+Enzyme and Microbial Technology

**Enhanced detection of RNA by MMLV reverse transcriptase coupled with
thermostable DNA polymerase and DNA/RNA helicase**

Hiroyuki Okano^a, Yuta Katano^a, Misato Baba^a, Ayako Fujiwara^b, Ryota Hidese^b,
Shinsuke Fujiwara^b, Itaru Yanagihara^c, Tsukasa Hayashi^d, Kenji Kojima^a,
Teisuke Takita^a, Kiyoshi Yasukawa^{a,*}

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

^b *Department of Bioscience, School of Science and Technology, Kwansei-Gakuin
University, 2-1 Gakuen, Sanda, Hyogo 669-1337, Japan*

^c *Department of Developmental Medicine, Osaka Medical Center and Research Institute
for Maternal and Child Health, 840 Murodo-cho, Izumi, Osaka 594-1101, Japan*

^d *Kainos Laboratories, Inc., 38-18, Hongo 2-chome, Bunkyo-ku, Tokyo 113-0033, Japan*

Abbreviations: MMLV, Moloney murine leukemia virus; RT, reverse transcriptase

Keywords: cDNA synthesis; DNA polymerase; Helicase; Reverse transcriptase;
Taguchi's method.

*Corresponding author. Fax: +81 75 753 6265.

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

ABSTRACT

Detection of mRNA is a valuable method for monitoring the specific gene expression. In this study, we devised a novel cDNA synthesis method using three enzymes, the genetically engineered thermostable variant of reverse transcriptase (RT), MM4 (E286R/E302K/L435R/D524A) from Moloney murine leukemia virus (MMLV), the genetically engineered variant of family A DNA polymerase with RT activity, K4pol_{L329A} from thermophilic *Thermotoga petrophila* K4, and the DNA/RNA helicase Tk-EshA from a hyperthermophilic archaeon *Thermococcus kodakarensis*. By optimizing assay conditions for three enzymes using Taguchi's method, 100 to 1000-fold higher sensitivity was achieved for cDNA synthesis than conventional assay condition using only RT. Our results suggest that DNA polymerase with RT activity and DNA/RNA helicase are useful to increase the sensitivity of cDNA synthesis.

1. Introduction

Detection of RNA has some merits over detection of DNA: it enables detection of the expression of a specific gene, high-copy-number ribosomal RNA, and RNA virus. Rapid and sensitive detection of a target RNA is important for optimal and efficient diagnosis, treatment, prevention, and observation as well as transcriptome analysis. The efficiency of cDNA synthesis strictly depends on the performance of reverse transcriptase (RT). Currently, RT from Moloney murine leukemia virus (MMLV) is widely used in cDNA synthesis. To increase the sensitivity in cDNA synthesis, stabilization of RT is an important subject because higher reaction temperature reduces RNA secondary structures and nonspecific primer binding. Various genetically engineered thermostable variants of MMLV RT have been generated, such as E286R/E302K/L435R/D524A [1, 2], E69K/E302R/W313F/L435G/N454K [3], and L139P/D200N/T330P/L603W/E607K [4]. However, their thermostabilities are still much lower than those of bacterial or archaeal DNA polymerases used in PCR.

Some bacterial or archaeal, wild-type or genetically engineered DNA polymerases have RT activity [5–8]. They include the wild-type enzymes of family A DNA polymerase from thermophilic bacteria *Thermus thermophilus* and *Thermus aquaticus*, the variant enzymes of family A DNA polymerase, such as K4pol_{L329A} [7], from hyperthermophilic bacteria *Thermotoga petrophila* K4, and the wild-type enzymes of family B DNA polymerase from hyperthermophilic archaeon *Thermococcus kodakarensis* and *Pyrococcus furiosus*. Because they are stable even at 90–100°C, cDNA synthesis and the subsequent PCR can be carried out in a single tube. However, their RT activities are considerably lower than that of retroviral RT [8]. Recently, Shi et al. reported that *Bst*

and Klenow DNA polymerases have RT activity [9].

DNA/RNA helicases play an important role in biological activities by eliminating undesired hydrogen bonds in double-stranded nucleic acids [10]. Various DNA/RNA helicases with different unwinding activities have been identified. Among them, helicases from *T. kodakarensis* have been well studied [11, 12]. Recently, Fujiwara et al reported that euryarchaeota specific superfamily 2 helicase (*Tk-EshA*) from *T. kodakarensis* decreased mis-amplified products in PCR, presumably by peeling off partially annealed primers from template DNA and/or unwinding template DNA [13].

cDNA synthesis, like PCR, is a key technology in life science research and clinical diagnosis. However, the sensitivity of cDNA synthesis is generally lower than that of PCR. Currently, wild-type or genetically engineered retroviral RT is used in cDNA synthesis. In this study, we hypothesized that DNA polymerase with RT activity and/or DNA/RNA helicase enhance the efficiency of retroviral RT-mediated cDNA synthesis assuming the following hypotheses: (i) DNA polymerase with RT activity synthesizes cDNA from a target RNA with a secondary structure, from which retroviral RT cannot synthesize cDNA. (ii) DNA/RNA helicase decreases a secondary structure in a target RNA. However, reaction conditions of these three enzymes are different, and thus optimization of reaction condition is required. In nucleic acid-related enzymes, such optimization has been reported in the RNA-specific amplification, an isothermal reaction (41-43°C) that specifically amplifies a target RNA sequence with RT, RNA polymerase, and RNase H [14, 15].

Taguchi's method is one of well-known statistical methods to optimize multiple factors in one reaction or process [16, 17]. The advantage of this method is that many factors can be simultaneously optimized with the minimum number of experiments. In

this study, we optimized the reaction condition of cDNA synthesis with MM4, K4pol_{L329A}, and Tk-EshA using Taguchi's method. The results have suggested that this novel cDNA synthesis method has higher sensitivity than that by retroviral RT alone.

