

Title	Enzymatic characterization of human immunodeficiency virus type 1 reverse transcriptase for use in cDNA synthesis.
Author(s)	Konishi, Atsushi; Shinomura, Mayu; Yasukawa, Kiyoshi
Citation	Applied biochemistry and biotechnology (2013), 169(1): 77-87
Issue Date	2013-01
URL	http://hdl.handle.net/2433/169949
Right	The final publication is available at www.springerlink.com
Type	Journal Article
Textversion	author

Enzymatic Characterization of Human Immunodeficiency Virus Type 1 Reverse Transcriptase for Use in cDNA Synthesis

Atsushi Konishi · Mayu Shinomura · Kiyoshi Yasukawa

Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

DMSO, dimethyl sulfoxide; HIV-1, human immunodeficiency virus type 1; HIV-1 M, HIV-1 group M; HIV-1 O, HIV-1 group O; MMLV, Moloney murine leukemia virus; PMSF, phenylmethylsulfonyl fluoride; RT, reverse transcriptase; T/P, template-primer; TCA, trichloroacetic acid.

*Correspondence author. Tel.: +81 75 753 6267; fax: +81 75 753 6265.

E-mail: yasukawa@kais.kyoto-u.ac.jp (K. Yasukawa).

Abstract The aim of this study is to explore the advantages of using human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) in cDNA synthesis. Recombinant HIV-1 group M (HIV-1 M) RT and HIV-1 group O (HIV-1 O) RT were produced in an *Escherichia coli* expression system. In the incorporation of dTTP into poly(rA)-p(dT)₁₅ (T/P), the K_m values for dTTP of HIV-1 M RT and HIV-1 O RT were 8 and 12% of that of Moloney murine leukemia virus (MMLV) RT, respectively, and the K_m values for T/P were 25 and 23% of that of MMLV RT, respectively. Compared with MMLV RT, HIV-1 M RT and HIV-1 O RT were less susceptible to formamide, which is frequently used for cDNA synthesis with a G+C-rich RNA to improve specificity. The high substrate affinity and low susceptibility to formamide of HIV-1 RT might be advantageous for its use in cDNA synthesis.

Keywords cDNA · formamide · HIV-1 · MMLV · reverse transcriptase

Introduction

Reverse transcriptase (RT) is the enzyme responsible for viral genome replication. Moloney murine leukemia virus (MMLV) RT and Avian myeloblastosis virus (AMV) RT are widely used in cDNA synthesis. MMLV RT is a 75-kDa monomer comprising the fingers, palm, thumb, and connection subdomains and the RNase H domain [1–3]. AMV RT is a heterodimer consisting of a 63-kDa α subunit and a 95-kDa β subunit. Human immunodeficiency virus type 1 (HIV-1) RT is widely used as a tool in the development of inhibitors for the treatment of HIV-1 infection. It is a heterodimer consisting of a 51-kDa subunit (p51) and 66-kDa subunit (p66). p66 comprises the same five domains as MMLV RT, and p51 comprises the fingers, palm, thumb, and connection subdomains but lacks the RNase H domain [4–6]. HIV-1 RT exists as a heterodimer as well as a homodimer, and the dissociation constants (K_d) of p51 and p66, p66 and p66, and p51 and p51 were reported to be 0.31, 4.2, and 230 μ M, respectively [7]. HIV-1 is classified into four phylogenetic major groups: M (main), O (outlier), N (non-M/non-O), and P. Group M infection is spread all over the world. Group O infection is restricted to Cameroon and neighboring countries in West Central Africa [8]. The amino acid sequence homology between HIV-1 group M (HIV-1 M) RT and HIV-1 group O (HIV-1 O) RT is 79% [8].

It is well known that the fidelity of HIV-1 RT is lower than those of MMLV RT and AMV RT [9]. Concerning this, Barrioluengo et al. reported that two single mutations (Lys65 \rightarrow Arg and Arg78 \rightarrow Ala) and one double mutation (Lys65 \rightarrow Arg/Val75 \rightarrow Ile) increase the fidelity of HIV-1 O RT to the same level as MMLV RT [9]. In addition, Álvarez et al. reported that HIV-1 O RT is more thermostable than MMLV RT [10].

These results indicate that the HIV-1 O RT variants K65R, R78A, and K65R/V75I have the same fidelity as, and higher stability than MMLV RT.

Low fidelity of RT is problematic for cloning of cDNA. However, extremely high fidelity is not required for detection of a target RNA in RT-PCR and RNA-specific amplification assays. RNA-specific amplification is an isothermal reaction (41-43 °C) that is widely used in clinical diagnosis [11–14], in which RT synthesizes promoter-bearing double-stranded DNA with the help of its RNase H activity, and RNA polymerase continues *in vitro* transcription to produce copies of RNA fragments that are subsequently recycled as RNA templates for synthesis of promoter-bearing double-stranded DNA. To increase the sensitivity and rapidness, the reaction is carried out in the presence of deoxy-inosine triphosphate (dITP) and dimethyl sulfoxide (DMSO). The use of dITP surely decreases the fidelity of RT.

In this study, we made enzymatic characterization of HIV-1 RT. The results have shown that HIV-1 RT has high substrate affinity and low susceptibility to formamide. These characteristics might be advantageous for using HIV-1 RT in cDNA synthesis.

Materials and methods

Materials

p(dT)₁₅ was purchased from Life Technologies Japan Ltd. (Tokyo, Japan). [methyl-³H]dTTP (1.52 TBq/mmol) and poly(rA) were from GE Healthcare (Buckinghamshire, UK). The RT concentration was determined by the method of

Bradford [15] using Protein Assay CBB Solution (Nacalai Tesque, Kyoto, Japan) with bovine serum albumin (Nacalai Tesque) as standard. Standard RNA, an RNA of 1014-nucleotides corresponding to DNA sequence 8353–9366 of the *cesA* gene of *Bacillus cereus* (GenBank accession no. DQ360825), was prepared by an *in vitro* transcription [14].

Plasmids

The 2,002-bp DNA fragment encoding MMLV RT was amplified from the pUC19 plasmid that contains the complete MMLV gene (8,332 bp) (GenBank accession no. J02255) [14], using the primers MRF and MRHISB. The 1,340-bp DNA fragment encoding HIV-1 M RT p51 gene was amplified using HIVRTM-HISF and HIVRTMp51-B, and the 1,706-bp DNA fragment encoding HIV-1 M RT p66 was amplified using HIVRTM-F and HIVRTMp66-B, both from the artificially synthesized HIV-1 M RT gene (Fig. S1A). The 1,340-bp DNA fragment encoding HIV-1 O RT p51 gene was amplified using HIVRTO-HISF and HIVRTOp51-B, and the 1,706-bp DNA fragment encoding HIV-1 O RT p66 was amplified using HIVRTO-F and HIVRTOp66-B, both from the artificially synthesized HIV-1 O RT gene (Fig. S1B). The nucleotide sequences of the primers are shown in Table S1. These amplified DNAs were digested with *NdeI* and *EcoRI*, and inserted in pET-22b(+) to produce pET-MRTHis, pET-HisHIVMp51, pET-HIVMp66, pET-HisHIVOp51, and pET-HIVOp66 (Fig. 1).

