of the gene into the pENTR/SD/TOPO entry vector. Meanwhile, reverse complement of stop codon UAG (CTA) was added to the reverse primer for correct expression in N-terminal fusion pDEST17 vector. PCR amplification was performed using *Pfx* DNA polymerase of *Thermococcus* sp that has low error rate and proof reading activity. Additional primers for sequencing were M13F (5'-TGTAAAACGACGGCCA GT-3'), M13R (5'-GGTCATAGCTGTTTCCTG-3'), SRV-2 RT 3751R (5'-GCCCAAGATTTTCTGATTGG -3'), SRV-2 RT 3799F (5'-GATTGGCGAACAAGTTT TGC-3'), SRV-2 RT 4247F (5'-TACTGGCCTCTTCTG G-3'), and SRV-2 RT 4604F (5'-GGCATAGCCGCATA CACTTT-3'). These primers were developed using Primer3 program (http://primer3.wi.mit.edu/) based on SRV-2 complete genome (Genbank AF126467.1).

SRV-2 RT Gene Cloning and Expression. SRV-2 RT gene was inserted to pENTR/SD/TOPO according to procedure from Invitrogen, USA and transformed to *E.coli* TOP10. Transformants were selected on LB agar plate containing 50 µg mL^{-1} kanamycin antibiotic. The clone was then cultured in LB broth and incubated at 37 °C for 18 h. Plasmid was extracted using QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany), then analyzed by PCR and sequencing techniques using M13 universal primer to prove the gene target was inserted correctly. SRV-2 RT gene in pENTR/SD/TOPO (Invitrogen, California, USA) was then sub-cloned to pDEST17 (Invitrogen, California, USA) destination vector between nucleotide positions 147 and 1830, downstream of 6× His-tag gene and transformed to *E. coli* DH5α. Analysis of recombination was performed by PCR and sequenced using T7 promotor forward primer and SRV-2 RT4925R primer. pDEST17 containing target gene was transformed to *E.coli* BL21AI using heat shock methods. The tranformant was then grown in 5 mL LB broth containing 100 µg mL^{-1} ampicillin until the OD$_{600}$ was 0.6-1.0. The culture was used to inoculate fresh LB medium containing 100 µg mL^{-1} ampicillin, which was then let to grow until mid-log phase (OD$_{600}$ of ~0.4) prior to induction with 20% L-Arabinose.

Purification and Expression Analysis of SRV-2 RT Recombinant Enzyme. *E. coli* BL21AI expressing SRV-2 RT recombinant protein was cultured overnight in 1 L of LB broth and then centrifuged at 10 000 × g for 10 min. Pelleted cells were lyzed using native binding buffer containing 50 mM potassium phosphate pH 7.8, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X 100, and 10 mM imidazole, and added with 8 mg lysozyme. The affinity

chromatography with Ni^{2+}-NTA chelating agent was used for further purification (Qiagen, Hilden, Germany). Recombinant enzyme concentration was determined using Bicinchoninic Acid (BCA) assay (Thermo Scientific, Illinois, USA). Expression of SRV-2 RT recombinant enzyme was detected by SDS-PAGE. Meanwhile, western blot was performed to analyze the serological reaction using antibody against 6× Histidine-tag (Invitrogen, California, USA) to SRV-2 RT.

SRV-2 RT Recombinant Enzyme Activity Analysis. SRV-2 RT enzyme was determined quantitatively using RT chemiluminescent assay (Roche, Penzberg, Germany) based on the ability of RT enzyme to synthesize new DNA from template/primer hybrid poly (A) × oligo $(dT)_{15}$. Assay was conducted in reaction mixture containing 46 mM Tris-HCl, 266 mM potassium phosphate, 27.5 mM magnesium chloride, 9.2 mM DDT, 10 mM dUTP/dTTP, and template/primer hybrid, at final pH of 7.8. The reaction mixture was incubated for 1 h at 37 °C. The amount of this synthesized DNA is used as a parameter of RT enzyme activity following a sandwich ELISA (enzyme-linked immunosorbent assay) protocol. Digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into the same DNA molecule, which is newly synthesized by the RT. Biotin-labeled DNA binds to the surface of microplate that have been precoated with streptavidin. An antibody to digoxigenin, conjugated to horseradish peroxidase (anti-DIG-POD), binds to the digoxigenin-labeled DNA. The chemiluminescent peroxidase substrate luminol/4-iodophenol is added. Peroxidase in the presence of hydrogen peroxide (H_2O_2) catalyzes the oxidation of luminol, resulting in reaction products that quantified using ELISA reader at the absorbance 490 nm that directly correlated to the level of RT activity in the sample. The specific activity of DNA polymerase were expressed in Unit (U) that means as the amount of enzyme required for the incorporation of 1 nmol of labeled dNTP in 10 min at 37 °C using poly (A)× oligo$(dT)_{15}$ as template/primer hybrid.

