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Triggered isothermal PCR by denaturation bubble-mediated strand exchange amplification†

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Here, we introduced the concept of strand exchange amplification (SEA) mediated by denaturation bubbles. Similar to traditional PCR, it only employed a DNA polymerase and a pair of common primers to realize a three-step cycle process, but the entire SEA reaction was performed at a single temperature.

Nucleic acid testing (NAT) is making increasingly important contributions to identify and potentially quantify specific nucleic acid sequences for life sciences and clinical diagnosis.[1,2] These specific nucleic acid targets are routinely located in low expression abundance genes, so nucleic acid amplification will be required to achieve good sensitivity for bioanalysis. The development of the polymerase chain reaction (PCR) has provided a powerful tool for nucleic acid amplification, and trace amounts of nucleic acid targets can be exponentially amplified by PCR.[3] However, all PCR-based techniques need electrically powered thermal cycling equipment for repeatedly heating and cooling processes, which has limited them to laboratory settings.[4] The advent of isothermal approaches for nucleic acid amplification circumvents the limitations of traditional PCR, providing the ability of DNA amplification under isothermal conditions without the need for a thermocycling apparatus.[5,6] Although the isothermal approaches can reduce system complexity by single-temperature incubation, they usually require various accessory proteins, chemicals (betaine or L-proline), or multiple primers to perform strand separation and amplification.[7–9] One of the future needs of bioanalysis is the development of isothermal approaches with simplicity, sensitivity, and robustness for global outbreak surveillance for emerging infectious diseases in remote or outlying regions and the developing world.[10] The strand exchange reaction (SER) of nucleic acids is a key natural process *in vivo* for genetic homologous recombination, DNA replication and DNA repair.[11–14] In a SER, a double-stranded DNA (dsDNA) exchanges one of its strands by a homologous single-stranded DNA (ssDNA) to form a heteroduplex product. The homologous recombination in living systems is dependent on the SER and this process is catalyzed by recombinases such as eukaryotic RAD51 and prokaryotic RecA.[15,16] In fact, SERs can still proceed without the aid of gene recombination enzymes due to DNA breathing.[17] Although the structure of dsDNA is very stable because of the complementarity of its many base pairs, in which the interaction of each base pair is relatively weak to allow transient opening and lead to DNA breathing to create a single-stranded denaturation bubble,[18] the local denaturation bubbles can occur even at room or physiological temperatures owing to intermittent breaking of the base pairs.[19–21] The forming rate of bubbles is impacted by the GC content and the temperature.[22] The key of PCR is that dsDNA is separated and amplified by repeatedly increasing and lowering the reaction temperature. Herein, we introduce the concept of isothermal nucleic acid amplification depending on the denaturation bubbles of dsDNA at a single temperature, termed denaturation bubble-mediated strand exchange amplification (SEA).

The reaction progress of SEA is illustrated in Fig. 1. The SEA reaction relies on the ability of the DNA duplex to dynamically dissociate at reaction temperature by allowing local opening of base pairs to create a single-stranded denaturation bubble. Then a short oligonucleotide primer is able to invade the denaturation bubble, allowing DNA polymerase to gain the extension. This procedure can be performed at a single temperature below the dsDNA melting temperature, obviating the need for a traditional heat denaturation process. Under thermophilic conditions, for example 65 °C, the forward primer P1 anneals to the denaturation bubble of double-stranded nucleic acids and extends by a DNA polymerase. Owing to the spontaneous local conformational fluctuations within the amplified product of P1, the backward primer P2 can invade the denaturation bubble of the amplified product and extend to provide the dsDNA amplification products

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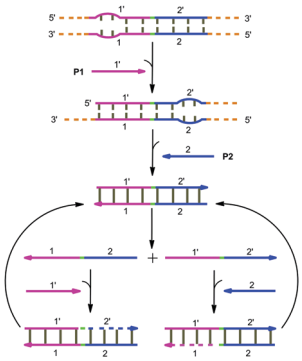