2. Materials and methods

2.1. Materials

The enzyme concentration was determined by the method of Bradford using Protein Assay CBB Solution (Nacalai Tesque, Kyoto, Japan) with bovine serum albumin (Nacalai Tesque) as standard. Standard RNA, which was the RNA of 267-nucleotides corresponding to the *cesD* gene positioned in 15,126–15,392 (+) bp in the *Bacillus cereus* genome (GenBank accession number NC010924), was prepared by an *in vitro* transcription. The concentration of purified RNA was determined spectrophotometrically at A_{260} and adjusted to 10^2 to 10^6 copies/ μ l with water and stored at -80°C until use. MMV (microarray with manageable volumes) chips were from Enplas (Tokyo, Japan). The automated liquid dispensing device was from Lifetech (Iruma, Japan).

2.2. Expression and purification of recombinant form of MM4, K4pol_{L329A}, and Tk-EshA

Preparation of recombinant MM4 was performed as described previously [1]. Briefly, *Escherichia coli* BL21(DE3) was transformed with the pET-22b(+) plasmid (Merck Bioscience, Tokyo, Japan) harboring the gene encoding MM4 with a C-terminal

(His)₆ tag. The MM4 was expressed in the soluble fraction of the transformants, from which active enzymes were purified. Purified MM4 was stored at –80°C in 50 mM Tris–HCl buffer (pH 8.3) containing 200 mM KCl and 50% (v/v) glycerol before use. For use in the activity measurement, the enzyme preparation was diluted with 10 mM potassium phosphate buffer (pH 7.6) including 2 mM dithiothreitol (DTT), 0.2% (v/v) Triton X-100, and 10% glycerol.

Preparation of recombinant K4pol_{L329A} was performed in the same manner as described previously [7]. Briefly, *E. coli* BL21-CodonPlus(DE3) was transformed with the pET-21a(+) plasmid harboring the K4pol_{L329A} gene. The enzyme was expressed in the soluble fraction of the transformants. After the heat treatment of the soluble fraction at 85°C for 30 min and centrifugation at 8,000g for 10 min., the enzyme was purified. Purified K4pol_{L329A} was stored at –30°C in 10 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl, 1 mM DTT, and 10% glycerol before use. For use, the enzyme preparation was diluted with 10 mM Tris-HCl buffer (pH 8.0).

Preparation of recombinant *Tk*-EshA was performed as described previously [13]. Briefly, *E. coli* BL21-CodonPlus(DE3)-RIL was transformed with the pET-28a(+) plasmid harboring the *Tk*-EshA gene with an N-terminal (His)₆ tag. The enzyme was expressed in the soluble fraction of the transformants. After the heat treatment of the soluble fraction at 70°C for 30 min and centrifugation at 14,000 rpm for 1 hour, the enzyme was purified. Purified *Tk*-EshA was stored at –30°C in 20 mM Tris–HCl buffer (pH 8.0) containing 500 mM NaCl, 1 mM DTT, and 10% glycerol before use. For use, the enzyme preparation was diluted with 20 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl and 1 mM DTT.

2.3. cDNA synthesis

The reaction mixture for cDNA synthesis was designed and prepared according to Taguchi's L27 orthogonal array consisting of 13 factors and three levels for each factor. The reaction was performed in a 0.2 ml PCR tube (Nippon Genetics, Tokyo, Japan) (10 µl per each condition) or a MMV chip (0.5 µl/well, 6 wells per each condition) in the presence of $5 - 5 \times 10^4$ copies/µl *cesD* RNA at 45°C for 30 min and stopped by heating at 65°C for 5 min. The reaction mixture for PCR (12.5 µl) was prepared by mixing water (8.6 µl), the product of the cDNA synthesis (0.75 µl), 10×PCR buffer (100 mM Tris-HCl buffer (pH 8.3) containing 500 mM KCl and 15 mM MgCl₂) (1.25 µl), 10 µM D2F2 primer 5'-AGCACATAGTAAAGGCTTA-3' (0.5 µl), 10 µM D4R4 primer 5'-CACCAAAGAGGTACGGTCTAATG-3' (0.5 µl), 2.0 mM dNTP (0.75 µl) and 1 U/µl recombinant *Taq* polymerase (0.15 µl) (Toyobo, Osaka, Japan). The reaction was performed in a 0.2 ml PCR tube for 30 cycles of 30 s at 95°C, 30 s at 57°C, and 30 s at 72°C in PCR Thermal Cycler Dice (Takarabio, Otsu, Japan). The amplified products were separated on 2.0% (w/v) agarose gels and stained with ethidium bromide (1 µg/ml).

Real-time PCR was performed in the following: the reaction mixture was prepared by mixing water (20.0 µl), the product of the cDNA synthesis (2.0 µl), 10 µM D2F2 primer (1.5 µl), 10 µM D4R4 primer (1.5 µl), and SYBR[®] *Premix Ex Taq*[™] (Tli RNaseH Plus) (25.0 µl) (Takarabio). The reaction was performed in a 0.2 ml PCR tube for 35 cycles of 30 s at 95°C, 30 s at 57°C, and 30 s at 72°C in PCR Thermal Cycler Dice Real Time System Single (Takarabio).

2.4. Statistical analysis

Each reaction condition for cDNA synthesis was scored as 2, 3, 4, 5, or 6, according to the lowest concentration (5×10^4 , 5×10^3 , 5×10^2 , 50, or 5 copies/ μ l, respectively) of *cesD* RNA in the cDNA synthesis reaction from which PCR product was detected. When no band was detected, the reaction was scored as 1. We analyzed the data supposing the following equation:

$$S/N_m = -10 \log (s_m^2) \quad (1)$$

where S/N_m is signal-to-noise ratio of and s_m is score of each reaction condition ($m=1, 2, \dots, 27$). The $S/N_{x,i}$ of level i ($= 1, 2$, or 3) of factor x ($= 1$ to 13) was the total of nine out of 27 S/N_m values where the level of factor x is i . For example, the $S/N_{1,1}$ and $S/N_{2,1}$ were calculated as follows:

