Alteration of enzymes and their application to nucleic acid amplification (Review)

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Abstract. Since the discovery of polymerase chain reaction (PCR) in 1985, several methods have been developed to achieve nucleic acid amplification, and are currently used in various fields including clinical diagnosis and life science research. Thus, a wealth of information has accumulated regarding nucleic acid-related enzymes. In this review, some nucleic acid-related enzymes were selected and the recent advances in their modification along with their application to nucleic acid amplification were described. The discussion also focused on optimization of the corresponding reaction conditions. Using newly developed enzymes under well-optimized reaction conditions, the sensitivity, specificity, and fidelity of nucleic acid tests can be improved successfully.

Contents

1. Introduction
2. Thermostabilization of reverse transcriptase

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3. Creation of the reverse transcriptase activity in thermostable DNA polymerase
4. Use of helicase to increase specificity
5. Fidelity evaluation with NGS
6. Use of recombinase and single-strand binding protein for isothermal DNA amplification
7. Other considerable factors involved in nucleic acid amplification
8. Conclusions and future perspectives

1. Introduction

Nucleic acid amplification tests are core technologies of clinical diagnosis. In pulmonary tuberculosis, such testing is capable of identifying *Mycobacterium* species in clinical respiratory samples more rapidly and accurately than sputum specimen examinations and culture-based methods. This advantage is key to appropriate treatment, prevention, and control of transmission of tuberculosis. In HIV detection, the nucleic acid amplification test is more sensitive and quantitative than other methods based on HIV-1-specific antibody or viral antigens, enabling the detection of HIV-1 at the initial stage of infection and the monitoring of disease progression (1,2).

Various nucleic acid amplification technologies have been devised, but the most widely used is PCR. In basic research, most researchers use PCR primarily for amplification, possibly because primer design is convenient and the enzymes are available at a reasonable price (3). In clinical diagnosis, on the other hand, isothermal nucleic acid amplification methods such as nucleic acid sequence-based amplification (NASBA) (4), strand displacement amplification (SDA) (5), rolling circle amplification (RCA) (6), helicase-dependent isothermal DNA amplification (HAD) (7), and loop-mediated isothermal amplification (LAMP) (8) are also used. The advantage of isothermal amplifications over PCR is that they do not require a complex device such as thermal cycler, improving throughput in situations when large numbers of clinical samples must be processed, as well as facilitating point-of-care diagnosis (4-9).

The performance of a nucleic acid amplification test depends largely on the performance of the enzymes involved. Thermostable DNA polymerase, first identified in

Thermus aquaticus (*Taq*) in 1976 (10), has become widely used since the discovery of PCR. Concerning performances of *Taq* polymerase, it was initially reported that the activity decreased to 50% at incubation at 95˚C for 1.6 h; the rate of processing was 60-150 nucleotides/sec; and the error rate was 0.38-1.32x10^4 errors/base (11). Since then, the performances of *Taq* polymerase were improved by genetic engineering. For example, the mutation of Phe667 into Tyr increased its efficiency of incorporation with ddNTP by 10^3-fold (12), and fusion of the helix-hairpin-helix motifs of DNA topoisomerase V to *Taq* polymerase increased the enzyme's stability and processivity (13). The performances of DNA polymerases from the hyperthermophilic archaeon *Thermococcus kodakarensis* (KOD) or *Pyrococcus furiosus* (*Pfu*) and that from thermophilic bacteria *Thermus thermophilus* (*Tth*) have also been improved by genetic engineering. Today, they are widely used in PCR along with *Taq* polymerase.

In addition to altering the enzymes, it is also important to optimize the reaction conditions. In the amplification techniques using multiple enzymes, such as RT-PCR and NASBA, this process is more complicated because each enzyme has its own optimal condition. Another concern is lowering the risk of contamination. In this regard, it is preferable to perform one-tube reactions with real-time monitoring (14).

The aim of the review is to outline recent advances in nucleic acid amplification technologies. The foci of the study are, reverse transcriptase as an example of an enzyme that has been markedly improved by genetic engineering; recombination polymerase amplification, an isothermal amplification which has attracted a great deal of recent attention; and focus helicase, an enzyme which increases specificity and decreases noise in the amplification. Next-generation sequencing (NGS) was used to evaluate the fidelity of cDNA synthesis and the statistical method to optimize the reaction conditions.

2. Thermostabilization of reverse transcriptase

Reverse transcriptase (RT) has RNA- and DNA-dependent DNA polymerase and ribonuclease (RNase) H activities. It is responsible for RNA viral genome replication. Moloney murine leukemia virus (MMLV) RT and avian myeloblastosis virus (AMV) RT are widely used in cDNA synthesis (15) (Table I). MMLV RT is a 75-kDa monomer, and AMV RT is a heterodimer consisting of an α subunit (63-kDa) and a β subunit (95-kDa) (16,17). The result of the homology search performed using the search program DNA Data Bank of Japan (DDBJ; https://www.ddbj.nig.ac.jp/index-e.html) CLUSTALW and the crystal stuructures of MMLV RT is shown in Fig. 1. MMLV RT and the α subunit of AMV RT comprise the fingers, palm, thumb, connection, and RNase H domains. The β subunit of AMV RT includes these five domains along with the C-terminal integrase domain. MMLV RT and AMV RT have two active sites. The active site for the DNA polymerase reaction is in the fingers/palm/thumb domain, and that for the RNase H reaction is in the RNase H domain.