Fig. 1 The schematic illustration of the isothermal SEA method. Numbers marked with a prime symbol (′) are complementary to the corresponding unmarked numbers. All base sequences of the target and two primers used are listed in the ESI,† Table S1.

of the two primers. Following the same reaction mechanism, the generated dsDNA amplification products dissociate, and subsequent primer binding and extension events yield the same two dsDNA amplification products. The generated dsDNA amplification products and primers continue to interact to produce a new generation of dsDNA amplification products as the traditional polymerase chain reaction (PCR) except for isothermal processes. When the reaction was performed under thermophilic conditions, for example 65 °C, we needed to choose suitable DNA polymerases, such as Vent or *Bst* DNA polymerase. Owing to the characteristics of these DNA polymerases, tandem repeat amplification products might be produced.

We investigated the feasibility of SEA by detecting the specific sequence of *Escherichia coli* (*E. coli*) 16S rDNA. Real-time fluorescence detection of *E. coli* 16S rDNA by different DNA polymerases is shown in Fig. 2A. When *Bst* 2.0 WarmStart DNA polymerase (WS *Bst* 2.0), Vent DNA polymerase (Vent), Vent (exo$^-$) DNA polymerase (Vent exo$^-$), and *Taq* DNA polymerase (*Taq*) were, respectively, used for the SEA reaction at their optimum reaction temperatures, all fluorescence intensities significantly increased compared with their blanks. So, each of the four aforementioned DNA polymerases could be used to perform the SEA reaction, in which WS *Bst* 2.0 was the best one to provide sensitive detection and exhibited a regular exponential amplification curve. The real-time fluorescence products were further subjected to PAGE analysis (Fig. 2B). There was a 52-bp DNA band in lanes 1–4 as expected, and the electrophoresis results further showed that the expected SEA reaction was effectively performed by each of the four DNA polymerases.

To further show the amplification efficiency of SEA, we used a 546-bp double-stranded PCR product from *E. coli* 16S rDNA as a template, and designed different primers to amplify different lengths of target DNA. As shown in Fig. 3A, all specific target fragments of 45-, 66-, and 87-bp could be successfully amplified, respectively. With the increase of length of target DNA, the amplification efficiency of SEA decreased according to the T_t value at the same concentration as the target (1.0×10^{-10} M). Therefore, SEA was better at detecting small fragments of nucleic acids. It is generally known that nucleic acid-based testing protocols require the ability to amplify a unique sequence on the target. However, it is very difficult to find a long unique sequence for infectious organisms with high variation rates such as influenza and human immunodeficiency viruses (HIV), which greatly limits the usefulness of most nucleic acid-based testing methods. With good amplification efficiency for short detection targets and simple primers, SEA would fill the gap of the existing nucleic acid-based testing methods to play an important role in surveillance, prevention and control of infectious organisms with high variation rates.

All amplification products from different target fragments were visualized by native PAGE (Fig. 3B). The expected 45-, 66-, and 87-bp amplification products, respectively, appeared on the gel. Besides, there were higher molecular weight amplification products observed on the gel that could not be predicted by the reaction scheme. The bands in lane 3 were excised from the gel and non-templated addition of adenine by *Taq* DNA polymerase was performed. The modified products were subcloned into a TA cloning vector, and sequenced. The sequenced result (Fig. 3C) of the 87-bp amplification product corresponded to the predicted final product. The amplification products with higher molecular weights represented 87-bp tandem repeats with variable numbers. The tandem repeats were separated from each other by a linker sequence that was related to adjacent sequences of the amplification target on the template by sequence alignment

