Study of the Amplification Capacity of A Two-Temperature PCR and its Application in Bovine Sex Identification

[1]Dong Wang, [1]Bo Lin, [1]Huabin Zhu, [1]Haisheng Hao, [2]Chengjiang Wu,
[1]Weihua Du and [1]Xueming Zhao
[1]The Key Laboratory for Farm Animal Genetic Resources and
Utilization of Ministry of Agriculture of China, Institute of Animal Sciences,
Chinese Academy of Agricultural Sciences, 100193 Beijing, China
[2]Jilin Agriculture University, 130118 Changchun, China

Abstract: To study the amplification potential and establish a two-temperature PCR sex identification method, DNA templates prepared from bovine blood, sperm and hair follicles were amplified through two-temperature PCR in this study. Fragments from 207-1413 bp could be amplified successfully while fragments of 1500 bp in length could not be amplified. In further studies we found that an extension step was necessary to amplify longer fragments and as the extension time decreased, the denaturation and annealing times were increased. After optimization we found the best two-temperature PCR to be a reaction using 1.0 mmol L^{-1} Mg^{2+}, 1.0 unit polymerase and 30 cycles. The amplification results using different equipment and Taq polymerases indicated that the two-temperature PCR can be used for any fragment equal to or <1413 bp with any common Taq polymerase. The kinetic rate of the polymerase was determined to be the factor limiting the amplification length and rate. Additionally, the PCR timing was shortened from 1.5-2.0 h to 30-38 min with the two-temperature PCR. The sex identification of bovine blood, fibroblasts and blastmeres were carried out by turns using this rapid PCR and a rapid sex identification method of cattle embryo was establishment. Both amplification time and cost were saved greatly with this method and we found that the two-temperature PCR was sensitive and fast enough for widespread use in many research areas such as genetics, sex identification, forensics and clinical medicine.

Key words: Bovine, rapid PCR, sex identification, two-temperature PCR, sensitive, genetics

INTRODUCTION

Polymerase Chain Reaction (PCR) has been widely used for sex identification, especially in the field of bovine embryo transfer (Manna *et al.*, 2003; Ekici *et al.*, 2006; Lu *et al.*, 2007). Some PCR methods were explored, including Primer Extension Preamplification-PCR (PEP-PCR) and nested PCR (Tominaga, 2004; Mapletoft and Hasler, 2005; Fu *et al.*, 2007). With these PCR methods, the stress reaction of the embryo outside the body is increased due to the longer amplification time. Though, the amplification time was markedly shortened after the Lamp method was invented (Zhang *et al.*, 2009), the false positive rates caused by contamination were problematic. Therefore, it is important to develop a rapid, sensitive and cost-effective PCR method for bovine sex identification.

Amplification times in PCR have been shortened greatly through polymerase studies (Mullis and Faloona, 1987; Angers *et al.*, 2001; Wang *et al.*, 2004).

Additionally, a variety of PCR machines with high thermal transfer efficiency have been invented such as infrared heated PCR systems (Easley *et al.*, 2006), shuttle PCR devices and Continuous-Flow Polymerase Chain Reaction (CF-PCR) instrumentation (Hashimoto *et al.*, 2004; Chen *et al.*, 2008). However, as the amplification efficiency increases, the cost of these special devices and the tendency of contamination increase.

In addition to the thermal transfer capability of the cycling instrument, the extension capacity of DNA polymerase is another key factor affecting PCR amplification efficiency. Innis *et al.* (1988) obtained an extension rate of 60.4 (nt sec^{-1}) at 70°C (Innis *et al.*, 1988). It was reported that the Taq polymerase showed an excellent extension activity and the amplification could be finished during the heating ramp (Benter *et al.*, 1995). So, the Taq polymerase is more popularly used than *Pyrococcus furiosus* exo-DNA polymerase and Sequenase 2.0 (Angers *et al.*, 2001). A 74 bp fragment was first successfully amplified using Taq polymerase with