$$S/N_{1,1} = S/N_1 + S/N_2 + S/N_3 + S/N_4 + S/N_5 + S/N_6 + S/N_7 + S/N_8 + S/N_9$$

$$S/N_{2,1} = S/N_1 + S/N_2 + S/N_3 + S/N_{10} + S/N_{11} + S/N_{12} + S/N_{19} + S/N_{20} + S/N_{21}$$

Accordingly, the lowest $S/N_{x,i}$ value of the three $S/N_{x,i}$ values ($S/N_{x,1}$, $S/N_{x,2}$, $S/N_{x,3}$) indicates that level i is the most appropriate. Variation (V_x ; $x = 1$ to 13) and percentage contribution (P_x) for factor x were calculated as the following equations:

$$V_x = (S/N_{x,1}^2 + S/N_{x,2}^2 + S/N_{x,3}^2)/9 - (S/N_1 + S/N_2 + \dots + S/N_{27})^2/27 \quad (2)$$

$$P_x = V_x / (V_1 + V_2 + \dots + V_{13}) \times 100 \quad (3)$$

Accordingly, high V_x and P_x values indicate that the effect of the difference in the three levels in factor x is high on the reaction efficiency for cDNA synthesis.

3. Results and discussion

3.1. Preparation of MM4, K4pol_{L329A}, and Tk-EshA

MM4, K4pol_{L329A}, and Tk-EshA were expressed in *E. coli* and purified to homogeneity. The SDS-PAGE analysis showed that the purified preparations of MM4, K4pol_{L329A}, and Tk-EshA exhibited 75-kDa, 95-kDa, and 100-kDa protein bands, respectively (Fig. 1).

3.2. Optimization of reaction condition using a 0.2 ml PCR tube

The reaction condition so far reported for cDNA synthesis by wild-type or variant of MMLV RT was 25 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 2 mM DTT, 5 mM MgCl₂ at 40-55°C for cDNA synthesis [1, 2]; that for cDNA synthesis by the variant of family A DNA polymerase with RT activity was 50 mM bicine-KOH buffer (pH 8.2), 1 mM Mn(OCOCH₃)₂, 115 mM CH₃COOK, 8% glycerol at 70°C [7]; and that for the ATPase reaction by DNA/RNA helicase was 50 mM HEPES buffer (pH 7.6), 1 mM ATP, 2 mM DTT, 2 mM MgCl₂ at 30–110°C [13]. To optimize the reaction condition, we designed 13 factors and three levels for each factor for the initial analysis (Table 1). Reaction temperature was set as 45°C because we thought that optimization of concentrations of reaction components is more important because optimal reaction

temperature depends on the melting temperatures of the primers to certain extent. Twenty-seven reaction conditions were designed according to Taguchi's L27 orthogonal array (Table 2), and the corresponding reaction mixtures were prepared.

Currently used assay methods for RT activity include a classical radioactive method using [^3H]dTTP and poly(rA)-p(dT₁₂₋₁₈), a fluorescence-based method using dTTP, poly(rA)-p(dT₁₂₋₁₈), and a fluorescent dye picogreen [18], and a cDNA synthesis method consisting of cDNA synthesis reaction using a template RNA of several hundred nucleotide and PCR [1, 3, 4]. In the radioactive and fluorescence-based methods, the product in the RT reaction can be directly analyzed. However, poly(rA) and p(dT₁₂₋₁₈) does not have a secondary structure. Considering that DNA/RNA helicase is used to decrease the secondary structure, we chose the cDNA synthesis method in this study.

Figure 2 shows the nucleotide sequence of *Bacillus cereus cesD* RNA used as a model RNA. The cDNA synthesis reaction (10 μl) was carried out with 5×10^5 , 5×10^4 , or 5×10^3 copies *cesD* RNA at 45°C for 30 min in a conventional 0.2 ml PCR tube. PCR was carried out using the cDNA synthesis product, and the PCR product was analyzed by agarose gel electrophoresis (Fig. 3). In the presence of 5×10^5 copies *cesD* RNA, reaction conditions 1, 2, 7, 12, 14, 16, 18–26 exhibited a 243-bp band (Fig. 3A). In the presence of 5×10^4 copies *cesD* RNA, reaction conditions 1, 12, 14, 16, 18, 19, 21, 24–26 did (Fig. 3B). In the presence of 5×10^3 copies *cesD* RNA, no reaction conditions produced products (Fig. 3C). Each reaction condition was scored, and its signal-to-noise ratio (S/N_m ; $m = 1$ to 27) was calculated (Table 3). The signal-to-noise ratio ($S/N_{x,i}$) was calculated for each level ($i = 1, 2$, or 3) of each factor ($x = 1$ to 13), and variation (V) and percentage contribution (P) were calculated for each factor (Table 4). The concentrations of MM4, MgCl_2 , and CH_3COOK exhibited higher V (110.7, 59.7, and

114.5, respectively) and P values (21.8%, 11.7%, and 22.5%, respectively) than other factors, suggesting that the differences in these three levels of these factors had a greater effect on the reaction efficiency for cDNA synthesis than the differences in the three levels of other factors. The concentration of D4R4 primer showed the lowest V and P values, suggesting that the difference in the three levels only minimally affected the reaction efficiency. The results of S/N values indicated that the most appropriate concentrations of MM4, MgCl₂, and CH₃COOK were 1 nM (level 3), 20 mM (level 3), and 100 mM (level 2), respectively.

Based on the results of the initial analysis, we designed 13 factors, in which D4R4 primer was replaced with ATP, and three levels for each factor for the second analysis (Table 1). Like the initial analysis, 27 reaction conditions were designed (Table 2). A cDNA synthesis reaction, PCR, and agarose gel electrophoresis were performed, and each reaction condition was scored. S/N, V, and P values were calculated in the same way as in the initial analysis (Tables 3 and 4). The results indicated that the concentration of *Tk*-EshA had highest V and P values (266.9 and 41.1%) and that its appropriate concentrations was 10 nM (level 2).

