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Full Length Research Paper

One-stop polymerase chain reaction (PCR): An improved PCR method with speedy operation and comparable efficiency

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The widely used polymerase chain reaction (PCR) protocol requires several post-cycling steps to visualize amplicons, decelerating PCR sample processing and result calling. "One-stop PCR" was developed by including both the loading buffer and nontoxic staining dye within a single PCR tube, allowing direct loading and simultaneous staining of amplicons for visualization. The efficiency and sensitivity of one-stop PCR were comparable to commercially available PCR kits and it is compatible with downstream uses and is not mutagenic. One-stop PCR robustly minimizes the time and labor required for PCR reaction setup and product visualization.

Key words: Polymerase chain reaction (PCR), post-cycling, compatible dye, Gelred.

INTRODUCTION

Polymerase chain reaction (PCR) has become the fundamental technique in molecular biology since the development in 1980s and has achieved remarkable innovations to enhance its efficiency, specificity and applicability (Alder et al., 2008), while there are still ever-increasing requirements for further improvements of the current PCR pipelines for safe, cost-effective and high-throughput manipulation (Ralser et al., 2006). With regards to the commonly used PCR protocols, several additional post-amplification steps are required to detect the PCR output, that is, inclusion and mixing of loading buffer as well as the preparation of agarose gels with staining dyes (ethidium bromide, SYBR Green I, etc) (Huang et al., 2005; Aziah et al., 2007). These post-PCR procedures not only increase the handling time but expose experimentalists to toxic reagents. Therefore, a novel PCR method is desired to compatibilize *Taq* DNA polymerase activity with specific additives that allows direct loading and simultaneous staining of PCR products for electrophoresis and visualization, so as to speed up the current PCR flowchart, especially when a large number of samples are needed to be analyzed (Chua et al., 2011).

Some compounds, such as glycerol and brom phenolblue that could serve as densifying agent and tracking dye, were found to be compatible with PCR (Haack and Vizuete-Forster, 2000). Recently，a novel nontoxic staining dye, Gelred, has been developed to substitute for ethidium bromide (Huang et al., 2005, 2010). These accumulating evidences converge on the possibility that a more convenient and safe PCR method is feasible to include specific additives in a single PCR mixture for speedy handlings while maintaining amplification efficacy and specificity. In this study, we developed a PCR method that incorporates densifying agent, monitoring and nontoxic staining dyes in a single PCR tube to permit efficient amplification, direct loading and simultaneous staining of PCR products. This method

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has been extensively used in our laboratory and we consistently obtained reproducible and reliable results.

MATERIALS AND METHODS

PCR master mix and cycling conditions

10×PCR reaction buffer consisted of $MgSO_4$ 20Mm, KCl 100 mM, $(NH_4)_2SO_4$ 80 mM, Tris-HCl (pH = 9.0) 100 mM and NP-40 0.5% (v/v). 5×compatible dye mix included 100g Ficoll400/L, 0.1 g cresol red/L, 0.8 g tartrazine/L, Gelred 1.25× (Original concentration is 10000× in DMSO; Biocompare, USA). 2×One-stop PCR master mix (2×Red mix) was prepared as follows: 10×PCR reaction buffer, 2 µl; 40 mM dNTP (10mM each), 0.5 µl; *Taq* DNA polymerase, 0.2 µl (1 unit); 5×compatible dye mix, 4 µl; PCR-grade water, 3.3 µl. The final pH of 2×Red mix was 7.5, when all the compounds were completely dissolved at 25°C. A typical 20 µl one-stop PCR mixture was composed of 10 µl 2×One-stop PCR master mix, 0.5 µl forward and reverse primer mix (10 µM total), 0.5 µl cDNA, 9 µl PCR-grade water. A 20 µl traditional PCR mixture included 10×PCR reaction buffer, 2 µl, 40 mM dNTP (10 mM each), 0.5 µl; *Taq* DNA polymerase, 0.2 µl (1 unit), 0.5 µl forward and reverse primer mix (10 µM total), 0.5 µl cDNA, 16.3 µl PCR-grade water. Three PCR kits (*Taq* DNA polymerase, *DreamTaq* DNA polymerase, 2× Blue master mix) were purchased from Shenergy (Shanghai, China), Fermentas (Lithuania) and DBI (Shanghai, China), respectively. All cycling condition were standardized as: 94°C, 2 min; 30 cycles of (94°C, 15 s, 55°C, 15 s, 72°C, 1 min); 72°C, 5 min. All cyclings were carried out in a Minicycler machine (MJ Research, USA). Unless indicated elsewhere, the products of traditional PCR and DNA ladders were separately mixed with 1×loading buffer containing 5% glycerol, 0.04% brom phenolblue, 0.04% xylene cyanol, 0.25× Gelred, before loading for electrophoresis.

