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Triggered isothermal PCR by denaturation bubble-mediated strand exchange amplification

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Abstract

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. These targets are routinely located in low expression abundance genes, so nucleic acid amplification is required for good sensitivity. The development of the polymerase chain reaction (PCR) has provided a powerful tool, but all PCR-based techniques need electrically powered thermal cycling equipment, which has limited them to laboratory settings. The advent of isothermal approaches circumvents the limitations of traditional PCR. Although 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. One of the future needs is the development of isothermal approaches with simplicity, sensitivity, and robustness for global outbreak surveillance in remote regions.

The strand exchange reaction (SER) of nucleic acids is a key natural process in vivo for genetic homologous recombination, DNA replication and DNA repair. 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. Homologous recombination is dependent on the SER, catalyzed by recombinases such as eukaryotic RAD51 and prokaryotic RecA. In fact, SERs can still proceed without the aid of recombination enzymes due to DNA breathing. Although the structure of dsDNA is very stable, the interaction of each base pair is relatively weak, allowing transient opening and DNA breathing to create a single-stranded denaturation bubble. Local denaturation bubbles can occur even at room or physiological temperatures owing to intermittent breaking of base pairs. The forming rate of bubbles is impacted by the GC content and temperature. 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).

Principle of SEA

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 spontaneous local conformation fluctuations within the amplified product, the backward primer P2 can invade the denaturation bubble of the amplified product and extend to provide the dsDNA amplification products of the two primers. The generated dsDNA products dissociate, and subsequent primer binding and extension events yield the same two dsDNA products, similar to traditional PCR except for isothermal processes. At 65° C, suitable DNA polymerases such as Vent or Bst DNA polymerase must be chosen.

Feasibility and performance

We investigated the feasibility of SEA by detecting the specific sequence of Escherichia coli 16S rDNA. Real-time fluorescence detection was performed with different DNA polymerases. Bst 2.0 WarmStart DNA polymerase (WS Bst 2.0), Vent DNA polymerase, Vent (exo-) DNA polymerase, and Taq DNA polymerase could all perform the SEA reaction, with WS Bst 2.0 providing the most sensitive detection and a regular exponential amplification curve. PAGE analysis showed a 52-bp DNA band as expected.

To show the amplification efficiency, we used a 546-bp double-stranded PCR product from E. coli 16S rDNA as a template and designed different primers to amplify target DNAs of 45-, 66-, and 87-bp. All three specific target fragments were successfully amplified. With increasing target length, the amplification efficiency of SEA decreased. Therefore, SEA was better at detecting small fragments of nucleic acids. This is valuable because it is very difficult to find a long unique sequence for infectious organisms with high variation rates (such as influenza and HIV). Sequencing of the 87-bp product corresponded to the predicted final product; higher molecular weight products represented tandem repeats, probably the result of self-folding extension related to DNA polymerase characteristics.

We optimized other assay conditions including reaction temperature, amount of DNA polymerase, and concentrations of primers, Mg^{2+} and PEG-200.

Generality and applications

For practical applications, we chose double-stranded pBluescript II KS(+) plasmid (pBlu2KSP) as a model target. Amplification reactions were carried out at 65° C with different amounts of dsDNA without any pre-treatment such as heat denaturation. The real-time fluorescence curves showed good regularity over a concentration range from 100 amol to 100 fmol, and the threshold time (Tt) value was linearly related to the negative logarithmic value of the target amount ($R^2 = 0.9968$). Considering the ability to detect circular pBlu2KSP, SEA could be well triggered by supercoiling-induced denaturation bubbles, showing good generality for both linear and circular DNA.

RNA is an important research target. Combining SEA with our previous finding that Bst DNA polymerase exhibits innate reverse transcriptase activity, we explored SEA for single-enzyme and one-step RNA detection. The 10-fold dilution of extracted 16S rRNA from E. coli was used as target, directly triggering SEA without heat denaturation or a separate reverse transcription step. As low as 100 amol of target RNA could be detected. SEA works at 65° C, which is beneficial for opening the secondary structure of RNA. We further performed RNA direct detection in 10% fetal bovine serum (FBS) without pre-treatment, and 100 amol of target RNA was well distinguished from the negative control, showing that SEA functioned in complex biological samples.

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.

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