

Cloning, Expression, Purification, Determining Activity of Recombinant HIV-1 Reverse Transcriptase

Kun Silprasit¹, Ratsupa Thammaporn², Supa Hannongbua^{1,2}
and Kiattawee Choowongkamon^{1,3*}

ABSTRACT

The spreading of HIV infection is still a serious epidemic disease in Asia, including Thailand, especially by transmission from mother to child and drug resistance HIV. Therefore, discovery of a new drug is the hope for curing the drug resistant strains. Drug discovery needs atomic resolution structure of the target protein, reverse transcriptase. In this paper, we reported the cloning, expression, purification and activity assay of HIV-1 reverse transcriptase in *E. coli*. The yield of homogeneous recombinant HIV-1 reverse transcriptase enzyme was 2 mg/liter culture. The enzyme activity assay using fluorometric method and PicoGreen dye was convenient and rapid, and K_m , determined by this method was $10.51 \pm 2.58 \mu\text{M}$ dTTP substrate. The fluorometric assay can be further used for high-throughput inhibitor screening application.

Key words: reverse transcriptase, HIV-1, cloning, expression, activity assay

INTRODUCTION

HIV is the cause of Acquired Immune Deficiency Syndrome (AIDS) which is the world serious epidemic disease. In Thailand, spreading of HIV infection is still a serious problem. Especially transmission of HIV from mothers to their children causes them to die at young age. To solve this problem, Thai government offers antiretroviral treatment regimens. The anti-HIV drugs such as nucleotide analogue and non-nucleotide analogue compounds have been used to inhibit reverse transcriptase (RT) HIV enzymes. However, the drugs-resistant strains of HIV still survive under antiviral drug-treated condition. Therefore, searching for novel and high efficiency

is indispensable, which require atomic detail of RT structures interacting with new drugs. To study the atomic structure, large quantities of highly purified active RT are generally required in protein X-ray crystallography technique. Although several cloning methods of HIV-1 RT heterodimer (p66 and p51) have been reported (Muller *et al.*, 1989; Hou *et al.*, 2004), there is none on the construction of plasmid for the expression of the HIV-1 RT in *E. coli*. Several advantages of RT expression in *E. coli* can be achieved such as easy expression, more economy, no biohazard, and ability to modify RT by point-mutations. Moreover, the conventional isotopic method is usually used for RT activity assay. This method is limited because the procedure is time consuming and require

¹ Interdisciplinary Graduated Program in Genetic Engineering, Kasetsart University, Bangkok 10900, Thailand.

² Department of Chemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.

³ Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.

* Corresponding author, e-mail: fsciktc@ku.ac.th.

radioactive isotope (Chavan and Prochaska, 1995). In this paper, we described the cloning and expression of HIV-1 RT and the detection of the enzyme activity using PicoGreen dye in fluoroscopic method. For the PicoGreen, it enhanced specifically upon binding to dsDNA or DNA: RNA hybrids and it is relatively nonfluorescent in the presence of ssDNA (Seville *et al.*, 1996). Therefore, the fluorometric method can be used to monitor the polymerase activity of HIV-1 reverse transcriptase. This protocol is the first report of using PicoGreen dye for a kinetic study. Because the routine investigation of enzyme/inhibitor activity is required, we are interested in developing a less cumbersome assay to examine the HIV-1 RT activity. The development of a new approach for measuring the HIV-1 RT activity was discussed in this paper.

