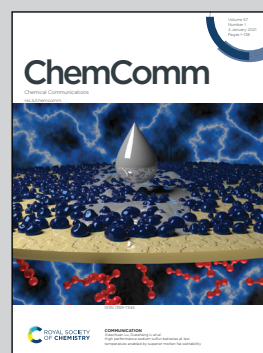**Showcasing research from Professor Zhen Huang's laboratory, College of Life Sciences, University of Sichuan, Chengdu, P. R. China.**

Highly convenient and highly specific-and-sensitive PCR using Se-atom modified dNTPs

A new strategy with Se-dNTPs can enhance PCR specificity (over 240-fold) and sensitivity (up to single-digit), effectively eliminate non-specific products, and simplify PCR design and optimization.

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Highly convenient and highly specific-and-sensitive PCR using Se-atom modified dNTPs†

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Primer design and condition optimization for PCR are tedious and labour-intensive. To conveniently achieve high selectivity, sensitivity and robustness, herein, we first report a new strategy with Se-dNTPs to enhance PCR specificity (over 240-fold) and sensitivity (up to single-digit), effectively eliminating non-specific products and simplifing PCR design and optimization.

The polymerase chain reaction (PCR) has become one of the most important techniques in modern life science.[1–4] From a small amount of DNA template, PCR can exponentially generate a large number of DNA copies in a short period of time. However, nonspecific DNA fragments are often produced, especially in multiple-round PCR, decreasing its amplification efficiency. Therefore, many steps in PCR are essential to obtain a successful experiment, including primer design and condition optimization. In aspect of primer design, available primers are characterized according to the following parameters:[5] (i) sufficient specificity for minimization of off-target annealing; (ii) appropriate melting-temperatures for balancing efficiency and specificity; (iii) a moderate GC content (40–60%) and absence of complementarity between primers, especially at their 3′ ends, for reducing nonspecific pairing between primers, which can significantly affect PCR efficiency and specificity; (iv) avoidance of a significant secondary structure, which can decrease amplification efficiency significantly. Despite availability of many free web-tools for primer design, their successful rate is not always satisfactory.[6–8]

The related verification and condition optimization are still necessary, including component quantities (such as DNA

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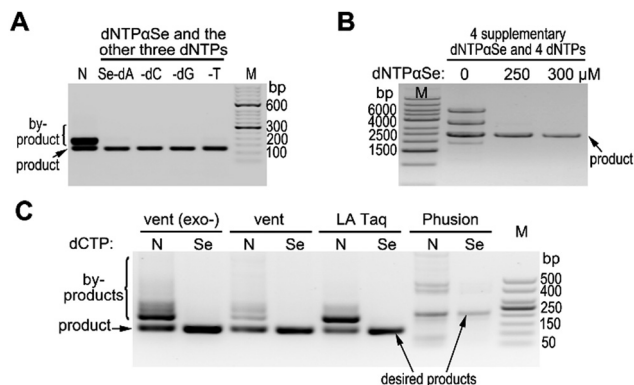
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polymerase, primers, templates and dNTPs) and thermal conditions (such as annealing and extension temperatures). In addition, many other efforts have been used to increase PCR efficiency, specificity and robustness, including hot-start strategy with heat-activated polymerase or primer,[9–11] directed-evolved DNA polymerase,[12] chemical additives (including DMSO, betaine, metal nanoparticles, quantum dots and nano-polymers),[13,14] supplementary recombinase and helicase,[15,16] and modified or immobilized primers.[17] These efforts are time-consuming and labour-intensive. Though many attentions have been paid to PCR, its non-specific amplification is often a challenge, especially when many primers are involved in an amplification, such as multiplex PCR.