Nucleic acid manipulation

Human cervical carcinoma Hela cell line cDNA was synthesized with 2 µg total RNA by RQ1 DNase (Promega) treatment and reverse transcribed with M-MLV (Promega), mainly based on the manufacture's instruction. Ethanol precipitation was performed essentially as previously described (D'Souza et al., 2005). *Silica* membrane filtration was carried out with a commercial PCR product purification kit (Generay, Shanghai), according to the manufacture's instruction. In brief, 50 µl PCR product was mixed thoroughly with binding buffer, and the resultant mixture was loaded directly onto a *silica* membrane Gelclean column. After filtration and washing, the attached DNA was dissolved by 50 µl elution buffer (Tris, pH = 7.5). An aliquot of 5 µl recovered DNA was sized in 2% agarose gel. A TA-cloning was performed essentially as previously described (Bi et al., 2007).

Primers

The forward and reverse primers (5'-3') used to amplify human β-actin mRNA (NCBI accession number NG_007992) were: F100, ATTGCCAATGGTGATGACCT; F200, ATGTCCACGTCACACTTCAT; F270, CGTGGACATCCGCAAAGAC; F300, GGGTGCCAGGGCAGTGATCT; F400, CGCCCTGGACTTCGAGCAAGAGAT; F500, TGTAACGCAACTAAGTCATA; F750, CTGTAACAACGCATCTCATA; R, TGGAAGGTGGACAGCGAGGC. Primers used to amplify H19 RNA (NCBI Accession number NR_002196.1) were: H19F, ACTCAGGAATCGGCTCTGGAA; H19R, ATGATGTGGTGGCTGGTGGTC. Primers used to amplify human GAPDH mRNA (NCBI Accession number NM_002046.3) were: GAPDH-F: GAGTCAACGGATTTGGTCGTATTG; GAPDH-R: ACAGTCTTCTGGGTGGCAGTGAT. A M13 reverse primer was used for sequencing of insert into pMD18-T vector (Takara, Japan).

RESULTS AND DISCUSSION

One-stop PCR does not affect amplification specificity and DNA mobility

In order to improve the current PCR protocols for more convenient handlings, we established an optimal PCR platform that includes density agent, tracking and staining dyes in a single PCR tube. We started with Ficoll400, cresol red, tartrazine and Gelred to upgrade the commonly used PCR methods due to their low cost and ready availability. As a proof-of-principle test, a series of fragments of human β-actin mRNA (NM_001101) ranging from 100 to 750 bp were amplified to examine the amplification specificity and migration pattern with the presence (at final concentration of 2% Ficoll400, 0.002% Cresol red, 0.016% Tartrazine, 0.25×Gelred in a 20 µl PCR mixture) or absence (traditional PCR) of these additives. As shown in Figure 1A and B, specific and sharp bands were detected in all PCR reactions, implying that the inclusion of the additives does not sacrifice its sensitivity and clarity. Furthermore, photocopiable electrophoresis profile shows that individual PCR products migrate strictly based on their size, irrespective of the inclusion of these additives or not, indicating that these compounds do not have adverse effect on DNA mobility. This test validates the concept that a mixture of densifying agent, tracking and staining dyes are compatible with PCR and it appeared to perform well under optimized conditions. We named it "one-stop PCR".

