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Polymerase Chain Reaction Can Run Faster by Parameter Optimization

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ABSTRACT

For a conventional PCR, the total reaction times of a certain DNA amplification using conventional PCR were minimized to more than a half. The extension time could be calculated as the formula: y=0.005x-5.2679 (y represented the extension time (sec) and x represented the DNA length (bp); y ≥ 1.0). The annealing time was minimized as short as 1 sec. However, the Ta was changed from Tm ± 6.0°C.

Keywords: PCR, Rapid, Parameter optimize

Highlights

- PCR annealing time was shortest in 1 second
- PCR extension time can be calculated as: y=0.005x-5.2679 (y: sec, x: bp)
- The optimal parameters could not work to mixed templates system

Abbreviations

PCR: Polymerase Chain Reaction; CAV16VP1: VP1 Gene of Coxsackievirus 16; ZKME: Zika Virus Membrane and Envelope Protein Gene; Tm: Primer Melting Temperature; Ta: Annealing Temperature

INTRODUCTION

The polymerase chain reaction (PCR) is a technique developed in 1983 by Kary Mullis and is used in molecular biology for the selective amplification of DNA or RNA segments [1]. The use of the heat-stable DNA polymerase from the *Thermus aquaticus* (Taq polymerase) makes the reaction amenable to automation and to amplify nearly 2.0 kilobase-pairs (kb) segment [2]. The reaction is simple, fast and extremely sensitive. The DNA or RNA in a single cell is sufficient to detect a specific sequence. Now, it is an easy, cheap, and reliable way to repeatedly amplify a focused segment of DNA of more than 5.0 kilobase-pairs (kb) *in vitro* [3]. Long-range PCR (exceeding 20 kb) could be efficiently achieved using a DNA polymerase fusion [4]. Therefore, PCR has become an essential tool in biological science. For example, PCR has been applied to a large number of medical procedures including detection of genetic disease mutations, sequence polymorphisms of various types in human genetics and forensic analysis, pathogenic components and disease diagnosis, DNA sequencing, and target gene isolation [1,5,6-9]. Even though the PCR machine and the heat-stable DNA polymerases have been upgraded and updated, the PCR parameters of annealing and extension reaction times recommended in the protocol remain unchanged [3,10]. Therefore, PCR parameter optimization is necessary to obtain better results in the shortest time. In this study, we minimized the PCR annealing and extension reaction time as short as possible to detect or amplify a target sequence.

PATIENTS AND METHODS

Plasmid and Bacterium

E. coli DH5α strain, pET32a, and pBEX vectors were conserved in Chongqing Medical University. pBEX-1AK

contains 400 bp artificial sequence and the recombinant was 3901 bp. The pET32a-CAV16VP1 plasmid contains 987 bp length of CAV16VP1 gene and pET32a-ZKME has 2097 bp length of ZKME gene.

Enzymes, Reagents, and Instruments

Agarose and 2x Premix Ex Taq (content of Taq DNA Polymerase, Buffer and dNTP mixture, cat# D332A) and DNA standard molecular (DL10,000 DNA Marker, cat# 3584A) were purchased from TaKaRa Company (TaKaRa, China, Dalian). GoldView nucleic acid stain (cat# D0125) was purchased from Biohao Company (China, Wuhan). Bio-Rad T100 thermal cycler PCR instrument and Gel Doc XR were purchased from Bio-Rad Company (USA, California). Electrophoresis apparatus (DYCP-32A) was purchased from Beijing LIUYI Company (China, Beijing).

PCR Parameters Optimization

Extension time optimization: As a general rule, all the Taq polymerase protocols are recommended to use extension times of one minute per 1000 base pairs (e.g. 2 min for a 2 kb product). However, it was reported that common Taq polymerase had an extension rate of 35-100 nucleotides/sec (nt/sec) per enzyme at 75°C. Therefore, we minimized the shortest extension times of the Ex Taq polymerase firstly. The DNA templates and primers were listed in Table 1.