Expression and Purification of MMLV RT

BL21(DE3) [*F*⁻, *ompT*, *hsdS_B*(*r_B*-*m_B*⁻) *gal dcm* (DE3)] cells were transformed with pET-HisMRT. The overnight culture of the transformants (8 mL) was added to 800 mL of L broth and incubated with shaking at 37°C. When *OD*₆₆₀ reached 0.6–0.8, 0.8 mL of 0.5 M IPTG was added and growth was continued at 30°C for 4 h. After centrifugation at 10,000 × *g* for 10 min, the cells were harvested, suspended with 20 mL of 10 mM Tris-HCl, 0.4% v/v Triton X-100, 10% v/v glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.5 and disrupted by sonication. After centrifugation at 15,000 × *g* for 20 min, the supernatant was collected, to which solid (NH₄)₂SO₄ was added to a final concentration of 40% saturation. After centrifugation at 20,000 × *g* for 20 min, the pellet was collected and dissolved in 6 mL of 0.02 M potassium phosphate, 2.0 mM dithiothreitol (DTT), 10% glycerol, pH 7.2 (0.02 M PDG) and dialyzed against the same buffer. The dialyzed solution was diluted to 60 mL with 2.0 mM DTT, 10% glycerol, pH 7.2 (DG) and applied to a column [25 mm (inner diameter) × 120 mm] packed with Toyopearl DEAE-650M gel (Tosoh, Tokyo, Japan) equilibrated with 0.02 M PDG. The bound RT was eluted with 0.3 M potassium phosphate, 2.0 mM DTT, 10% glycerol, pH 7.2 (0.3 M PDG). The eluate (10–15 mL) was diluted to 100 mL with 0.02 M PDG containing 20 mM imidazole and applied to the column packed with a Ni²⁺-sepharose (HisTrap HP 1 mL, GE Healthcare, Buckinghamshire, UK) equilibrated with 0.02 M PDG containing 20 mM imidazole. The bound RT was eluted with 0.02 M PDG containing 500 mM imidazole. The eluate was dialyzed against 20 mM potassium phosphate, 2.0 mM DTT, 50% glycerol, pH 7.2, and stored at -80°C.

Expression and Purification of HIV-1 M RT and HIV-1 O RT

BL21(DE3) cells were transformed with pET-HisHIVMp51, pET-HIVMp66, pET-HisHIVOp51, or pET-HIVOp66, cultured, harvested, and suspended by the same method used for MMLV RT. For the production of HIV-1 M RT, the suspension containing the N-terminally (His)₆-tagged HIV-1 M p51 and that containing the untagged HIV-1 M p66 were mixed. For the production of HIV-1 O RT, the suspension containing the N-terminally (His)₆-tagged HIV-1 O p51 and that containing the untagged HIV-1 O p66 were mixed. HIV-1 M RT and HIV-1 O RT were purified and stored by the same methods used for MMLV RT.

Measurement of RT activity to Incorporate dTTP into poly(rA)-p(dT)₁₅

The activity to incorporate dTTP into poly(rA)-p(dT)₁₅ was measured as described previously [16]. Briefly, the reaction was carried out in 25 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM DTT, 5.0 mM MgCl₂, 25 μM poly(rA)-p(dT)₁₅ (this concentration expressed as that of p(dT)₁₅), 0.2 mM [³H]dTTP, and active fractions or purified preparations of RT at 37°C. An aliquot (20 μL) was taken from the reaction mixture at a predetermined time and immediately spotted onto the glass filter GF/C 2.5 cm (Whatman, Middlesex, UK). The amounts of [³H]dTTP incorporated was counted, and the initial reaction rate was determined.

Measurement of RT Activity for cDNA Synthesis

The activity for cDNA synthesis was measured as described previously [14]. Briefly, the reaction (20 μL) was carried out in 25 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM

DTT, 0.1 mM dNTP, 0.5 μ M DNA primer (R12), 0.8 pg/ μ L (corresponding to 2 pM) standard RNA, 0.05 μ g/ μ L *E. coli* RNA, and various concentrations (0.1–1000 nM) of RT at 46°C for 30 min and stopped by heating at 95°C for 5 min. The PCR reaction (30 μ L) was then carried out in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.5 μ M (each) DNA primers (F5 and RV), 0.1 mM dNTP, 10% v/v product of the reverse transcriptase reaction, and 0.05 U/ μ l *Taq* polymerase. The cycling parameters were 95°C for 30 s, followed by 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s. The amplified products were separated on 1.0% agarose gels and stained with ethidium bromide (1 μ g/mL).

Analysis of Irreversible Thermal Inactivation of RT

Irreversible thermal inactivation of RT was analyzed as described previously [16]. Briefly, the thermal inactivation was carried out in 10 mM potassium phosphate (pH 7.6), 2.0 mM DTT, 0.2% v/v Triton X-100, 10% glycerol, 28 μ M poly(rA)-p(dT)₁₅ and 20 nM MMLV RT, 500 nM HIV-1 M RT, or 200 nM HIV-1 O RT at a range of temperatures from 42 to 50°C for 10 min followed by incubation on ice for 30–60 min. The remaining RT activity to incorporate dTTP into poly(rA)-p(dT)₁₅ was determined at 37°C as described above.

Results

Production of Recombinant MMLV RT, HIV-1 M RT, and HIV-1 O RT

To achieve a high-level production, we selected the C-terminus for MMLV RT and the N-terminus for HIV-1 M RT and HIV-1 O RT as the (His)₆ binding site and 30°C for all RTs as the *E. coli* culture temperature after IPTG induction. Figure 1 shows five expression plasmids, pET-MRTHis for C-terminally (His)₆-tagged MMLV RT, pET-HIVMp66 for untagged HIV-1 M p66, pET-HisHIVMp51 for N-terminally (His)₆-tagged HIV-1 M p51, pET-HIVOp66 for untagged HIV-1 O p66, and pET-HisHIVOp51 for N-terminally (His)₆-tagged HIV-1 O p51.

Purification of MMLV RT was carried out from the *E. coli* cells containing the C-terminally (His)₆-tagged MMLV RT. Purification of HIV-1 M RT and HIV-1 O RT was carried out from the mixture of the cells containing the untagged p66 and those containing the N-terminally (His)₆-tagged p51. Ammonium sulfate fractionation was used as the first step, ion-exchange column chromatography as the second step, and Ni²⁺ affinity column chromatography as the third step. The active fraction at the third step was dialyzed against the solution containing 50% glycerol. Figure 2 shows the results of SDS-PAGE analysis of the active fractions at each purification stage and the purified enzyme preparations. MMLV RT yielded a single band with a molecular mass of 75 kDa, and HIV-1 M RT and HIV-1 O RT yielded two bands with molecular masses of 51 and 66 kDa. Representative purification is shown in Table S2.

Activities of Recombinant MMLV RT, HIV-1 M RT, and HIV-1 O RT

Figure 3 shows the results of steady-state kinetic analysis of RT in the incorporation of

dTTP into poly(rA)-p(dT)₁₅ (T/P). The dependences of the initial reaction rate (v_o) on dTTP (Fig. 3A) and T/P (Fig. 3B) concentrations exhibited the Michaelis-Menten curves. The $K_{m,dTTP}$, $K_{m,T/P}$, and k_{cat} values are summarized in Table 1. The $K_{m,dTTP}$ and $K_{m,T/P}$ values of the HIV-1 M RT and HIV-1 O RT were in the range of 8–25% of those of MMLV RT, suggesting that HIV-1 RT has higher affinity for dTTP and T/P than MMLV RT. The k_{cat} values of HIV-1 M RT and HIV-1 O RT were 1.2 and 3.8% of that of MMLV RT, respectively. Consequently, the k_{cat}/K_m values of HIV-1 M RT and HIV-1 O RT were in the range of 5–32% of those of MMLV RT.

Figure 4 shows cDNA synthesis activities of RT. The reaction was carried out at 46°C for 30 min with varying enzyme concentrations of 0–1,000 nM, followed by PCR of the cDNA synthesis reaction products and agarose gel electrophoresis of the PCR products. In all RTs, the amplified product with the expected size of 601 bp was detected for the enzyme concentrations of 0.1–10 nM, but not for those of 50–1,000 nM. This indicates that all RTs have similar cDNA synthesis activities.

Formamide and dimethyl sulfoxide (DMSO) are frequently used for the polymerase chain reaction (PCR) with a G+C-rich DNA to improve specificity [18–20]. Figure 5 shows the activities of RT to incorporate dTTP into T/P in the presence of various concentrations (0–30% v/v) of formamide or DMSO at 42°C. The relative activity was defined as the ratio of the initial reaction rate in the presence of the indicated organic solvent to that in its absence (61.8 nM s⁻¹ for MMLV RT, 34.0 nM s⁻¹ for HIV-1 M RT, and 33.9 nM s⁻¹ for HIV-1 O RT). Formamide and DMSO inhibited the activity in a dose-dependent manner. The HIV-1 M RT and HIV-1 O RT activities were slightly enhanced in the presence of 4–18% formamide, and the MMLV RT activity was slightly enhanced in the presence of 2–16% DMSO. The concentrations required to decrease the

activities of MMLV RT, HIV-1 M RT, and HIV-1 O RT to 50% of the maximum activity (IC_{50}) of formamide were 8, 22, and 22%, respectively, and those of DMSO were 21, 16, and 16%, respectively. This indicates that Formamide affects MMLV RT more potently than HIV-1 M RT and HIV-1 O RT, while DMSO affects HIV-1 M RT and HIV-1 O RT more potently than MMLV RT.