MATERIALS AND METHODS

Materials

The pGEX3X vector was used for cloning and protein expression in bacteria *Escherichia coli* strain JM109 and BL21 (DE3). Then, protein samples were purified by using DEAE cellulose and Phosphor cellulose P11 (Whatman). RT activity of the purified RT protein was determined by using EnzChek RT Assay Kit (Molecular Probe)

Construction and expression of recombinant HIV RT plasmid

The cDNA of the full length HIV genome was kindly provided by the Department of Medical Sciences, Ministry of Public Health, Thailand (HIVHXB2CG, Accession number K03455). The HIV RT gene was amplified from cDNA of the HIV genome by the Polymerase Chain Reaction (PCR) using a forward primer, p66F (5' GTCCCCATACTAGGTCCCATTAGCCCT ATTGAGACTGTA'3) for both hetero subunits coding sequences, and two reverse primers, p66R

(5' CGGGGATCCTTATAGTACTTTTCCT GATTCCAGCACTGA'3) and p51R (5' CACGAATTCTTAGAAGGTTT CTGCTCCTAC TATGGGTTCTTT'3) to amplify RT66 and RT51 open reading frames (ORF), respectively. The primers were designed from a sequence of HIV RT gene in the database (Gene Bank, Accession number K03455) and restriction enzyme sites were added into the sequences. The *Eco*N I site was added into a 5' end of forward primer, while *Bam*H I and *Eco*R I and a termination, TAA, codon was added into 5' end of the reverse primers for cloning RT66 and RT51 ORFs, respectively. Both PCR products, RT66 and RT51 DNA fragments, were purified by an ethanol precipitation. The expression plasmid (pGEX3X) and the DNA fragments were cut by using appropriate restriction enzymes. The GST encoding region in the pGEX3X was also removed. The excised DNA fragments were ligated into the expression vector. The recombinant plasmid harboring HIV RT genes were called pGXRT66 or pGXRT51, which were under control of *tac* (T7/Lac) promoter and carried an ampicillin resistant gene. Each plasmid was individually transformed into *Escherichia coli* strain BL21 (DE3). The plasmids DNA containing RT66 and RT51 were isolated from the recombinant *E. coli* and the RT sequences were confirmed by DNA sequencing (Macrogen, Korea). The cells containing plasmid pGXRT66 or pGXRT51 were cultured in 5 ml Luria-Bertani (LB) containing ampicillin (100µg/ml) with shaking at 220 rpm at 37 °C, overnight. Then, 5 ml of cell suspension was inoculated in a flask containing 500 ml LB with ampicillin and cultured with shaking at 220 rpm at room temperature until O.D.₆₀₀ reached 0.4. The culture was placed at 25 °C with shaking at 220 rpm and the gene expression was induced by adding lactose into the cultures with a final concentration of 0.5% (w/v). After 4 h of induction, the induced cells were harvested by centrifugation at 4,000 g for 10 minutes and the expressed protein was detected

by SDS-PAGE.

Purification of recombinant HIV RT

The purification steps were adapted from North *et al.* (1994). Both pGXRT66 and pGXRT51 expressed cells were suspended in a lysis buffer composing of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF and 5% glycerol and lysed by sonication. Cell debris and inclusion bodies were removed by centrifugation at 10,000 g for 30 minutes, and then protein in supernatant was precipitated in 30 % saturated ammonium sulfate. After centrifugation at 10,000 g for 30 minutes, the pellet was dissolved in column buffer and dialyzed for 3 h against 1 l of the column buffer pH 7.5 (50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, 5% glycerol and 75 mM NaCl). The protein solution was applied onto a DEAE-cellulose column (Whatman) pre-equilibrated with the column buffer pH 7.5, followed by elution with 0.5 M NaCl in the same buffer at 4 °C. The fractions containing both 66 and 51 kDa subunits of RT were pooled and mixed to get an active HIV-1 RT. The protein solution was dialyzed against column buffer, adjusted pH to 8.9 and then applied to a Phosphor cellulose P11 column (Whatman) equilibrated with the same column buffer pH 8.9 at 4 °C. Heterodimeric RT was eluted by gradient elution with 0-0.3 M NaCl in column buffer pH 8.9. Then, the active RT was further purified by Q-sepharose anion-exchange column and gradient eluted with the gradient 0-0.5 M NaCl in the column buffer pH 8.9 at 4 °C. The enzyme purity was determined by SDS-PAGE.