Thermostability of DNA polymerases is important for their wide-range practical use. For cDNA synthesis, an elevated reaction temperature is highly desirable because it reduces RNA secondary structure and nonspecific binding of the primer. However, RT is thermolabile. The initial

activities of MMLV RT and AMV RT are reduced by 50% at 44 and 47˚C, respectively, during a 10-min incubation (18). Thus, improving the thermostability of RT has been an important subject. The thermostabilities of MMLV RT (19-21) and AMV RT (20) were first improved by eliminating the RNase H activity. The thermostability of MMLV RT was improved by introducing the triple mutation E286R/E302K/L435R or E286R/E302K/L435R/D524A in which the negatively charged (Glu286 and Glu302) and hydrophobic (Leu435) residues that were thought to interact with a template-primer were replaced with positively charged residues, and the catalytic residue responsible for RNase H activity Asp524 was replaced with Ala (22). The thermostability of MMLV RT was also improved by the mutation of Val433 present on the molecular surface to Arg (23). Finally, a highly thermostable MMLV variant A32V/L72R/E286R/E302K/W388R/L435R was generated by combining the triple mutation E286R/E302K/L435R with the following mutations: The mutation of the internal residue, Ala32 to Val in order to stabilize the hydrophobic core, the mutation of the hydrophobic surface residue, Leu72 to Arg, and the mutation of Trp388 which is close to the negatively charged residues to Arg in order to introduce a salt bridge (24). In a random mutation assay followed by a combination of stabilizing mutations, E69K/E302R/W313F/L435G/N454K was generated using a filter assay (25), L139P/D200N/T330P/L603W/E607K was generated using emulsion PCR (26), and D200C was obtained by screening an amino acid scanning library (27). The amino acid residues mutated for thermostabilization are widespread throughout the molecule (Fig. 1B).

Recombinant MMLV RT is well expressed in the soluble fractions in *Escherichia coli*, from which sufficient amounts of active enzymes are purified. On the contrary, AMV RT has been barely expressed in the soluble fractions of *E. coli*. Instead, the active AMV RT α subunit was expressed in insect cells (28), and its thermostability was improved by introducing the triple mutation V238R/L388R/D450A, corresponding to E286R/W388R/D524A in MMLV RT (29). Notably, recombinant AMV RT has been successfully expressed in the soluble fractions in *E. coli* since then, and is now commercially available.

cDNA synthesis, as with PCR, is a key technology both in clinical diagnosis and basic research. However, cDNA synthesis is less sensitive than PCR. To circumvent this problem, a cDNA synthesis method using three enzymes, the thermostable MMLV RT quadruple variant E286R/E302K/W388R/D524A (described above), the genetically engineered family A DNA polymerase variant with RT activity from the hyperthermophile *Thermotoga petrophila* K4 (K4pol$_{L329A}$) which will be described in the next section and the DNA/RNA helicase from a hyperthermophilic archaeon *Thermococcus kodakarensis* (*Tk*-EshA), was developed (Table I). K4pol$_{L329A}$ and *Tk*-EshA will be described later. In amplification techniques using multiple enzymes such as NASBA (1,30), optimization is more complicated than when using a single enzyme as in the case of PCR. In this case, statistical methods such as Taguchi's method have been successfully used for optimization (31) (Fig. 2). The merit of statistical methods is that many factors can be optimized at the same time with the minimum number of experiments.

Stabilization of RT is desirable for cDNA synthesis. Improvement in the thermostability of MMLV RT and AMV

Table I. Enzymes used for nucleic acid amplification.

Enzyme	Application	(Refs.)
Reverse transcriptase (RT)		
AMV RT	cDNA synthesis, NASBA	(15,18,20,28,59)
MMLV RT	cDNA synthesis	(16-27,29,59,60)
DNA polymerase		
Taq polymerase	PCR	(10-13)
Tth polymerase	PCR, cDNA synthesis	(37,38)
K4pol$_{L329A}$[a]	PCR, cDNA synthesis	(43,48)
RTX[b]	PCR, cDNA synthesis	(47,48)
Bacillus subtilis polymerase	RPA	(70)
DNA helicase		
Tk-EshA	PCR, cDNA synthesis	(48,54,56)
Tk-Upf1	PCR	(56)
Single-strand DNA-binding protein		
T4 gp32	RPA	(68,70)
Recombinase		
T4 uvsY	RPA	(69,70)
T4 uvsX	RPA	(70)

[a]L329A variant of DNA polymerase from *Thermotoga petrophila* K4, [b]16-tuple variant of KOD DNA polymerase. KOD, *Thermococcus kodakarensis*; RPA, recombinase polymerase amplification.

RT is an important subject. Characterization of about 700 variants of phage T4 lysozyme revealed that there can be various kinds of effective stabilizing methods such as disulfide bridge, salt-bridge interaction, metal binding, and hydrophobic stabilization (32). We consider that the thermostabilities of MMLV RT and AMV RT may be further improved by combining stabilizing mutations.