We conducted the analysis six times. Three levels of eight out of the total 16 factors (13 factors listed in Table 1, ATP, trehalose, and reaction temperature) for each analysis are shown in Fig. 4. The final optimal reaction condition was 10 nM MM4, 50 nM K4pol_{L329A}, 20 nM *Tk*-EshA, 0.2 mM dNTP, 50 mM KCl, 20 mM Tris-HCl (pH 8.3), 50 mM Bicine-NaOH (pH 8.2), 5 mM MgCl₂, 1 mM Mn(OCOCH₃)₂, 100 mM CH₃COOK, 100 mM trehalose, 1 mM ATP, 10 µg/ml *E. coli* RNA, 500 nM primer, and 0% glycerol at 50°C.

3.3. Optimization of reaction condition using a microarray chip

MMV (microarray with manageable volumes) is a novel microarray technology, enabling the sample transfer between chips or between a chip and a conventional sample tube for multistep reactions [19, 20]. One of the advantages of the optimization of reaction condition using such microarray chips is that many conditions can be simultaneously compared.

Like the case with the optimization using a 0.2 ml PCR tube, thirteen factors, three levels of each factor, and 27 reaction conditions were designed for the first analysis (Tables 1 and 2). The cDNA synthesis reaction (0.5 μ l) was carried out in a MMV chip. The results were scored, and S/N, V, and P values were calculated and analyzed. Such analyses were conducted six times. The three levels of eight factors for each analysis are shown in Fig. S1. The results indicated that the patterns obtained by the optimizations using 0.2 ml tube and MMV chip were almost the same. Finally, same optimal reaction condition was obtained as the case with the optimization using a PCR tube.

Finally we compared the sensitivity between the optimized and conventional reaction conditions (Fig. 5). The optimized condition contains MM4, K4pol_{L329A}, and *Tk*-EshA, while the conventional reaction condition contains MM4 alone. The concentrations of Bicine-NaOH, MgCl₂, Mn(OCOCH₃)₂, CH₃COOK, trehalose, ATP, and *E. coli* RNA were also different between the two conditions. The cDNA synthesis reaction was carried out with 50–500,000 copies *cesD* RNA at 50°C for 30 min in a conventional 0.2 ml PCR tube (Fig. 5A, B, E, and F) or a MMV chip (Fig. 5C and D). In the analysis of the cDNA synthesis products with PCR and subsequent electrophoresis (Fig. 5A–D), the optimized condition detected 500 (Fig. 5B) or 25

copies (Fig. 5D) *cesD* RNA, while the conventional condition did not detect 50,000 (Fig. 5A) or 2500 copies (Fig. 5C) *cesD* RNA. In the analysis of the cDNA synthesis products with real-time PCR (Fig. 5E and F), the optimized condition exhibited Ct values, which were defined as the number of cycles required for the fluorescent signals to reach the threshold, of 17.6, 21.2, 27.3, 30.8, and 32.1 for the cDNA synthesis products from 500,000, 50,000, 5,000, 500, and 50 copies *cesD* RNA, respectively (Fig. 5F), while the conventional condition exhibited the Ct values of 24.9, 26.5, 31.3, and 34.6 for the products from 500,000, 50,000, 5,000, and 500 copies *cesD* RNA, respectively. (Fig. 5E). These results indicate that by optimizing assay conditions for three enzymes, 100 to 1000-fold higher sensitivity was achieved for cDNA synthesis than conventional assay condition using only RT.

3.4. Insights into the effects of each factor on the sensitivity of cDNA synthesis

We specifically examined the effects of K4pol_{L329A} and *Tk*-EshA (Fig. 6). The cDNA synthesis reaction was carried out with 50–500,000 copies *cesD* RNA at 50°C for 30 min in a conventional 0.2 ml PCR tube. PCR was carried out using the cDNA synthesis product, and the PCR product was analyzed by agarose gel electrophoresis. In the presence of MM4 and the absence of K4pol_{L329A}, 500 copies were not detected in the absence of *Tk*-EshA (Fig. 6A), but were detected in its presence (Fig. 6B). In the absence of MM4 and the presence of K4pol_{L329A}, 5×10^5 copies were not detected in the absence of *Tk*-EshA (Fig. 6C) but were detected in its presence (Fig. 6D). This suggests that *Tk*-EshA increases the sensitivity of MM4- or K4pol_{L329A}-mediated cDNA synthesis.

In Taguchi's method, percentage contribution (P) indicates relative magnitude of effect of the difference in three levels of each factor. In the following, we discuss the effects of MM4, K4pol_{L329A}, *Tk*-EshA, Bicine-NaOH, MgCl₂, Mn(OCOCH₃)₂, trehalose, ATP, and *E. coli* RNA on the reaction efficiency of cDNA synthesis. Manganese ion is required for RT activity of K4pol_{L329A} [7]. In this study, the final optimal K4pol_{L329A} and Mn(OCOCH₃)₂ concentrations were 50 nM and 1 mM, respectively. The percentage contribution (P) values of Mn(OCOCH₃)₂ were low in the initial (4.2%), second (9.6%) analyses (Table 4) but high (10–34 %) in the third to sixth analyses. Indeed high concentration (5 mM) of Mn(OCOCH₃)₂ decreased the efficiency. We think that this is due to the competition of Mn²⁺ and Mg²⁺ for the binding to the active site of MM4, as pointed previously [5]. We confirmed that cDNA synthesis reaction proceeded in the presence of 50 mM K4pol_{L329A} in the absence of MM4 (Fig. 7). However P values of K4pol_{L329A} were relatively low in the initial (4.2%), second (7.4%), and the third to sixth (1.7–11.8 %) analyses, suggesting that the effect of K4pol_{L329A} was smaller than MM4. This is in agreement with the results as shown in Fig. 5.