Meanwhile, this SRV-2 RT rombinant enzyme was applied to reverse transcription-PCR (RT-PCR) technique to qualitatively analyze the enzyme's ability in reverse transcribing the RNA template to the cDNA. The methods was referred to Superscript III reverse transcriptase (Invitrogen, California, USA) and the cDNA PCR amplification was performed to amplify β-globin and β-actin as house-keeping genes.

RESULTS

SRV-2 RT Gene Isolation and Cloning. In this study, reverse transcriptase gene was isolated from SRV-2 *pol* located at positions 3284-4925 specific band of about 1600 bp was shown in gel electrophoresis (Fig 1), indicating the presence of the targeted gene. PCR analysis using M13 forward and reverse primers was performed to verify the SRV-2 RT in pENTR/SD/TOPO plasmid, resulted the specific amplicon of about 2000 bp (Fig 2). The nucleotides sequence analysis was analyzed by BLAST program (http://blast.ncbi.nlm.nih.gov/), indicating the presence of the gene target, with 99% (1631 of 1641 nucleotides) similarities to SRV-2 RT D2/RHE/OR isolate (Genbank AF126467.1). The gene of interest was inserted to the vector in correct direction without any nucleotide shifts nor mutations in the reading frame. The insertion site was downstream of the ribosome binding site (RBS) and flanked by *att*L1 and *att*L2. It was proven that the target gene had been successfully inserted in the plasmid (Fig 3).