Fluorometric assay of RT activity

An EnzChek RT Assay Kit (Molecular Probes) was used for detecting the RT activity. The mixture containing 5 µl of 350 base-poly (A) ribonucleotide template and 5 µl of oligo d(T)16 primer in a nuclease-free microcentrifuge tube was incubated at room temperature for 1 h to allow

the primer to anneal to the template. Then, 0.1 µl of the mixture was added into the new tube containing 19.9 µl of polymerizing buffer (63 mM Tris-HCl, pH 8.1, 8 mM MgCl₂, 132 mM NaCl, and 13 mM DTT). The reaction was started by adding 5 µl of the enzyme solution (20 ng/µl) of each purification steps or purified enzyme into reaction mixture. The samples reaction was incubated at 37 °C for 30 minutes and stopped by adding 2 µl of 200 mM EDTA. The background fluorescence was normalized by subtracting a control reaction which was prepared by adding the stopping solution before incubation. The enzyme kinetic assay was also determined by using the same protocol. The appropriated time course and amount of enzyme in the reaction were determined by adding varied amounts of 12-100 ng purified HIV-1 RT and incubated at 37 °C for 150 seconds. After that, at every 30 second, 5 µl of the reaction mixture was pipetted into a new tube containing EDTA to stop the reaction. For K_m determination, the polymerizing reaction was performed by adding a varied amount of 10-80 µM dTTP substrates, 60 µl reaction mixtures were used and the reaction process was followed for 12 minutes. Every 1 minute, 5 µl of the reaction mixture was pipetted into a new tube containing EDTA to stop the reaction. Then, the polymerizing activity in each tube was measured using a fluorometric assay by adding 600 µl of PicoGreen at 1:400 dilution in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) to the EDTA-terminated reaction mixture and incubated for 5-15 minutes on ice, in the dark. The mixture solution was pipetted into a fluorometric micro cuvette and the fluorescence intensity was measured by using excitation and emission at 502 nm and 523 nm, respectively.

RESULTS AND DISCUSSION

Construction, design, and expression of RT

Since instability of HIV-1 RT heterodimerization in *E. coli* had been reported

(Muller *et al.*, 1991), we constructed individual plasmids to express HIV-1 RT subunit p66 and p51. The p66 and p51 coding sequences of HXB2 HIV-1 RT were amplified from a plasmid containing *pol-gag* gene using PCR with specific primers. The PCR products showed the expected DNA fragments of approximately 1,700 bp and 1,400 bp for p66 and p51 heterosubunit, respectively (Figure 1A). After digestion and purification of the PCR products, the DNA fragments were ligated into plasmid pGEX3X. The resulting plasmid, pGXRT66 and pGXRT51 (Figure 1B and C), were individually transformed into the *E. coli* strain BL21 (DE3).

The expression of plasmids containing the p66 or p51 ORFs under the control of the *tac* promoter was induced by 0.5% (w/v) lactose. Upon induction, significant amounts of new proteins appear as major bands with molar masses of 66 kDa or 51 kDa, respectively (Figure 2, lane A). However, the overexpression of reverse transcriptases can be found in both soluble and inclusion bodies fractions as previously reported (Hizi *et al.*, 1988; Koller *et al.*, 1995). The protein seems to be distributed in both fractions equally, we only used the soluble fractions for further purification and got enough yield for kinetics studies.

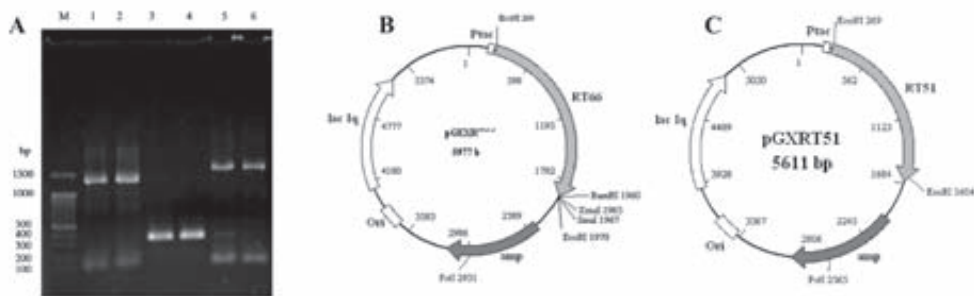


Figure 1 The PCR amplified DNA fragments (A), RT p51 coding DNA (lane 1 and 2), RT p66 coding DNA (lane 5 and 6), and RT expression plasmid constructions, pGXRT66 (B) and pGXRT51(C).