Performance of one-stop PCR

We then sought to assess the efficiency of one-stop PCR and other three commercially available PCR kits by amplifying a 400 bp fragment of human β-actin mRNA (NM_001101) at gradient template quantities (1/1, 1/10, 1/100). As indicated in Figure 2A, the efficiency of one-stop PCR was definitely comparable to any of these three PCR kits. Also, one-stop PCR manifested good specificity and sensitivity with decremental template amount. Next, we carried out a triplex PCR to further examine the amplification efficiency. Three fragments of human GAPDH (600 bp, NM_002046), human β-actin (270 bp, NM_001101) and human H19 RNA (170 bp, NR_002196.1) were simultaneously amplified in a single reaction mix (Novak Kujundzic et al., 2008). We found that one-stop PCR was more sensitive and efficient than traditional PCR in the triplex condition, as visualized in Figure 2B. The two lines of evidence demonstrate that

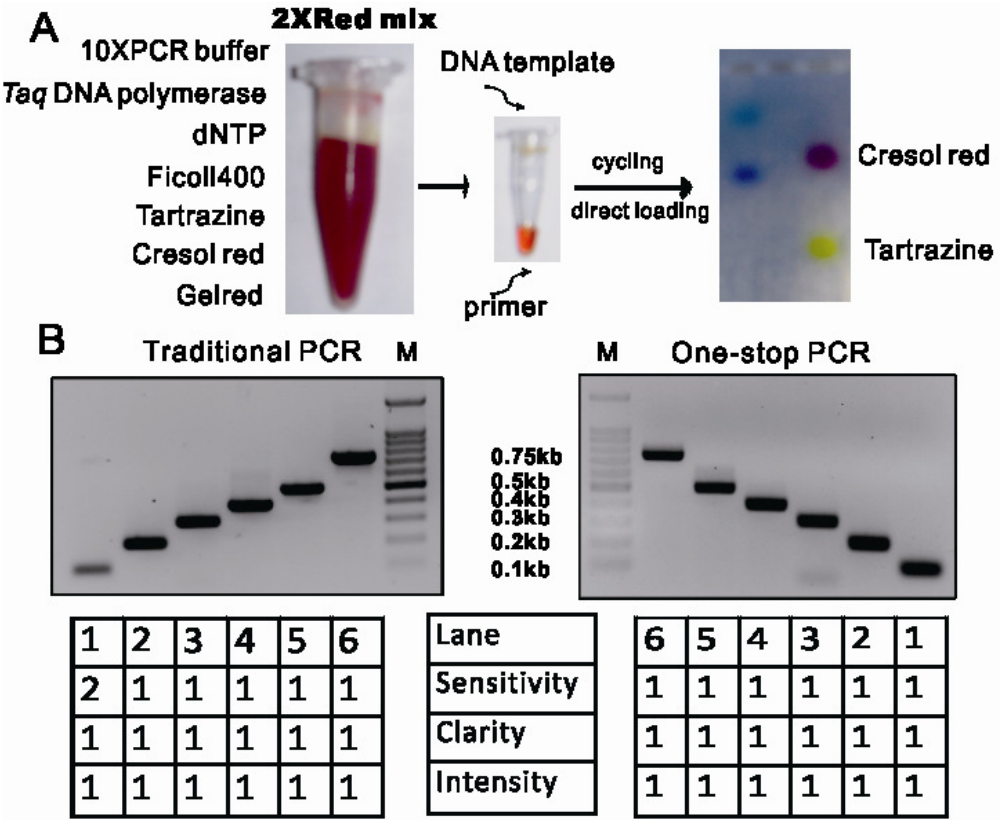

Figure 1. Illustration of one-stop PCR and its applicability. (A) Briefing of one-stop PCR: A 2×Redmix is prepared by adding cresol red, tartrazine, ficoll400 and Gelred to conventional PCR reaction at desired concentration. After cycling, the PCR products can be directly loaded into gel for electrophoresis and visualization. A typical 20 µl one-stop PCR mixture was composed of 10 µl 2×One-stop PCR master mix, 0.5 µl forward and reverse primer mix (10 µM total), 0.5 µl cDNA, 9 µl PCR-grade water. 2×One-stop PCR master mix (2×Red mix) was prepared as follows: 10×PCR reaction buffer, 2 µl; 40 mM dNTP (10 mM each), 0.5 µl; *Taq* DNA polymerase, 0.2 µl (1 unit); 5×compatible dye mix, 4 µl; PCR-grade water, 3.3 µl. 5×compatible dye mix included 100 g Ficoll400/L, 0.1 g cresol