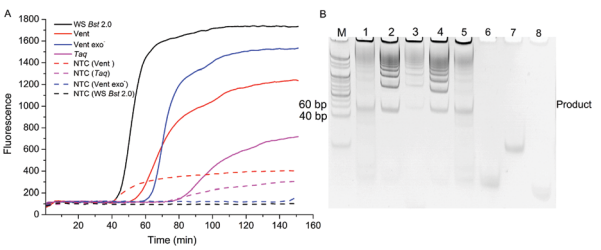

Fig. 2 Verification of SEA by different DNA polymerases. (A) The verification of SEA by real-time fluorescence detection. (B) 17.5% native PAGE of the corresponding amplification reaction products from (A). (M) 20-bp DNA ladder. (1) *Taq* DNA polymerase. (2) Vent DNA polymerase. (3) Vent (exo$^-$) DNA polymerase. (4) *Bst* 2.0 WarmStart DNA polymerase (WS *Bst* 2.0). (5–8) Corresponding NTC of lanes 1–4, respectively. The NTC was a no-template control to be used as the blank.

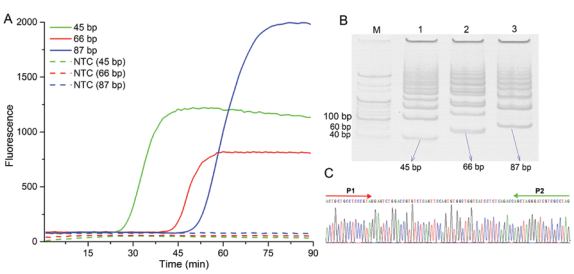

Fig. 3 (A) The real-time fluorescence curves for different lengths of amplification products. The concentrations of primers were 1.0×10^{-6} M. NTC means no template control. (B) Native PAGE of the corresponding amplification products from (A). (C) Sequencing of the 87-bp product. All sequences used are listed in the ESI,† Table S1.

(the sequencing data are provided in the ESI,† Table S2). These tandem repeats probably were the result of self-folding extension, which may be related to the characteristics of some DNA polymerases, such as Vent and *Bst* DNA polymerases, used in isothermal nucleic acid amplification reactions.[23–25]

To make SEA work well, we have successfully optimized other assay conditions including the reaction temperature, the amount of DNA polymerase, and the concentrations of primers, Mg^{2+} and PEG-200 (ESI,† Fig. S1). The optimum assay conditions were used for the following experiments.

For a nucleic acid-based testing method, generality is very important for practical applications where DNAs are mostly double-stranded. After the feasibility of SEA was verified, we subsequently chose the double-stranded pBluescript II KS (+) plasmid (referred to as pBlu2KSP) as a model target to show the practical application ability of SEA. The corresponding primers were designed according to the specific sequence of pBlu2KSP (sequences are listed in the ESI,† Table S1). Amplification reactions were carried out at 65 °C with different amounts of the extracted pBlu2KSP dsDNA target without any pre-treatment such as the heat denaturation step. Real-time fluorescence detection of SEA for different concentrations of the pBlu2KSP dsDNA target is shown in Fig. 4A. The real-time fluorescence curves showed good regularity with the increase of the amount of pBlu2KSP DNA in the presence of a tested concentration range from 100 amol to 100 fmol. The threshold time (T_t) value was linearly related to the negative logarithmic value of the amount of pBlu2KSP DNA in the range from 100 amol to 100 fmol (Fig. 4B). The regression equation was $T_t = -112.448 + 17.222\ (-\lg C_{PBS})$ (C_{PBS} was the concentration of pBlu2KSP DNA, $R^2 = 0.9968$). pBlu2KSP is a circular double-stranded DNA molecule. Considering the ability to detect pBlu2KSP, SEA could be well triggered by supercoiling-induced denaturation bubbles. Native DNA is usually subjected to topological constraints as eukaryotic linear DNA and prokaryotic circular DNA.[26] According to the aforementioned results, it can be concluded that the SEA method has good generality for both a free linear DNA and a circular DNA.