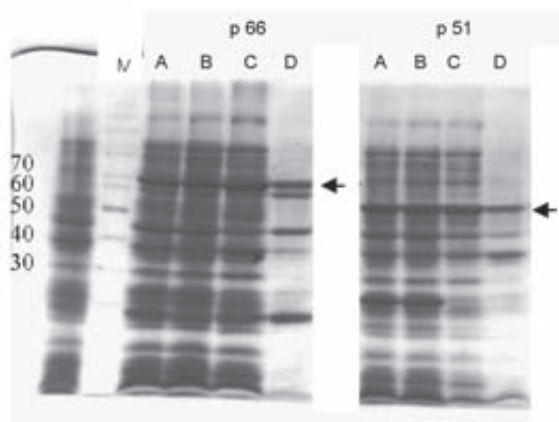


Figure 2 The individual expression of heterodimeric p66 and p51 proteins in BL21 (DE3) cell, protein ladder (M), total cell lysate (A), soluble fraction (B), 30% ammonium sulfate precipitation (C) and inclusion bodies (D).

Purification of heterodimeric forms of RT

Purification of heterodimeric RT was adapted from North *et al.* (1994). The expressed recombinant cells were lysed by sonication, inclusion body fractions were removed by centrifugation at 12,000 g for 30 minutes. The protein p66 and p51 were obtained from 30% saturated ammonium sulfate protein precipitation from soluble protein fraction (Figure 2). Each dialyzed protein solution was applied to anion

exchange DEAE cellulose. Under these conditions, the p66 and p51 were found in the unbound fraction (Figure 3A and B). The fractions of targeted proteins were pooled and further purified by Phosphor cellulose P11 column which can bind to nucleic acid binding proteins. The heterodimeric enzyme was detected in the bound fraction which was eluted by gradient NaCl (Figure 4), and then fractions containing both subunits were pooled together.