Table 1 Property of DNA templates and primers

Plasmids	Primers	Tm (°C)	Tm	GC%	Length (bp)
pBEX-1AK	pAKF: 5'-tcgagggatccccggaattc-3'	56	60.5	60%	3901
	pAKR: 5'-catagatgccaggaggaatgcgatgttctttttc-3'	65		44%	
ZKME	pZKEF: 5'-acggatccatggcagatactag-3'	55	52.5	50%	2097
	pZKER: 5'-cagtgaattcccccacatc-3'	50		52%	
CAV16VP1	pCAV16F: 5'-cataggatccatgggagatcctattgcag-3'	61	61.0	48%	987
	pCAV16R: 5'-cgtatgtcgactcacaacgttgttatcttg-3'	61		43%	

Amplification was performed in a Bio-Rad DNA Engine 2 as follows: initial heating to 94°C for 3 min; then denaturation for 30 sec at 92.3°C, annealing for 30 sec at 50°C for pBEX-1AK and pET32a-CAV16VP1 plasmids and 30 sec at 48°C for pET32a-ZKME plasmid, respectively. The extension times of pBEX-1AK were followed as 10 sec, 11sec, 12 sec, 13 sec, 14 sec, 15 sec, 20 sec, 21 sec, 22 sec, 23 sec, 24 sec, and 3 min at 72°C for 20 cycles, respectively. The extension times of CAV16VP1 were followed as 1 sec, 2 sec, 3 sec, 4 sec, 5 sec, and 6 sec at 72°C for 20 cycles, respectively. The extension times of ZKME were followed as 2 sec, 3 sec, and 4 sec at 72°C for 20 cycles, respectively. A final extension was added to all reactions at 72°C for 5 min prior to cooling to 4°C. The reaction was amplified starting with 1.0 µl of DNA vectors (30 ng/µl), 25 pM of each PCR primer, 2.5 units of Ex Taq polymerase, 2.5 mM of each dNTP and 2 mM Mg^{2+}. ddH_2O was added to a final volume of 20 µl. The products (5.0 µl) were run in 0.8% agarose gel. The gels were visualized and scanned by Gel Doc XR.

Annealing time optimization: The amplification was performed in a Bio-Rad T100 instrument as follows: initial heating to 94°C for 3 min; then denaturation for 30 sec at 92°C. The annealing times of pBEX-1AK were for 30 sec, 25 sec, 20 sec, 15 sec, 10 sec, 4 sec, 3 sec, 2 sec, and 1 sec at 50°C, respectively and 3 min at 72°C for 20 cycles. A final extension was added to all reactions at 72°C for 5 min prior to cooling to 4°C. The reaction system was same as described in the extension time optimization. The products were run in 0.8% agarose gel. The gels were visualized and scanned by Gel Doc XR.

Ta value optimization: Generally, the specificity of primer-template annealing of PCR was critically determined by the annealing temperature (Ta). Thus, it was necessary to determine the range of Ta value for a certain primer and template. The reaction was performed as follows: initial heating to 94°C for 3 min; then denaturation for 30 sec at 92°C. The Ta of pBEX-1AK was for 30 sec at 53°C-66°C and 66°C-70°C gradients, respectively, and 3 min at 72°C for 20 cycles. A final extension was added to all reactions at 72°C for 5 min prior to cooling to 4°C. The reaction system was same as described previously. The Ta of pET32a-ZKME was 60°C-70°C gradients, and the Ta of pET32a-CAV16VP1 was 55°C-65°C and 66°C-69°C gradients, respectively. The products were detected and scanned as described previously.

Mixture Templates Detection

In order to detect whether the optimal parameters work in a mixture templates system, each 10 µl of PCR mixture

was taken from the 40 µl reaction mixture of CAV16VP1, ZKME, and pBEX-1AK and was mixed in a tube. The reaction volume was all 30 µl. The PCR was performed as follows: initial heating to 94°C for 3 min; then the 20 cycles reaction was performed as the parameters: denaturation for 30 sec at 92°C, the Ta of mixture, CAV16VP1, ZKME, and pBEX-1AK were for 5 sec at 50°C and extension at 72°C for 30 sec. The products were detected and scanned as described previously.