red/L, 0.8 g tartrazine/L, Gelred 1.25× (Original concentration is 10000× in DMSO; Biocompare, USA). (B) Six DNA sequences ranging in size from 100 to 750 bp were amplified by conventional PCR (left) and one-stop PCR (right), respectively, and sized by 2% agarose gel. One-stop PCR generated unique products that migrated strictly based on their molecular weight. Gelred is capable of staining DNA simultaneously for immediate visualization. Sensitivity, clarity and intensity are scored on a ranking system where 1 indicates the best performance，2 good, 3 fair, and 0 the worst (same below). The cycling was run with: 94℃, 2 min; 30 cycles of (94℃, 15 s, 55℃, 15 s, 72℃, 1 min); 72℃, 5 min, in a Minicycler machine (MJ Research, USA). Unless indicated elsewhere, the products of traditional PCR and DNA ladders were separately mixed with 1×loading buffer containing 5% glycerol, 0.04% brom phenolblue, 0.04% Xylene Cyanol, 0.25× Gelred, before loading for electrophoresis.

one-stop PCR is an efficient and reliable PCR method.

Compatibility with downstream uses

Three lines of evidence were presented to evaluate the compatibility of one-stop PCR with downstream applications. We precipitated a 0.4 kb human β-actin products of one-stop PCR and traditional PCR products with NaAc and pure ethanol. As shown in Figure 2C, the two PCR systems performed almost equally, implying that these additives do not compromise DNA recovery by ethanol precipitation. Next, we sought to use *silica* membrane filtration to further assess its applicability. The 0.4 kb humanβ-actin products of one-stop PCR and traditional PCR products were processed to flow through *silica* membrane column for purification, and the attached DNA was dissolved and separated by electrophoresis

