# Betaine Improves LA-PCR Amplification

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**Abstract** PCR is a powerful tool for the amplification of genetic sequences. It has been widely applied in molecular biology. It is generally used to amplify short segments (several hundreds basepairs to several kilobasepairs). It is difficult to amplify a long DNA segment. Based on the sequenced genes, it is known that most intact genes are very long. And intact gene is very important for the gene to express specially and effectively. Long PCR is a very useful tool to amplify intact genes for constructing special expression vectors. We have tried several chemicals to optimize long PCR system and found betaine was the best. Betaine, as an amino acid analogue with small tetraalkylammonium ions, could remarkably improve the amplification of long targets from the plant genome. The suitable concentration of betaine was between 1.0 mol/L and 2.5mol/L. We could effectively amplify a 9 kb DNA segment from maize genome DNA and a 16 kb DNA segment from plasmid. It was shown that different primers and different targets (different GC content) needed different concentrations of betaine. Betaine can reduce or eliminate non-special amplification. In the meantime we tried other additive chemicals, such as DMSO, glycerin, formamide. They were no notable results in long PCR.

**Key words** Betaine, LA-PCR

Since it was invented in 1988[1], polymerase chain reaction (PCR) has been widely applied in molecular biological research. Usually, it is suitable for amplification of short and medium DNA sequence. However, it is difficult to amplify DNA sequence longer than 6 kilobasepairs through normal Taq DNA polymerase. Until 1994, Barnes et al and Cheng et al made a great progress in long and accurate PCR (LA-PCR) by combining a high level of an exonuclease-free, N-terminal deletion mutant of Taq DNA polymerase, Klentaq1, with a very low level of a thermostable DNA polymerase exhibiting a 3′-exonuclease activity(Pfu, Vent, or Deep Vent)[2,3]. By the combination of these two DNA polymerase, they amplified 35kb DNA with high fidelity and high yield from λ-bacteriophage genome DNA[2] and made the LA-PCR possible. In 2000, Fromenty *et al*. found the template integrity was one of the critical factors for the success of LA-PCR[4]. It was thought the apurinic/apyrimidinic (AP) sites of DNA templates were able to block progression of the DNA polymerase. They utilized *Escherichia coli* exonuclease Ⅲ, a major repair enzyme in bacteria, to cleave DNA at AP sites before amplification and to improve the long PCR amplification of damaged DNA templates.

We recently tried to use LA-PCR to amplify phosphoenolpyruvate carboxylase gene (*pepc* 6.7 kb, GeneBank accession E17154) and pyruvate orthophosphate dikinase gene (*ppdk* about 13.8 kb, GeneBank accession M58656 M36283) of maize. We couldn't amplify those genes based on the above long PCR methods (combination of two kinds of DNA polymerase and using exonuclease Ⅲ to treat template). At the same time, we used several PCR additives such as formamide, glycerol, dimethyl sulfoxide (DMSO). But they don't improve the PCR results. At last, we found betaine could improve those genes amplification with high fidelity and high yield. Henke et al reported bataine could improve the PCR amplification of GC-rich DNA sequences[5]. Baskaran et al recommended improving LA-PCR efficiency by combining 1.3mol/L of betaine and 1.3% of DMSO[6]. Based on our result, betaine should be a very useful additive of LA-PCR.

## 1 Materials and Methods

### 1.1 LA-PCR

LA-PCR was usually performed with two kinds of Taq DNA polymerase, TaKaRa Taq and LA-Taq DNA polymerases which were bought from TaKaRa Biotechnology (Dalian) Co., Ltd. There were four kinds of reaction buffers with TaKaRa LA-Taq DNA polymerase (10 × LA-PCR Buffer Ⅰ, 10 × LA-PCR Buffer Ⅱ, 2 × GC Buffer Ⅰ, 2 × GC Buffer Ⅱ). The LA-PCR mixture contained 300μmol/L of

Received: 03-02-2004

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dNTPs, 3mmol/L of $MgCl_2$, 1 × PCR buffer(10mmol/L of Tris-HCl pH8.3 and 50mmol/L of KCl), 0.2μmol/L of each primer p5′ and primer p3′, 2.5u of LA-Taq DNA polymerase and 1μg of maize genome DNA with variable concentrations of betaine. All PCR were performed in a volume of 50μL in 0.2 mL thin wall PCR tubes through Biometra Tgradient system (Whatman Co).