Data Analysis

PCR products were stained with GoldView nucleic acid stain, and the gels were visualized and scanned by Bio-Rad Gel Doc XR. DNA marker (DL10000) contained 10,000 bp, 7,000 bp, 4,000 bp, 2,000 bp, 1,000 bp, 500 bp and 250 bp bands.

RESULTS

Extension Time Optimization

Extension step was the longest stage during PCR perform. It is generally recommended amplifying 1.0 kb DNA segment per 1 min as a standard extension time parameter. Therefore, a 3.9 kb DNA segment need more than 2 hours and 22 min to run a 25 cycles PCR. However, it only took 1 h and 3 min using the optimal parameters. Our results suggested that PCR could run faster than the normally recommended protocols (Tables 2 and 3).

Table 2 PCR efficiency of optimal parameters

Template	Methods	Ta	Annealing time	Extension time	20 cycles	25 cycles	30 cycles
CAV16 (987 bp)	Normal	55°C	30 sec	1 min	1:16:37	1:31:17	1:45:57
	Optimal		5 sec	1 sec	0:46:52	0:54:27	1:02:02
ZKME (2097 bp)	Normal	50°C	30 sec	2 min	1:40:25	2:00:45	2:21:05
	Optimal		5 sec	7 sec	0:51:46	1:00:36	1:09:16
pBEX-1AK (3901 bp)	Normal	50°C	30 sec	4 min	2:22:25	2:52:45	3:23:05
	Optimal		5 sec	15 sec	0:54:34	1:03:59	1:13:24

Table 3 The length of DNA templates and the shortest extension time

Plasmids	Ta°C	GC%	Length (bp)	Extension Time Sec	Polymerization Speed nt/sec	Formula*
pBEX-1AK	50	47.9%	3901	15	260	y=0.005x-5.2679
ZKME	50	49.6%	2097	3	699	
CAV16VP1	55	47.4%	987	1	987	
*y: polymerization speed, sec; x: DNA length, bp						

In this study, we found that the full length of 3901 bp pBEX-1AK plasmid could be amplified in 15 sec with Ex Taq polymerase. That meant Ex Taq polymerase could polymerize 260 nt/sec. The band was sharp enough for target-DNA detection. Thus, a single reaction cycle could be finished only one-twelfth of the recommended standard time. However, the 2097 bp length of ZKME (zika virus membrane and envelope protein gene, GenBank KU365778.1) could be amplified in 3 sec with a polymerization speed of 699 nt/sec. A length of 987 bp CAV16VP1 (VP1 gene of coxsackievirus 16, GenBank GQ429252.1) could be amplified in 1 sec with a polymerization speed of 987 nt/sec (Figure 1).