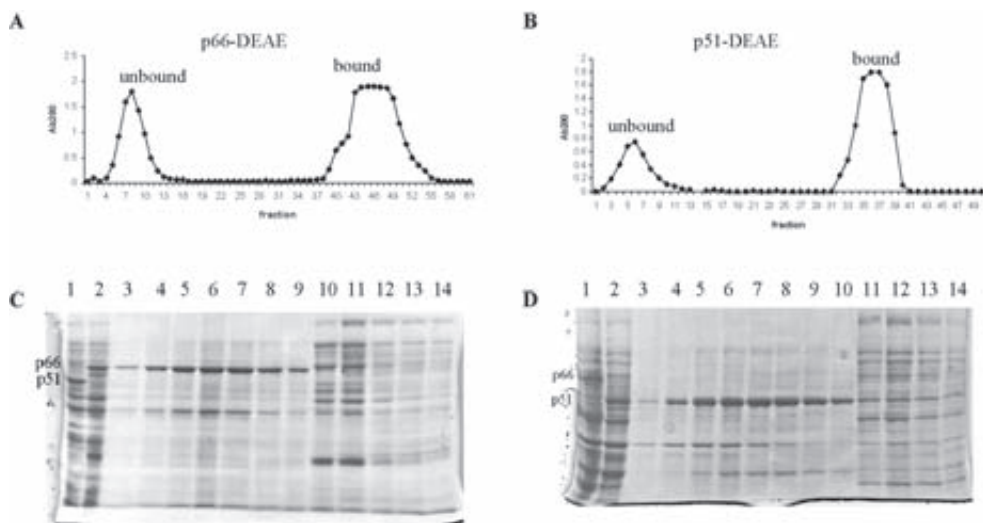


Figure 3 DEAE Purification of HIV-1 RT subunit p66 and p51. The elution profiles from DEAE cellulose column, p66 (A) and p51 (B) and SDS-PAGE (12%) of p66 (C) and p51 (D), lanes 1 and 2 (C and D) are crude proteins. C, unbound fractions in lanes 3-9 and bound fractions in lanes 10-14 for p66 purification. D, unbound fractions in lanes 3-10, bound fractions in lanes 11-14 for p51 purification.

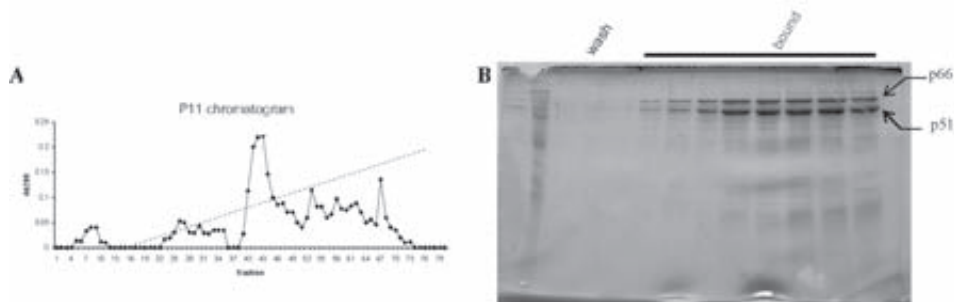


Figure 4 Purification of heterodimeric RT using Phosphor cellulose P11 column. A, the elution profile from the column by a linear gradient of NaCl concentration, 0-0.3M (spot line). B, SDS-PAGE of representative fractions (fractions 40-47) showing heterodimeric RT protein eluting from the column.

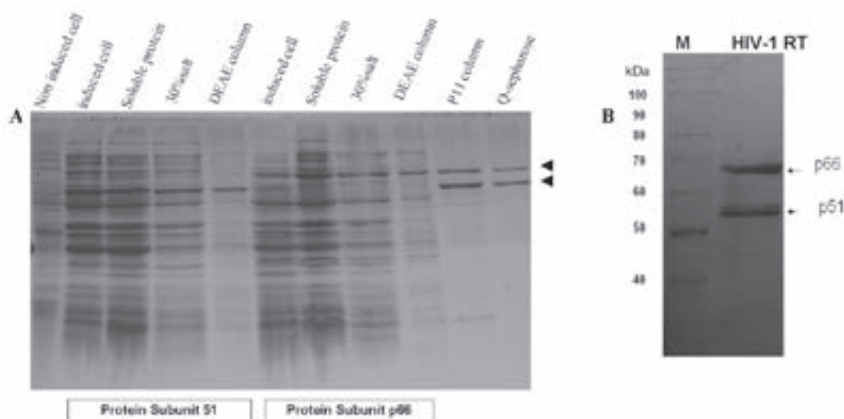


Figure 5 SDS-PAGE (12%) electrophoresis of purified recombinant HIV-1 RT. A, showing the protein after passing each purification step. B, representative molecular weights of the purified heterodimeric RT (66 kDa for p66 and 51 kDa for p51).

The last step of the purification protocol (anion exchange chromatography) was carried out mainly to ensure the removal of contaminating proteins. The heterodimeric RT was applied to the anion exchange Q-sepharose column and the purity of the protein in purification steps are shown in Figure 5. The purified heterodimeric RT showed appropriated sizes, 66 kDa and 51 kDa (Figure 5B). Using these purification protocols, we were able to prepare approximately 2 mg of the heterodimeric forms of RT from 1 liter of

E. coli culture. The protein yield was lower than that from previously reported (6-10 mg) (Hou *et al.*, 2004), which may be due to the multiple purification steps. However, the homogeneity and purity of the recombinant HIV-1 RT are the most important issue since the RT will be used for further kinetics study and X-ray crystallography. A summary of the purification and yield after passing each purification step is shown in Figure 5 and Table 1. The specific activities of the HIV-1 RT heterodimer preparations were defined

Table 1 Purification of the recombinant HIV-1 reverse transcriptase (RT) expressed in *E. coli*.