In our preliminary research, DNA polymerases can recognize dNTPαSe and incorporate it into DNA with high specificity and similar product yield.[18] Based on these discoveries, we decided to explore Se-atom-assisted PCR (Se-PCR) for suppression of non-specific products, simplification of primer design, and ease of condition optimization. In order to investigate whether nonspecific products can be suppressed in PCR, we performed the reactions in the presence or absence of individual dNTPαSe replacing corresponding canonical dNTP (Fig. 1 and Table S1, ESI†). Excitingly, we found that even after 30 cycles of PCR reaction (using Taq DNA polymerase), nonspecific product was barely observed in the presence of any dNTPαSe, while nonspecific products were formed in the absence of dNTPαSe (Fig. 1A). Further, the PCR product yields were generally similar in the presence or absence of dNTPαSe. Furthermore, another strategy is to add four supplementary dNTPαSe analogues (as low as 1.0 equiv.) into PCR reactions. When PCR amplifying relatively long DNA sequences, the four supplementary analogues completely supressed nonspecific product formation as well (with similar yield, Fig. 1B). Excitingly, the specificity of PCR with high-fidelity DNA polymerases can be further improved (Fig. 1C), though they [vent (exo-), vent, LA Taq, Phusion polymerases] are of 2-, 5-, 6.5-, 52-fold higher fidelity than currently used Taq, respectively.[19,20]

To study whether in the presence of dNTPαSe, the PCR reaction conditions can be easily optimized, we decided to

Fig. 1 Highly-specific PCR using Se-atom modified dNTPs. (A and B) Two strategies (the replacement and supplement) using dNTPαSe as substrates. (A) One of four dNTPαSe triphosphates replaced the corresponding canonical dNTP. (B) Supplementary four dNTPαSe triphosphates were added into the PCR reactions with canonical dNTPs. (C) Additional specificity enhancement of high-fidelity DNA polymerases with dNTPαSe (dNTPαSe representing dCTPαSe, except in LA Taq reaction using dATPαSe and dCTPαSe). Rates of the by-product suppression using dNTPαSe were approximately 19.7 [vent (exo-)], 9.0 (vent), 61.6 (LA Taq) and 9.8 (Phusion) folds.

explore a broad range of component concentrations. We carried out the gene cloning experiments by the primer concentration optimization, followed by 16 different sets of optimizing experiments using human genome. We found that at various concentrations of primers, all reactions with canonical dNTPs produced nonspecific products, while dNTPαSe produced barely any by-products (Fig. 2A). Further, when canonical dNTPs were used and primer concentration increased, more by-products were formed proportionally, while the reactions

with supplementary dNTPαSe produced barely any by-products (Fig. 2A). Furthermore, we found that in the 16 different conditions for optimization, canonical dNTPs caused formation of many by-products, while dNTPαSe significantly reduced by-product formation (Fig. 2B). Clearly, dNTPαSe is better than the canonical dNTPs and can allow easy optimization.

To investigate whether PCR thermal conditions can be easily optimized,[21,22] in the presence of dNTPαSe, we performed a series of PCRs at various annealing temperatures (T_a) and extension temperatures (T_e). We found that the reactions with canonical dNTPs produced many by-products under various T_a and T_e combinations (Fig. 3A and B), while supplementary dNTPαSe offered clean products under the same conditions (Fig. 3C and D). These results indicated that the supplementary dNTPαSe can effectively improve the specificity of PCR, provide a broader range of thermal conditions, and simplify the optimization steps for highly specific PCR amplification.

Based on our results above, we decided to explore whether we can choose primers randomly for a PCR reaction, namely simplifying primer design. With 20 different primer pairs chosen arbitrarily, we performed PCR reactions on the same template. We found that only using canonical dNTPs, 6 out of 20 primer pairs were satisfactory (successful rate 30%), while in the presence of dCTPαSe, 19 out of 20 were successful (rate 95%, Fig. 4 and Table S2, ESI†).