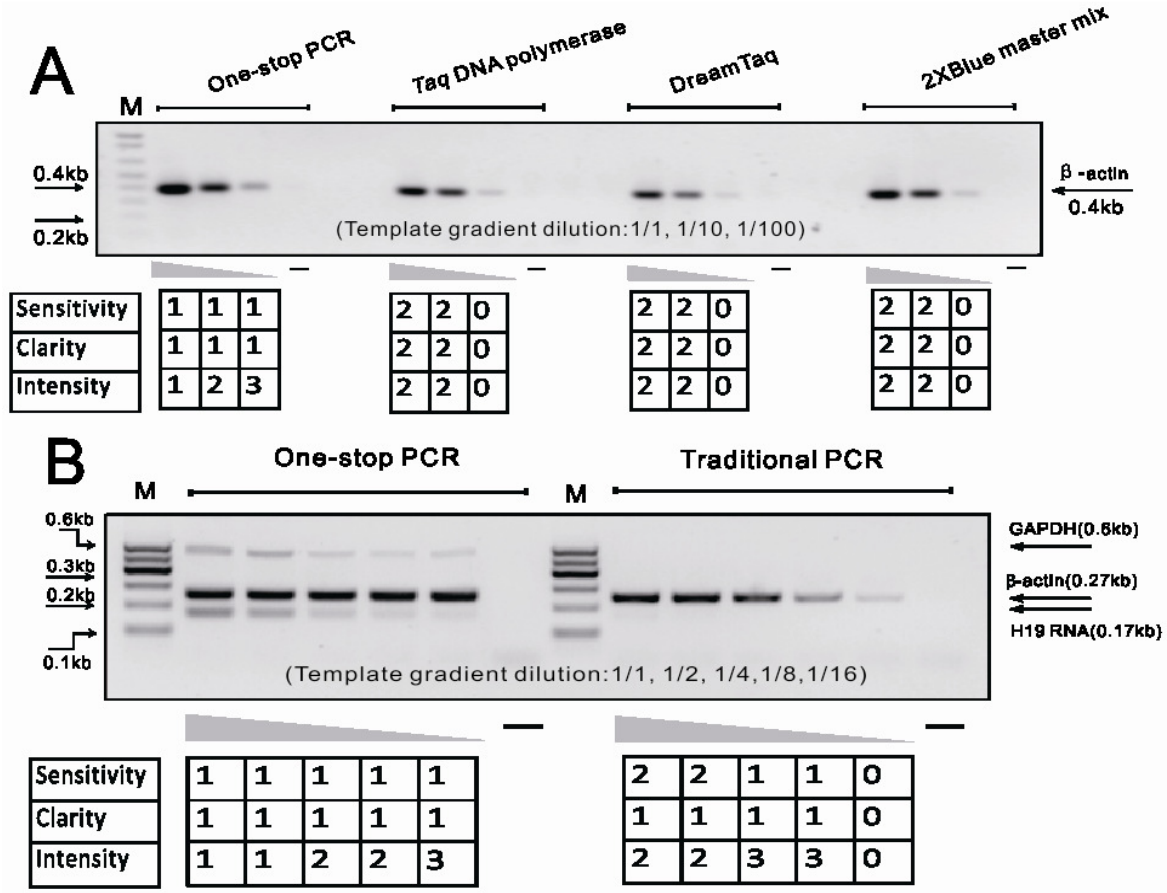

Figure 2. Efficiency and effect of one-stop PCR on nucleic acid amplification. (A) One-stop PCR is comparable to commercially available PCR kits. A 0.4 kb fragment of human β-actin mRNA was amplified with gradient template amount at dilutions of 1/1, 1/10, 1/100. All the PCR products were separated on 2% agarose gel. "-" was negative control with the absence of template. Three PCR kits (*Taq* DNA polymerase, *DreamTaq* DNA polymerase and 2× Blue master mix) were purchased from Shenergy (Shanghai, China), Fermentas (Lithuania) and DBI (Shanghai, China), respectively. (B) One-stop PCR performs well under the condition of triplex PCR. Three fragments of human GAPDH mRNA (0.6 kb), β-actin mRNA (0.25 kb), H19 mRNA (0.17 kb) were simultaneously amplified as triplex PCR by one-stop PCR and traditional PCR. The final concentrations of primers used in this test were 50, 250 and 50 nM for GAPDH, β-actin and H19, respectively. The cDNA was proportionally diluted at 1/1, 1/2, 1/4, 1/8 and 1/16 and 1 µl aliquot was used as template. A 20 ul traditional PCR mixture included 10×PCR reaction buffer, 2 µl, 40 mM dNTP (10mM each), 0.5 µl; *Taq* DNA polymerase, 0.2 µl (1 unit), 0.5 µl forward and reverse primer mix (10 µM total), 0.5 µl cDNA, 16.3 µl PCR-grade water. (C) One-stop PCR is compatible with downstream applications. 0.4 kb human β-actin products of one-stop PCR and traditional PCR were treated with either ethanol precipitation or *silica* membrane filtration. The recovered DNA were run in parallel with input (1/10 of the total 50µl PCR products). *Silica* membrane filtration was carried out with a commercial PCR product purification kit (Generay, Shanghai), according to the manufacture's instruction. Ethanol precipitation was performed essentially according to previously described method (D'Souza et al., 2005). (D) By TA-cloning and sequencing the 0.4 kb human β-actin DNA products in (C), it revealed that one-stop PCR is not mutagenic. A TA-cloning was performed essentially according to previously described method (Bi et al., 2007). (E) One-stop PCR is time- and labour-saving. The time required to add and mix loading buffer after PCR cycling is viewed as the time saved where one-stop PCR is adopted otherwise. When 10, 20, 40 and 60 individual PCR samples were processed, an appreciable time and labour was saved as shown. P1, P2 and P3 stands for three experimentalists tested in the time-saving assay.