Protein fraction	Total protein (mg)	Fluorescence (Intensity)	Specific activity ² (Intensity/mg)	Purification ratio ³ (Specific activity of purification step/ Specific activity of cell lysate)
Cell lysate ¹	61.9	63.56	1.03	1.00
Soluble fraction ¹	60.2	243.81	4.05	3.93
30% NH ₂ SO ₄ precipitate ¹	49.34	42.86	0.87	0.85*
DEAE column ¹	28.59	200.89	7.03	6.83
P11 column	7.75	100.09	12.91	12.53
Q-sepharose column	1.9	190.55	100.29	97.37

¹ the subunit p51 and p66 protein solutions were mixed together (1:1) and used for RT activity measurement.

² specific activities were calculated by Fluorescence intensity per amount of protein (mg).

³ purification ratios can be calculated as specific activity of each purification step per specific activity of cell lysate.

* the value was decreased by (NH₄)₂SO₄.

by enzyme activity as fluorescence intensity per amount of protein (mg) and showed 97-fold increasing compared to the crude protein.

Since the expression of HIV-1 RT using bacterial system had been first reported in 1987 (Larder *et al.*, 1987), many purification protocols were improved, for example, single step purification using HPLC (Fletcher *et al.*, 1996), two steps using metal-binding column with His-tag (Stuart *et al.*, 1990, Maier *et al.*, 1999), but the purification yield and the effect of tag-RT on protein kinetic activity are not satisfactorily obtained. Using our purification protocol, the purified HIV-1 RT gave enough yields for kinetic study and can be improved for conventional protocol for RT purification.

Fluorometric assay of RT activity

To avoid the disadvantage of isotopic method, we applied a fluorescence dye, PicoGreen, for the determination of recombinant HIV-1 RT activity. PicoGreen can interact with heteroduplex RNA/DNA as previously reported (Singer *et al.*, 1997). The fluorometric assay can detect enzyme activity after each purification step as shown in Table 1. However, the purification by precipitation with ammonium sulfate precipitation showed the abnormal activity value in which the specific activity and purification ratio were decreased. It possible that $(\text{NH}_4)_2\text{SO}_4$ salt can affect the assay,

the decreasing of fluorescence signal by ammonium sulfate was described (Singer *et al.*, 1997). The purified RT activity assay showed the increase of fluorescent signals related to time and RT amount as shown in Figure 6A. To determine relationship between reaction rate and amount of RT, the reaction rate (fluorescent intensity/second) from figure 6A were plotted with amount of RT. The increasing of RT causes elevation of the reaction rate as showed in Figure 6B. The relationship between amount of substrate dTTP and reaction rate was determined by varying amount of dTTP between 10 and 80 μM , the Figure 7A showed increasing of reaction rate (V) related to dTTP concentration (S). The K_m of dTTP was obtained by *Lineweaver-Burk plot* ($1/V$ versus $1/S$, Y axis and X axis, respectively) (Figure 7B) and X co-ordinate showed $-1/K_m$, while Y co-ordinate showed $1/V_{\text{max}}$. By three-repeated experiments, the results showed an average value for K_m as $10.51 \pm 2.58 \mu\text{M}$ for dTTP. This value was within the range reported by other investigations, for example, the K_m for dTTP was 3.05-18.7 μM and $7.7 \pm 1.56 \mu\text{M}$ by using the isotopic method (Kasai *et al.*, 2002) and, the fluorometric assay with 4', 6'-diamidino-2-phenylindole (Chavan and Prochaska, 1995). The different assays and reagent compositions could cause variation in kinetic parameters.