Encouraged by the high specificity on simplex PCR with dNTPαSe, we explored multiplex PCR (MP-PCR), a powerful tool for simultaneously detecting many target sequences in one tube.[23–25] Our results indicated that using canonical dNTPs, the conventional MP-PCRs still formed many by-products even after the time-consuming optimization (Fig. 5), while in the presence of dCTPαSe, MP-PCRs conveniently offered clean reactions without significant by-products, indicating the suppression of nonspecific amplifications. Further, we found that the Se-modified products moved faster than the corresponding
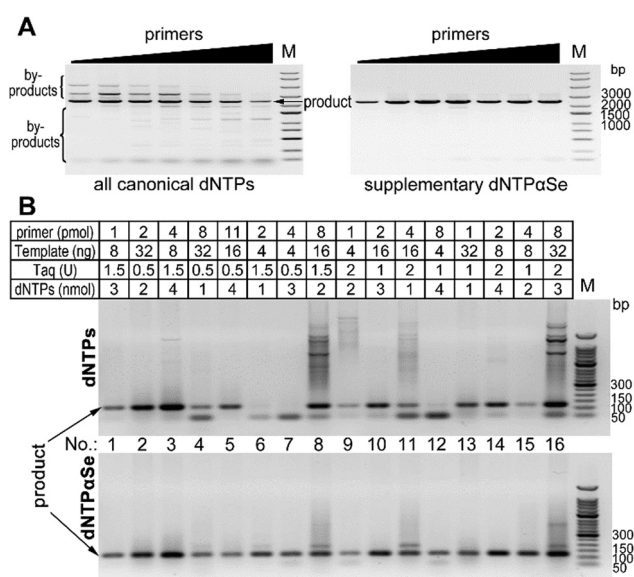

Fig. 2 PCR with various concentrations of components. (A) Reactions with various concentrations of primers (from left to right: 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0 μM). (B) Reactions with various combinations of primers, template, Taq DNA polymerase and dNTPs (total volume: 10 μL). Specific products were indicated with arrows.
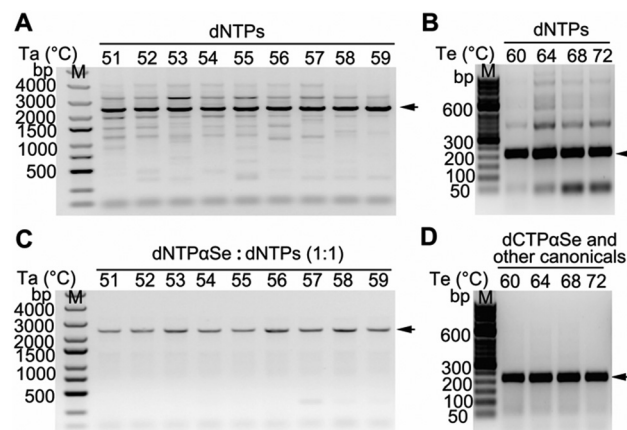