SRV-2 RT Recombination into pDEST17 *E. coli* Expression System. The SRV-2 RT cloned in pENTR/SD/TOPO entry plasmid had been subcloned to pDEST17 destination vector by LR recombination reaction. pDEST17 is a vector, which is N-terminally fused with 6× His tags for purification purposes. ATG initiation codon lies upstream of the 6× His tags. Analysis of recombination in the pDEST17 vector by PCR amplification using the T7 promoter primer to SRV-2 RT 4925R produced a specific fragment of of about 1800 bp (Fig 4). Nucleotide sequence analysis indicated that the SRV-2 RT gene had been inserted properly, in frame within the vector.

SRV-2 RT Enzyme Expression Analysis. SDS-PAGE analysis of SRV-2 RT recombinant protein showed the presence of a band at molecular weight approximately 64.9 kDa (Fig 5). This result was confirmed by molecular weight calculation of 547 amino acids SRV-2 RT and six histidines, using the software from website http://www.expasy.org/compute_pi/Mw. SRV-2 RT recombinant protein has been successfully purified using Ni^{2+}-NTA spin column affinity chromatography. The purified expressed protein was eluted using imidazole, which binds nickle ion more tightly than 6xHis. In this research, purification method was performed under native conditions that the 6× His-SRV-2 RT enzymes was in soluble condition. However, under this condition, the non-tagged proteins will also interact with Ni^{2+}-NTA
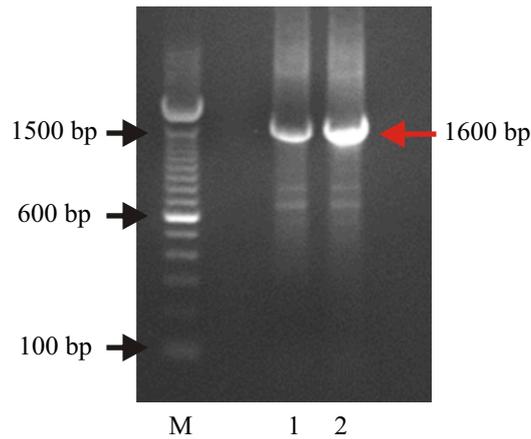
Fig 1 SRV-2 RT gene amplification using specific primer SRV-2 RT 3284F and 4925R. M: 100 bp DNA ladder (Invitrogen); 1-2: 1600 bp PCR product of SRV-2 RT.
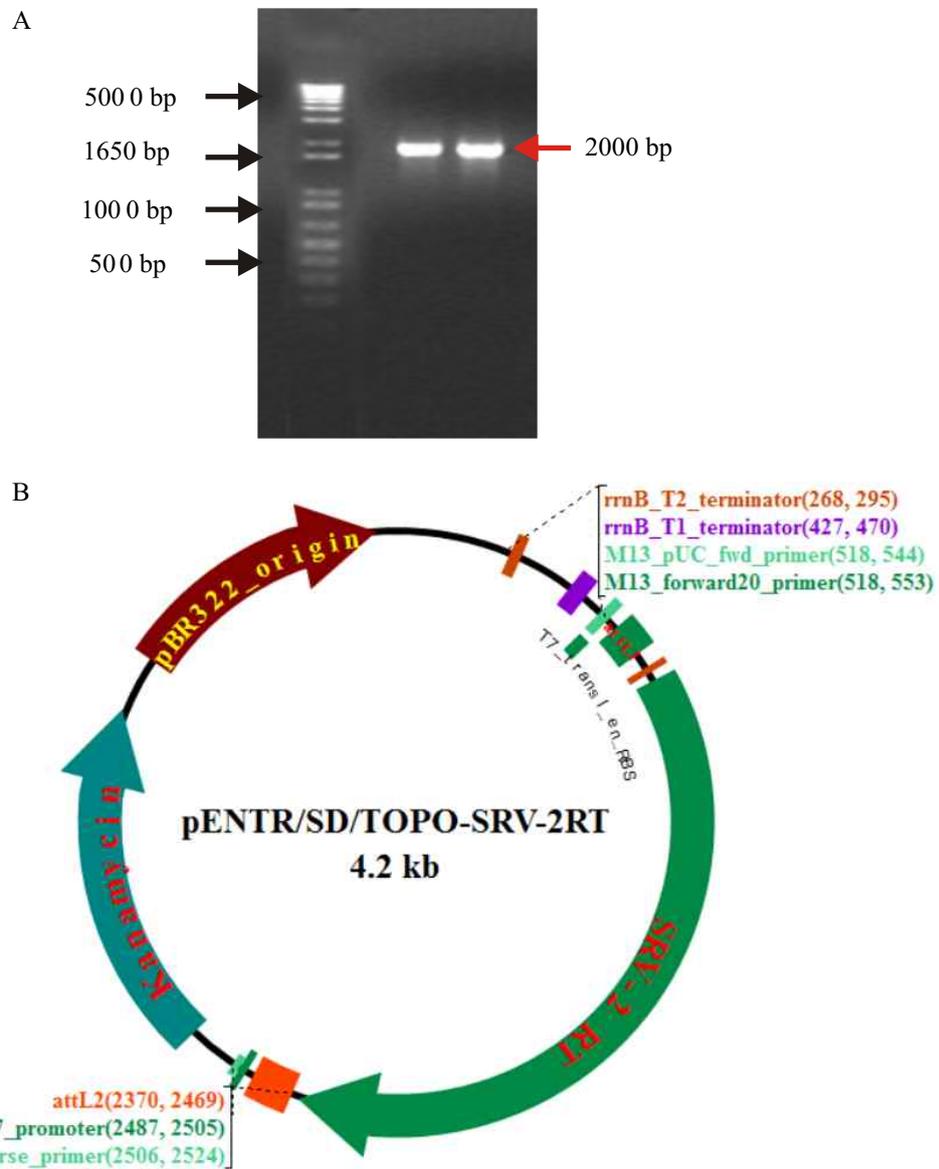