Corresponding Author: Dong Wang, The Key Laboratory for Farm Animal Genetic Resources and
Utilization of Ministry of Agriculture of China, Institute of Animal Sciences,
Chinese Academy of Agricultural Sciences, 100193 Beijing, China

two-temperature PCR (Cha *et al.*, 1992). Wittwer amplified a 536 bp fragment of human β-globin with 30 sec cycles of rapid PCR but the extension rate was only about 36 nt sec^{-1}. So, they pointed out that primer extension was not instantaneous, the time required for elongation depended on the length of the amplicon. What is more, the time became the limiting factor for PCR with very short cycle (Wittwer *et al.*, 1990). It was believed that the polymerization rate of Taq polymerase is ~33 nt sec^{-1} at the optimal temperature and the extension time should be 1 min for each 1000 bp fragment (Sambrook and Russsell, 2001). The sequence length and extension time are crucial for the amplification yield of Taq polymerase, the amplification yield of 297 bp amplicon by rapid PCR with 10 sec extension time is <45.3% of the theoretical yield, the better amplification result for 600 bp amplicon was obtained with 30 sec extension time while the yield with 12 sec extension time is only 36.8% of the theoretical yield.

These evidences demonstrated the extension time should be lengthened as the length of fragment sequence increases (Whitney *et al.*, 2004). Thereafter, few studies about the two-temperature PCR were reported. The detection of the respiratory virus and the human PPARG gene demonstrated once more the high sensitivity and efficiency of the two-temperature PCR for fragments equal to or <717 bp (Lam *et al.*, 2007; http://tools. invitrogen. com/content/sfs/brochures/cms_040563. pdf). In their study, special equipment and reagents for rapid PCR were used, the Applied Biosystems 9800 Fast PCR System and the GeneAmp Fast PCR Master Mix are essential (Applied Biosystems, CA). However, the components of the Master Mix were not provided and the potential amplification capacity of the fast PCR system was not shown. Furthermore, it was not showed if the rapid PCR depends on the properties of the Taq polymerase or the special reagents and equipment (Lam *et al.*, 2007) and no experiments showed whether fast DNA amplification can be obtained using common equipment and reagents. Exploring the amplification potential may play a very important role in rapid sex determination and other genetic detection areas.

Therefore, we investigated the amplification potential of the two-temperature PCR method and set up a rapid PCR detection technique using common equipment and reagents, provided an alternative of current rapid PCR systems involved expensive devices and reagents. In this study, two-temperature PCR was used to amplify DNA fragments longer than 200 bp using common PCR controls. Longer fragments were amplified until no PCR results were detected. The factors crucial to rapid PCR was investigated and optimized as were the effects of different kinds of Taq polymerase and PCR cycler. Finally, the application of this method to sex identification of the blood, fibroblast and blastmere was also demonstrated.

MATERIALS AND METHODS

Enzymes: Taq DNA Polymerase® (20 mmol L^{-1} Tris-HCl (pH 8.0), 0.1 mmol L^{-1} EDTA, 1 mmol L^{-1} DTT, 100 mmol L^{-1} KCl and 50% glycerol; Lot: H6721) and Taq reaction buffer (10x; 200 mmol L^{-1} Tris-HCl (pH 8.4), 200 mmol L^{-1} KCl, 100 mmol L^{-1} (NH$_4$)2SO$_4$ and 15 mmol L^{-1} MgCl$_2$; Lot: H6512) were purchased from Tiangen Co. (Beijing, China). To investigate the effect of different Taq polymerase products on the two-temperature PCR, other polymerases and reaction buffers were purchased from the following companies: Takara, Taq polymerase® (100 mmol L^{-1} Tris-HCl, 500 mmol L^{-1} KCl and 15 mmol L^{-1} MgCl$_2$) and 10×buffer (200 mmol L^{-1} Tris-HCl (pH 8.4), 200 mmol L^{-1} Kcl, 100 mmol L^{-1} (NH$_4$) 2SO$_4$ and 15 mmol L^{-1} MgCl$_2$); Dongsheng, Taq polymerase® (20 mmol L^{-1} Tris-HCl, 0.1 mmol L^{-1} EDTA, 1 mmol L^{-1} DTT, 100 mmol L^{-1} KCl, 50% glycerol and 1% Triton X-100) and 10×buffer (500 mmol L^{-1} KCl, 100 mmol L^{-1} Tris-HCl and 15 mmol L^{-1} MgCl$_2$) and Dingguo Company, Taq polymerase® (50 mmol L^{-1} Tris-HCl, 1 mmol L^{-1} DTT, 0.1 mmol L^{-1} EDTA, 100 mmol L^{-1} KCl, 1% Triton X-100 and 50% glycerol (pH 8.0)) and 10×buffer (100 mmol L^{-1} Tris-HCl, 100 mmol L^{-1} (NH$_4$) 2SO$_4$, 100 mmol L^{-1} KCl and 1% Triton X-100 (pH 8.8)).