Three different LA-PCR protocols were used.

Protocol 1 was employed to amplify *pepc* gene from maize genome DNA. The following primers were applied to amplify a 6767 bp DNA fragment: Forward primer pepc-5′ 5′-GGTGTTAGGACACGTGGTTAGCTAC-3′ (nt 15-39 of maize *pepc* gene E17154) and backward primer pepc-3′ 5′-GAAGCTTCCCTAAGCTCCAATCACT TGC-3′ (nt 6781-6761 of maize *pepc* gene E17154). The thermocycler profile for *pepc* gene was 1 cycle of 94℃ for 3 min, 60℃ for 1 min and 72℃ for 8 min, 35 cycles of 94℃ for 30 s, 62℃ for 1 min, and 72℃ for 8 min, with a final extension at 72℃ for 10 min.

Protocol 2 was employed to amplify partial *ppdk* gene from maize genome DNA. The following primers were applied to amplify an 8917 bp DNA fragment: Forward primer ppdk-5′ 5′-GTTGTTAGGAAGGAGGTGGATAGAA-3′ (nt from -1471 to -1447 of maize *ppdk* gene M58655 M36283) and backward primer ppdk-3′ 5′-GTCGAGGAAGCGGCGGAAGGAGTCG-3′ (nt 7445-7421 of maize *ppdk* gene M58655 M36283). The thermocycler profile for *ppdk* gene was 1 cycle of 94℃ for 3 min, 60℃ for 1 min and 72℃ for 500 s, 35 cycles of 94℃ for 30 s, 60.6℃, 63℃ or 65℃ for 1 min, and 72℃ for 500 s, with a final extension at 72℃ for 10 min.

Protocol 3 was employed to amplify the plasmid pBAC216 (18688 bp), which contains the whole maize *ppdk* gene (13390 bp), bar gene with 35s promoter, Adh1 intron and Nos3′ terminator (1881 bp) and modified pGreen0029 (3417 bp). The following primers were applied to amplify a 16807 bp fragment: Forward primer plasmid-5′ 5′-TATGCCATGGTCATGTATCGATAACATTAACGTTT-3′ (nt 2006-2040 of pBAC216) and backward primer plasmid-3′ 5′-GTTTGCGCGCTATATTTTGTTTTCTCATA-3′ (nt 124-96 of pBAC216). The thermocycler profile for pBAC216 was 1 cycle of 94℃ for 3 min, 62℃ for 1 min and 72℃ for 800 s, 35 cycles of 94℃ for 30 s, 65℃ for 1 min, and 72℃ for 800 s.

### 1.2 Isolation of maize DNA and plasmid DNA

Maize DNA was extracted by CTAB method as describing in Short Protocols in Molecular Biology[7]. Five grams of maize leaves were ground to powder in liquid nitrogen and transferred into a fresh 50mL tube. 15mL of CTAB extracting buffer were added into the tube which contained samples and mixed. 15mL of chloroform were added and mixed. Separate the organic and aqueous phases by centrifuge. Transfer the upper aqueous phase to a fresh 50mL tube. Precipitate the DNA by adding an equal volume of isopropanol. Transfer the DNA precipitation to a fresh 1.5mL Eppendorf tube by glass rod. Dry the DNA in room temperature. Dissolve the DNA pellet in 200μL of TE buffer. Plasmid DNA prepared by alkaline lysis with SDS as describing in Molecular Cloning[8]. Pour 1.5mL of the bacterial culture into a fresh 1.5mL tube. Centrifuge at 13000r/min at 4℃. Remove the supernatant and dry the bacterial pellet. Resuspend the bacterial pellet in 200μL of solution Ⅰ. Add 200μL of solution Ⅱ to the bacterial suspension and mix. Add 150μL of ice-cold solution Ⅲ and mix. Centrifuge the bacterial lysate at 13000r/min for 15 minutes at 4℃. Transfer the supernatant to a fresh tube. Precipitate the plasmid DNA from the supernatant by adding an equal volume of isopropanol. Collect the precipitation by centrifuging at 13000r/min for 5 minutes at 4℃. Remove the supernatant and dry the pellet at room temperature. Dissolve the pellet in 150μL of TE buffer.

### 1.3 Primer and betaine

Primers were synthesized by ABI 3900 High-Throughput DNA Synthesizer. Betaine was bought from Sigma.