RNA is always an important research target in life sciences for playing key roles in biological entities.[27,28] Conventional RNA detection methods usually consist of two steps: reverse transcription step followed by amplification reactions. Combining SEA with our previous finding that *Bst* DNA polymerase exhibits innate reverse transcriptase activity,[29] we sought to explore SEA

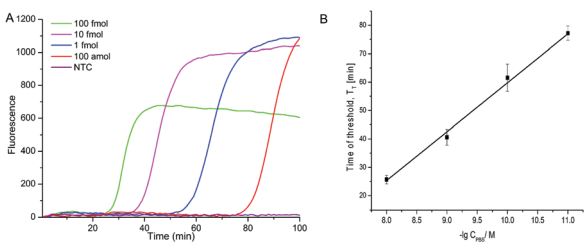

Fig. 4 (A) The real-time fluorescence detection for 10-fold dilution of the pBlu2KSP dsDNA target. (B) The relationship between the T_t values and the negative logarithmic values of the amount of the pBlu2KSP dsDNA target (error bars show mean standard deviations of three determinations).

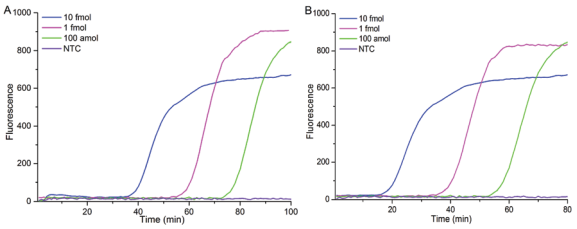

Fig. 5 The real-time fluorescence detection of the *E. coli* 16S rRNA target by SEA. (A) In a buffer solution. (B) In 10% (v/v) fetal bovine serum. The experimental conditions were consistent with those for the buffer, except for the serum added.

for single enzyme and one-step RNA detection that made the inverse transcription step and the next amplification reaction occur together. The 10-fold dilution of extracted 16S rRNA from *E. coli* was used as the target and was directly used to trigger the SEA reaction without requiring both heat denaturation to open the secondary structure of RNA and the reverse transcription step. Real-time fluorescence detection of SEA for different concentrations of the RNA target is shown in Fig. 5A. The fluorescence curves showed good regularity with the increase of the amount of the RNA target, and as low as 100 amol of target RNA could be detected by this method, demonstrating that SEA could be used for RNA direct detection. SEA works at 65 °C, which would be beneficial for opening the secondary structure of RNA. This makes it easier for SEA to detect highly structured bacterial and viral targets under isothermal conditions. To further improve the detection sensitivity and reduce the assay time, we could add some small chemical molecules that could promote the opening up of dsDNA in the reaction system. On the other hand, this method could also be coupled with other isothermal nucleic acid amplification technologies, such as exponential isothermal amplification reaction (EXPAR) to increase detection sensitivity.

We further performed RNA direct detection in 10% fetal bovine serum (FBS) without any pre-treatment to investigate the feasibility of SEA in biological samples. We measured the fluorescence signals of different concentrations of the RNA target in 10% FBS (Fig. 5B). The fluorescence curves also showed good regularity corresponding to the amount of the RNA target, and 100 amol of target RNA was well distinguished from the negative control, showing that SEA functioned in 10% FBS too.

In addition, the T_t value of the same concentration of the target in 10% FBS was smaller than that of standard buffer. This result indicated that SEA was feasible for the direct detection of RNA in such a complex biological sample. The system presented was successfully implemented for RNA detection, which would spur the speedy development of RNA one-step detection technologies to just meet the urgent need for rapid detection of RNA in clinical diagnosis and inspection and quarantine. Moreover, the specificity of the amplification was tested by detecting hepatitis C virus (HCV) (ESI,† Fig. S2) and the sequences used are listed in the ESI,† Table S1.

In conclusion, we introduced the concept of strand exchange amplification (SEA) mediated by denaturation bubbles, which utilizes natural strand "breathing" to open up dsDNA rather

than heat denaturation. Since the dsDNA target could naturally melt at reaction temperature, the entire SEA reaction could be performed at a single temperature. It is possible to envision more isothermal nucleic acid amplification technologies based on the concept of SEA, which will greatly benefit POCT/NAT devices due to the simplicity of the reaction system and the isothermal process.[30]

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