Tk-EshA exhibited relatively high P values in the first (8.4%), second (41.1%), and the third to sixth (5.0–45.8%) analyses. According to the recent report on the noise reduction in PCR using an archaeal DNA/RNA helicase [13], low concentration (2–10 nM) of helicase did not decrease mis-amplified products sufficiently, while high concentration (100 nM) decreased not only mis-amplified but also original amplified products, and that the optimal concentration was 50 nM. We think that this observation applies to our result that P values were high and the optimal concentration was 20 nM. DNA/RNA helicase eliminates hydrogen bonds between DNA or RNA bases using the energy of ATP hydrolysis. In the second analysis, we examined the effect of ATP with

three levels of 0.01, 0.03, and 0.1 mM (Table 1). After the six analyses, the optimal condition was determined as 1 mM, but P values were low (0.4–4.3%). We think that the effect of ATP concentration was marginal.

Certain organic chemicals such as dimethyl sulfoxide (DMSO) [21] and formamide [22] are sometimes used for PCR reaction with a G+C-rich DNA to improve specificity, by decreasing the melting temperatures of the primers. DMSO is also used in RNA amplification reaction using RT, RNA polymerase, and RNase H [14, 15]. We reported that low concentration of DMSO (12%, v/v) or formamide (6–8%) improved the efficiency of cDNA synthesis, however, high concentration (24% or 6–8%, respectively) inhibited it [23]. Considering that enzymes, in general, are inactivated in the presence of organic solvents, helicase is better than DMSO or formamide for use in cDNA synthesis.

We previously reported that in the presence of high concentrations (50–1,000 nM) of MMLV RT or HIV-1, the reaction efficiency of cDNA synthesis was decreased [24]. We speculated that this defect is due to increased nonspecific binding of RT to template-primer rather than enhanced RNA cleavage because 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 [24]. The optimal concentration of MM4 and *E. coli* RNA were determined as 10 nM and 10 µg/ml, respectively (Figs. 4 and S1), which seemed high considering our previous observation [24]. This might be explained by that *E. coli* RNA binds RT, inhibiting nonspecific binding of RT to template-primer.

The activation and stabilization of enzymes by sugars have been widely studied. Among sugars, trehalose, a natural non-reducing disaccharide in which two glucose

units are linked in an $\alpha 1 \rightarrow \alpha 1$ linkage, has been found to be the most effective. Effects of trehalose on RT activity and stability were previously reported [25, 26]. In the fourth analysis, we examined the effect of trehalose with three levels of 0, 0.1, and 1 M. The result indicated that the optimal condition was 0.1 M, while the P value was low (0.8%) (data not shown). In this study, the length of synthesized cDNA was short (300 nt) (Fig. 2). We think that the effect of trehalose will be evident for long-chain cDNA synthesis.

3.4. Conclusion

The reaction condition of cDNA synthesis with MM4, K4pol_{L329A}, and *Tk*-EshA was optimized. The sensitivity increased by 100 to 1000-fold compared to conventional assay condition using only RT. The results suggest that DNA polymerase with RT activity and DNA/RNA helicase are useful to increase the sensitivity of cDNA synthesis. The results also suggest that a comprehensive optimization is more useful than a limited optimization targeting one or a few reaction components.

Acknowledgments

This study was supported by SENTAN, Japan Science and Technology Agency. We also appreciate Dr. Koichi Nishigaki, Emeritus professor of Saitama University and Dr. Yuzuru Husimi of the Graduate University for Advanced Studies.

References

- 376 1. K. Yasukawa, M. Mizuno, A. Konishi, K. Inouye, Increase in thermal stability of
377 Moloney murine leukaemia virus reverse transcriptase by site-directed mutagenesis,
378 J. Biotechnol. 150 (2010) 299–306.
- 379 2. Y. Katano, T. Hisayoshi, I. Kuze, H. Okano, M. Ito, K. Nishigaki, T. Takita, K.
380 Yasukawa, Expression of Moloney murine leukemia virus reverse transcriptase in a
381 cell-free protein expression system, Biotechnol. Lett. 38 (2016) 1203-1211.
- 382 3. B. Arezi, H. Hogrefe, Novel mutations in Moloney Murine Leukemia Virus reverse
383 transcriptase increase thermostability through tighter binding to template-primer,
384 Nucleic Acids Res. 37 (2009) 473–481.
- 385 4. A. Baranauskas, S. Paliksa, G. Alzbutas, M. Vaitkevicius, J. Lubiene, V. Letukiene,
386 S. Burinskas, G. Sasnauskas, R. Skirgaila, Generation and characterization of new
387 highly thermostable and processive M-MuLV reverse transcriptase variants, Protein
388 Eng. Des. Sel. 25 (2012) 657–668.
- 389 5. T.W. Myers, D.H. Gelfand, Reverse transcription and DNA amplification by a
390 *Thermus thermophilus* DNA polymerase, Biochemistry 30 (1991) 7661–7666.
- 391 6. H. Shandilya, K. Griffiths, E.K. Flynn, M. Astatke, P.J. Shih, J.E. Lee, G.F. Gerard,
392 M.D. Gibbs, P.L. Bergquist, Thermophilic bacterial DNA polymerases with
393 reverse-transcriptase activity, Extremophiles 8 (2004) 243–251.
- 394 7. S. Sano, Y. Yamada, T. Shinkawa, S. Kato, T. Okada, H. Higashibata, S. Fujiwara,
395 Mutations to create thermostable reverse transcriptase with bacterial family A DNA
396 polymerase from *Thermotoga petrophila* K4, J. Biosci. Bioeng. 113 (2012)
397 315-321.
- 398 8. K. Yasukawa, A. Konishi, M. Shinomura, E. Nagaoka, S. Fujiwara, Kinetic analysis
399 of reverse transcriptase activity of bacterial family A DNA polymerases, Biochem.

Biophys. Res. Commun. 427 (2012) 654–658.