Fig 2 (A) PCR amplification to pENTR/SD/TOPO SRV-2 RT plasmids using M13 F and M13 R primer. M: 1kb DNA ladder (Invitrogen); 1-2: 2000 bp amplicon contained SRV-2 RT gene. (B) Genetic map illustration of pENTR/SD/TOPO-SRV-2 RT vector.

M13 Forward primer
TGTAAAACGACGGCCAGTCTTAAGCTCGGGCCCCAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCA
*att*L1
ACAAATTGATGAGCAATGCTTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCTCCGCGGCCGCCTTGTTTAACT
RBS   **SRV-2 RT3284F**   **SRV-2 RT gene**
TTAAGAAGGAGCCCTTCACCCCTGTTTGGGTTGATCAATGGCCCCTAACTCAAGAAAAACTTGCTGCTGCCCAACAGT
TAGTGCAAGAACAATTACAGGCAGGGCATATTATAGAAAGTAATTCTCCCTGGAATACACCTATATTTGTCATAAAAA
AGAAGTCTGGTAAATGGAGGCTTTTGCAAGATTTAAGGGCGGTAAATGCCACCATGGTATTAATGGGAGCTCTCCAAC
CTGGGCTGCCCTCACCAGTGGCTATTCCTCAGGGATATTTTAAAATAGTCATTGATCTTAAAGATTGTTTTTTTACTA
TCCCCCTTCAGCCCGTTGATCAAAAGCGATTTGCTTTTAGTCTCCCGTCTACCAACTTTAAACAACCAATAAAACGTT
ATCAATGGAAAGTGTTGCCTCAAGGCATGGCCAATAGTCCTACCTTGTGTCAAAAATATGTAGCTGCTGCTATAGAGC
CAATCAGAAATCTTGGGCACAAATGTACATTATACACTATATGGATGACATTCTAATAGCAGGAAAGATTGGCGAAC
AAGTTTTGCAGTGTTTTGCTCAACTTAAACAAGCATTGACAACTACTGGGTTACAAATAGCTCCAGAAAAGGTACAGC
TACAAGATCCATATACCTACCTTGGGTTTCAACTTAATGGTCCCAAAATTACCAATCATAAGGCAGTTATTCGTAGAG
ATAAGTTACAAACCCTTAATGATTTTCAAAAACTTTTGGGAGATATCAATTGGCTTAGACCCTATTTACACCTCACTA
CAGGAGATCTAAAACCTCTTTTTGATATCCTAAAAGGGGATTCTAATCCTAACTCACCCAGATTTTTATCTGAAGCAG
CCCTTACGTCTCTTAAAAAGGTAGAAACAGCTATTGCTGAACAATTTGTTACACAAATAGATTATACACAGCCATTGA
CCTTTTTAATTTTTAATACTACACTGACACCTACTGGCCTCTTCTGGCAAAATAATCCTGTCATGTGGGTTCACTTGC
CTGCATCGCCAAAAAAGGTATTGTTCCCCTATTATGATGCTATAGCAGATCTTATTATCCTTGGAAGGGACAACAGTA
AAAAATATTTTGGACTTGAACCATCTACTATTATACAACCCTATTCTAAATCTCAAATCCATTGGTTAATGCAAAACA
CAGAAACATGGCCAATTGCCTGCGCTTCCTATGCAGGCAACATTGATAATCATTATCCACCCAATAAACTTATTCAAT
TTTGCAAACTCCATGCAGTTGTTTTTCCCCGAATCATTAGTAAAACTCCTCTAGACAATGCCTTACTGGTATTCACTG
ATGGATCCTCTACCGGCATAGCCGCATACACTTTTGAAAAGACCACTGTCAAATTTAAAACCTCCCATACGTCAGCCC
AATTAGTAGAATTACAAGCCCTAATTGCAGTGTTATCGGCCTTTCCTCATCGGGCCCTCAATATTTATACAGATAGTG
CATACTTAGCTCATTCTATACCTCTACTTGAGACAGTATCGCAAATCAAACATATCTCAGACACAGCAAAATTATTTT
TACAGTGCCAACAACTAATATGCAACAGGTCTATACCCTTTTATTTAGGACATATCAGGGCCCATTCAGGATTACCAG
GACCTTTATCTCAAGGTAATCACTAGAAGGGTGGGCGCGCCGACCCAGCTTTCTTGTACAAAGTTGGCATTATAAGAA
SRV-2 RT4925R
AGCATTGCTTATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTATTTGCCATCCAGCTGATA
*att*L2
TCCCCTATAGTGAGTCGTATTACATGGTCATAGCTGTTTCCTG
M13 reverse primer

Fig 3 Nucleotide sequences of Indonesian isolate's SRV-2 RT at nucleotide positions 3284-4925 inserted in the pENTR/SD/TOPO vector. Nucleotide sequences of vector and SRV-2 RT gene were highlighted in yellow and grey, respectively. Primer positions were indicated with red letters.

resin at higher affinity than in denaturing condition. This was shown by the large number of proteins appearing in the binding buffer (Fig 5A). The result of the BCA assay showed that protein concentration was 2280 µg mL^{-1}. This non-specific binding could have been reduced by adding low concentration of imidazole in the washing buffers. The concentration of purified RT-SRV-2 eluted with acidic buffer containing high concentration (250 mM) of imidazole was 306 µg mL^{-1} protein (Table 1). For further expression analysis, Western blot was performed using anti-6xHis-tag antibody showed the presence of a specific band around 64.9 kDa (Fig 5B).

Analysis of SRV-2 RT Recombinant Enzyme Activities. Purified SRV-2 RT recombinant enzyme had the activity of 1420 Unit in 200 µL elution buffer, therefore, the enzyme volumetric activity was 7.1 U µL^{-1} and the enzyme specific activity was 22.76 U mg^{-1} total protein (Table 1). The purified SRV-2 RT recombinant enzyme (3 µL of 21 U is the optimum reaction) was then applied in RT-PCR, to create in comparison to 1 µL of 200 U commercial MMLV-RT

from Invitrogen, cDNA of β-globin and β-actin using Hela cell as mRNA template (Invitrogen). The PCR produced 206 bp and 350 bp fragments corresponding to β-globin and β-actin, respectively (Fig 6). The result proved the functionality of the expressed SRV-2 RT.