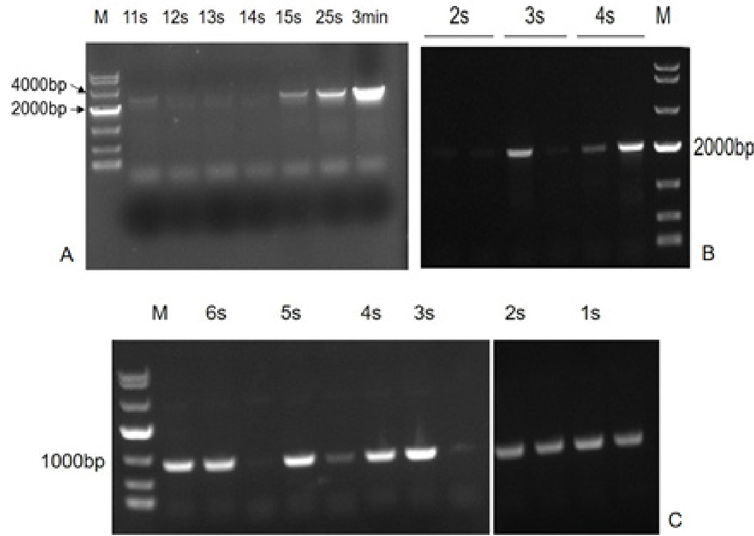

Figure 1 Extension times for the different length of DNA templates. Each tube contains 2.5 units of Ex Taq polymerase, 2.5 mM of each dNTP and 2 mM Mg^{2+}, respectively. 1.0 µl (30 ng/µl) of pBEX-1AK DNA and 25 pM of each primer (A), 1.0 µl (30 ng/µl) of ZKME DNA and 25 pM of each primer (B) and 1.0 µl (30 ng/µl) of CAV16VP1 DNA and 25 pM of each primer (C) are added into the tubes, respectively, and ddH_2O is added to a final volume of 20 µl. The PCR performs with initial heating at 94°C for 3 min; then denaturation for 30 sec at 92.3°C, the annealing times are 30 sec and the annealing temperature is at 50°C for pBEX-1AK and ZKME, and 55°C for CAV16VP1, respectively. The different extension times are labeled above the bands and react for 20 cycles

That meant the shorter DNA template, the faster polymerization speed of Ex Taq polymerase. Therefore, the Ex Taq polymerization was not a constant speed reaction. The formula of polymerization speed was: y=0.005x-0.2679 (y represented the polymerization speed (sec) and x represented DNA length (bp)) (Figure 2).

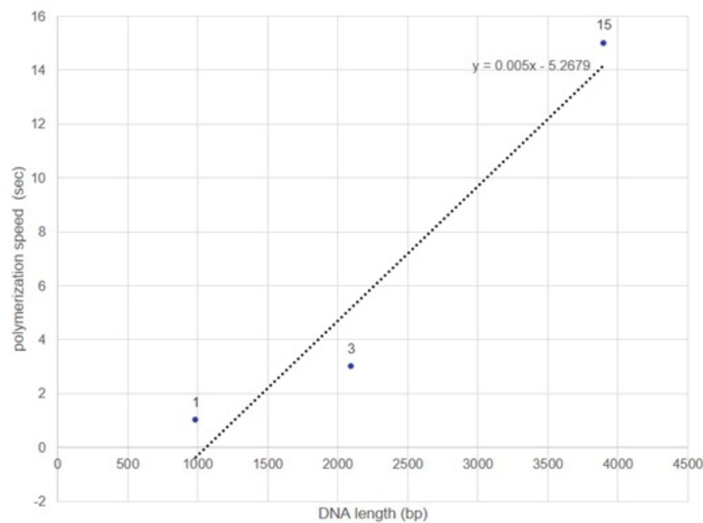

Figure 2 Ex Taq polymerization speed curve, the y-axis represents the extension time (sec) and the x-axis represents DNA length (bp). y ≥ 1.0

That was the longer length of the DNA template, the slower Ex Taq polymerization speed. However, the PCR speed was faster than 1.0 kb/sec for a long PCR. Of course, the production of long PCR in minimized times was less than in the normal extension time protocol (Table 2). However, if the 1 sec at 72°C extension step was omitted from CAV16VP1 template PCR, the CAV16VP1 could not be amplified (data not shown). That meant, the 1 sec was the lower limit extension time for DNA template even though it was less than 1.0 kb.

Annealing Time Optimization

Annealing step allows for hybridization of the primers to each of the single-stranded DNA templates. It was finished rapidly in 1 sec in this study. Notably, for the PCR efficiency, specificity and production there was no difference between the different annealing time reactions for a 3.9 kb template of pBEX-1AK. The shortest annealing times of ZKME and CAV16VP1 segments were also 1 sec. However, the bands of non-specific products were sharper if the annealing time was less than 5 sec for a long PCR. Thus, the results suggested that 5 sec to 10 sec annealing was enough for primer-template hybridization of PCR (Figure 3).