9. C. Shi, X. Shen, S. Niu, C. Ma, Innate reverse transcriptase activity of DNA polymerase for isothermal RNA direct detection, *J. Am. Chem. Soc.* 137 (2015) 13804–13806.
10. M.R. Singleton, M.S. Dillingham, D.B. Wigley, Structure and mechanisms of helicases and nucleic acid translocases, *Annu. Rev. Biochem.* 76 (2007) 23–50.
11. H. Atomi, T. Fukui, T. Kanai, M. Morikawa, T. Imanaka, Description of *Thermococcus kodakarensis* sp. nov., a well studied hyperthermophilic archaeon previously reported as *Pyrococcus* sp. KOD1, *Archaea* 1 (2004) 263–267.
12. Y. Shimada, W. Fukuda, Y. Akada, M. Ishida, J. Nakayama, T. Imanaka, S. Fujiwara, Property of cold inducible DEAD-box RNA helicase in hyperthermophilic archaea, *Biochem. Biophys. Res. Commun.* 389 (2009) 622–627.
13. A. Fujiwara, K. Kawato, S. Kato, K. Yasukawa, R. Hidese, S. Fujiwara, Application of a *Euryarchaeota*-specific helicase from *Thermococcus kodakarensis* for noise reduction in PCR, *Appl. Environ. Microbiol.* 82 (2016) 3022–3031.
14. T. Kievits, B. van Gemen, D. van Strijp, R. Schkink, M. Dircks, H. Adriaanse, L. Malek, R. Sooknanan, P. Lens, NASBA isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection, *J. Virol. Methods* 35 (1991) 273–286.
15. K. Yasukawa, N. Agata, K. Inouye, Detection of *cesA* mRNA from *Bacillus cereus* by RNA-specific amplification, *Enzyme Microb. Technol.* 46 (2009) 391–396.
16. P. Rathee, A. Kamboj, S. Sidhu, Optimization and development of Nisoldipine nano-bioenhancers by novel orthogonal array (L27 array), *Int. J. Biol. Macromol.* 86 (2016) 556–561.

17. R.N. Dash, H. Mohammed, T. Humaira, An integrated Taguchi and response surface methodological approach for the optimization of an HPLC method to determine glimepiride in a supersaturatable self-nanoemulsifying formulation, Saudi. Pharm. J. 24 (2016) 92–103.
18. K. Silprasit, R. Thammaporn, S. Tecchasakul, S. Hannongbua, K. Choowongkamon, Simple and rapid determination of the enzyme kinetics of HIV-1 reverse transcriptase and anti-HIV-1 agents by a fluorescence based method, J. Virol. Methods 171 (2011) 381–387.
19. Y. Kinoshita, T. Tayama, K. Kitamura, M. Salimullah, H. Uchida, M. Suzuki, Y. Husimi, K. Nishigaki, Novel concept microarray enabling PCR and multistep reactions through pipette-free aperture-to-aperture parallel transfer, BMC Biotechnol. 10 (2010) 71.
20. H. Sharma, Y. Kinoshita, S. Fujiu, et al., Establishment of a reborn MMV-microarray technology: realization of microbionme analysis and other hitherto inaccessible technologies, BMC Biotechnol. 14 (2014) 78.
21. N. Chester, D.R. Marshak, Dimethyl sulfoxide-mediated primer T_m reduction: a method for analyzing the role of renaturation temperature in the polymerase chain reaction, Anal. Biochem. 209 (1993) 284–290.
22. F. Hubé, P. Reverdiau, S. Iochmann, Y. Gruel, Improved PCR method for amplification of GC-rich DNA sequences, Mol. Biotechnol. 31 (2005) 81–84.
23. K. Yasukawa, A. Konishi, K. Inouye, Effects of organic solvents on the reverse transcription reaction catalyzed by reverse transcriptases from avian myeloblastosis virus and Moloney murine leukaemia virus, Biosci. Biotechnol. Biochem. 74 (2010) 1925–1930.

- 448 24. A. Konishi, K. Yasukawa, K. Inouye, Improving the thermal stability of avian
449 myeloblastosis virus reverse transcriptase α subunit by site-directed mutagenesis,
450 Biotechnol. Lett. 34 (2012) 1209–1215.
- 451 25. P. Carninci, Y. Nishiyama, A. Westover, M. Itoh, S. Nagaoka, N. Sasaki, Y.
452 Okazaki, M. Muramatsu, Y. Hayashizaki, Thermostabilization and
453 thermoactivation of thermolabile enzymes by trehalose and its application for the
454 synthesis of full length cDNA, Proc. Natl. Acad. Sci. USA 95 (1998) 520–524.
- 455 26. A.N. Spiess, R. Ivell, A highly efficient method for long-chain cDNA synthesis
456 using trehalose and betaine, Anal. Biochem. 30 (2002) 1168–1174.
- 457

Figure legends

Fig. 1. SDS-PAGE under reducing conditions. Coomassie Brilliant Blue-stained 10% SDS-polyacrylamide gel is shown. Lanes: The Protein Molecular Weight Marker (Bio-rad) (lane 1), purified enzyme preparations of MM4 (lane 2), K4pol_{L329A} (lane 3), and *Tk*-EshA (lane 4).

Fig. 2. Nucleotide sequence of the target RNA and the primers. The base position corresponds to that described in the sequences 15,126–15,392 of the *cesD* gene of *Bacillus cereus* (GenBank accession number NC010924)

Fig. 3. Comparison of the sensitivities of the 27 reaction conditions. cDNA synthesis (10 µl in a PCR tube) was carried out in 500–50,000 copies/µl of *cesD* RNA at 45°C for 30 min in a PCR tube. PCR was carried out with the D2F2 and D4R4 primers. Amplified products were applied to a 2% agarose gel, followed by staining with ethidium bromide (1 µg/ml). Lanes 1–27 correspond to the reaction conditions for cDNA synthesis according to Taguchi's L27 orthogonal array.

Fig. 4. Optimization of reaction conditions for cDNA synthesis in a PCR tube. cDNA synthesis was carried out at 45°C for 30 min at 45°C (Round 1–3) or 50°C (Round 4–6). PCR was performed using the D2F2 and D4R4 primers. The sensitivities of the 27 reaction conditions were evaluated, and three concentration levels of each of the 13 factors were ranked. Concentration levels of MM4, K4pol_{L329A}, *Tk*-EshA, KCl, MgCl₂, Mn(OCOCH₃)₂, CH₃COOK, and *E. coli* RNA are plotted, respectively, against round of

analysis. Filled circles imply the highest rank. Note that because KCl was not included in 13 factors in the fourth analysis, only closed circle was plotted.