DISCUSSIONS

SRV-2 RT gene was cloned in pENTR/SD/TOPO entry vector before being subcloned by site specific recombination into pDEST/17destination *E.coli* expression system. The recombination was mediated by LR Clonase II enzyme mix, a mixture of the bacteriophage λ integrase (Int) and excisionase (Xis), and the *E. coli* integration host factor (IHF) protein (Shuman 1994; Landy 1989; Hunt 2005). This vector was designed to facilitate high-level, inducible expression of recombinant proteins in *E. coli* using the pET system. The system takes advantage of the high activity and specificity of the bacteriophage T7 RNA polymerase to allow regulated expression of heterologous genes in *E. coli* from the T7 promoter
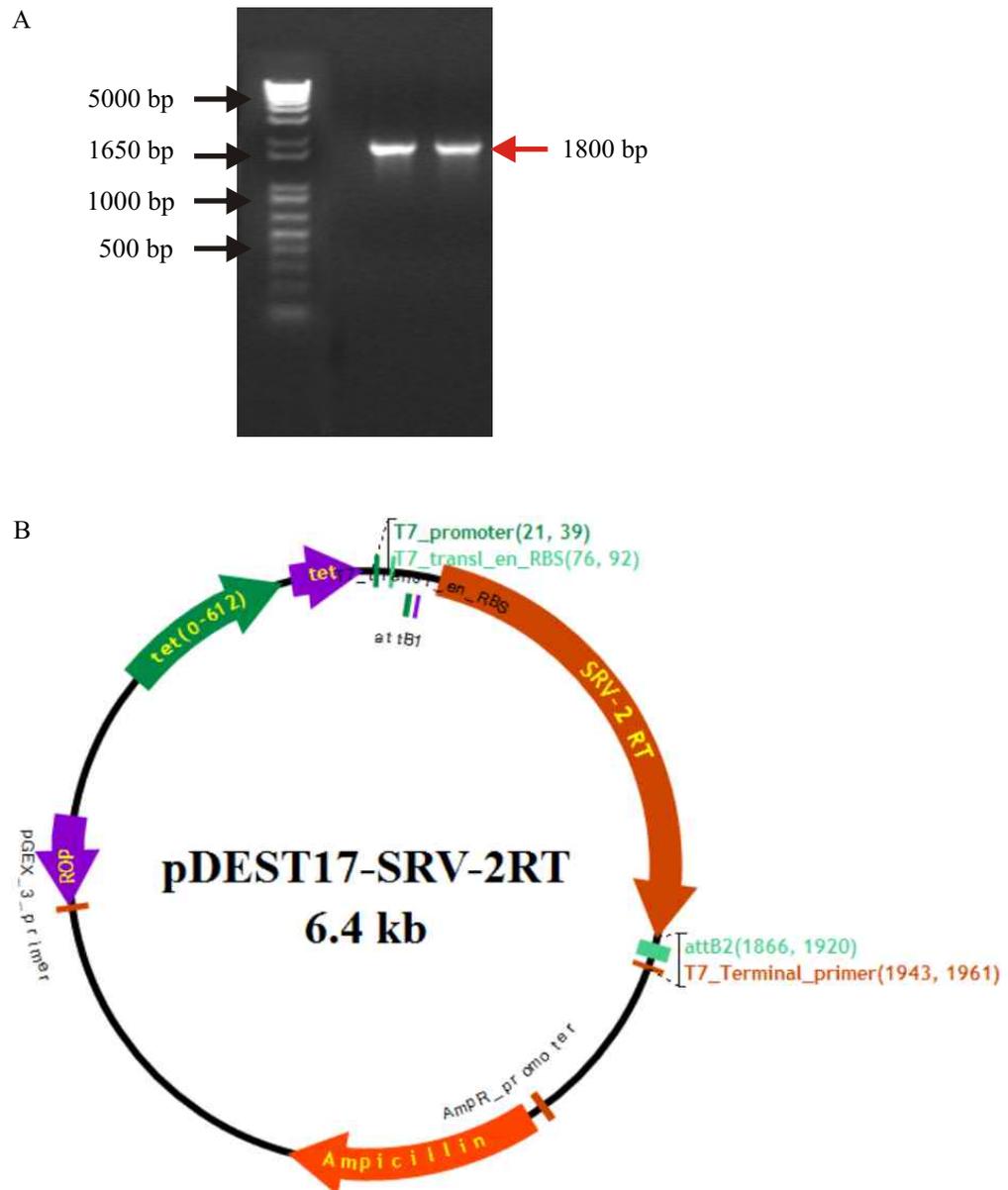
Fig 4 (A) PCR amplification to pDEST17 SRV-2 RT plasmids using T7 forward primer and SRV-2 RT4148 reverse primer. M: 1 kb DNA ladder (Invitrogen); 1-2: 1800 bp amplicon containing SRV-2 RT gene. (B) Genetic map illustration of pENTR/SD/TOPO-SRV-2 RT vector.

(Rosenberg *et al.* 1987).

SDS-PAGE and western blot analyses indicated that the molecular weight of our SRV-2 RT 6× His N-terminal fusion protein was 64.9 kDa. The 6× His tag is small enough that it does not affect the structure and functionality of the expressed protein. In addition the tag allows the protein to be purified under denaturing and native conditions, which is useful if refolding of insoluble proteins is to be attempted. However, N-terminal 6× His tags does not only plays a role in protein recombinant purification but also seems to have stabilizing effect on the mRNA structure in the translation initiation region (Svensson *et al.* 2006).