3. Creation of the reverse transcriptase activity in thermostable DNA polymerase

The DNA-dependent DNA polymerase distinguishes suitable substrates DNA and dNTPs from unsuitable RNA and rNTPs. The exact mechanisms of this distinction are unknown, but two mechanisms have been proposed. One mechanism is for rNTP/dNTP distinction. In Klenow polymerase, the bulky 2' hydroxyl group of ribose interferes with the substrate-binding region of Klenow polymerase: Glu710 sterically blocks the 2' hydroxyl group of rNTP. As a result, the enzyme accepts dNTP but excludes rNTP (33). A similar hindrance effect was reported in archaeon *Thermococcus litoralis* family B DNA polymerase: Tyr412 exludes rNTP by acting as a steric gate for the 2' hydroxyl group of ribose (34). The other mechanism is for template distinction. Archaeal family B DNA polymerase excludes uracil-containing templates, and DNA synthesis is prematurely arrested at the position where uracil is contained. By contrast, bacterial DNA polymerase I ignores the absence of the 5' methyl group in uracil, and accepts a uracil-containing template. Therefore, the 2' hydroxyl group of ribose is considered a key factor for the distinction of RNA/DNA for bacterial DNA-dependent DNA polymerase (35). A similar effect was reported in Klenow polymerase: Asn420 and Tyr423 in the 3'-5' exonuclease domain play a role in RNA exlusion by interfering with the 2' hydroxyl group of the template molecule (36).

To generate thermostable RT using DNA polymerases from thermophilic bacteria and archaea, several approaches have been taken (37-42). Some bacterial DNA polymerases (Pol I) show reverse transcriptase activity in the presence of Mn^{2+}. The *Tth* DNA polymerase from *Thermus thermophilus* also shows the RT activity (37,38). It lacks a 3'-5' exonuclease domain, which contributes to fidelity in PCR. DNA polymerase I from the hyperthermophilic bacterium, *Thermotoga* sp, possesses a 3'-5' exonuclease domain. A study on chimeric DNA polymerases from *Thermotoga* sp and *Thermus* sp showed that chimeric DNA polymerases with RT activity possessed attenuated 3'-5' exonuclease activity (42). Mutations were introduced into another DNA polymerase from *Thermotoga petrophila* K4 (K4PolI) to allow K4PolI to accept an RNA. Among the variants constructed, T326A, L329A, Q384A, F388A, M408A, and Y438A exhibited RT activity while their 3'-5' exonuclease activites were reduced. By contrast, K4Pol$_{N422A}$ and K4Pol$_{F451A}$ did not exhibit RT activity but possessed full 3'-5' exonuclease activity (43). These results suggest that there is a correlation between the gain of RT activity and the loss of 3'-5' exonuclease activity. On the other hand, introduction of random mutations into *Taq* polymerase showed that mutations in domains other than the 3'-5' exonuclease domain generated the mutants with RT activity (39). Further structural studies are needed to exlopre the mechnasim connecting RT and 3'-5' exonuclease activities.

Archaeal family B DNA polymerases, such as those from *Pyrococcus furiosus* (44) or *Thermococcus kodakarensis* (45),
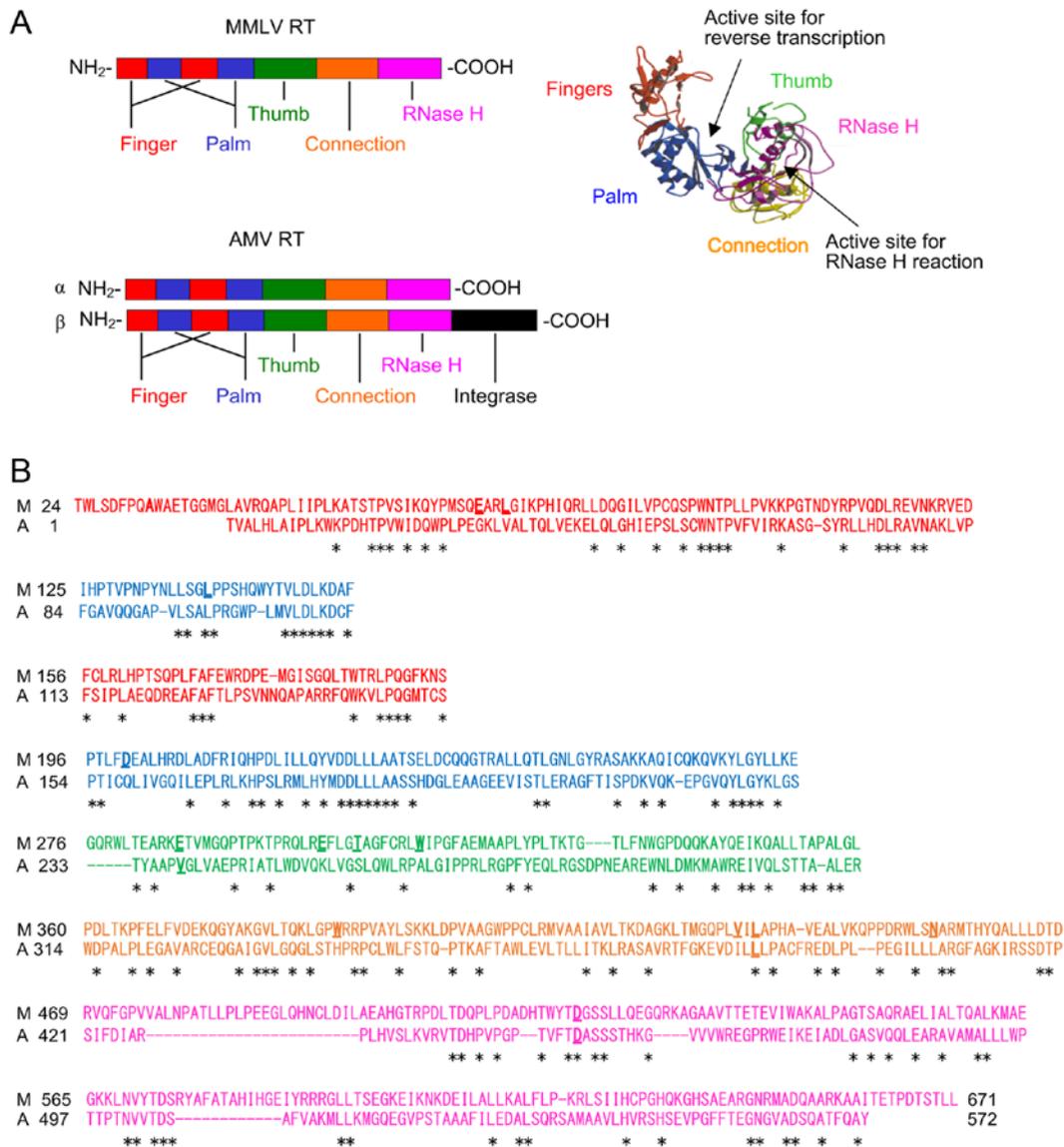
Figure 1. (A) Domain structures of MMLV RT and AMV RT. (B) A sequence alignment of MMLV RT and AMV RT. A homology search was performed using the search program DNA Data Bank of Japan (DDBJ) CLUSTALW and was revised based on the data of X-ray crystallographic analysis of MMLV RT. Asterisks show homologous amino acid residues. The amino acid residues to be mutated for thermostabilization are marked in bold and are underlined. Leu603 and Glu607 in ref. 26 correspond to Leu593 and Glu597, respectively.