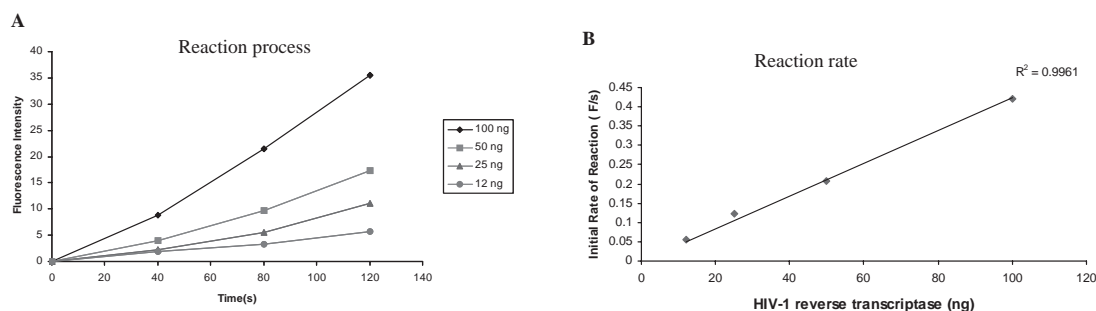
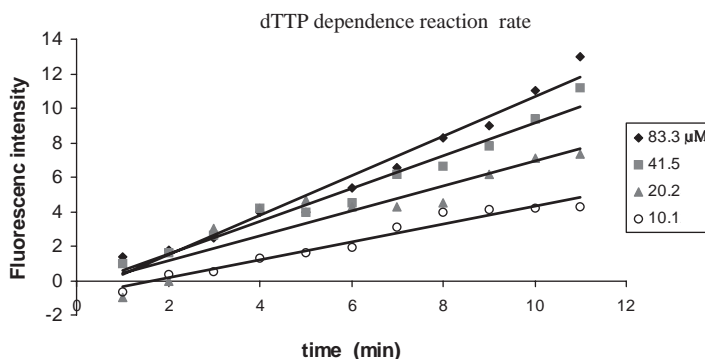
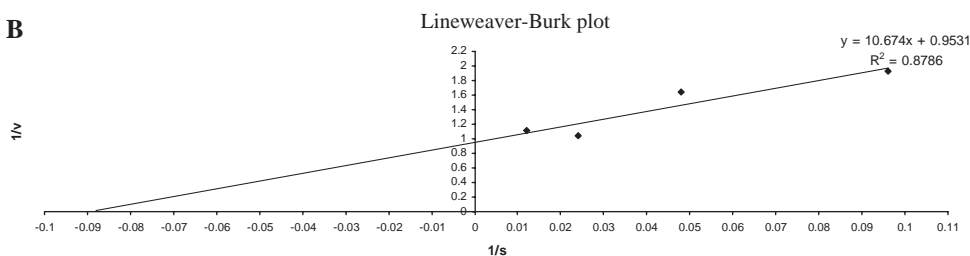


Figure 6 The recombinant HIV-1 RT activity measured by the fluorometric method. A, The reaction rates depend on the amounts of RT. B, The linear relationship between enzyme concentration and reaction rate.

A



B



* Slope (V) from Figure A and dTTP concentration (S) were plotted with Lineweaver-burk plot

Figure 7 Relationship between substrate concentration and reaction rate. A, showing the effect of varying the concentration of dTTP on the reaction rate of the RNA-dependent DNA polymerase activity of HIV-1 reverse transcriptase. B, a Lineweaver-Burk plots determining the K_m for dTTP.

CONCLUSION

We reported cloning, expression and purification of HIV-1 RT in *E. coli*. The homogeneous recombinant HIV-1 RT enzyme could be obtained after four steps of purification, but the yield was less than other reports. However, our purification yielded the higher purity and homogeneity of the enzyme which is suitable for further experiments such as enzyme/inhibitor kinetics study and protein X-ray crystallography experiment. This paper also showed that the enzyme kinetics and activity could be rapidly determined by the fluorometric method using PicoGreen dye. This method is easy for an enzyme activity assay and high-throughput for drug screening.