PCR equipment: Instead of using expensive equipment specifically designed for rapid PCR the researchers used the following common commercial equipment for the two-temperature PCR: a Mastercycler® (Eppendorf) and a Thermal Cycler TC-3000® (Techne). The heating rate was 3°C sec^{-1} for the Mastercycler and 3.6°C sec^{-1} for the TC-3000.

DNA preparation: DNA templates were prepared from bovine blood, hair follicles and sperm samples. An anticoagulant blood sample (100 μL) was fully mixed with 900 μL of ddH$_2$O. After a 10 min incubation in an ice bath, the mixture was centrifuged at 13000 g for about 1 min. The supernatant was discarded and the pellet was washed once with ddH$_2$O. Next, 120 μL of ddH$_2$O was added and mixed with the pellet, after which the sample tube was boiled for 10 min. This mixture was cooled in an ice bath for 5 min and recentrifuged for 5 min. Then the supernatant was extracted. The supernatant was either used immediately in the PCR reaction or stored at -20°C for future use.

The hair follicle sample was mixed with 20 μL of Proteinase K lysate and incubated at 65°C for 30 min then 95°C for 15 min and 4°C for 10 min. The mixture was centrifuged and the supernatant was used in the PCR reaction immediately or stored at -20°C for future use.

About 1 μL of semen sample (about 30000 sperm) was fully mixed with 200 μL of ddH_2O and then centrifuged at 13000 g for 2 min. After the supernatant was discarded, 100 μL of alkaline lysis buffer (100 μL 10×PCR buffer, 20 μL $MgCl_2$, 10 μL protease and 870 μL ddH_2O) was added to the pellet and the sample was then incubated at 65°C for 15 min. Finally, 100 μL of neutralizer fluid (900 mmol L^{-1} Tris-HCl (pH = 8.3)) was added to inactivate the reaction. The mixture was then centrifuged at 13,000 g for about 1 min. The supernatant was then immediately used in the PCR reaction or stored at -20°C for future use.

Design and synthesis of primers: Primer C34 was designed with Oligo 6.0, according to the gene sequence of bovine chromosome 2 (gi: 119951937). Primers Y34, Y56 and Y684 were designed according to the sequence of the SRY bovine gene (gi: 4878004). Primer 923 was designed according to the MYF5 bovine gene (gi: 281335) and primer 1371 was designed according to the MSTN bovine gene (gi: AB076403).

The rest of the primers were designed according to the gene sequence of bovine euchromosome 3 (gi: 119890289). Primer information is shown in Table 1. All primers were synthesized by Invitrogen Company (Shanghai, China).

PCR procedures: Unless otherwise noted, all fragments were amplified using an Eppendorf Mastercycler and a control PCR program: preheating at 94°C for 5 min, cycling for 30 cycles and extending at 72°C for 8 min. Each cycle consisted of a denaturation step for 45 sec at 94°C, an annealing step for 30 sec at the temperature shown in Table 1 and an extension step for 1 min at 72°C. The two-temperature PCR program was as follows: preheating at 94°C for 30 sec, cycling for 30 cycles and extending at 72°C for 1 min.

Each cycle consisted of a denaturation step for 1 sec at 94°C and an annealing step for 1 sec at the temperature shown in Table 1. PCR products longer than 1000 bp were analyzed by 1% agarose gel electrophoresis while other products shorter than 1000 bp were analyzed by 2% agarose gel electrophoresis.