Stabilities of Recombinant MMLV RT, HIV-1 M RT, and HIV-1 O RT

Figure 6 shows the remaining activities of RT to incorporate dTTP into T/P after thermal treatment at 42–50°C for 10 min. The relative activities, which were defined as the ratio of the initial reaction rate with incubation at the indicated temperature for 10 min to that without incubation, of all RTs decreased with increasing temperature. The relative activities of HIV-1 O RT were higher than those of MMLV RT and HIV-1 M RT at all temperatures examined. The temperatures reducing initial activity by 50% in 10-min incubation, T_{50} , of MMLV RT, HIV-1 M RT, and HIV-1 O RT were estimated about 44, 42, and 46°C. This suggests that the thermal stability of HIV-1 O RT is slightly higher than those of MMLV RT and HIV-1 M RT.

Discussion

In the dTTP incorporation reaction, the K_m and k_{cat} values of HIV-1 M RT and HIV-1 O RT were lower than those of MMLV RT (Fig. 3, Table 1). This finding suggests that HIV-1 RT has higher substrate affinity but lower enzyme processivity than

MMLV RT. All three RTs exhibited similar cDNA synthesis activities (Fig. 4). In the dTTP incorporation reaction, the initial substrate concentrations (0.2 mM dTTP and 25 μ M T/P) were higher than the enzyme concentration (2–20 nM), whereas in the cDNA synthesis reaction, the initial substrate concentration (2 pM target RNA) was lower than the enzyme concentration (0.1–10 nM). In most cDNA synthesis reactions, the initial concentration of target RNA is considerably lower than that of the RT. Thus, HIV-1 RT has an advantage over MMLV RT because it has a higher substrate affinity. HIV-1 can infect both dividing and non-dividing cells, whereas, MMLV can infect only dividing cells. The clear difference in kinetic parameters can be explained by the fact that RT has evolved to facilitate viral replication under specific cellular environments with different substrate concentrations. Such differences for recombinant RTs were reported in feline leukemia virus and feline immunodeficiency virus [21].

All RTs exhibited similar cDNA synthesis defects at high enzyme concentrations (50–1,000 nM). Similar result was obtained for the MMLV RT variant D524A, wherein the catalytically important residue for RNase H activity, Asp524, was substituted with Ala to abolish RNase H activity and increase stability (data not shown). We speculate that this defect is due to increased nonspecific binding of RT to T/P rather than enhanced RNA cleavage.

We previously reported that 6–8% formamide and 12% DMSO improved the efficiency of MMLV RT-catalyzed cDNA synthesis at low temperatures (25–34°C) [20]. We presume this effect is due to the increase in primer specificity caused by reducing secondary RNA structure and nonspecific binding of the primer, as previously reported [18, 19]. Formamide is also used in RNA isolation [22] and storage [23]. In this study, compared with MMLV RT, HIV-1 M RT and HIV-1 O RT were less susceptible to

formamide but more susceptible to DMSO (Fig. 5), suggesting that formamide more effectively enhances cDNA synthesis by HIV-1 RT. The mechanism underlying the difference in the inhibitory effects of DMSO and formamide on MMLV RT and HIV-1 RT activity is unknown.

HIV-1 O RT exhibited slightly higher thermal stability than MMLV RT and HIV-1 M RT (Fig. 6), which is in agreement with the previous report [10]. The reason for the difference in stability of HIV-1 M RT and HIV-1 O RT is unknown. We generated a highly thermostable MMLV RT variant, E286R/E302K/L435R/D524A, by introducing positive charges into the positions that have been implicated in the interaction with T/P by site-directed mutagenesis [24]. We also generated a highly stable AMV RT α subunit variant V238R/L388R/D450A by introducing the triple mutation (Val238→Arg/Leu388→Arg/Asp450→Ala) corresponding to the mutation introduced into MMLV RT (Glu286→Arg/Leu435→Arg/Asp524→Ala) [25]. Same strategy might be applicable to stabilize HIV-1 M RT and HIV-1 O RT.

In conclusion, both HIV-1 M RT and HIV-1 O RT exhibited high affinity for DNA-primed RNA template and low susceptibility to formamide. Our results suggest that the use of HIV-1 RT in cDNA synthesis is attractive for detection of a small number of target RNA molecules.

Acknowledgments

This study was supported in part (K.Y.) by Grants-in-Aid for Scientific Research (nos. 19580104 and 21580110) from the Japan Society for the Promotion of Science and Daiwa Securities Health Foundation.

References

- 1 Kimmel, A. R., & Berger, S. L. (1987) *Methods in Enzymology*, 152, 307–316.
- 2 Georgiadis, M. M., Jessen, S. M., Ogata, C. M., Telesnitsky, A., Goff, S. P., & Hendrickson, W. A. (1995) *Structure*, 3, 879–892.
- 3 Lim, D., Gregorio, G. G., Bingman, C., Martinez-Hackert, E., Hendrickson, W. A., & Goff, S. P. (2006) *Journal of Virology*, 80, 8379–8389.
- 4 di Marzo Veronese, F., Copeland, T. D., DeVico, A. L., Rahman, R., Oroszlan, S., Gallo, R. C., & Sarngadharan, M. G. (1986) *Science*, 231, 1289–1291.
- 5 Patel, P. H., Jacobo-Molina, A., Ding, J., Tantillo, C., Clark, A. D. Jr., Raag, R., Nanni, R. G., Hughes, S. H., & Arnold, E. (1995) *Biochemistry*, 34, 5351–5363.
- 6 Ding, J., Das, K., Hsiou, Y., Sarafianos, S. G., Clark, A. D. Jr., Jacobo-Molina, A., Tantillo, C., Hughes, S. H., & Arnold, E. (1998) *Journal of Molecular Biology*, 284, 1095–1111.
- 7 Venezia, C. F., Meany, B. J., Braz, V. A., & Barkley, M. D. (2006) *Biochemistry*, 48, 9084–9093.
- 8 Buonaguro, L., Tornesello, M. L., & Buonaguro, F. M. (2007) *Journal of Virology*, 81, 10209–10219.
- 9 Barrioluengo, V., Álvarez, M., Barbieri, D., & Menéndez-Arias, L. (2011) *Biochemical Journal*, 436, 599–607.
- 10 Álvarez, M., Matamoros, T., & Menéndez-Arias, L. (2009) *Journal of Molecular Biology*, 392, 872–884.
- 11 Kievits, T., van Gemen, B., van Strijp, D., Schukkink, R., Dircks, M., Adriaanse,

- H., Malek, L., Sooknanan, R., & Lens, P. (1991) *Journal of Virological Methods*, 35, 273–286.
- 12 Ishiguro, T., Saitoh, J., Horie, R., Hayashi, T., Ishizuka, T., Tsuchiya, S., Yasukawa, K., Kido, T., Nakaguchi, Y., Nishibuchi, M. & Ueda, K. (2003) *Analytical Biochemistry*, 314, 77–86.
- 13 Masuda, T., Yasukawa, K., Isawa, Y., Horie, R., Saitoh, J., Ishiguro, T., Nakaguchi, Y., Nishibuchi, M., & Hayashi, T. (2004) *Journal of Bioscience and Bioengineering*, 98, 236–243.
- 14 Yasukawa, K., Agata, N., & Inouye, K. (2010) *Enzyme Microbial Technology*, 46, 391–396.
- 15 Bradford, M. M. (1976) *Analytical Biochemistry*, 72, 248–254.
- 16 Yasukawa, K., Nemoto, D., & Inouye, K. (2008) *Journal of Biochemistry*, 143, 261–268.
- 17 Konishi, A., Nemoto, D., Yasukawa, K., & Inouye, K. (2011) *Bioscience, Biotechnology, and Biochemistry* 75, 1618–1620.
- 18 Chester, N., & Marshak, D. R. (1993) *Analytical Biochemistry*, 209, 284–290.
- 19 Sarkar, G., Kapelner, S., & Sommer, S. S. (1990) *Nucleic Acids Research*, 18, 7465.
- 20 Yasukawa, K., Konishi, A., & Inouye, K. (2010) *Bioscience, Biotechnology, and Biochemistry*, 74, 1925–1930.
- 21 Operario, D. J., Reynolds, H. M., & Kim, B. (2005) *Virology*, 335, 106–121.
- 22 Bowrin, V., Rouse-Miller, J., Sutton, F., & Sirju-Charran, G. (2012) *Phytochemical Analysis*, doi 10.1002/pca.2390.
- 23 Swinson, K., & Koban, M. (2005) *Journal of Biochemical and Biophysical*

Methods, 63, 149–153.

