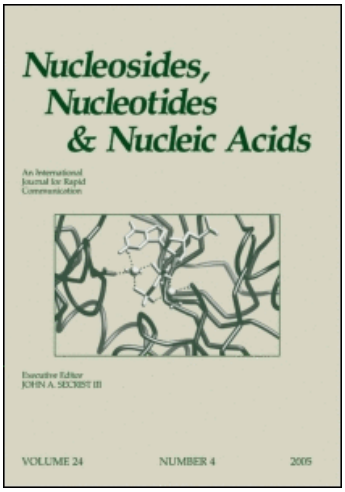

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HIGH GC CONTENTS OF PRIMER 5′-END INCREASES REACTION EFFICIENCY IN POLYMERASE CHAIN REACTION

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□ *Many studies have suggested that regulation of the polymerase chain reaction (PCR) is influenced by several factors. However, the understanding of reaction efficiency factors is not sufficient. Here we propose that high GC contents of primer 5′-end increases reaction efficiency in PCR. Using 71 primers (45 pairs), we analyzed factors that affect reaction efficiency, and statistically tested the correlation between the amplification signals and several factors. As a result, there were significant correlations between the amplification signals and the GC contents in the first 1∼3 bps of primer 5′-end.*

Keywords Polymerase chain reaction; primer sequence; amplification efficiency

INTRODUCTION

Polymerase chain reaction[1] is an important tool for rapid amplification of a specific DNA fragment, the target sequence. The PCR amplification usually consists of 20 to 50 repeated amplification cycles. For the success of PCR, several studies have reported various PCR influence factors: buffer reagents, primer length, GC contents of primers and template, complexity of primer, annealing temperature, product size, resisting structure such as dimer, etc.[2–6] Recently, some studies focused on the nucleotide composition of the primer 3′ end[7–11] because elongation starts from the 3′ end of the primer. Taking account of the stability and originality of the 3′ end, statistical models predicting failure rate of PCR[12] and a program to design a group-specific primer[13] have been developed. On another front, several

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studies have reported that a certain kind of compound conjugating primer 5′ end can be used to amplify DNA with good reaction efficiency.[14,15] In PCR for detection of a specific species, reaction efficiency is very important because high reaction efficiency results in a strong electrophoresis signal that then increases the reliability and robustness of the inspection. Although a strong signal is obtained by lengthening PCR product size, that is, increasing places for intercalating, a primer of short product size better detects fragmented DNA such as meat-and-bone meal[16] and fish meal.[17] In this study, we used computationally-designed 71 primers (45 pairs) whose sequences were selected randomly. Among many factors, we changed only the sequence of primer, while fixing the other conditions such as PCR buffer, melting temperature (Tm), PCR amplification condition and electrophoresis condition. For the purpose of designing primers which produce a strong electrophoresis signal, we statistically analyzed several factors to determine their influence on reaction efficiency.

MATERIALS AND METHODS

Raw meat samples from cattle, sheep, goats, deer, and swine, obtained from commercial sources, were used in the present study. All samples were confirmed to produce amplification signals of the same strength using a primer pair for mammals.[18] Primers were designed by a computer program GSPRIMER as previously described,[13] with the following conditions; Tm value: 55°C, product size: 80∼200 bp, primer size: 18∼24 bp, GC contents of primer: 24∼60%. We designed 71 primers (45 primer pairs) for several species of mammals in various regions of the sequences. We tried to select primer sequences as randomly as possible from the output of the program, that is, no factors were considered other than that all primers were designed to avoid pair/self dimer, since understanding of the impact is not sufficient. Mitochondrial DNA (mtDNA) extraction and PCR amplification were performed as previously described, using an mtDNA extractor CT kit (Wako, Osaka, Japan).[18] Amplification was performed with the following cycling conditions: after an initial heat activation at 95°C for 9 minutes, 45 cycles were programmed at the conditions of 92°C for 30 seconds, 55°C 30 seconds, 72°C 30 seconds, and final extension at 72°C for 5 minutes.