possess a higher fidelity than thermophilic bacteria enzymes, such as those from *T. aquaticus* and *T. thermophilus*. However, as mentioned above, archaeal family B DNA polymerase excludes a template containing uracil, which is different from bacterial DNA polymerase (46). Family B DNA polymerase recognizes DNA more precisely than bacterial DNA polymerase I. Modified family B DNA polymerases with a Pol ζ fingers domain that displayed RT activity were developed by the mutation experiment into the 3'-5' exonuclease domain of hybrid archaeal family B DNA polymerases with a Pol ζ fingers domain (41). Recently, Ellefson *et al* generated a 16-tuple variant of KOD DNA polymerase known as RTX with RT activity from the hyperthermophilic archaeon, *Thermococcus kodakarensis*, by a directed evolution method (47). In this method, emulsion PCR was carried out with primers containing various numbers of ribonucleotides so that only DNA polymerase with RT activity enabled self-replication (47). These results indicate that family B

DNA polymerases can be used as a source to create reverse transcriptase.

DNA polymerases with RT activity enable one-step RT-PCR without retroviral RT. The merit of one-step RT-PCR over two-step RT-PCR is that multiple openings of reaction tubes and reagent delivery are not necessary, leading to a decrease in DNA contamination risk. Furthermore, artificially created reverse transcriptase K4pol$_{L329A}$ and RTX are applicable for high sensitive RNA detection by one-step RT-PCR combining with the genetically engineered MMLV-RT and thermostable DNA/RNA helicase (48). COVID-19 RNA was also detected from clinical samples by using the system (data not shown). Details of the helicase role are mentioned below.

4. Use of helicase to increase specificity

DNA/RNA helicases exhibit nucleic acid binding, ATP hydrolysis, translocation, and unwinding of nucleic acid duplex by
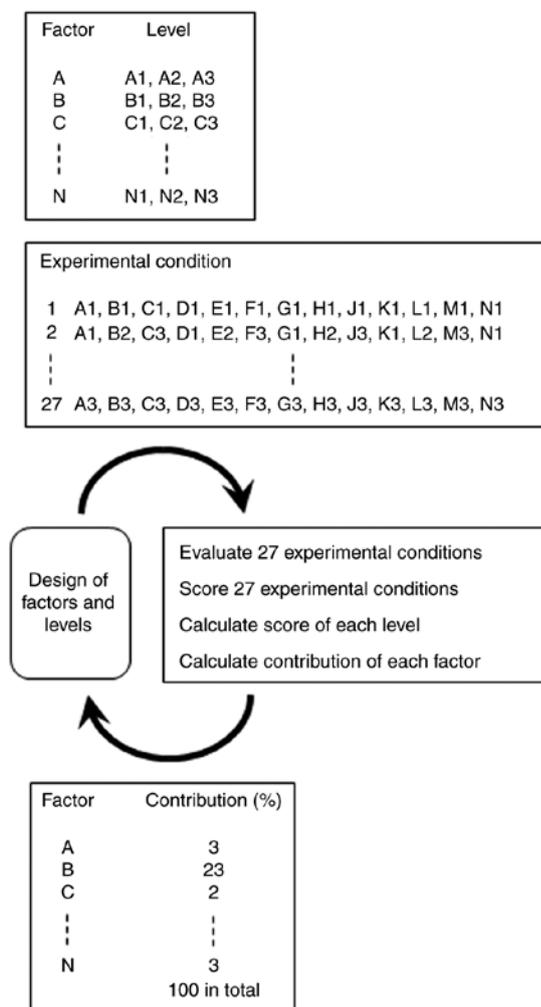
Figure 2. Application of Taguchi's method to optimize the reaction conditions with multiple enzymes. Schematic illustration of the cycle to optimize the reaction conditions is shown.

eliminating hydrogen bonds from the base-pairing between DNA/DNA, DNA/RNA, and RNA/RNA hybrids from the 3' or 5' unpaired end utilizing the energy generated upon ATP hydrolysis. Therefore, helicases are expected to unwind the secondary structured template and partially annealed primer/template duplexes in DNA and RNA synthesis. DNA/RNA helicases are classified into several superfamilies (SFs) according to their amino acid sequences (49). The SF1 and SF2 helicases are large and diverse groups, sharing catalytic cores with almost identical folds and extensive structural similarities. UvrD, an SF1 DNA helicase that unwinds blunt-end substrates as well as nicked circular DNA, was used in an isothermal DNA amplification at low temperature, called helicase-dependent amplification (50-53). In this amplification, a mesophilic DNA polymerase is applicable.