## 2 Results

Preliminary experiments showed that it was failed to amplify the intact *pepc* gene with primers pepc-5′ and pepc-3′ by TaKaRa Taq or LA-Taq with any kinds of reaction buffers. We could only amplify partial *pepc* gene with some non-specific bands by LA-Taq with GC Ⅰ or GC Ⅱ reaction buffer through primers designed for partial *pepc* gene amplification (data not shown). To improve the specificity and efficiency, DMSO, formamide, glycerol and betaine were added to the PCR mixture respectively. Our data showed that 5% of formamide improved the specificity of partial *pepc* gene amplification. 5% of DMSO and 10% of glycerol had no notable effect on the specificity or efficiency of partial *pepc* gene amplification. Moreover, 5% of formamide did not improve the amplification of intact *pepc* gene. But betaine remarkably improved the PCR amplification of partial and whole *pepc* gene from maize genome DNA. We found the concentration of betaine was very important for the specificity of PCR amplification. Both 1.5 and 2mol/L of betaine improved the specificity of whole *pepc* gene amplification. At the mean time, 1.5mol/L of betaine could enhance the efficiency of PCR amplification (Comparing Lane 1.5 and Lane 2.0 in Figure 1). However 0.5mol/L or 1mol/L of betaine didn't amended long PCR amplification (see Figure 1 and 2). When amplifying partial *ppdk* gene (about 8.9kb) from maize genome, we optimized the concentration of betaine and annealing temperature (see Figure 2). We found high annealing temperature was requisite but not sufficient to improve the amplification specificity of partial *ppdk* gene. Combining high annealing temperature with suitable concentration of betaine, the specificity and efficiency of PCR amplification were improved remarkably. Using 1.5 or 2.0mol/L betaine, a 16807 bp fragment was amplified from plasmid pBAC216 (see Figure 3). It's possible to amplify much longer fragments from plasmid or λDNA by this PCR system.

## 3 Discussion

We show here that betaine notablely improve the PCR amplifica-

tion of single copy long gene from plant genome. Betaine seems a better PCR additive than other additives such as formamide, glycerol,

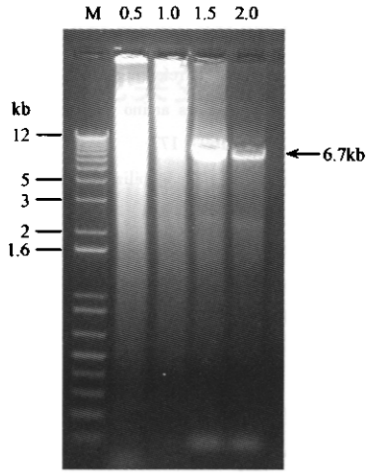


Fig.1 Agarose gel electrophoresis map of amplified maize *pepc* gene with variable concentration of betaine (Electrophoresis with agarose gel in 1 × TAE buffer. 20μL of PCR samples were loaded.)

M: 1 kb plus DNA molecular weight marker from invitrogen; (12kb, 11kb, 10kb, 9kb, 8kb, 7kb, 6kb, 5kb, 4kb, 3kb, 2kb, 1.65kb, 1kb, 850bp, 650bp, 500bp, 400bp, 300bp, 200bp, 100bp)

0.5: Amplification of maize *pepc* gene with 0.5M betaine; 1.0: Amplification of maize *pepc* gene with 1mol/L betaine; 1.5: Amplification of maize *pepc* gene with 1.5mol/L betaine; 2.0: Amplification of maize *pepc* gene with 2mol/L betaine

DMSO for long sequence amplification. Betaine can even improve the amplification of normal TaKaRa Taq polymerase for long sequence (see Figure 1). We consider there are four kinds of possible mechanism of betaine to improve LA-PCR amplification. Firstly, betaine possibly decreases the Tm of GC-rich sequences. Rees *et al*[9] found betaine eliminated the base pair composition dependence of DNA melting. Although we haven't used so high concentration of betaine (5.2mol/L), 1.5 to 2.0mol/L of betaine is at least able to decrease the Tm or destruct secondary structures of maize genome DNA. And betaine maybe isostabilizes the two primers nearly (the Tm of primer ppdk-5′ is 54℃ and the Tm of primer ppdk-3′ is 89.2℃), although the "isostabilizing" concentration of betaine (at which AT and GC base pairs are equally stable) is around 5.2mol/L[9]. Secondly, betaine maybe protects template DNA from apurinic/apyrumidinic effects of high temperature. An Apurinic/apyrumidinic (AP) site is one factor that blocks the elongation of DNA polymerase[4]. Thirdly, betaine maybe protects Taq DNA polymerase from high temperature. Arakawa *et al* have reported that various proteins are stabilized against thermal denaturizing and preferentially hydrated in 0.5 ~ 2mol/L betaine in 1983 and 1985[10, 11]. Fourthly, betaine eliminates pause by Taq DNA polymerase. Mytelka *et al* have report that T7 DNA polymerase tends to pause at a set of sequences[12]. We think it is another factor that affects the long PCR amplification.