Comparison of N- versus C-terminal positioning of His tag on several proteins expressed in bacteria and eukaryotes as well as analyses of the protein expression level and solubility indicated that, in most cases, the N-terminal tags improved protein expression (Busso *et al*. 2003; Svensson *et al.* 2006). However, in this study we had only the soluble active SRV-2 RT enzyme, while any attempts to recover enzymatically active preparation of the insoluble inclusion bodies had been unsuccessful. In the case of the Gateway expression vector pDEST-17, the N-terminal 6× His tag is followed by an additional 21 amino acids that arise from transcription through the Gateway recombination and topoisomerase sites.
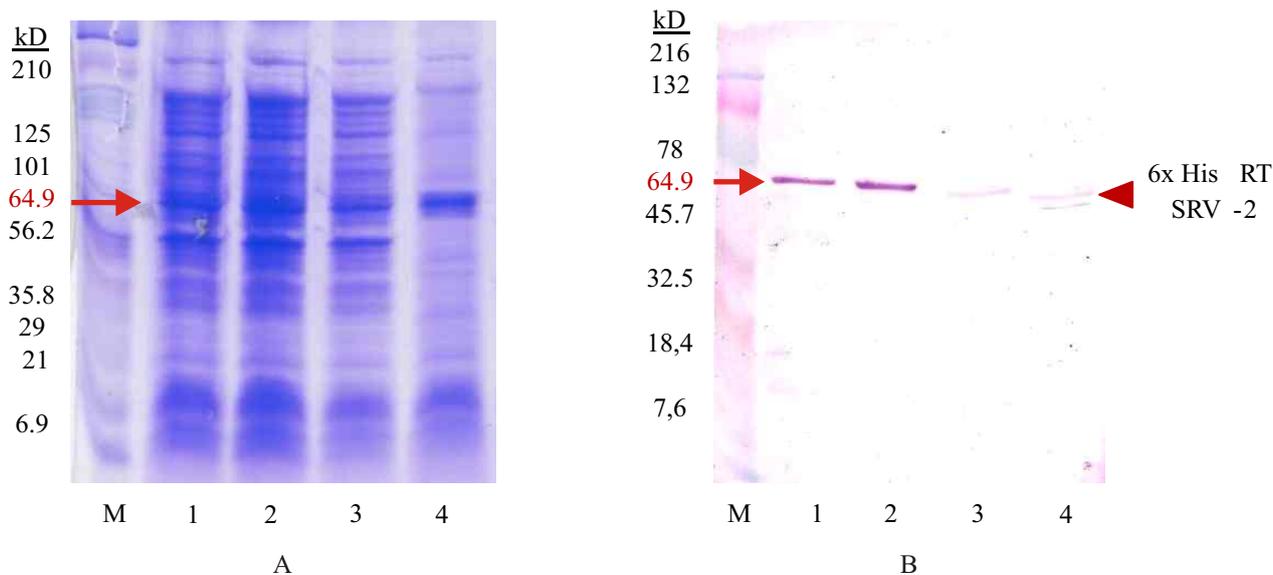
Fig 5 SRV-2 RT recombinant enzyme expression analysis of pre- and post- Ni^{2+}-NTA purification produced specific band at 64.9 kDa. (A) 10% SDS PAGE stained with Coomassie brilliant blue. M: Broad range prestained protein marker (BioRad); 1: pre-purified, 2: binding buffer, 3: washing buffer, 4: elution buffer. (B) Western blot analysis using HRP conjugated anti-polypeptide Histidine antibody. M: Kaleidoskop™ prestained protein marker (BioRad); 1: pre-purified; 2: elution buffer; 3: washing buffer; 4: binding buffer.

Table 1 Protein concentration and activities of the Ni^{2+}-NTA purified RT SRV-2

Fraction of Purification Step	Protein Concentration (µg mL^{-1})	Total Protein (mg)	Enzyme Concentration (U µL^{-1})	Total Enzyme Activity (U)	Enzyme Specific Activity (U mg^{-1} protein)
Pre-purified	2760	1656	0.10	60	0.04
Binding buffer	2280	1368	0.15	90	0.07
Washing buffer	578	346.8	0.24	144	0.42
Elution buffer	312	62.4	7.1	1420	22.76

The protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Scientific) and the enzyme activity was measured by Reverse Transcriptase Assay chemiluminescent Kit (Roche).
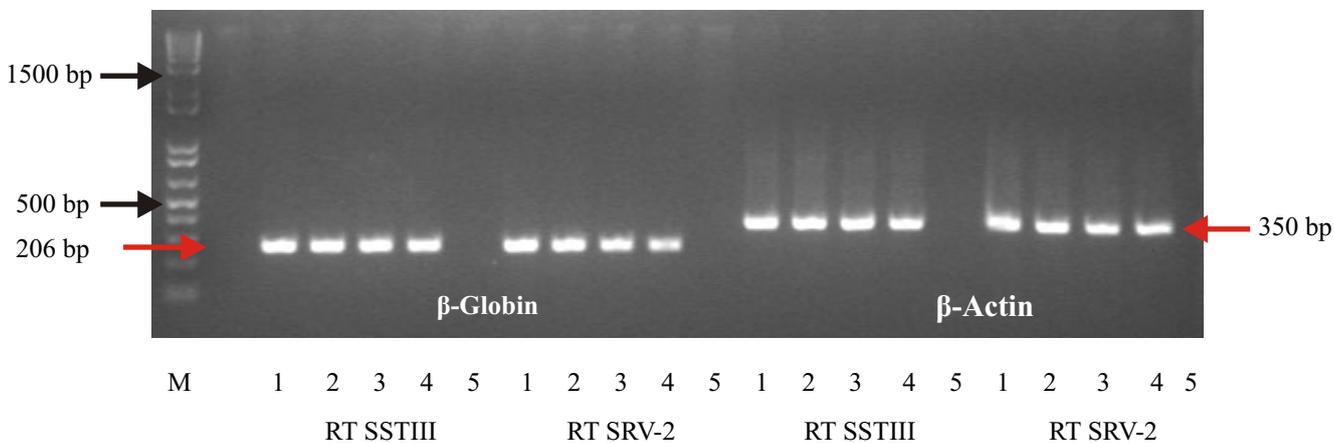