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LITERATURE CITED

- Chavan, S. J. and H. J. Prochaska. 1995. Fluorometric measurement of reverse transcriptase activity with 4', 6-diamidino-2-phenylindole. **Anal. Biochem.** 225: 54-59.
- Fletcher, R. S., G. Holleschak, E. Nagy, D. Arion, G. Borkow, Z. Gu, M. A. Wainberg and M. A. Parniak. 1996. Single-step purification of

- recombinant wild-type and mutant HIV-1 reverse transcriptase. **Protein Expr. Purif.** 7: 27-32.
- Hizi, A., C. McGill and S. H. Hughes. 1988. Expression of soluble, enzymatically active, human immunodeficiency virus reverse transcriptase in *Escherichia coli* and analysis of mutants. **Proc. Natl. Acad. Sci. USA.** 85: 1218-1222.
- Hou, E. W., R. Prasad, W. A. Beard and S. H. Wilson. 2004. High-level expression and purification of untagged and histidine-tagged HIV-1 reverse transcriptase. **Protein Expr. Purif.** 34: 75-86.
- Kasai, N., Y. Mizushima, F. Sugawara and K. Sakaguchi. 2002. Three-dimensional structural model analysis of the binding site of an inhibitor, nervonic acid, of both DNA polymerase β and HIV-1 reverse transcriptase. **J. Biochem.** 132: 819-828.
- Koller, G., K. Graumann, W. Kramer, M. Sara and A. Jungbauer. 1995. Laboratory-scale production and purification of recombinant HIV-1 reverse transcriptase. **J. Chromatogr.** 664: 107-118.
- Larder, B., D. Purifoy, K. Powell and G. Darby. 1987. AIDS virus reverse transcriptase defined by high level expression in *Escherichia coli*. **EMBO J.** 6(10): 3133-3137.
- Maier, G., U. Dietrich, B. Panhans, B. Schroder, H. Rubsamen-Waigmann, L. Cellai, T. Hermann and H. Heumann. 1999. Mixed reconstitution of mutated subunits of HIV-1 reverse transcriptase co-expressed in *Escherichia coli*: two tags tie it up. **Eur. J. Biochem.** 261: 10-18.
- Muller, B., T. Restle, H. Kuhnel and R. S. Goody. 1991. Expression of the heterodimeric form of human immunodeficiency virus type 2 reverse transcriptase in *Escherichia coli* and characterization of the enzyme. **J. Biol. Chem.** 266: 14709-14713.
- Muller, B., T. Restle, S. Weiss, M. Gautel, G. Sczakiel and R. S. Goody. 1989. Co-expression of the subunits of the heterodimer of HIV-1 reverse transcriptase in *Escherichia coli*. **J. Biol. Chem.** 264: 13975-13978.
- North, T. W., G. L. Hansen, Y. Zhu, J. A. Griffin and C. K. Shih. 1994. Expression of reverse transcriptase from feline immunodeficiency virus in *Escherichia coli*. **Antimicrob. Agents Chemother.** 38(2):388-391.
- Seville, M., A. B. West, M. G. Cull and C. S. McHenry. 1996. Fluorometric assay for DNA polymerases and reverse transcriptase. **Biotechniques** 21: 664-672.
- Singer, V. L., L. J. Jones, S. T. Yue and R. P. Haugland. 1997. Characterization of PicoGreen reagent and development of a fluorescence-based solution assay for double-stranded DNA quantitation. **Anal. Biochem.** 249: 228-238.
- Stuart, F., J. Le Grice and F. Gruninger-Leitch. 1990. Rapid purification of homodimer and heterodimer HIV-1 reverse transcriptase by metal chelate affinity chromatography. **Eur. J. Biochem.** 187: 307-314.