Unexpected DNAs sometimes get amplified due to primer mis-annealing during PCR. In order to efficiently reduce such mis-amplified products, an approach using helicase was devised (54). *Tk*-EshA, a euryarchaeota-specific SF2 helicase EshA from the hyperthermophilic archaeon *Thermococcus kodakarensis*, was first used for this purpose. In the presence of RNA, *Tk*-EshA exhibited maximal ATPase activity at 80˚C. *Tk*-EshA unwinds forked and 3' overhung

DNAs (54). *Tk*-EshA also possesses euryarchaeal termination activity (Eta), which disrupts the transcription elongation complex (55). We hypothesized that *Tk*-EshA unwinds the structured template and peels off mis-annealed primers during PCR. To address this issue, PCR was performed using various DNAs as a substrate. When 16S rDNA was used, several mis-amplified products (noise DNAs) were detected in the absence of *Tk*-EshA. However, they were eliminated in the presence of *Tk*-EshA. These effects of *Tk*-EshA were confirmed whether *Taq* DNA polymerase (a family A DNA polymerase, PolI type) or KOD DNA polymerase (a family B DNA polymerase, α type) was used. When *toxA* gene from *Pseudomonas aeruginosa* DNA, which possesses high GC content (69%), was used, mis-amplified bands were also eliminated by the addition of *Tk*-EshA, suggesting that *Tk*-EshA was more effective than increasing the annealing temperature to reduce mis-amplified DNAs in the *toxA* amplification (54). The action of *Tk*-EshA is shown in Fig. 3. Another type (superfamily 1B) of helicase, *Tk*-Upf1 (TK0178) from *T. kodakarensis*, was examined for the effects on conventional PCR and digital PCR and compared with those of *Tk*-EshA. It is important to eliminate nonspecific amplification for identification of SNPs. Of four double-stranded DNA substrates, forked, 5' overhung, 3' overhung, and blunt-ended DNAs, the unwinding activity of *Tk*-Upf1 was the highest towards 5' overhung DNAs (56). The concentration of *Tk*-Upf1 required for noise DNA elimination was 10-fold lower than that of *Tk*-EshA. The addition of *Tk*-Upf1 also eliminated noise DNAs derived from the misannealed primer when a 5' or 3' overhung misannealed primer was included as a competitive primer along with specific primers. In digital PCR, *Tk*-EshA and *Tk*-Upf1 functioned as signal enhancers: *Tk*-EshA or *Tk*-Upf1 increased the fluorescent intensities, improving separation between the common and risk allele clusters. The amount of *Tk*-Upf1 required to improve the performance of digital PCR was smaller than that of *Tk*-EshA.

5. Fidelity evaluation with NGS

Fidelity indicates the performance in the incorporation of correct nucleotides. Various methods have been applied to analyze DNA polymerase fidelity such as misincorporation (57), misextension (57), primer extension (58), and M13 *lacZ* mutation (59) assays. In a misincorporation assay, the reaction rates to incorporate correct and incorrect nucleotides are compared, while in a misextension assay, the reaction rates for extension from the mispaired end (i.e., A:G) and from the paired end (i.e., A:T) are compared (57). In these two assays, the reactions are carried out under single-turnover conditions. In a primer extension assay, the reaction in the absence of one dNTP is compared with that in the presence of all four dNTPs (58). In the M13 *lacZ* mutation assay, the error rates are calculated from the mutation frequency, which is determined as the ratio of mutant plaques to all plaques (59). The error rates of MMLV RT and AMV RT determined by this assay were $3.3-5.9 \times 10^{-4}$ errors/base and that of HIV-1 RT was 5.9×10^{-3} errors/base (59). The M13 *lacZ* mutation assay has been the only method used to determine the error rate. However, it has some issues. Silent mutation affects the calculation of error rates. Identification of plaque color depends on
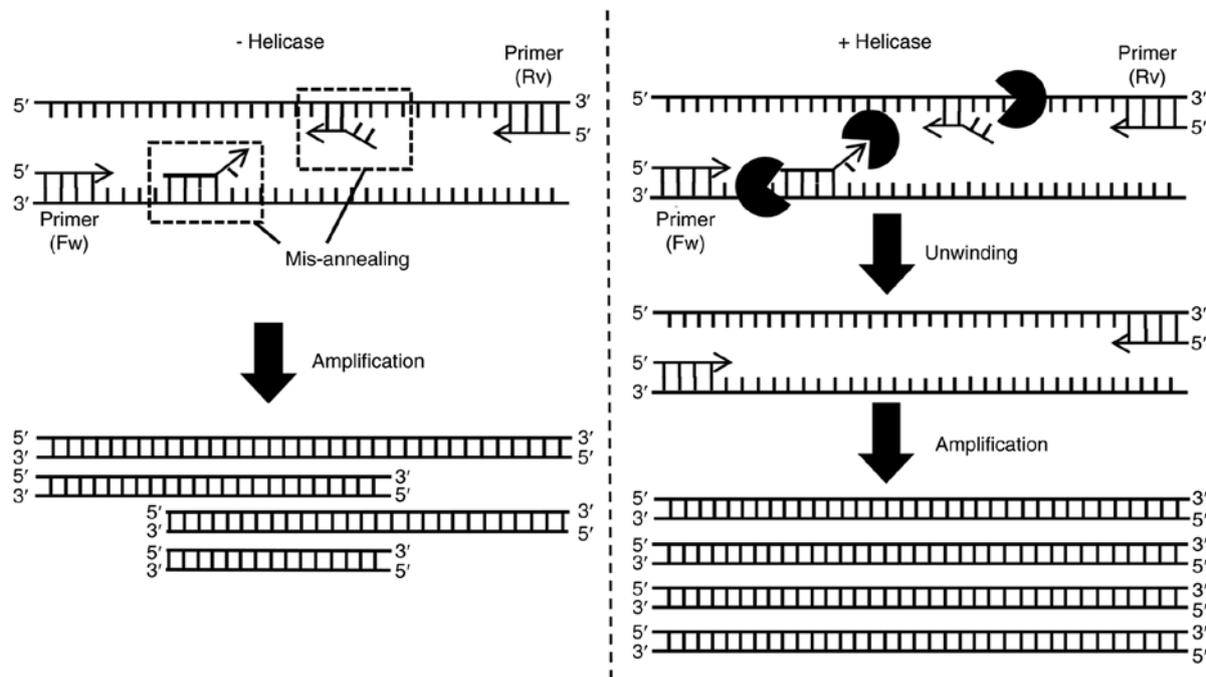
Figure 3. Model for noise reduction in PCR by helicase. Image of noise reduction in PCR by helicase such as a Tk-EshA is shown in the figure. In the absence of helicase, primers mis-anneal to various homologous regions, resulting in the amplification of noise DNAs. In the presence of helicase shown as a pacman, mis-annealed primers are peeled off and specific primers dominantly anneal to the target region, resulting in the reduced amplification of noise DNAs.