The optimization and analysis of different factors: After different fragments were amplified with the two-temperature PCR, the effects of the concentration of Mg^{2+}, the amount of Taq DNA polymerase and the number of cycles were assessed. The results were used to determine the two-temperature PCR program that could efficiently amplify DNA fragments range in length in minimal time.

Table 1: Primers used in this study

Name	Sequences	Melting temperature (°C)	Length (bp)
C34	F:TTGCTGCTCTTGCCTTTGCTT	62.0	207
	R:GTCCACCTGCCACAACTAAAT	62.0	
Y34	F:CGCCTTCATTGTGTGGTCTC	62.0	402
	R:CTGCTGTGATGCTCCTTTTG	60.0	
Y56	F:TTGACCGACGATGTTTACAG	58.0	503
	R:GGCTTTCCATTTGTGAGTAT	56.0	
Y684	F:TCCGCTATGTTCAGAGTATT	56.0	684
	R:TAAGCACAAGAAAGTCCAGG	58.0	
G923	F:ATATGCCACGGATAAAAGC	58.0	923
	R:GCGAGATGCTATTCAGATTT	58.0	
G1371	F:GCTCCCAGACCTTACCCCAA	72.3	1371
	R:CACAGCCCCTCTTTTTCCAC	70.3	
G1413	F:ATGGTGAAGGTCGGAGTGAACGG	72.0	1413
	R:AAAGGGCAAGAGCAAAGGTCAGCA	72.0	
G1500	F:TCCTTCCTTAGCCTTGCCGTCTTT	72.0	1500
	R:CCTTGACTGTGCCGTTGAACT	74.0	

Sex identification with rapid PCR: Sex identification from blood, fibroblast and blastmere samples for both male and female Holstein bovines was carried out using two-temperature PCR. For great difference in the length between the amplicons will help to the rapid sex identification, the primer Y56 for 503 bp fragment of sex-specific Sry gene and primer G1371 for 1371 bp internal fragment of Myostatin gene were used in the duplex PCR system. The sensitivity of the two-temperature PCR was determined using two template groups, one using 1 cell and the other using 5 cells.

The cultured fibroblast was centrifuged then the cell pellet was washed with PBS and adjust cell concentration to 1×10^3 cells mL^{-1}. Under the microscope, the fibroblast were separated using suctionpipe in 1 cell or 5 cells each time, the separated fibroblast were transferred into different centrifuge tube labeled with cell number and the sex, respectively.

Then the cloned cattle embryos from fibroblast were identified. Embryos after cultured 7 days were washed 3 times in sterile 0.2 M saccharose PBS containing no bovine serum albumin to avoid the potential contamination of bovine DNA in Bovine Serum Albumin (BSA). Each embryo were placed in a disposable, plastic Petri dish with 0.4 mL 0.2 M saccharose PBS, the splitting was carried out using a×120 stereomicroscope and a micromanipulator with a metal microblade. The 1 and 3 blastomeres were biopsied from trophectoderm of each blastocysts and were transferred to the PCR tube and labeled for sex identification, respectively while the embryo was returned to culture.

A 20 μL PCR reaction was performed for sexing, firstly with blood DNA samples and different number of fibroblast samples then with different number of blastmeres of the cloned embryos. Each reaction system included 2 μL of 10×PCR buffer, 0.35 μL of 62.5 mmol L^{-1}

$Mg^{+2}+$, 2 μL DNA template (or fibroblast or blastmere), 0.4 μL of 2.5 U μL^{-1} Taq DNA polymerase, 2.25 μL of 2 μmol μL^{-1} primer Y56, 2.25 μL of 2 μmol μL^{-1} primer G1371, 4 μL of 1 mmol L^{-1} dNTP and 6.75 μL ddH2O. The two-temperature PCR cycle program was as follows: 94°C for 30 sec followed by 30 cycles of 94°C for 1 sec, 55°C for 1 sec and then a final 1 min extension at 72°C. At the same time, the control PCR program was used to amplify the blood DNA samples, the parameter was described in section 2.5. The PCR products were detected with 1.5% agarose gel electrophoresis and the amplification time was recorded at once.