Fig. 3 Effects of thermal conditions on PCR specificity. (A and B) Reaction specificity at various annealing temperatures (T_a) and extension temperatures (T_e) with all canonical dNTPs. (C and D) Reaction specificity at various T_a and T_e with four dNTPαSe analogues. Specific products were indicated with arrows.
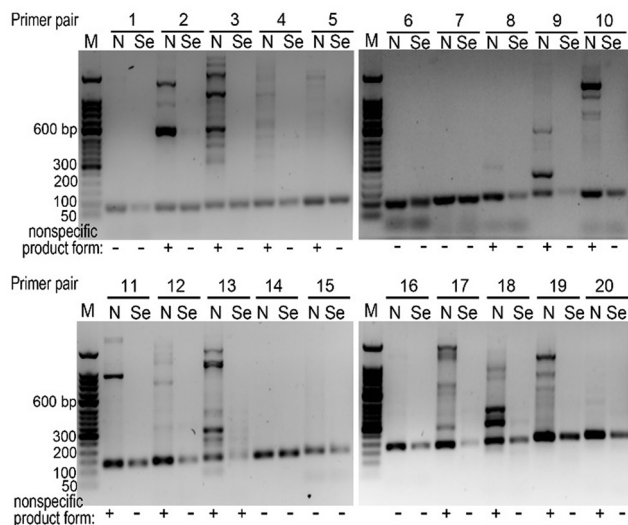
Fig. 4 The Se-PCR specificity with randomly selected primers. "N" indicates the reactions with all canonical dNTPs, while "Se" indicates reactions with dCTPαSe and other three canonical dNTPs.
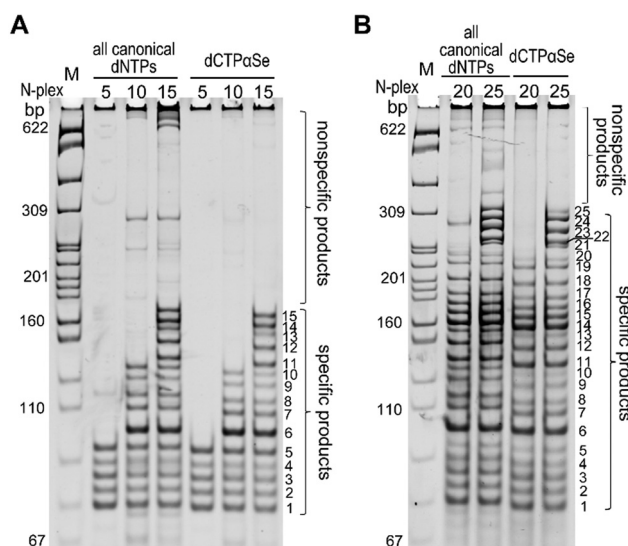

Fig. 5 Multiplex PCR with dCTPαSe and the other three canonicals. (A) 5-, 10- and 15-plex PCRs. (B) 20- and 25-plex PCRs.

canonical ones on native PAGE (Fig. 5), which is probably caused by the hydrophobic nature of the Se-modifications.

Effective suppression of nonspecific amplification with dNTPαSe is necessary, as multiplex PCR can amplify multiple target sequences by using multiple primer pairs simultaneously and the complexity of primers can trigger formation of primer-dimers and other nonspecific products. In order to understand the nonspecific suppression, we performed the melting-temperature (T_m) study with the Se-PCR products, namely Se-DNAs. Interestingly, we have found that the Se-DNAs have lower T_m values, compared to the corresponding unmodified DNAs (Table S3, ESI†). The lower T_m can discourage the mis-annealing of the primers and mis-extension of dNTPs.
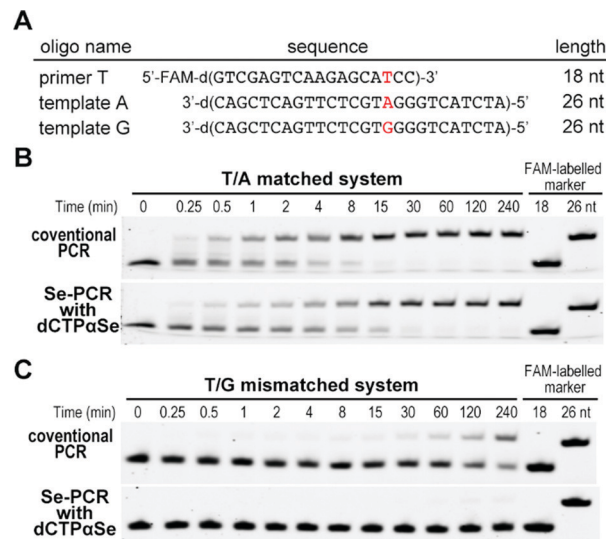

Fig. 6 Suppression of mismatched DNA polymerization in the presence of dNTPαSe. (A) DNA sequences used in B and C. (B) Matched primer extension: ratio of the reaction rates between dCTP and dCTPαSe was 1.3. (C) Mis-matched primer extension: rate of the discrimination against T/G by dCTPαSe was 315.