the individual. In addition, the reaction is DNA-dependent DNA synthesis, but not RNA-dependent DNA synthesis, even for RT.

In NGS, hundreds of million sequences are obtained in one NGS run. NGS has been widely used to identify rare mutations, misincorporations, and base modifications introduced in genomic DNA (60,61). One of the problems of NGS is that a number of errors are introduced. To address this issue, a method to identify ultra-rare mutations in the genomic DNA using NGS was devised (62), which uses adaptors containing two tags of 12 randomized bases for the ligation of DNA fragments containing the sequences to be analyzed. All sequence reads are grouped based on tag sequences and orientations. By analyzing whether all sequence reads in the same group had the same mutation or not, each mutation that was observed via NGS indicated whether the error was already present in the genome or was incorporated by PCR or NGS (62).

We used NGS to determine the error rate of cDNA synthesis (63,64). As shown in Fig. 4, cDNA was synthesized from a standard RNA with a primer possessing a tag of 14 randomized bases. All sequence reads are grouped based on tag sequences. By analyzing all sequence reads in the same group, each mutation revealed whether the error was incorporated by cDNA synthesis or not. The error rate obtained using this method of MMLV RT was 1.0×10^{-4} errors/base and that with HIV-1 RT was 2.6×10^{-4} errors/base (63), which was approximately 20% of those reported using the M13 *lacZ* mutation assay (59). Notably, unlike the M13 *lacZ* mutation assay, the NGS-based mutation assay reveals the mutation species and the frequency at each nucleotide position (63). This method may be effective in the assessment of the fidelity of various RTs with different reaction conditions: We reported that high concentrations of dNTP, $MgCl_2$, and $Mn(OCOCH_3)_2$

decreased the fidelity, and these effects were obvious in reactions using HIV-1 RT (64).

Fidelity of cDNA synthesis is important in clinical diagnosis and in life science research. The issue raised is how fidelity of RT and DNA polymerase can be ameliorated. One strategy is to optimize the concentrations of the enzyme, salts, and dNTP in the reaction solution. Another strategy is based on the studies conducted on HIV-1 RT (65-67). The fidelity of HIV-1 RT is lower than that of MMLV RT and AMV RT. One of the consequences of low fidelity of HIV-1 RT is the emergence of drug-resistant HIV-1 RT variants, such as K65R, R78A, and V75I. Interestingly, the mutations that confer drug resitance to these variants increase the fidelity of HIV-1 RT (65-67). This suggests that introduction of the corresponding mutations in MMLV RT or AMV RT may increase the fidelity, although such evidence has not yet been reported.