- 24 Yasukawa, K., Mizuno, M., Konishi, A., & Inouye, K. (2010) *Journal of Biotechnology*, 150, 299–306.
- 25 Konishi, A., Yasukawa, K., & Inouye, K. (2012) *Biotechnology Letters*, 34, 1209–1215.

Figure legends

Fig. 1 Expression plasmids. The structures of pET-MRTHis, pET-HIVMp66, pET-HisHIVMp51, pET-HIVOp66, and pET-HisHIVOp51 are shown. The asterisk indicates the termination codon

Fig. 2 SDS-PAGE analysis. CBB-stained 10% SDS-polyacryl amide gel is shown. (A-C) Active fractions of each purification stage for MMLV RT (A), HIV-1 M RT (B), and HIV-1 O RT (C). Lanes: marker proteins (lane 1), soluble fractions of the total extracts (lane 2), the centrifuged pellets after fractionation by ammonium sulfate at 40% saturation (lane 3), active fractions of ion-exchange chromatography (lane 4) and Ni²⁺ affinity chromatography before (lane 5) and after (lane 6) dialysis

Fig. 3 Dependence on substrate concentration of the initial reaction rate (v_o) in the incorporation of dTTP into poly(rA)-p(dT)₁₅. (A) Dependence on dTTP concentration. (B) Dependence on poly(rA)-p(dT)₁₅ concentration. The initial concentrations of MMLV RT, HIV-1 M RT, and HIV-1 O RT were 2, 50, and 20 nM, respectively. The initial concentration of poly(rA)-p(dT)₁₅ was 25 μ M (A), and that of dTTP was 200 μ M (B). Solid lines represent the best fit of the Michaelis-Menten equation with the non-linear least squares method. Symbols for the enzymes: MMLV RT (open circle), HIV-1 M RT (open triangle), and HIV-1 O RT (open square)

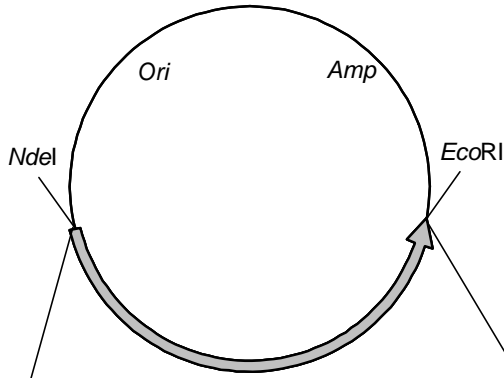
Fig. 4 Dependence on enzyme concentration of RT-catalyzed cDNA synthesis. (A) MMLV RT. (B) HIV-1 M RT. (C) HIV-1 O RT. cDNA synthesis was carried out with 0

(lane 2), 0.1 (lane 3), 0.5 (lane 4), 1.0 (lane 5), 5.0 (lane 6), 10 (lane 7), 50 (lane 8), 100 (lane 9), 500 (lane 10), 1,000 (lane 11) nM MMLV RT (A), HIV-1 M RT (B), and HIV-1 O RT (C) at 46°C. Then, PCR was carried out. Marker DNAs (lane 1) and the amplified products (lanes 2-11) were separated on 1.0% agarose gels and stained with ethidium bromide. The arrow indicates the expected size (601 bp) of the amplified products

Fig. 5 Dependence on organic solvent concentration of RT-catalyzed incorporation of dTTP into poly(rA)-p(dT)₁₅. The reaction was carried out at 42°C with the initial enzyme concentrations of 2 nM for MMLV RT, 50 nM for HIV-1 M RT, and 20 nM for HIV-1 O RT and the initial poly(rA)-p(dT)₁₅ and [³H]dTTP concentrations of 25 μM and 0.2 mM, respectively, in the presence of 0–30% formamide (A) or DMSO (B). Symbols for the enzymes: MMLV RT (open circle), HIV-1 M RT (open triangle), and HIV-1 O RT (open square)

Fig. 6 Irreversible thermal inactivation of RT. MMLV RT (20 nM), HIV-1 M RT (500 nM), and HIV-1 O RT (200 nM) were incubated at 42–50°C in the presence or absence of poly(rA)-p(dT)₁₅ (T/P) (28 μM) for 10 min. Then, the reaction to incorporate dTTP into poly(rA)-p(dT)₁₅ was carried out at 37°C with the initial enzyme concentrations of 2 nM for MMLV RT, 50 nM for HIV-1 M RT, and 20 nM for HIV-1 O RT

pET-MRTHis	(7,818 bp)
pET-HIVMp66	(7,537 bp)
pET-HisHIVMp51	(7,153 bp)
pET-HIVOp66	(7,537 bp)
pET-HisHIVOp51	(7,153 bp)