RESULTS

The primers were divided into three groups according to the strength of signals (brightness of the electrophoresis band) that they produced. In determining the strength of signal, we used control signals indicating a weak, medium, and strong reaction (Figure 1). We assumed that a low amplification is caused by the low amplification primer of a pair even if

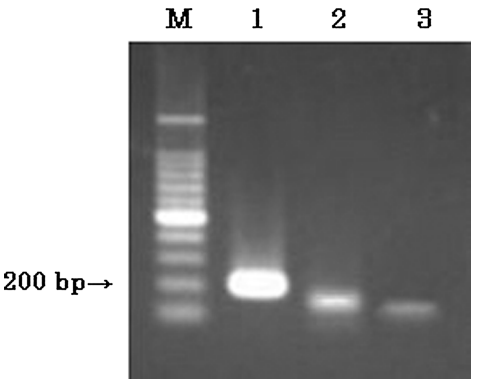

FIGURE 1 Controls of amplification signals. Lane M, DNA size markers (100 bp ladder); lane 1, control of strong signal; lane 2, control of medium signal; lane 3, control of weak signal.

the other primer is a high amplification primer, that is, whichever is the lower decides amplification efficiency. This assumption that only the low amplification primer of a pair is responsible for the low PCR amplification was substantiated by creating strong signals using a primer pair consisting of two strong signal primers and then replacing a primer of the pair with another primer (Figure 2; Table 1). Measurements were taken of the number of G+C in the first 1∼5 bps at each of the 5′ and 3′ ends, GC contents of the

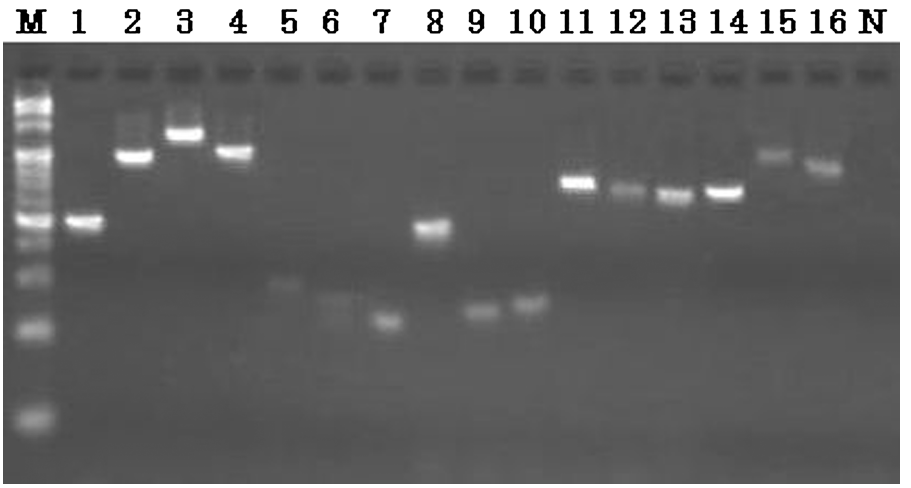

FIGURE 2 Amplification signals of primers. Lane M, DNA size markers (20 bp ladder); lane 1, Amplification by primer set F1 plus R1 (sorted into strong signal); lane 2, primer set F1 plus R2 (strong); lane 3, primer set F1 plus R3 (strong); lane 4, primer set F1 plus R4 (strong); lane 5, primer set F1 plus R5 (weak); lane 6, primer set F1 plus R6 (weak); lane 7, primer set F1 plus R7 (medium); lane 8, primer set F2 plus R8 (strong); lane 9, primer set F3 plus R8 (weak); lane 10, primer set F4 plus R8 (weak); lane 11, primer set F5 plus R9 (strong); lane 12, primer set F5 plus R10 (weak); lane 13, primer set F5 plus R11 (medium); lane 14, primer set F5 plus R12 (strong); lane 15, primer set F6 plus R9 (weak); lane 16, primer set F6 plus R10 (weak); lane N, negative control (water).

TABLE 1 Primer sequences and signal strength used in Figure 2

Name	Seqence	Signal
F1	TCCTCCGAGCGATTTTAAAGA	strong
F2	CAATTGAATGCAAATCAAC	strong
F3	AGATTGTTGGGCTCCAC	weak
F4	ACTAGATTGGTGGGCTC	weak
F5	GACCCTATGGAGCTTTAAC	strong
F6	CTTCCAATCAGTGAAATTGACC	weak
R1	CTTTCGTACTGGGAGAAATAC	strong
R2	CTTTGATAGCGGTTGCAC	strong
R3	AACTAAGCACTCTATTCTTAGTT	strong
R4	TAATAATTAGTGTAGAGAGGCG	strong
R5	GTTAAGGAGAGGTTTTGAAT	weak
R6	TATGGGCCCGATAGCTT	weak
R7	AGTGAGATGTGTCATATACATAGA	medium
R8	ATAAATAATGTTATAATTATTGATG	strong
R9	AGTTATGTTTTATCCCTTTG	strong
R10	CTAAGAGAGAGTTCTACGGTC	weak
R11	GCACGAGATTTACCAACT	medium
R12	CGTATGTAGAAATAGAGGTTTAGC	strong