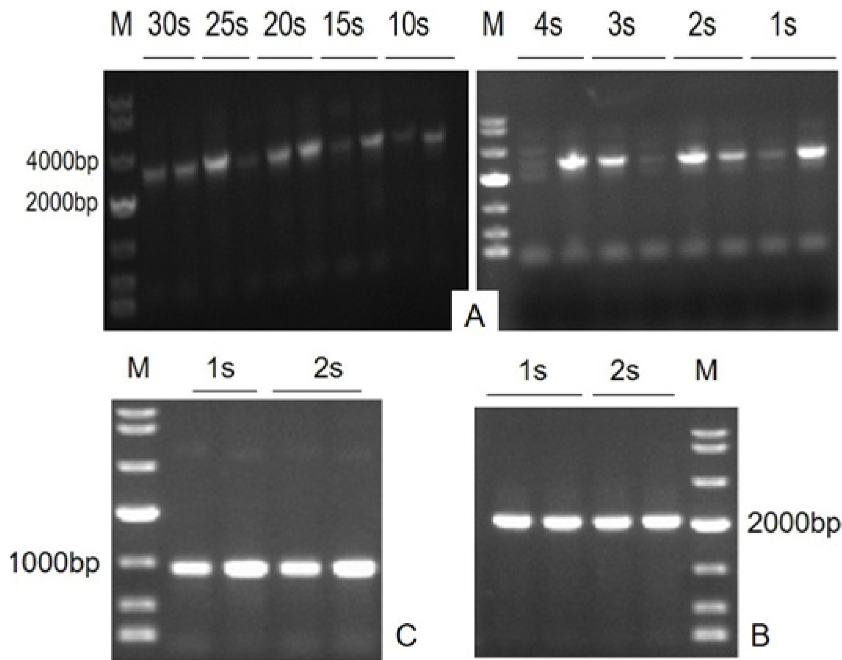

Figure 3 Annealing times for the different length of DNA templates. Each tube contains 2.5 units of Ex Taq polymerase, 2.5 mM of each dNTP and 2 mM Mg^{2+}, respectively. 1.0 µl (30 ng/µl) of pBEX-1AK DNA and 25 pM of each primer (A), 1.0 µl (30 ng/µl) of ZKME DNA and 25 pM of each primer (B) and 1.0 µl (30 ng/µl) of CAV16VP1 DNA and 25 pM of each primer (C) are added into the tubes, respectively, and ddH_2O is added to a final volume of 20 µl. The PCR performs with initial heating at 94°C for 3 min; then denaturation for 30 sec at 92.3°C, the different annealing times are labeled above the bands and the annealing temperature is at 50°C for pBEX-1AK and ZKME, 55°C for CAV16VP1, respectively. The extension times is designed as 1.0 kb/ min at 72°C for 20 cycles

Ta Value Optimization

It was suggested that the annealing time does not affect the efficiency and specificity of PCR in this study. Therefore, the specificity of primer-template annealing was critically determined by the annealing temperature (Ta). Generally, the primer melting temperature (Tm) was calculated according to the following equation:

$$Tm = (T + A) \times 2 + \left(G + C\right) \times 4$$

PCR Ta was rapidly calculated according to the following formula [11]:

Ta=Tm ± 3.5°C

In this study, the primers of pBEX-1AK could work from 53°C to 66.8°C. The average Tm (Tm) of pAKF and pAKR was 60.5°C (Table 2). That meant Ta of pAKF and pAKR was changed from Tm-7.5°C to Tm+6.3°C. However, the 66.3°C annealing reaction produced sharper non-specific products than the other reaction annealing from 53°C-66°C reaction for pBEX-1AK amplification. Thus, the optimal upper Ta was 66°C (Figure 4). Therefore, the Ta of pAK primers was Tm ± 5.5°C. Similarly, Ta of pZKME primers was changed from Tm-7.5°C to Tm+11.3°C. However, the PCR production was decreased from 62°C. That meant the optimal upper Ta of pZKME primers was 60°C (Figure