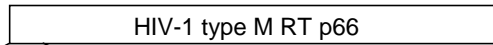
pET-MRTHis



ATGACATGGCTGTCTGATTTTCCTCAG
M T W L S D F P Q

CCAGACACCTCTACCCTCCTCCACCACCACCATCACCCTAG
P D T S T L L H H H H H *

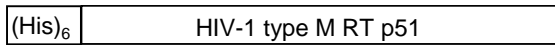
pET-HIVMp66



ATGCCGATTAGCCCGATTGAAACT
M P I S P I E T

GCAGGTATTCGTAAAGTTTTATAA
A G I R K V L *

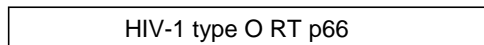
pET-HisHIVMp51



ATGCATCATCATCATCATCCGATTAGCCCGATTGAAACT
M H H H H H H P I S P I E T

CCACCATTAGTTAAACTCTGGTAA
P P L V K L W *

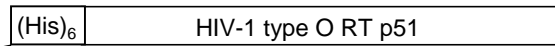
pET-HIVOp66



ATGCCGATCTCGCCGATTGCTCCG
M P I S P I A P

AAAGACATTCGTCTGTTTATAA
K D I R R V L *

pET-HisHIVOp51



ATGCATCATCATCATCATCCGATCTCGCCGATTGCTCCG
M H H H H H H P I S P I A P

CCGCCGCTGATTAAACTGTGGTAA
P P L I K L W *

Fig. 1

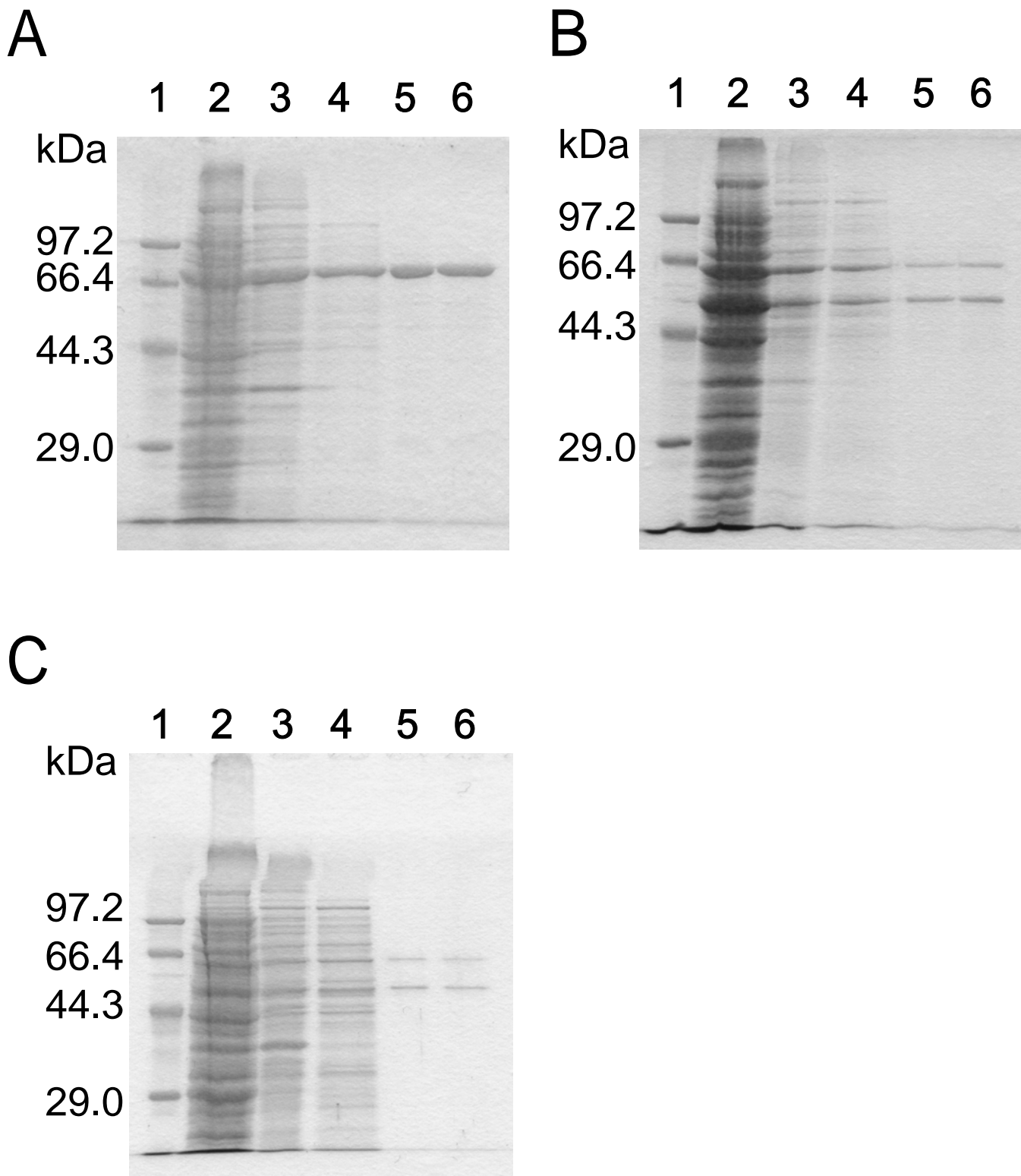


Fig. 2

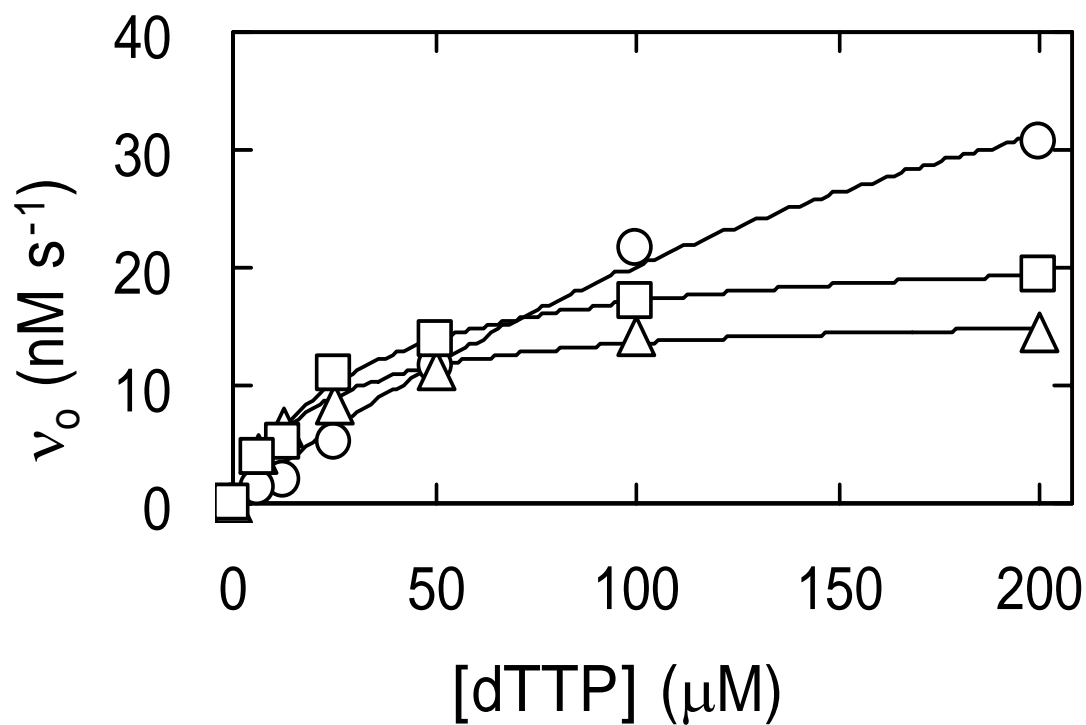
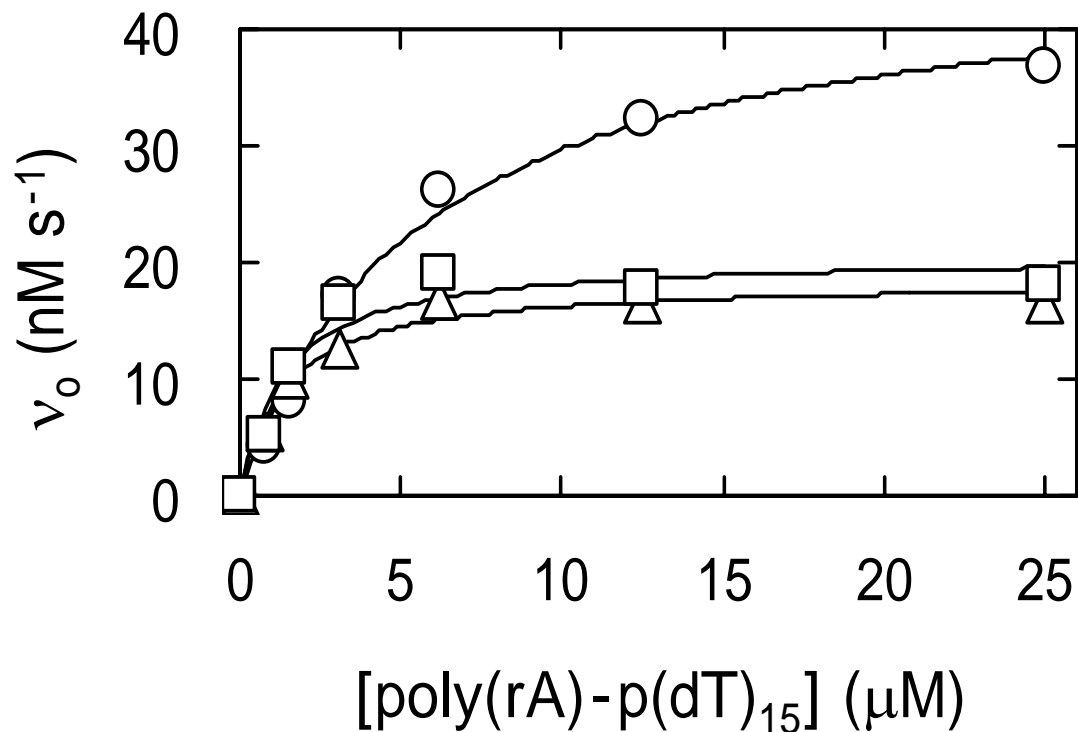
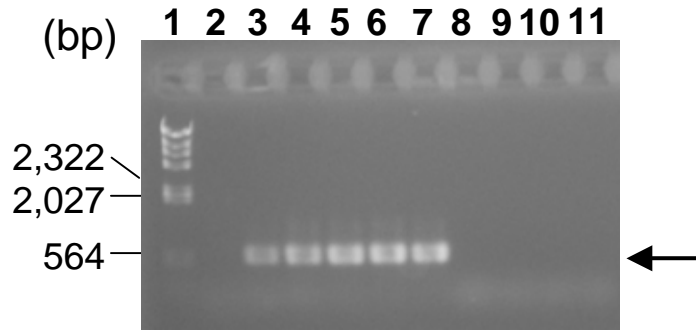
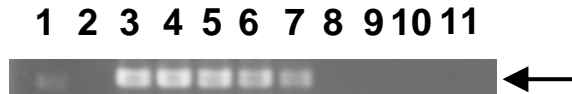
A**B**