Fig 6 Comparison of two steps RT PCR products of β-globin and β-actin house keeping genes using SRV-2 RT enzyme and SSIII RT commercial enzyme (Invitrogen). The PCR produced 206 bp and 350 bp bands representing the β-globin and β-actin, respectively. 1: 100 bp DNA ladder (Invitrogen); 1-4: repeated samples; 5: master mix control.

Although we did not remove the additional amino acids, we were quite certain that the properties of the recombinant SRV-2 RT are similar, if not identical, to those of the native RT enzyme. Western blot analysis of this soluble N-terminal 6× His SRV-2 RT using anti-6× His antibody indicated the presence of a specific band of 64.9 kDa, confirming the presence of the tagged protein.

Three dimensional protein modelling demonstrated that, like other RTs, SRV-2 was a multi-functional enzyme possessing the RNA-dependent DNA polymerase (RDDP), DNA-dependent DNA polymerase (DDDP), and RNase H activities (Saepuloh, submitted for publication). In this study, we only analyzed quantitatively the RDDP catalytic activities based on oligo(dT)$_{15}$ primers elongation on poly(rA)n templates (Table 1). As already found for most RTs, among the synthetic template-primers suitable for the RDDP activity, poly (rA)n (oligo(dT)$_{12-18}$ is the most efficient (Taube *et al.* 1998). Divalent cations are required for the catalytic activities of most DNA polymerases, including the SRV-2 RT, which used Mg^{2+} as an enzyme cofactor. SRV-2 RT enzyme activity was also measured qualitatively by using the enzyme in a two-step reverse transcription PCR. The ability of this enzyme in reverse transcribing mRNA template into cDNA followed by PCR amplification using a pair specific primer designed for coding sequences gene, indicated that this SRV-2 RT has the RDDP and DDDP activities.

The volumetric activity of the purified SRV-2 RT recombinant enzyme was 7.1 U µL^{-1}. The activity was lower compared to the commercial enzymes, for examples theMMLV RT Superscript III (Invitrogen) and *avian myoblastosis virus* (AMV) RT (Promega, Wisconsin, USA), of which the activities were 200 U µL^{-1} and 20-25 U µL^{-1}, respectively. It is possible that the one step Ni^{2+}-NTA chromatography was not enough to remove protein contaminants from the enzyme eluate, thus causing the low enzymatic activity. On the other hands, it is also possible that different groups of retrovirus indeed have different activities. If this is the case, then we will also have to bear in mind that the RT enzymes whose activities we were comparing came from different groups, MMLV is a gammaretrovirus, AMV is an alpharetrovirus, and SRV-2 is a betaretrovirus. It is known that although RTs from the different groups of retrovirus share similar functional catalytic activities, they differ significantly in various parameters, such as structure and subunit compositions, molecular weights, catalytic properties, biochemical and biophysical characteristics and sensitivity to different inhibitors (Herschhorn and Hizi 2010).

In conclusion, SRV-2 RT gene was successfully isolated, cloned and expressed in *E. coli.* The expressed protein had also been purified and successfully used in RT-PCR. Although the functionality of this recombinant SRV-2 RT enzyme was proven indeed the activity was lower than commercial enzymes. Therefore, it seems still necessary to optimize the expression of the recombinant SRV-2 RT enzyme and to concentrate the product to increase the activity. In addition, it is also necessary to characterize the biochemical and kinetic properties of the enzyme, if we intend to further study the enzyme or to use it for commercial production. This preliminary result was for us very intriguing to explore this enzyme in depth in the next study.

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REFERENCES

Busso D, Delagotte-Busso B, Moras D. 2005. Construction of a set gateway-based destination vectors for high-throughput cloning and expression screening in *Escherichia coli*. J Anal Biochem. 343(2):313-321. doi:10.1016/j.ab.2005.05.015.

Gardner MB, Luciw P, Lerche N, Marx P. 1988. Nonhuman primate retrovirus isolates and AIDS. Adv Vet Sci Comp Med. 32:171-190.