Fig. 5. Comparison of the sensitivities of the cDNA synthesis reactions catalyzed by MM4 and by MM4, K4pol_{L329A}, and *Tk*-EshA. cDNA synthesis reaction (10 µl in a PCR tube (A, B, E, F) or 0.5 µl in a MMV chip (C, D)) was carried out at 50°C for 30 min. The concentrations of Bicine-NaOH (pH 8.2), MgCl₂, Mn(OCOCH₃)₂, CH₃COOK, trehalose, and ATP were 0, 5, 0, 0, 0, 0, and 0 mM, respectively (A, C, E) and 50, 5, 1, 100, 100, and 1 mM, respectively (B, D, F). The concentration of *E. coli* RNA was 0 (A, C, E) and 10 µg/ml (B, D, F). The concentrations of MM4, dNTP, KCl, Tris-HCl (pH 8.3), D4R4 primer, glycerol, and *cesD* RNA were 10 nM, 0.2 mM, 50 mM, 20 mM, and 500 nM, 0%, and 5–50,000 copies/µl (A–F). PCR and subsequent electrophoresis were carried out as described in Fig. 3 (A–D). Real-time PCR was carried out as described in the Materials and methods (E, F).

Fig. 6. Comparison of the sensitivities of the cDNA synthesis reactions catalyzed by MM4 and by K4pol_{L329A}. cDNA synthesis reaction (10 µl in a PCR tube) was carried out at 50°C for 30 min. The concentrations of Bicine-NaOH (pH 8.2), Tris-HCl (pH 8.3), KCl, MgCl₂, Mn(OCOCH₃)₂, and CH₃COOK were 0, 20, 50, 5, 0, and 0 mM, respectively (A, B) and 50, 0, 0, 0, 1, and 100 mM, respectively (C, D). The concentration of ATP was 0 (A, C) and 1 mM (B, D). The concentrations of dNTP, D4R4 primer, trehalose, *E. coli* RNA, and *cesD* RNA were 0.2 mM, 500 nM, 100 mM, 10 µg/ml, and 5–50,000 copies/µl (A–D). PCR and subsequent electrophoresis were carried out as described in Fig. 3.

Table 1

Factors and levels.

Initial analysis				
Factor		Level		
		1	2	3
1	MM4 (nM)	0.01	0.1	1
2	K4pol _{L329A} (nM)	0	1	100
3	<i>Tk</i> -EshA (nM)	0	1	50
4	dNTP (each) (mM)	0.05	0.25	0.5
5	KCl (mM)	10	50	200
6	Tris-HCl (pH 8.3) (mM)	5	25	100
7	Bicine-NaOH (pH 8.2) (mM)	10	50	200
8	MgCl ₂ (mM)	1	5	20
9	Mn(OCOCH ₃) ₂ (mM)	0.2	1	5
10	CH ₃ COOK (mM)	30	100	300
11	D4R4 primer (μM)	0.1	0.5	1
12	<i>E. coli</i> RNA (μg/ml)	0	1	10
13	glycerol (% , v/v)	0	3	9

Second analysis				
Factor		Level		
		1	2	3
1	MM4 (nM)	1	10	100
2	K4pol _{L329A} (nM)	10	50	200
3	<i>Tk</i> -EshA (nM)	10	50	200
4	dNTP (each) (mM)	0.1	0.2	0.4
5	KCl (mM)	10	50	200
6	Tris-HCl (pH 8.3) (mM)	5	25	100
7	Bicine-NaOH (pH 8.2) (mM)	10	50	200
8	MgCl ₂ (mM)	1	5	20
9	Mn(OCOCH ₃) ₂ (mM)	0.2	1	5
10	CH ₃ COOK (mM)	10	30	100
11	ATP (μM)	10	30	100
12	<i>E. coli</i> RNA (μg/ml)	0	1	10
13	glycerol (% , v/v)	0	3	9

Table 2
Taguchi's L27 orthogonal array.

Reaction condition	Factor ^a												
	1	2	3	4	5	6	7	8	9	10	11	12	13
	Level ^a												
1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	2	2	2	2	2	2	2	2	2
3	1	1	1	1	3	3	3	3	3	3	3	3	3
4	1	2	2	2	1	1	1	2	2	2	3	3	3
5	1	2	2	2	2	2	2	3	3	3	1	1	1
6	1	2	2	2	3	3	3	1	1	1	2	2	2
7	1	3	3	3	1	1	1	3	3	3	2	2	2
8	1	3	3	3	2	2	2	1	1	1	3	3	3
9	1	3	3	3	3	3	3	2	2	2	1	1	1
10	2	1	2	3	1	2	3	1	2	3	1	2	3
11	2	1	2	3	2	3	1	2	3	1	2	3	1
12	2	1	2	3	3	1	2	3	1	2	3	1	2
13	2	2	3	1	1	2	3	2	3	1	3	1	2
14	2	2	3	1	2	3	1	3	1	2	1	2	3
15	2	2	3	1	3	1	2	1	2	3	2	3	1
16	2	3	1	2	1	2	3	3	1	2	2	3	1
17	2	3	1	2	2	3	1	1	2	3	3	1	2
18	2	3	1	2	3	1	2	2	3	1	1	2	3
19	3	1	3	2	1	3	2	1	3	2	1	3	2
20	3	1	3	2	2	1	3	2	1	3	2	1	3
21	3	1	3	2	3	2	1	3	2	1	3	2	1
22	3	2	1	3	1	3	2	2	1	3	3	2	1
23	3	2	1	3	2	1	3	3	2	1	1	3	2
24	3	2	1	3	3	2	1	1	3	2	2	1	3
25	3	3	2	1	1	3	2	3	2	1	2	1	3
26	3	3	2	1	2	1	3	1	3	2	3	2	1
27	3	3	2	1	3	2	1	2	1	3	1	3	2

^a Factors 1–9 and levels 1–3 of each factor in the initial and second analyses are described in Table 1.