Fig. 3

A



B



C

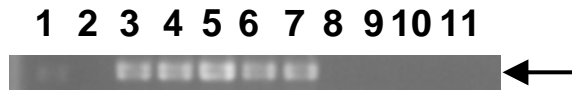
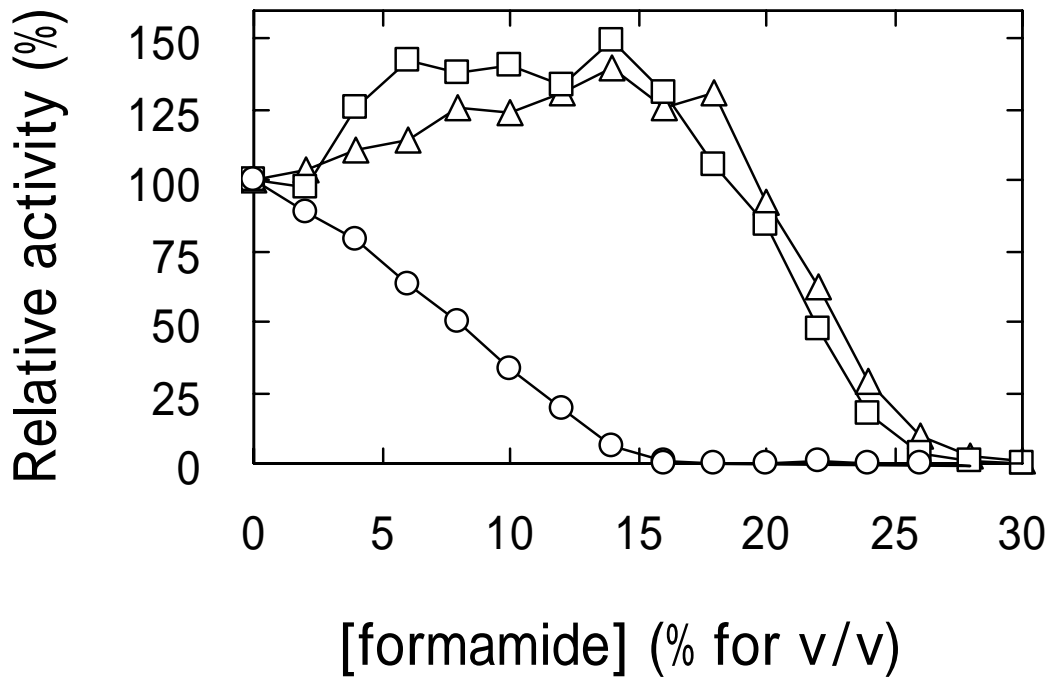
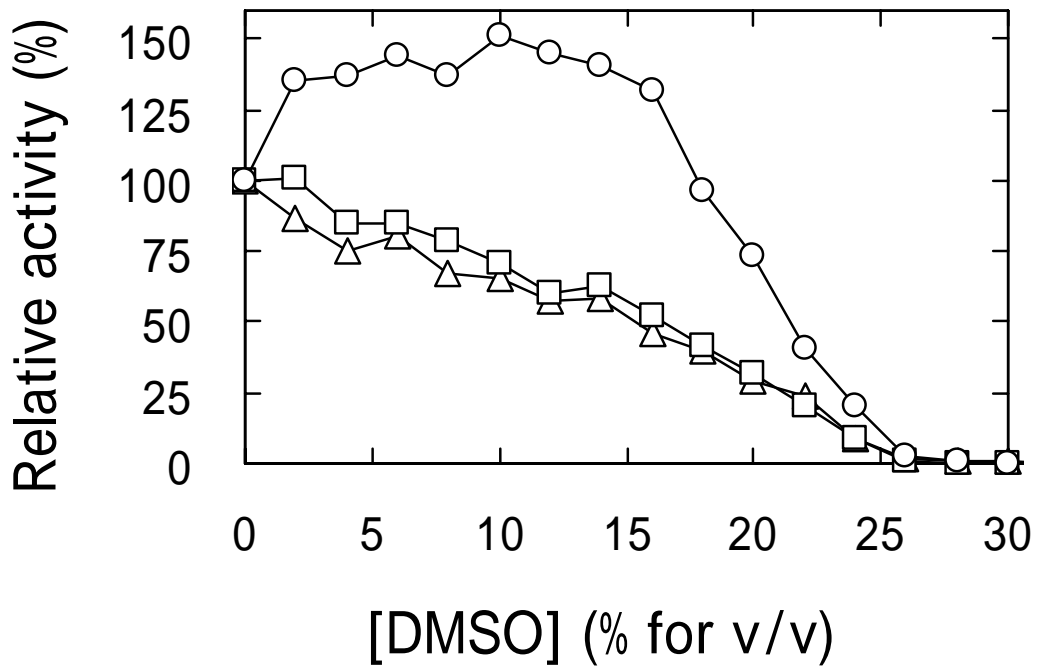