RESULTS AND DISCUSSION

The amplification capacity of two-temperature PCR: In order to determine the amplification capacity of the two-temperature PCR, DNA fragments of different lengths were amplified; the length was increased until no product could be detected. A common PCR was performed as control to confirm the feasibility of the two-temperature program. To elucidate the application of the two-temperature PCR, DNA templates from blood, sperm and hair follicle were amplified. As shown in Fig. 1 for
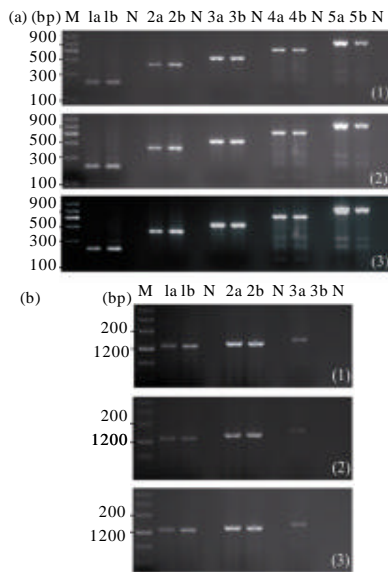

Fig. 1: Comparison of the amplification results of 208-1500 bp fragments from (1) blood, (2) hair follicle and (3) sperm DNA templates M: DNA marker. N: Negative control. Lanes na are fragments amplified with common cycling PCR and lanes nb are fragments amplified with two-temperature PCR. Lanes 1-5 in (A): 207, 402, 503, 684 and 923 bp fragments, respectively. Lanes 1-3 in (B): 1 371, 1413 and 1500 bp fragments, respectively

fragments <1500 bp, the bands obtained with two-temperature PCR were as clear as those obtained through the common program. This result indicates that the two-temperature PCR efficiently amplified DNA fragments from 207-1413 bp. It took 38 and 120 min, respectively for the two-temperature PCR and common PCR to finish the entire amplification process; furthermore, 1500 bp fragments was only amplified by the common PCR program.

The above result implied that the extension stage is very important for the amplification of fragments longer than 1413 bp. In order to obtain the amplification products of 1500 bp DNA sequence, the common PCR program was modified, the extension duration was shortened from 60-20 sec^{-1} while the time for denaturation and annealing were set at 15, 10, 5 and 1 sec, respectively (Table 2). Interestingly as showed in Fig. 2, the running time of denaturation and annealing influenced the lasting of the extension. Therefore, the extension time could be reduced when the time for denaturation and annealing was increased.

Optimization of the two-temperature PCR method: As shown in Fig. 3, the best PCR results were obtained when the concentration of Mg^{2+} was set as 1.0 mmol L^{-1}, the

Table 2: Different durations of the denaturation, annealing and extension stages for the amplification of 1500 bp fragments

Lanes	Denaturation (sec)	Annealing (sec)	Extension (sec)
1	15	15	50
2	15	15	40
3	15	15	35
4	15	15	30 (/)
5	15	15	20 (/)
6	10	10	50
7	10	10	40
8	10	10	35 (/)
9	10	10	30 (/)
10	5	5	60
11	5	5	55
12	5	5	50 (/)
13	5	5	40 (/)
14	1	1	60
15	1	1	55 (/)