6. Use of recombinase and single-strand binding protein for isothermal DNA amplification

Recombinase polymerase amplification (RPA) is an isothermal reaction that is conducted at a temperature between 37 and 42˚C. RPA specifically amplifies a target DNA sequence with a recombinase, a single-stranded DNA-binding protein (SSB), and a strand-displacing polymerase (68). SSB binds to the primers and prevents oligonucleotide primers from forming secondary structures. Recombinase binds to the primers in the presence of ATP and with the assistance of the loading factor, T4 UvsY, which was originally identified as the T4 recombination mediator protein (69). The primers of the resulting complex bind to the homologous sequences of the DNA template using the ATP hydrolyzing activity of recombinase. In addition, SSB binds to the dispatched strand,
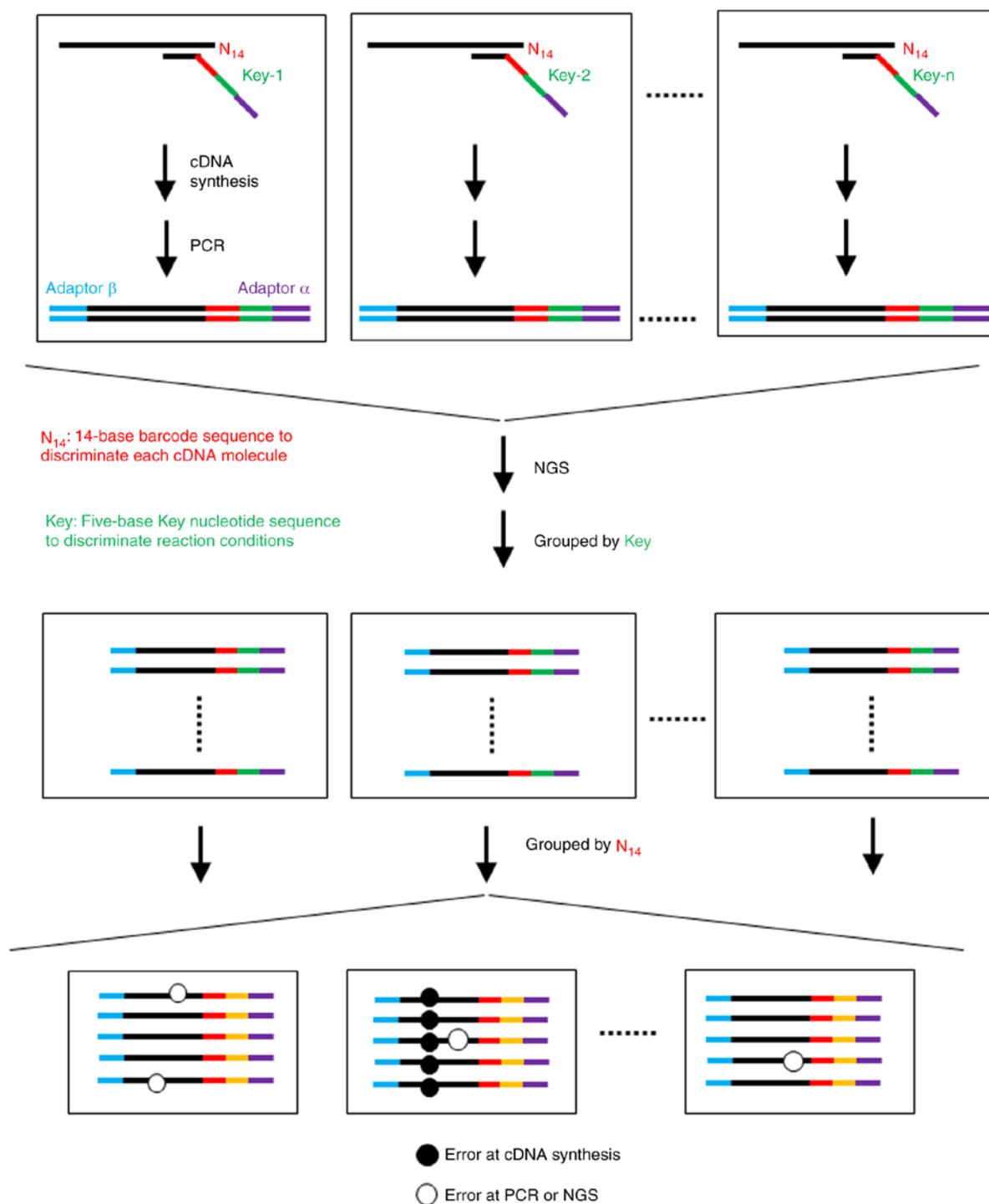
Figure 4. Workflow of the analysis of the error rate in the cDNA synthesis reaction. N_{14}, Key-n, and adaptor α and β indicate the 14-base randomized barcode sequence, five-base key nucleotide sequence, and Ion Proton sequencing adaptor α and β sequences, respectively.

and strand-displacing polymerase extends the primer. Thus, the synthesis of a new DNA strand occurs (Fig. 5A).

In the first report of RPA in 2006 (70), T4 uvsX and T4 uvsY were used as recombinase, T4 gp32 was used as SSB, and *Bacillus subtilis* polymerase was used as strand-displacement DNA polymerase (Table I). Since then, RPA has been widely used to detect various targets. At present, the RPA kit is commercially available from TwistDX (Cambridge). One of the merits of RPA over other isothermal nucleic acid amplification methods is that the reaction occurs at the human body

temperature (37˚C). RPA has the potential to eliminate the use of specialized equipment to provide the required temperature. Thus, RPA may be the most ideal nucleic acid amplification method for use in point-of-care diagnosis. Indeed, a number of RPA targets reported to date are pathogenic organisms including *Mycobacterium tuberculosis* (71,72), *Chlamydia trachomatis* (73), *Streptococcus pneumoniae* (74), and *Leishmania donovani* (75).