Table 3

Score and signal-to-noise ratio (S/N) of each reaction condition.

Initial analysis			Second analysis		
Reaction condition	Score	S/N	Reaction condition	Score	S/N
1	3	−9.542	1	2	−6.021
2	2	−6.021	2	4	−12.041
3	1	0	3	1	0
4	1	0	4	3	−9.542
5	1	0	5	2	−6.021
6	1	0	6	1	0
7	2	−6.021	7	1	0
8	1	0	8	1	0
9	1	0	9	1	0
10	1	0	10	1	0
11	1	0	11	1	0
12	3	−9.542	12	1	0
13	1	0	13	1	0
14	3	−9.542	14	2	−6.021
15	1	0	15	1	0
16	3	−9.542	16	4	−12.041
17	1	0	17	3	−9.542
18	3	−9.542	18	3	−9.542
19	3	−9.542	19	1	0
20	2	−6.021	20	1	0
21	3	−9.542	21	1	0
22	2	−6.021	22	4	−12.041
23	2	−6.021	23	4	−12.041
24	3	−9.542	24	1	0
25	3	−9.542	25	1	0
26	3	−9.542	26	1	0
27	1	0	27	3	−9.542
		(Total) −125.527			(Total) −104.396

Table 4

Signal-to-noise ratio (S/N), variation (V) and percentage contribution (P).

Initial analysis						
Factor		S/N			V	P (%)
		Level 1	Level 2	Level 3		
1	MM4	−21.58	−38.17	−65.77	110.7	21.8
2	K4pol _{L329A}	−50.21	−31.13	−44.19	21.2	4.2
3	<i>Tk</i> -EshA	−56.23	−28.63	−40.67	42.6	8.4
4	dNTP (each)	−44.19	−44.19	−37.15	3.7	0.7
5	KCl	−50.21	−37.15	−38.17	11.7	2.3
6	Tris-HCl (pH 8.3)	−56.23	−34.65	−34.65	34.5	6.8
7	Bicine-NaOH (pH 8.2)	−44.19	−50.21	−31.13	21.2	4.2
8	MgCl ₂	−38.17	−27.60	−59.75	59.7	11.7
9	Mn(CH ₃ COO) ₂	−50.21	−31.13	−44.19	21.2	4.2
10	CH ₃ COOK	−44.19	−63.28	−18.06	114.5	22.5
11	D4R4 primer	−44.19	−46.69	−34.65	9.0	1.8
12	<i>E. coli</i> RNA	−44.19	−56.23	−25.11	54.7	10.8
13	glycerol	−44.19	−37.15	−44.19	3.7	0.7
					(Total) 508.2	(Total) 100

Second analysis						
Factor		S/N			V	P (%)
		Level 1	Level 2	Level 3		
1	MM4	−33.62	−37.15	−33.62	0.9	0.1
2	K4pol _{L329A}	−18.06	−45.67	−40.67	48.1	7.4
3	<i>Tk</i> -EshA	−73.27	−25.11	−6.02	266.9	41.1
4	dNTP (each)	−33.62	−46.69	−24.08	28.6	4.4
5	KCl	−39.65	−45.67	−19.08	43.2	6.6
6	Tris-HCl (pH 8.3)	−37.15	−39.65	−27.60	9.0	1.4
7	Bicine-NaOH (pH 8.2)	−40.67	−39.65	−24.08	19.2	3.0
8	MgCl ₂	−15.56	−52.71	−36.12	77.0	11.9
9	Mn(OCOCH ₃) ₂	−45.67	−43.17	−15.56	62.0	9.6
10	CH ₃ COOK	−27.60	−39.65	−37.15	9.0	1.4
11	ATP	−49.19	−24.08	−31.13	37.3	5.7
12	<i>E. coli</i> RNA	−21.58	−39.65	−43.17	29.8	4.6
13	glycerol	−36.12	−43.17	−25.11	18.4	2.8
					(Total) 649.3	(Total) 100

Fig. 1

D2F2:5' –AGCACATAGTAAAGGCTTA–3'

1 AAAGCAUCUCUAAAAGCACAUAGUAAAGGCUUAUCUCUUUAUCUAUCUAGUAUUUAUCAUCUAUUAU

66 GAUUGGUUUUGUUUCUUGUUUUUCCGAGUGUUUCAAAAAGUAGUGGUUUUCAGAUUUAAUGAAAU

131 CCUUACCCCUGGUCUAAUGAAAUCAUUAGGAAUCGAAGGGAAUAUGGCAAUUUAAAUGACUAU

196 UUAAAUAUUAAUUUCUUUAAUUCAUUGUUUUUAUACAUUUUAAUGGCCUAUUGUAUAAUGACAAC

261 GAUUAAGUUGGUGACAAGACCAUUAGACCGUACCUCUUUGGUGUAUUUUUUUAUCUUCACCUGUUU

D4R4:3' –GTAATCTGGCATGGAGAAACCAC–5'

326 CAAAUCAAAGGUACUUUUCACGCAUUUUAUGGUGUUUUUUACAGGGUUAUUUUGAUUUCCCUA

381 GUAACGGUUCUUUCUGGUUUUUUAGGAGC

Fig. 2

Initial *cesD* RNA in
a PCR tube (copies)

(A) 5×10^5

(B) 5×10^4

(C) 5×10^3

Fig. 3

Fig. 4

			MM4 (nM)	K4pol _{L329A} (nM)	<i>Tk</i> -EshA (nM)
(A)	(C)	(E)	10	0	0
(B)	(D)	(F)	10	50	20

Fig. 5

Fig. 6

Supplementary Material

Fig. S1. Optimization of reaction conditions for cDNA synthesis in a MMV chip. Experiments were carried out and data were processed as described in Fig. 4.