(/): no band observed

Fig. 2: Amplification results of the 1500 bp fragment using a blood DNA template M: DNA marker N: Negative control. Lanes 1-15: 1500 bp fragment amplified with different denaturation, annealing and extension times as shown in Table 2
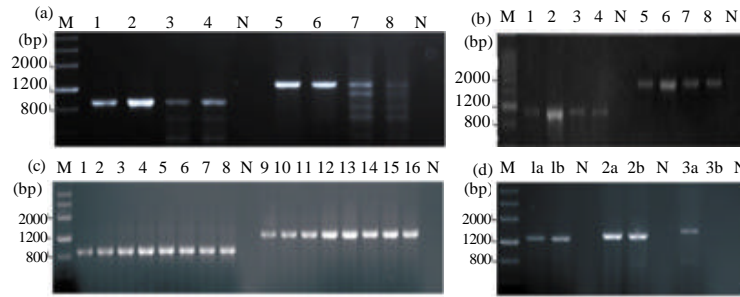
Fig. 3: Optimization of the Mg^{2+} concentration (a) the Taq polymerase concentration (b) and number of cycles (c) and amplification results when using the optimized conditions (d) for the amplification of a blood DNA template M: DNA marker. N: negative control. a) Lanes 1-4: A 923 bp fragment amplified with Mg^{2+} concentrations of 0.5, 1.0, 1.5 or 2.0 mmol L^{-1}, respectively. Lanes 5-8: A 1371 bp fragment amplified with Mg^{2+} concentrations of 0.5, 1.0, 1.5 or 2.0 mmol L mmol L^{-1}, respectively. b) Lanes 1-4: A 923 bp fragment amplified with 0.5, 1.0, 1.5 or 2.0 units of Taq polymerase, respectively; Lanes 5-8: A 1 371 bp fragment amplified with 0.5, 1.0, 1.5 or 2.0 units of Taq polymerase, respectively. c) Lanes 1-8: A 923 bp fragment amplified for 24, 26, 28, 30, 32, 34, 36 or 38 cycles, respectively. Lanes 9-16: A 1371 bp fragment amplified for 24, 26, 28, 30, 32, 34, 36 or 38 cycles, respectively. d): Lanes 1-3: 1371, 1413, 1500 bp fragments, respectively. Lanes na show fragments amplified using the common cycling PCR. Lanes nb show fragments amplified with the two-temperature PCR

PCR product declined as well as a series of non-specific amplification bands appeared when Mg^{2+} concentration was above 1.0 mmol L^{-1}. While less clear band was detected when 0.5 mmol L^{-1} Mg^{2+} was used. As showed in Fig. 3, clear bands were obtained after two-temperature PCR reaction using 1.0 unit polymerase. The amplification yield was less by PCR reaction with 1.5 and 2.0 units of polymerase. Figure 3 shows that a much clearer band was obtained with PCR reaction of 30 cycles. Beyond 30 cycles, the brightness of the bands did not increase. In order to determine whether the failure of the 1500 bp fragment amplification was related to the PCR or to the amplification capacity of the two-temperature PCR, the 1500 bp fragment and other fragments were amplified using optimized conditions in both the common and the two-temperature PCRs. As showed in Fig. 3, under optimized two-temperature PCR conditions, clear bands were only obtained for fragments shorter than 1500 bp, no bands detected for 1500 bp fragment.

A comparison of polymerases and equipment: To study whether different Taq polymerase differently impact the two-temperature PCR process, Taq DNA polymerase products from 4 different companies were used in the two-temperature PCR. As shown in Fig. 4, the 923 and 1371 bp fragments were successfully amplified with Taq polymerases from Tiangen, Takara, Dongsheng and Dingguo. A TC-3000 Thermal Cycler with a heating/cooling rate up to 3.6°C sec^{-1} was used, compared parameters to the Eppendorf Mastercycler. The amplification results in Fig. 4 show that ideal amplification
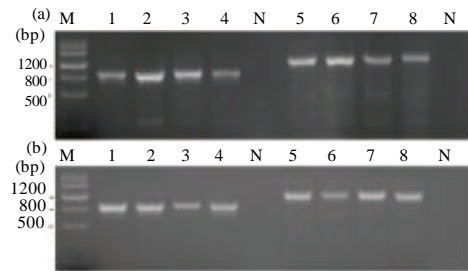

Fig. 4: Amplification results for 923 and 1371 bp fragments using a blood DNA template and different equipment and Taq DNA polymerases. a: Amplification results using the Eppendorf Mastercycler and b: Amplification results using the TC-3000 Thermal Cycler. M: DNA marker. N: negative control. Lanes 1-4: A 923 bp fragment amplified with Taq polymerases from Tiangen, Takara, Dongsheng or Dingguo Co., respectively. Lanes 5-8: A 1371 bp fragment amplified with Taq polymerases from Tiangen, Takara, Dongsheng or Dingguo Co., respectively

results were obtained with the two different types of PCR machines. It took around 30 min to finish the whole amplification procedure with the TC-3000 Thermal Cycler.