In accordance with this trend, various technologies have been combined with RPA. For example, cutaneous
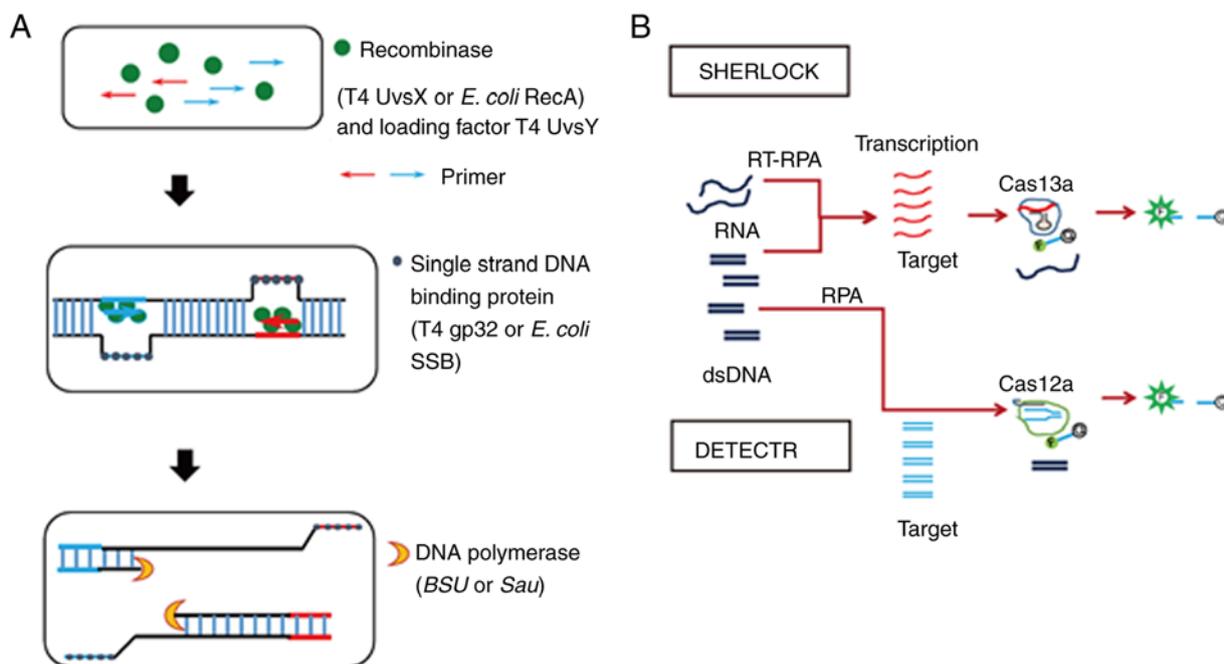
Figure 5. (A) Schematic illustration of the RPA process. (B) Models of SHERLOCK and DETECTR nucleic acid detection systems.

leishmaniasis was detected using an FTA card, a paper-based card commercialized by GE Healthcare for the isolation and storage of nucleic acids, and loop-mediated isothermal amplification (LAMP) (76,77). Lateral flow assay (78), enzyme-linked oligonucleotide assay (79), and electrochemical method (80) were used for end-point detection of RPA amplicons, whereas solid phase amplification was used for the real-time detection of RPA amplicons (81).

Clustered, regularly interspaced, short, palindromic repeats (CRISPR)/CRISPR-associated (CAS) systems were originally identified as an RNA-guided genetic silencing system in bacteria and archaea (82). At present, CRISPR/CAS9 is widely used in genome engineering. CRISPR-Cas13a and CRISPR-Cas12a have been applied to RPA (Fig. 5B). Specific high sensitivity enzymatic reporter unlocking (SHERLOCK) was established using Cas13a, an RNA-guided RNase that cleaves its specific target as well as the nearby non-targeted RNAs (collateral effect). The collateral cleavage enables release of the quenched fluorescent reporter (83). A multiplexed detection system was also established using Cas13, Cas12a, and Csm6 (84). Use of SHERLOCK allowed detection of Zika virus (sensitivity 2 aM) and that of a single nucleotide polymorphism of a human gene (83,84). DNA endonuclease-targeted CRISPR transreporter (DETECTR) was established using CAS12a, an RNA-guided DNase. The DETECTR detected human papillomavirus (HPV) 16 and 18 at attomolar levels (85). These approaches may thus serve as valuable tools to increase the sensitivity of RPA and provide a means for developing novel point-of-care diagnosis with high sensitivity and rapidness.

7. Other considerable factors involved in nucleic acid amplification

Various factors are known to be involved in enzymatic reactions, and such factors include organic solvents. Enzymes are generally inactivated by organic solvents, but use of organic additives in enzymatic reactions can sometimes make previously problematic processes feasible. Indeed, various organic additives have been used to improve reaction efficiency and specificity in PCR (86,87). Dimethyl sulfoxide (DMSO) and formamide have been used to improve specificity for the reaction with a G+C-rich DNA (88,89). In cDNA synthesis, DMSO and formamide increased the reaction efficiency to some extent (90).

Since nucleic acids are highly negatively charged, they may be affected by positively charged small molecules such as polyamines. It was initially reported that spermidine was not beneficial in PCR (91). However, subsequent reports showed that spermidine prevents PCR inhibition problems encountered while analyzing clinical stool samples (92,93). By optimizing the effects of these polar molecules, the efficiency of nucleic acid amplification is expected to further improve.

8. Conclusions and future perspectives

Despite being a widespread analytical method both in fundamental research and clinical diagnosis, there are limitations in nucleic acid amplification, which are represented by false-positive and false-negative results. Many efforts are still being devoted to improve the sensitivity, specificity, rapidness, and accuracy of nucleic acid amplification. The catalytic mechanism of nucleic acid-related enzymes has been extensively investigated by means of X-ray crystallography, kinetic analysis, and site-directed mutagenesis, leading to the generation of enzymes exhibiting extremely high activity and stability. Such enzymes and optimized reaction conditions offer many advantages that can be expected to enhance the efficiency of nucleic acid amplification tests, which may meet the increasing demand of point-of-care diagnosis both in developed and developing countries.

Availability of data and materials

Not applicable.

Authors' contributions

KY, IY and SF contributed to conceiving and designing the study, drafted and wrote the manuscript. All authors have read and approved the final version ofthe manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have not competing interests.

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