Fig. 4

A**B****Fig. 5**

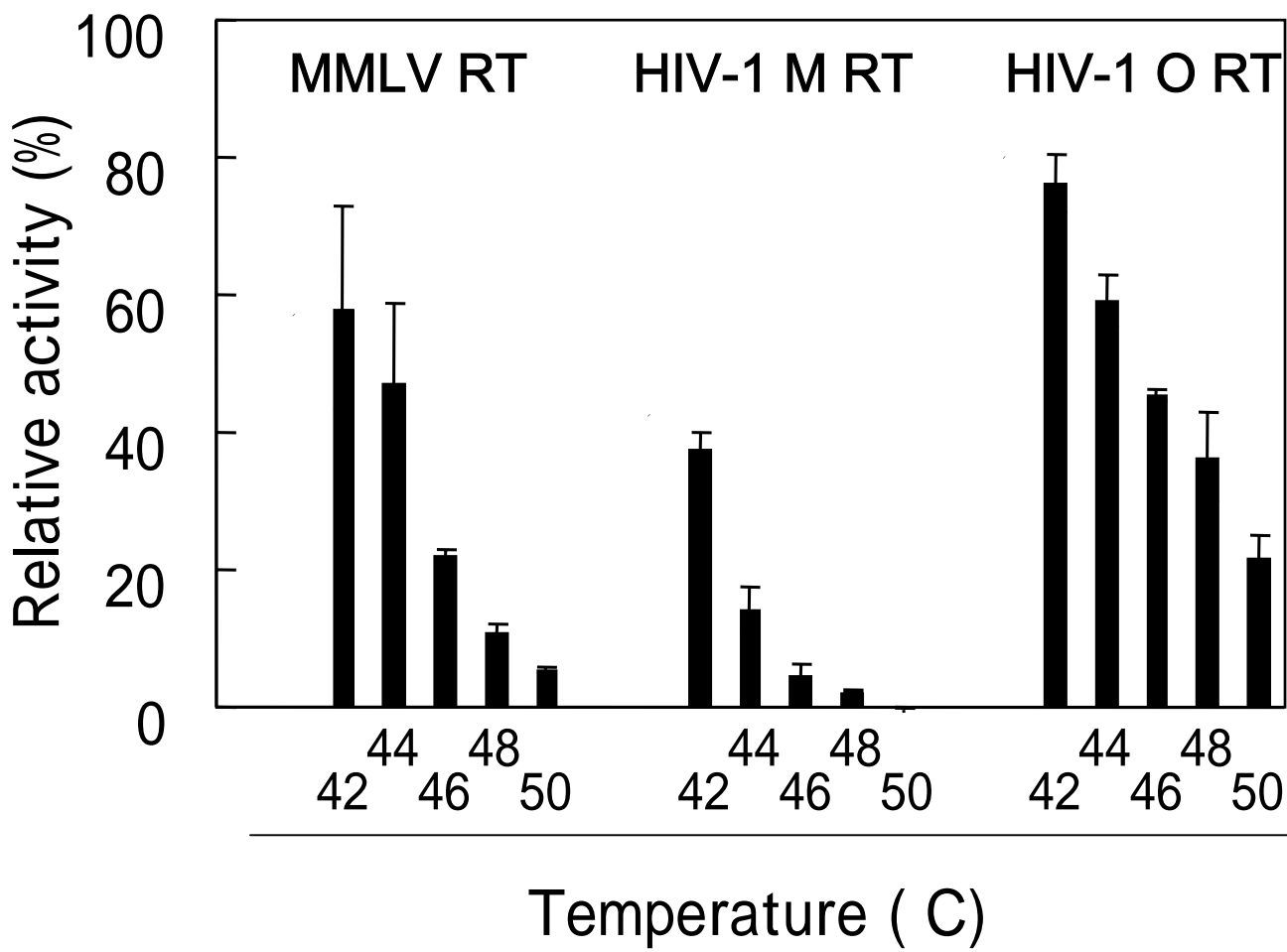


Fig. 6

Table 1

Kinetic parameters of RT in the to incorporation of dTTP into poly(rA)-p(dT)₁₅ (T/P) at 37°C.

RT	$K_{m, \text{dTTP}}$ (μM)	$K_{m, \text{T/P}}$ (μM)	k_{cat} (s^{-1})
MMLV RT	250 ± 60 (1.0)	5.6 ± 0.8 (1.0)	29 ± 6 (1.0)
HIV-1 M RT	20 ± 2 (0.080)	1.4 ± 0.2 (0.25)	0.35 ± 0.02 (0.012)
HIV-1 O RT	30 ± 4 (0.12)	1.3 ± 0.4 (0.23)	1.1 ± 0.1 (0.038)

Average of triplicate determinations with SD value is shown. Numbers in parentheses indicate values relative to MMLV RT.

1 CCGATTAGCCCGATTGAAACTGTGCCCGTTAAACTGAAACCAGGTATGGATGGACCGAAAAGTAAAACAATGGCCACTGACAGAAGAAAAAG
1 P I S P I E T V P V K L K P G M D G P K V K Q W P L T E E K
91 ATTAAGCACTGGTTGAAATTTGCACCGAAATGGAAAAAGAAGGCAAAAATCTCTAAAATGGGCCGAGAACCCGTATAACACCCCGCTG
31 I K A L V E I C T E M E K E G K I S K I G P E N P Y N T P V
181 TTTGCAATTAATAAAAAAGATTCTACAAAGTGGCGTAAATTTGGTAGATTCCGCGAGCTTAATAAACGCACTCAAGATTTCTGGGAAGTT
61 F A I K K K D S T K W R K L V D F R E L N K R T Q D F W E V
271 CAACTTGGGATTCTCACCCGCGCGTCTGAAAAAGAAAAAATCAGTGACTGTTTTGGATGTAGGAGATGCATATTTTAGCGTCCCCTTG
91 Q L G I P H P A G L K K K K S V T V L D V G D A Y F S V P L
361 GATTTCGATTTTCGTAAATATACCGCATTCACTATCCCGTCTATTAACAATGAAACACCAGGCATTCGTTACCAATATAACGTACTTCTT
121 D F D F R K Y T A F T I P S I N N E T P G I R Y Q Y N V L P
451 CAAGGTTGAAAGGCTCTCCCGCATTTTTTCAGTCGAGTATGACTAAAACTTTGGAACCTTTCCGCAACAAAATCCGGACATTGTTATC
151 Q G W K G S P A I F Q S S M T K I L E P F R K Q N P D I V I
541 TACCAGTACATGGATGATCTGTATGTTGGATCCGACCTGGAAATCGGACAACATCGCACAAAATCGAAGAGCTGCGTCAACACCTTCTG
181 Y Q Y M D D L Y V G S D L E I G Q H R T K I E E L R Q H L L
601 CGCTGGGCTTGACTACACCTGACAAAAACATCAAAAAGAACCACCGTTCCTGTGGATGGGTTATGAATTGCACCCTGACAAATGGACC
201 R W G L T T P D K K H Q K E P P F L W M G Y E L H P D K W T
721 GTTCAACCTATCGTCCCTGCCGAAAAGACAGCTGGACGGTTAATGACATTCAAAGTTGGTAGGCAAGTAAATTTGGGCATCTCAGATT
241 V Q P I V L P E K D S W T V N D I Q K L V G K L N W A S Q I
811 TACCCGGGCATTAAGTGCGCCAACTTTGTAAACTGCTGCGCGGCACCAAGGCTCTGACCGAAGTCATCCCTCTGACCGAAGAAGCTGAA
271 Y P G I K V R Q L C K L L R G T K A L T E V I P L T E E A E
901 CTGGAACCTGACAGAGAACCAGCAAAATTTCTGAAAGAACCCTCCATGGAGTGTATTATGACCCAAGTAAAGATTTGATTGCCGAAATTCAA
301 L E L A E N R E I L K E P V H G V Y Y D P S K D L I A E I Q
991 AAACAAGGACAAGGCCAATGGACCTATCAGATTTATCAAGAACCTTTTAAAACTTAAAACTGGAAAATATGCACGCATGCGCGGTGCG
331 K Q G Q G Q W T Y Q I Y Q E P F K N L K T G K Y A R M R G A
1081 CATACAAACGATGTTAAACAGCTGACAGAAGCCGTACAAAAAATTACAACAGAATCCATTGTAATCTGGGGAAAAACCCGAAATTTAAA
361 H T N D V K Q L T E A V Q K I T T E S I V I W G K T P K F K
1171 TTACCGATTCAAAAAGAGACGTGGGAAACGTGGTGGACCGAATACTGGCAGGCAACCTGGATTCCGGAATGGGAATTTGTAACACCCCA
391 L P I Q K E T W E T W W T E Y W Q A T W I P E W E F V N T P
1261 CCATTAGTTAAACTCTGGTATCAACTCGAAAAAGAACCAGTTGTAGGGGCAGAACTTTTTATGTGGACGGAGCTGCGAATCGCGAAACG
421 P L V K L W Y Q L E K E P I V G A E T F Y V D G A A N R E T
1351 AAATTAGGTAAGCTGGATATGTGACGAATCGCGGGCGCAAAAAGTAGTAACCCCTACCGATACCACTAATCAAAAAACCGAACTCCAA
451 K L G K A G Y V T N R G R Q K V V T L T D T T N Q K T E L Q
1441 GCAATTTACCTCGCCCTGCAAGATTGAGGCTTAGAAGTTAACATTGTCCTGATAGCCAATACGCCCTTGGTATTATTCAAGCACAGCCG
481 A I Y L A L Q D S G L E V N I V T D S Q Y A L G I I Q A Q P
1531 GATCAAAGCGAATCAGAATTAGTCAATCAGATTATTGAACAATTAATAAAAAAGAGAAAGTATATTTAGCCTGGGTACCAGCCCAATAA
511 D Q S E S E L V N Q I I E Q L I K K E K V Y L A W V P A H K
1621 AAAGGAATTTGGTGGCAACGAACAGGTAGACAAACTGGTATCTGCAGGTATTCGTAAGTTTTATAA
541 K G I G G N E Q V D K L V S A G I R K V L *

Fig. S1 Nucleotide sequences of artificially synthesized HIV-1 M RT DNA. The amino acid sequences is also shown