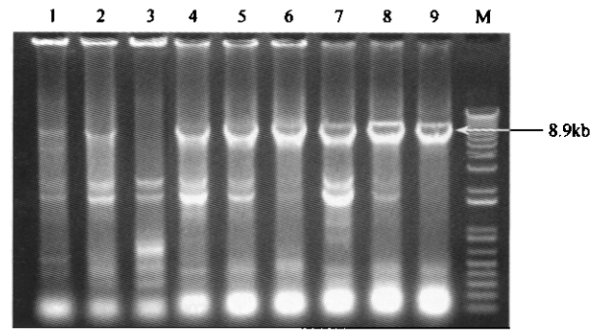


Fig.2 Agarose gel electrophoresis map of amplified maize *ppdk* gene with variable concentrations of betaine and annealing temperature (0.9% Agarose gel 1 × TAE buffer, 20μL of PCR samples were loaded.)

1: 1mol/L betaine with 60.6 ℃; 2: 1mol/L betaine with 63 ℃; 3: 1mol/L betaine with 65 ℃; 4: 1.5mol/L betaine with 60.6 ℃; 5: 1.5mol/L betaine with 63 ℃; 6: 1.5mol/L betaine with 65 ℃; 7: 2mol/L betaine with 60.6 ℃; 8: 2mol/L betaine with 63 ℃; 9: 2mol/L betaine with 65 ℃; M: 1 kb plus DNA molecular weight marker from invitrogen(see Figure 1)

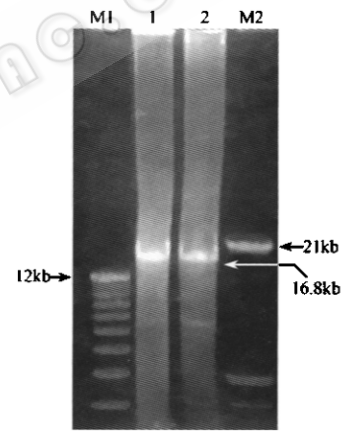


Fig.3 Agarose gel electrophoresis map of amplified plasmid pBAC216

(0.9% Agarose gel 1 × TAE buffer, 20μL of PCR samples were loaded.)

M1: 1 kb plus DNA molecular weight marker from invitrogen(the upper band is 12kb); 1: 1.5mol/L betaine; 2: 2.0mol/L betaine; M2: λ DNA/*Eco*R Ⅰ + *Hind* Ⅲ markers (the upper band is 21kb)

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# 甜菜碱增强长片段 PCR 的扩增

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**摘 要** 聚合酶链式反应(PCR)作为一项非常成熟的技术可以用于基因组序列的扩增。普通的 PCR 技术只适合于短片段 DNA 的扩增，一般在 6 kb 以下。对于 6kb 至十几 kb 甚至几十 kb 以上的 DNA 片段的扩增就非常困难。通过添加不同化学物质，发现甜菜碱对长片段 PCR 的扩增有非常有效的增强作用。通过对玉米总 DNA 以及质粒 DNA 的扩增，发现 1mol/L 到 2.5mol/L 甜菜碱对改进 PCR 扩增效果明显。通过添加甜菜碱，可以从玉米基因组中扩增出 9 kb 以上的单拷贝片段，从质粒中扩增出 16 kb 以上片段。经过试验，发现不同 GC 含量的引物需要使用不同浓度的甜菜碱。甜菜碱可以减少甚至消除长片段 PCR 中的非特异性扩增。同时，我们发现其它的添加物，如 DMSO，甘油，甲酰胺对长片段 PCR 的作用不明显。

**关键词** 甜菜碱，长片段 PCR

**中图分类号** Q75 **文献标识码** A **文章编号** 1000-3061(2004)05-0715-04

收稿日期：2004-03-02，修回日期：2004-05-12。

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