Further, with dNTPαSe, we performed the extension on a mis-matched template, where the mis-matched site (T/G) is four nucleotides away from the elongation site (Fig. 6A). Our study has revealed that compared with canonical dCTP, dCTPαSe enhanced the mis-match discrimination of DNA polymerase. Furthermore, our steady-state kinetic study has indicated that dCTPαSe is over 240-fold more discriminative than the canonical (Fig. 6B, C and Fig. S1, ESI†), thereby revealing its PCR specificity enhancement on a mis-paired template. As the incorporation of dNTPαSe is slower than that of the canonical one,[18] the step of dNTPαSe incorporation can offer sufficient time to DNA polymerase for discriminating against mismatched primer/template (such as T/G wobble pair) and enhancing PCR polymerization specificity.

In addition, we explored the effect of dNTPαSe on sensitivity, and we found that compared with canonical PCR strategy, efficiency and yield of Se-PCR (two strategies) might vary (Fig. 1–4), and sensitivity differences among these three strategies were small in the absence of background DNAs (Fig. S2, ESI†). However, in the presence of background and low number of copies of target molecules, Se-PCR is more specific and sensitive than the canonical, because of its suppression on nonspecific products (Fig. 7), which cause the consumption of the substrates and primers, therefore reducing the sensitivity and specificity. We performed qPCR for detecting human papilloma virus type 16 (HPV-16), in the presence of human genome as background DNA (Fig. 7). Further, we measured the high-resolution melting (Fig. 7C) and carried out agarose gel electrophoresis (Fig. 7D). We found that using the supplement strategy, dNTPαSe could largely increase specificity by eliminating by-products formed with the commercial kits (Fig. 7C and D). Furthermore, we discovered that being consistent with the suppression of nonspecific products and background noise, the
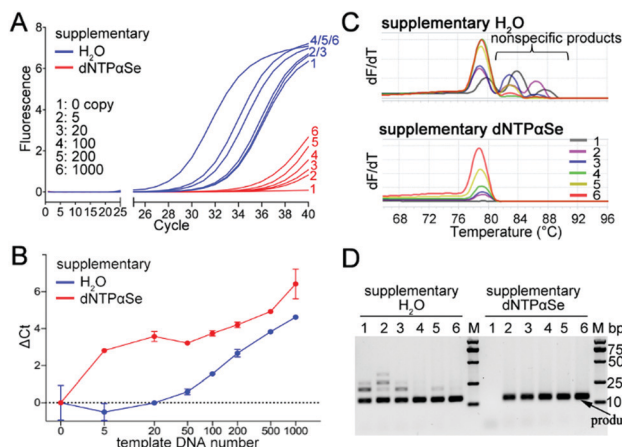
Fig. 7 Sensitivity improvement of HPV PCR detection by dNTPαSe in the presence of human genomic DNA. (A) Amplification curves; single-digit copies of DNA molecules were detected. (B) Relationship between ΔC_t and viral DNA copies in qPCR (SYBR Green I); ΔC_t is PCR C_t (Cycle of threshold) difference between those of background and sample; $\Delta C_t < 1$ was defined as negative detection result. (C) High-resolution melting analyses of the corresponding reaction products in A. (D) Agarose gel analysis of reaction products in A.

dNTPαSe-supplemented system allowed the detection up to 5 copies of targets (Fig. 7B), approximately 20-fold more sensitive than the original kit, in the presence of human DNA.

In conclusion, we have discovered that dNTPαSe can significantly reduce the off-target amplification in PCR. We have established two strategies for highly-specific DNA polymerization and synthesis: (i) replacement of canonical dNTP(s) with dNTPαSe and (ii) supplement with four dNTPαSe analogues. In addition, we have found that dNTPαSe can effectively improve the specificity (over 240 folds) and in turn enhance sensitivity (up to single-digit copies of targets) *via* inhibiting nonspecific polymerization. Moreover, dNTPαSe can usefully simplify design and optimization of highly-specific PCR and MP-PCR.

Conflicts of interest

There are no conflicts to declare.

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