whole and middle sequence of the primer, and complexity[19] of the primer. We statistically analyzed the relationship between the amplification signals and these measurements. The number of primers with a weak, medium or strong amplification signal having no or one G+C in the first one base pair; having no, one or two G+C in the first two base pairs; and having no, one, two, or three G+C in the first three base pairs of the 5′ end are shown in Table 2. For example, 12 primers that had a weak amplification signal had no G or C in the first base pair of the 5′ end. The number of G+C in the first 1∼3 bps of the 5′ end seems to have a positive correlation with the strength of amplification signals. We calculated the correlation coefficient between the amplification signals and the measurements of each factor and applied a statistical test. The results showed there were significant correlations between amplification signal and the number of G+C in the first 1∼3 bps of the 5′ end (Table 3). While the other measurements did not show significant differences against amplification signal, the correlation coefficients for the measurements of the 5′ end were all positive and those of the 3′ end were negative. We also compared the GC contents of the 5′ and 3′ ends of the primer to determine the relationship between the balance and the signal strength by applying a t-test. There were no significant differences.

DISCUSSION

The results of this study indicate that the number of G+C in the 5′ end of a primer affects amplification signal, regardless of the sequence of the

TABLE 2 Number of primers with different signals and different GC contents in the first one, two, and three base pairs of the 5′ end

Strength of signal	Number of primers whose first bp of 5′end has G+C counts of:		Number of primers whose first two bps of 5′end has G+C counts of:			Number of primers whose first three bps of 5′end has G+C counts of:			
	0	1	0	1	2	0	1	2	3
Weak	12	7	7	12	0	4	11	4	0
Medium	9	11	7	7	6	4	8	6	2
Strong	11	21	4	17	11	0	18	13	1

TABLE 3 Correlation coefficients between factors and amplification signals

	Correlation coefficient with signal	Significant correlation test (p value)
G+C in first 1 bp of 5′ end	0.235	significant (0.049)
2 bp of 5′ end	0.344	significant (0.003)
3 bp of 5′ end	0.264	significant (0.026)
4 bp of 5′ end	0.227	not significant (0.056)
5 bp of 5′ end	0.229	not significant (0.055)
G+C in middle sequence	0.049	not significant (0.685)
G+C in first 1 bp of 3′ end	−0.170	not significant (0.157)
2 bp of 3′ end	−0.131	not significant (0.276)
3 bp of 3′ end	−0.040	not significant (0.743)
4 bp of 3′ end	−0.073	not significant (0.543)
5 bp of 3′ end	−0.106	not significant (0.380)
complexity	−0.091	not significant (0.449)

A correlation was assumed to be significant if the $p < 0.05$. "Middle sequence" is the part of the primer sequence remaining after 5 bps have been removed from each end.

3′ end. However there were some cases when the signal was weak despite being 5′- GC rich and cases when the signal was strong despite being 5′- GC poor. These cases were probably due to the existence of a sequence similar to the primer in the reaction system which might have resulted in a conflict with target sequence, thereby affecting reaction efficiency. There was no significant correlation between GC contents of whole sequences of primer and the signal. This suggests that the GC contents of primers do not have a significant effect on reaction efficiency, despite the general observation in the past that they do so.[3–7] Considering the fact that GC forming has three hydrogen bonds as against two bonds of AT forming, the results of our study supports a hypothesis that the primers with high GC contents in the 5′ end anneal from the 5′ end: If primers anneal from the 5′ end, primers annealing at an incorrect position would stop annealing to the 3′ end of the primer without starting a chain elongation reaction. This would keep the primers intact and allow them to be used for a second time for the next cycle. (On the contrary, if primers anneal from the 3′ end, primers annealing at the wrong position would start a chain elongation reaction anyway, resulting in the formation of a junk fragment of sequence. This would not allow the primers to be used for the next cycle.) Based on this hypothesis, even if the Tm value is raised by increasing the number of G+C in the 5′ end for reaction efficiency, the specificity would be unaffected as long as the primer has high originality of the 3′ end.[8] In conclusion, primers which produce a strong signal can be designed by increasing the G+C contents in the 5′ end.