(Figure 2C). The recovery efficiency of *silica* membrane was similar to both methods. Finally, TA-cloning and sequencing of these products revealed that one-stop PCR is not mutagenic (Figure 2D). We thus summarize that one-stop PCR is completely compatible with downstream experiments, expanding its applicability in a

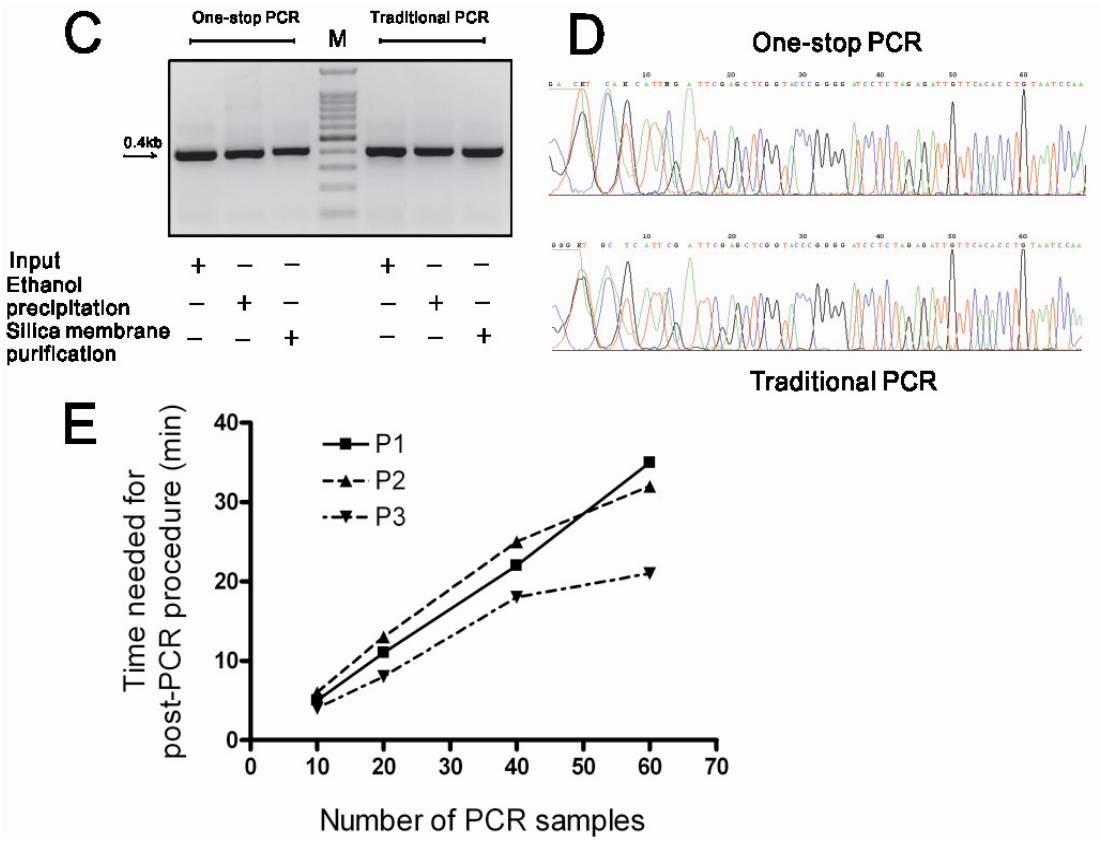

Figure 2. Contd.

wide context of molecular manipulations.

Conclusion

One-stop PCR integrates density agent, tracking nontoxic staining dyes with other PCR components into a single PCR mixture and takes experimentalists from gel to visualization in one easy step. It would be helpful to accelerate nucleic acid detection in biological laboratories.

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