4). Therefore, the Ta of pZKME primers was Tm ± 7.5°C. However, Ta of pCAV16VP1 primers was changed from Tm-6.0°C to Tm+6.0°C. Similarly, the PCR production was reduced from 67°C. Thus, the optimal upper Ta of pCAV16VP1 primers was 66°C (Figure 4). The Ta of pCAV16VP1 primers was Tm ± 5.0°C. We found that the mean of Ta in this study was Tm ± 6.0°C ((7.5+6.0+5.0)/3=6.0). Therefore, the results suggested that the general formula of Ta in conventional PCR value was: Ta=Tm ± 6.0°C.

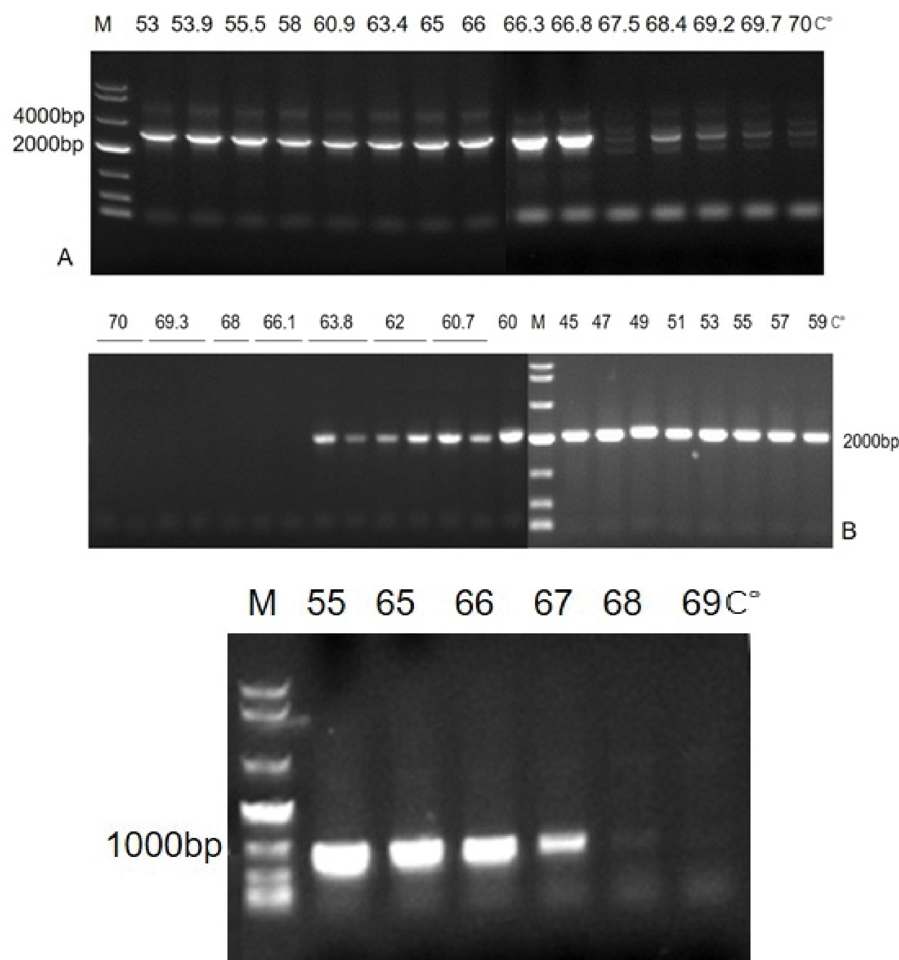

Figure 4 Annealing temperatures (Ta) for different primers. Each tube contains 2.5 units of Ex Taq polymerase, 2.5 mM of each dNTP and 2 mM Mg^{2+}, respectively. 1.0 µl (30 ng/µl) of pBEX-1AK DNA and 25 pM of each primer (A), 1.0 µl (30 ng/µl) of ZKME DNA and 25 pM of each primer (B) and 1.0 µl (30 ng/µl) of VP1 DNA and 25 pM of each primer (C) are added into the tubes, respectively, and ddH_2O is added to a final volume of 20 µl. The PCR performs with initial heating at 94°C for 3 min; then denaturation for 30 sec at 92.3°C, annealing times is 30 sec and the different Ta's are labeled above the bands and extension times is designed as 1.0 kb/ min at 72°C for 20 cycles