1 CCGATCTCGCCGATTGCTCCGGTGCCGGTGAACCTGAAACCGGGTATGGATGGTCCGAAAAGTGAACAATGGCCGCTGTCTCGTGAAAAA
1 P I S P I A P V P V K L K P G M D G P K V K Q W P L S R E K
91 ATTGAAGCACTGACCGCTATCTGCCAGGAAATGGAACAAGAAGGCAAAATTTACGTATCGGTCCGAAAAACCGTATAATACGCCGATT
31 I E A L T A I C Q E M E Q E G K I S R I G P E N P Y N T P I
181 TTTGCCATTAAGAAAAAAGATTCCGACCAAATGGCGCAAACCTGGTGGACTTTTCGTGAACTGAACAACGCACGCAGGATTCTGGGAAGTT
61 F A I K K K D S T K W R K L V D F R E L N K R T Q D F W E V
271 CAACTGGGCATTCCGCATCCGGGCGGTCTGAAACAGCGTCAATCAGTTACCGTCCCTGGATGTGGGTGACGCCTATTTCTCGTGTCCGCTG
91 Q L G I P H P G G L K Q R Q S V T V L D V G D A Y F S C P L
361 GATCCGGACTTTTCGTAATACACCGCATTACAGATCCCGAGCGTTAAACAATGAAACCCCGGGCGTGCCTATCAGTACAACGTTCTGCCG
121 D P D F R K Y T A F T I P S V N N E T P G V R Y Q Y N V L P
451 CAAGGCTGGAAGGTTCTCCGGCTATTTTCAGAGCTCTATGACCAAAATCCTGGATCCGTTCCGTAAGTAATCCGGAAGTGAAATT
151 Q G W K G S P A I F Q S S M T K I L D P F R K S N P E V E I
541 TATCAGTACATCGATGACCTGTATGTGGTTCGATCTGCCGCTGGCAGAACATCGTAAACCGGTGGAAGTCTGCGCGAACACCTGTAC
181 Y Q Y I D D L Y V G S D L P L A E H R K R V E L L R E H L Y
631 CAGTGGGGCTTTACCACGCCGGATAAAAAACATCAGAAAACCGCGTTCCTGTGGATGGGTTATGAACTGCACCCGGACAAATGGAGC
211 Q W G F T T P D K K H Q K E P P F L W M G Y E L H P D K W T
721 GTTCAGCCGATTCAACTGCCGGATAAAGAAGTGTGGACCGTTAACGACATCCAGAAACTGGTCGGCAAACCTGAATTGGGCCAGCCAGATT
241 V Q P I Q L P D K E V W T V N D I Q K L V G K L N W A S Q I
811 TACCAAGGTATCCGTGTGAAAGAAGTGTGCAAACCTGATTCCGCGCACGAAAAGCCTGACCGAAGTGGTTCCGCTGTCTAAGAAGCAGAA
271 Y Q G I R V K E L C K L I R G T K S L T E V V P L S K E A E
901 CTGGAAGTGAAGAAAACCGCGAAAAACTGAAAGAACCAGTCCATGGTGTGATTACCAGCCGGATAAAGACCTGTGGGTTAGCATTAG
301 L E L E E N R E K L K E P V H G V Y Y Q P D K D L W V S I Q
991 AAACATGGCGAAGGTCATGACCTATCAGGTCTACCAAGTGAACACAAAAACCTGAAAACGGGCAAAATACGCGCTCAGAAAGCCAGC
331 K H G E G Q W T Y Q V Y Q D E H K N L K T G K Y A R Q K A S
1081 CACACCAATGACATCCGCCAGCTGGCTGAAGTCTGCAGAAAGTCTCTCAAGAAGCGATTGTGATCTGGGGTAAACTGCCGAAATTTCTG
361 H T N D I R Q L A E V V Q K V S Q E A I V I W G K L P K F R
1171 CTGCCGGTGACCCGGAACCTGGTGGGCGAATATTGGCAGGCAACGTGGATTCCGGAATGGGAATTTGTTAGTACCCCG
391 L P V T R E T W E T W W A E Y W Q A T W I P E W E F V S T P
1261 CCGCTGATTAACCTGTGGTATCAGCTGGAACCGAACCATCGTTGGCGCTGAAACCTTCTACGTCGATGGTGCGCCAACCGTAATACG
421 P L I K L W Y Q L E T E P I V G A E T F Y V D G A A N R N T
1351 AAACCTGGCAAGCGGGTTATGTGACCGAACAGGCAAAACAAACATTATCAAACCTGGAAGAAACCACGAATCAGAAAGCAGAAGTATGAT
451 K L G K A G Y V T E Q G K Q N I I K L E E T T N Q K A E L M
1441 GCTGTTCTGATTGCGCTGCAGGATAGTAAAGAACAAGTTAATATCGTCACCGACTCCAGTACGTCCTGGGTATTATCAGTTCCAGCCG
481 A V L I A L Q D S K E Q V N I V T D S Q Y V L G I I S S Q P
1531 ACCCAATCAGATTCCCGATTGTCCAGCAAATTTATCGAAGAACTGACCAAAAAAGAACCGGTGTATCTGACCTGGGTTCCGGCACACAAA
511 T Q S D S P I V Q Q I I E E L T K K E R V Y L T W V P A H K
1621 AAAGGCATCGGCGTAACGAAAAAATTGATAAACTGGTTTCTAAGACATTCGTCGTGTTTATAA
541 K G I G G N E K I D K L V S K D I R R V L *

Fig. S2 Nucleotide sequences of artificially synthesized HIV-1 M RT DNA. The amino acid sequences is also shown

Table S1

Primers.

Primers	Sequences (5'-3')
MRF	5'- <u>AAGGAGATATACATATGACATGGCTGTCTG</u> -3'
MRHISB	5'- <u>CTGAATTCTAGTGGTGATGGTGGTGGTGGAGGAGGG</u> <u>TAGAGGTGTCT</u> -3'
HIVRTM-HISF	5'- <u>CCTTTTCATATGCATCATCATCATCATCCGATTA</u> <u>GCCCGATTGAAACTG</u> -3'
HIVRTMp51-B	5'- <u>TTTTCGAATTCTTACCAGAGTTTAACTAATGGTG</u> -3'
HIVRTM-F	5'- <u>CCTTTTCATATGCCGATTAGCCCGATTG</u> -3'
HIVRTMp66-B	5'- <u>TTTTGAATTCTTATAAACTTTACGAATACC</u> -3'
HIVRTO-HISF	5'- <u>CCTTTTCATATGCATCATCATCATCATCCGATC</u> <u>TCGCCGATTGCTCCGG</u> -3'
HIVRTOp51-B	5'- <u>TTTTCGAATTCTTACCACAGTTTAAATCAGCG</u> -3'
HIVRTO-F	5'- <u>CCTTTTCATATGCCGATCTCGCCGATTG</u> -3'
HIVRTOp66-B	5'- <u>TTTTGAATTCTTATTATAAAACACGACGAATGTCT</u> <u>TTAGAAACCAGTTTATCAATTTTTTCG</u> -3'

The mismatched nucleotides with the sequence of the MMLV RT, HIV-1 M RT, or HIV-1 O RT gene are underlined.

Table S2

Representative purification of RT.

Stage	Volume (mL)	Activity (units ^a)	Recovery (%)	Protein (mg)	Specific activity (units/mg)	Purification (fold)
i) MMLV RT						
(NH ₄) ₂ SO ₄ fractionation	6.0	928,000	100	21	44,000	1.0
DEAE Toyopearl	16	553,000	60	13	43,000	1.0
Ni ²⁺ Sepharose	2.5	629,000	68	4.3	150,000	3.4
Purified enzyme	0.7	372,000	40	2.0	190,000	4.3
ii) HIV-1 group M RT						
(NH ₄) ₂ SO ₄ fractionation	8.0	6,310	100	24	260	1.0
DEAE Toyopearl	16	5,070	80	7.5	680	2.6
Ni ²⁺ Sepharose	2.5	2,080	33	1.3	1,500	5.8
Purified enzyme	0.7	1,760	28	0.9	1,900	7.3
iii) HIV-1 group O RT						
(NH ₄) ₂ SO ₄ fractionation	12	16,800	100	52	320	1.0
DEAE Toyopearl	28	20,400	120	21	970	3.0
Ni ²⁺ Sepharose	2.5	5,190	31	1.3	4,000	13
Purified enzyme	0.7	3,910	23	0.8	5,000	16

^aOne unit of RT is defined as the amount which incorporates 1 nmol of dTTP into poly(rA)-p(dT)₁₅ in 10 min at 37°C.