REFERENCES

1. Mullis, K.B.; Faloona, F.A. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol* **1987**, 155, 335–350.

2. Haas, S.; Vingron, M.; Poustka, A.; Wiemann, S. Primer design for large scale sequencing. *Nucleic Acids Res.* **1998**, 26, 3006–3012.
3. Housley, D.J.; Zalewski, Z.A.; Beckett, S.E.; Venta, P.J. Design factors that influence PCR amplification success of cross-species primers among 1147 mammalian primer pairs. *BMC Genomics* **2006**, 7. [Article no. 253 online]
4. Sahdev, S.; Sini, S.; Tiwari, P.; Saxena, S.; Saini, K.S. Amplification of GC-rich genes by following a combination strategy of primer design, enhancers and modified PCR cycle conditions. *Mol Cell Probes* **2007**, 21, 302–307.
5. Varadaraj, K.; Skinner, D.M. Denaturants or cosolvents improve the specificity of PCR amplification of a G + C-rich DNA using genetically engineered DNA polymerases. *Gene* **1994**, 140, 1–5.
6. Vondrusková, J.; Parizkova, N.; Kypr, J. Factors influencing DNA expansion in the course of polymerase chain reaction. *Nucleos Nucleot Nucleic* **2007**, 26, 65–82.
7. Benita, Y.; Oosting, R.S.; Lok, M.C.; Wise, M.J.; Humphery-Smith, I. Regionalized GC content of template DNA as a predictor of PCR success. *Nucleic Acids Res.* **2003**, 31, e99.
8. Miura, F.; Uematsu, C.; Sakaki, Y.; Ito, T. A novel strategy to design highly specific PCR primers based on the stability and uniqueness of 3′-end subsequences. *Bioinformatics* **2005**, 21, 4363–4370.
9. Onodera, K.; Melcher, U. Selection for 3′ end triplets for polymerase chain reaction primers. *Mol. Cell Probes* **2004**, 18, 369–372.
10. Yuryev, A.; Huang, J.; Pohl, M.; Patch, R.; Watson, F.; Bell, P.; Donaldson, M.; Phillips, M.S.; Boyce-Jacino, M.T. Predicting the success of primer extension genotyping assays using statistical modeling. *Nucleic Acids Res.* **2002**, 30, e131.
11. Yuryev, A.; Huang, J.; Scott, K.E.; Kuebler, J.; Donaldson, M.; Phillips, M.S.; Pohl, M.; Boyce-Jacino, M.T. Primer design and marker clustering for multiplex SNP-IT primer extension genotyping assay using statistical modeling. *Bioinformatics* **2004**, 20, 3526–3532.
12. Andereson, R.; Mölls, T.; Remm, M. Predicting failure rate of PCR in large genomes. *Nucleic Acids Res.* **2008**, 36, e66.
13. Shinoda, N.; Kusama, T.; Yoshida, T.; Sugiura, T.; Kadowaki, K.; Onodera, T.; Sugiura, K. Developing PCR primers using a new computer program for detection of multiple animal derived materials in feed. *J. Food Prot.* **2008**, 71, 2257–2262
14. Adessi, C.; Matton, G.; Ayala, G.; Turcatti, G.; Mermod, J.J.; Mayer, P.; Kawashima, E. Solid phase DNA amplification: characterisation of primer attachment and amplification mechanisms. *Nucleic Acids Res.* **2000**, 28, e87.
15. Afonina, I.; Zivarts, M.; Kutyavin, I.; Lukhtanov, E.; Gamper, H.; Meyer, R.B. Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder. *Nucleic Acids Res.* **1997**, 25, 2657–2660.
16. Frezza, D.; Favaro, M.; Vaccari, G.; von-Holst, C.; Giambra, V.; Anklam, E.; Bove, D.; Battaglia, P.A.; Agrimi, U.; Brambilla, G.; Ajmone-Marsan, P.; Tartaglia, M. A competitive polymerase chain reaction-based approach for the identification and semiquantification of mitochondrial DNA in differently heat-treated bovine meat and bone meal. *J. Food Prot.* **2003**, 66, 103–109.
17. Nomura, T.; Kusama, T.; Kadowaki, K. Detection of fish DNA in ruminant feed by PCR amplification. *Shokuhin Eiseigaku Zasshi* **2006**, 47, 222–224.
18. Kusama, T.; Nomura, T.; Kadowaki, K. Development of primers for detection of meat and bone meal in ruminant feed and identification of animal origin. *J. Food Prot.* **2004**, 67, 1289–1292.
19. Orlov, Y.L.; Potapov, V.N. Complexity: an internet resource for analysis of DNA sequence complexity. *Nucleic Acids Res.* **2004**, 32, W628–W633.