Herschhorn A, Hizi A. 2010. Retroviral reverse transcriptase. Cell Mol Life Sci. 67(16):2717-2747. doi:10.1007/s00018-010-0346-2.

Hizi A, Herschhorn A. 2008. Retroviral reverse transcriptases (other than those of HIV-1 and murine leukemia virus): A comparison of their molecular and biochemical properties. Virus Res. 134(1-2):203-220. doi:10.1016/j.virusres.2007.12.008.

Hunt I. 2005. From gene to protein: A review of new and enabling technologies for multi parallel protein expression. Protein Expr Purif. 40(1):1-22. doi:10.1016/j.pep.2004.10.018.

Iskandriati D, Saepuloh U, Mariya S, Grant RF, Solihin DD, Sajuthi D, Pamungkas J. 2010. Isolation and characterization of simian retrovirus type D from *Macaca fascicularis* and *M. nemestrina* in Indonesia. Microbiol Indones. 4(3):132-136. doi: 10.5454/mi.4.3.6.

Kohlstaedt LA, Wang J, Friedman JM, Rice PA, Steitz TA. 1992. Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science 256(5065):1783-1790. doi:10.1126/science.1377403.

Kotewitcz M, Sampson CM, D'Alessio M, Gerard GF. 1988. Isolation of cloned moloney murine leukemia virus reverse transcriptase lacking ribonuclease H activity. Nucleic Acid Res. 16(1):265-277. doi:10.1093/nar/16.1.265.

Landy, A. 1989. Dynamic, structural, and regulatory aspect of site-specific recombination. Annu Rev Biochem. 58:913-949. doi:10.1146/annurev.bi.58.070189.004405.

Lerche NW. 2010. Simian retroviruses: Infection and disease-implications for immunotoxicology research in primates. J Immunotoxicol. 7(2):93-101. doi:10.3109/15476911003657406.

Lerche NW, Osborn KG. 2003. Simian retrovirus infections. Potential confounding variables in primate toxicology studies. Toxicol Pathol. 31(Suppl):103-111.

Marracci GH, Avery NA, Shiigi SM, Couch G, Palmer H, Pilcher KY, Nichols H, Hallick LM, Axthelm MK, Machida CA. 1999. Molecular cloning and cell-specific growth characterization of polymorphic variants of type D serogroup 2 simian retroviruses. Virology 261(1):43-58. doi:10.1006/viro.1999.9858.

Marracci GH, Kelley RD, Pilcher KY, Crabtree L, Shiigi SM, Avery N, Leo G, Webb MC, Hallick LM, Axthelm MK. 1995. Simian AIDS type D serogroup 2 retrovirus: isolation of an infectious molecular clone and sequence analyses of its envelope glycoprotein gene and 3' long terminal repeat. J Virol. 69(4):2621-2628.

Marx PA, Maul DH, Osborne KG. 1984. Simian AIDS: isolation of type D retrovirus and disease transmission. Science 223(4640):1083-1086. doi:10.1126/science.6695196.

Montiel NA. 2010. An updated review of simian betaretrovirus (SRV) in macaque hosts. J Med Primatol. 39(5):303-314. doi:10.1111/j.1600-0684.2010.00412.x.

Morton WR, Agy MB, Capuano SV, Grant RF. 2008. Specific pathogen free macaques: definition, history and current production. ILAR J. 49(2):137-144. doi:10.1093/ilar.49.2.137.

Rosenberg AH, Lade BN, Chui DS, Lin SW, Dunn JJ, Studier FW. 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. Gene 56(1):125-135. doi:10.1016/0378-1119(87)90165-X.

Sarafianos SG, Marchand B, Das K, Himmel D, Parniak MA, Hughes SH, Arnold E. 2009. Structure and function of HIV-1 reverse transcriptase: molecular mechanisms of polymerization and inhibition. J Mol Biol. 385(3): 693-713. doi:10.1016/j.jmb.2008.10.071.

Shuman S. 1994. Novel approach to molecular cloning and polynucleotide synthesis using vaccinia DNA Topoisomerase. J Biol Chem. 269(51):32678-32684.

Svensson J, Andersson C, Reseland JE, Lyngstadaas P, Bulow L. 2006. Histidine tag fusions increases expression levels of active recombinant amelogenin in *Escherichia coli*. Protein Expr Purif. 48(1):134-141. doi:10.1016/j.pep.2006.01.005.

Taube R, Loya S, Avidan O, Perach M, Hizi A. 1998. Reverse transcriptase of mouse mammary tumour virus: expression in bacteria, purification and biochemical characterization. Biochem J. 329(3): 579-587.

Telesnitsky A, Goff SP. 1997. Reverse transcriptase and the generation of retroviral DNA. In Coffin JM, Hughes SH, Varmus HE, editors. Retroviruses. New York: Cold Spring Harbor Laboratory Press. p 121-160.