Application of the two-temperature PCR method: To demonstrate the potential use of two-temperature PCR, the sex identification of bovines was conducted with two-temperature PCR using blood samples. The Y
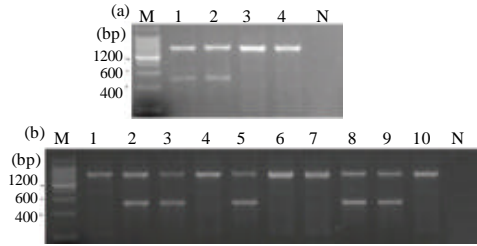
Fig. 5: The sex identification results using the two-temperature duplex PCR for the 503 bp (Sry) and 1371 bp (Myostatin) fragments in bovine blood samples (a) and the sensitivity of the sex identification method using the fibroblast DNA template (b). M: DNA marker. N: negative control. (a) Lanes 1 and 2 are blood from a male and lanes 3 and 4 are blood from a female. (b) Lanes 1 and 2 are blood samples from a female and a male using control PCR program, respectively Lanes 3-6: single cells. Lanes 7-10: five cells. Lanes 1, 4, 6, 7, and 10 are from females and Lanes 2, 3, 5, 8, 9 are from males
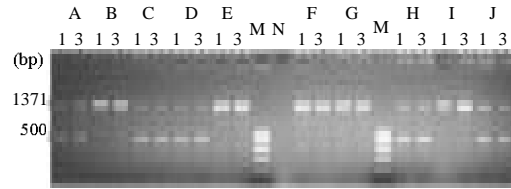

Fig. 6: The sex identification results using the two-temperature duplex PCR for the 503 and 1371 bp fragments in cloned bovine embryos M: DNA marker from 100- 600 bp. N: negative control in saccharose PBS. The letters from A-J represent different cloned embryos, the number 1 represents the sex identification results of samples in 1 blastmere while 3 represents the sex identification results of samples in 3 blastmeres. Embryo A, C, D, H, J are males and embryo B, E, F, G, I are females

chromosome specific primer Y56 and the internal primer G1371 were used in this duplex PCR system. The sexing shown in Fig. 5 showed that all samples had clear bands, the identified results were all consistent with the known sex of the animals. Furthermore, it took 30 min to finish the PCR amplification using the TC-3000 Thermal Cycler while it took 38 min for the Eppendorf Mastercycler.

After the one and five-cell templates were amplified using this duplex two-temperature PCR, clear, bright and exact amplification results were obtained from both groups. As expected, two bands were obtained in all male samples and one band was obtained in all female samples. The ideal identification results shown in Fig. 5 imply that the specificity could be increased by removing the extension step and shortening the annealing step. About 10 cloned embryos were biopsied and 1 and 3 blastmeres were sampled from each embryo separately. The identification results with this rapid PCR were shown in Fig. 6 and the clear and bright bands indicated that the ideal results were obtained from all embryos. The same identification results were obtained from both blastmere number groups and they are all consistent with the known sex of the cloned embryos.

Exploring of the extension capacity of the Taq polymerase is the key of rapid PCR: The optimal extension temperature for Taq polymerase is variable, it changes between 70 and 79°C, depending on specific PCR system

used (Innis *et al.*, 1988; Wittwer and Garling, 1991; Sambrook and Russell, 2001). Correspondingly, the extension rate was also different from 33.3-60.4 nt sec^{-1}, according to the extension temperature applied. In the previous study we obtained an extension rate of 191 nt sec^{-1} (Zhu *et al.*, 2005). Wittwer *et al.* (1990) suggested that the PCR yield increased when longer elongating time selected moreover, little change happened when the extension time was above 10~20 sec (Wittwer and Garling 1991). The extension time was only the transition time from the annealing step to the denaturation step in the study. If the optimal extension temperature was between 70 and 80°C and the extension could be negligible at other temperature, it took 3.33 sec to go from 70-80°C, using the Eppendorf Mastercycler with a heating rate of 3°C sec^{-1}. The average incorporating speed would be around 424 nt sec^{-1} (1413/3.33 sec^{-1}). Actually, the synthesis speed is not uniform, the greatest amplification capacity was far greater than the results reported previously (Hashimoto *et al.*, 2004; Chen *et al.*, 2008). This high amplification capacity might be the result of the specific kinetics of the polymerase but not the equipment or reagents used. The kinetics of the polymerase controls the length of fragment could be amplified. Therefore, further studies about the characteristics of Taq polymerase will improve the amplification potential of the two-temperature PCR.