Mixture Templates could not Amplify using Optimal Parameters

There was only CAV16VP1 amplified from the mixture of CAV16VP1, ZKME and pBEX-1AK templates using optimal parameters. If the template was longer than 2.0 kb, the PCR could not work using the optimal parameters founded in this study (Figure 5).

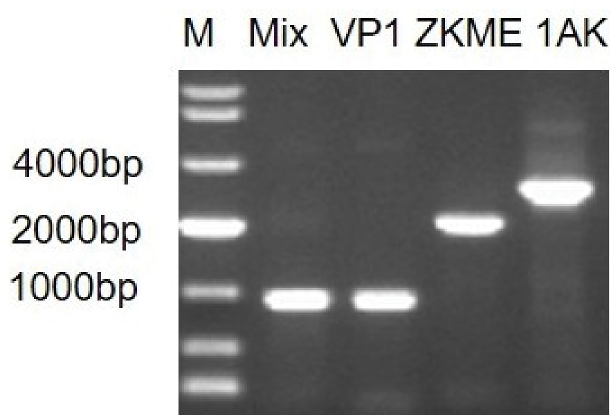

Figure 5 PCR of mixture templates using optimal parameters. VP1, ZKME and 1AK tubes (bands) contain 1.0 µl of DNA templates (30 ng/µl), 25 pM of each primer, 2.5 units of Ex Taq polymerase, 2.5 mM of each dNTP and 2 mM Mg^{2+}, respectively. ddH_2O is added to a final volume of 20 µl. Mix tube (band) contains each 1.0 µl of CAV16VP1, ZKME and pBEX-1AK mixture and their primers (25 pM each) and the final volume is 20 µl. The PCR starts with initial heating at 94°C for 3 min; then denaturation for 30 sec at 92.3°C, the annealing times is 5 sec at 55°C and extended for 30 sec as optimal parameters for 20 cycles

DISCUSSION

PCR was a rapid, specific, and sensitive method for the amplification of nucleic acid sequences and widely used in molecular biology research and clinical diagnosis [12-16]. Therefore, most of PCR optimizations were focused on real-time PCR for molecular diagnostic [17-19]. For example, the multiplex PCR using 3 sets of primers could detect the targeted viruses in 109 of the 235 (46.4%) stool samples in a single tube [17]. However, the optimizations of conventional PCR was reported a decade ago and was ignored in recent years [20,21]. Of course, DNA polymerases with different features were a crucial factor for rapid, specific, and sensitive amplifying target DNA [22,23]. The efficiency of DNA polymerases for the extension of genomic fragments was affected by the amount of DNA template. The optimal DNA (µg)/polymerase (U) ratio was 1.25/1.0 (*Thermococcus litoralis* exo- DNA polymerase (Vent exo)) [20].

In a certain PCR system, the extension time and annealing time affect the polymerization speed. Therefore, to minimize the conventional PCR cycle time was necessary for the rapid and specific detection of the target gene in the laboratory. Our results suggested that 20 cycles PCR with 10 sec annealing were enough for positive clone detection and the extension time was calculated as the following formula: $y=0.005x-5.2679$ (y represented the polymerization speed (sec) and x represented DNA length (bp). $y \geq 1.0$ sec). Thus, the total reaction times of a 3.9 kb length PCR were minimized to a half of normal protocols (Table 2).

CONCLUSION

The annealing temperature affects the fidelity of the amplicon. For a conventional PCR, we found a general formula of Ta value: $Ta=Tm \pm 6.0$°C. The results indicated that certain primers could work in a broad range of Tm value.

DECLARATIONS

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Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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