The limitation of the knowledge influenced the exploring of the rapid PCR: Mg^{2+} influences enzyme activity and forms soluble complexes with dNTPs during DNA synthesis (Zippelius *et al.*, 2000). As the concentration of

Mg^{2+} increased, the dNTP incorporation and enzyme activity were enhanced while the sensitivity of the PCR reaction decreased and non-specific products were obtained when Mg^{2+} beyond 1.0 mmol L^{-1}. The optimal reaction conditions were affected by the presence of too many ions introduced by high concentrations of the Taq DNA polymerase and the result about the effects of different amount of polymerase were not consistent with other studies (Akane *et al.*, 1994; Zippelius *et al.*, 2000). The fact that the 1500 bp fragment still could not be amplificated with this PCR even using the optimized two-temperature PCR system, implies that the kinetic activity of the polymerase was contributed to the inability of long fragments amplification in the two-temperature PCR.

Many studies showed that the extension rate is very limited (Angers *et al.*, 2001; Sambrook and Russell, 2001), even in the most fast rate of 60.4 nt sec^{-1} found before (Innis *et al.*, 1988), the uniform extension rate was obtained in the optimal temperature interval during increasing form 70-80°C (Innis *et al.*, 1988; Wittwer and Garling, 1991; Sambrook and Russell, 2001) and the heating rate of the cycler is about 2.5°C sec^{-1}, the extension length is only up to 241.6 bp (60.4 nt sec^{-1} (80-70°C) 2.5°C sec^{-1}). The comparison between theoretical and experiment yield of different length fragments showed that, the amplicon length is a key factor limiting the amplification of the long fragment and the longer fragments amplified, the lower extension rate obtained (Whitney *et al.*, 2004). So, the studies on the rapid PCR later were focused on the special machines and the reagents. The studies about two-temperature PCR and the amplification capacity of the Taq polymerase were scarce. We obtained an extension rate at 424 nt sec^{-1}, helping to explore the kinetics of Taq polymerase deeply. The two-temperature PCR could be completed successfully using common Taq polymerases and equipment, the ramp time, between annealing step to denature step is enough to finish the extension for amplicons ≤1413 bp. This evidence may be beneficial to researchers in any genetics laboratory. Using the two-temperature PCR program without the special reagents and equipment would save time and money costs.

Sex identification demonstrated the potential use of two-temperature PCR in more areas: The two-temperature PCR showed sufficient sensitivity in sex identification of cattle embryo using a single blastmere as the template and the false positive result was avoided. Furthermore, it greatly decreased the detection time, compared with the tedious PCR methods previously used

(Pomp *et al.*, 1995; Tominaga and Hamada, 2004; Fu *et al.*, 2007; Lu *et al.*, 2007). Using common cycler, it took only 30-38 min to finish the amplification process with two-temperature PCR while this process lasted for 2 h using the common PCR program. More than two third of time was saved by two-temperature PCR and the detecting cost was decreased sharply.

CONCLUSION

A rapid, simple, efficient and cost-effective bovine sex identification method was established using a two-temperature PCR method that requires between 30 and 38 min for the amplification process. The two-temperature PCR which could be completed with common PCR reagents and equipment is the inherent reaction characteristic of Taq polymerases. The potential amplification ability could be greater and faster if the amplification properties of the Taq polymerase were better understood. The two-temperature PCR method is efficient in time and money, also of high sensitivity, simplicity and accuracy, it might contribute to the renovation of detection methods in many fields such as forensic medicine, clinical medicine and sex identification.

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