High GC Amplification: A Comparative Study of Betaine, DMSO, Formamide and Glycerol as Additives

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Abstract: High GC content is proved to be an obstacle for successful PCR when following standard protocols recommended by Mullis KB. To avoid this false negative, additives are often used. However, among those easily accessible additives such as betaine, dimethylsulfoxide (DMSO), formamide and glycerol, their concentrations used in the PCR mixture were reported to be in a wide range, and no systematic researches exist either about the most optimal concentration of each additive or about which additive is the most powerful. Using human leucocyte antigen-B (HLA-B) PCR amplification as a model, we evaluated the effects of these 4 additives with a KCl-contained or KCl-free reaction buffer, and with different additive concentrations. We found that 0.6 M for betaine, 10% for DMSO, 5% for formamide and 10% for glycerol were their optimal concentrations, respectively, and the 0.6 M betaine had the highest successful rate. We also found that KCl will greatly improve the amplification yields when the template GC content was moderate, while zero KCl could improve the yields when the template GC content was high. We thus recommended a 0.6 M betaine and KCl-free buffer as the first choice in high GC amplifications. [Life Science Journal. 2006;3(1):67-71] (ISSN: 1097-8135).

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1 Theory

The polymerase chain reaction (PCR) method invented by Mullis, et al (Mullis, 1987) greatly enhanced the biologists' capacity to isolate, characterize, and produce large quantities of DNA *in vitro*. Since its emergence, no one has ever doubted the effectiveness of the procedures recommended by Mullis, especially when the GC content of amplified DNA sequence is between 40% - 60%. While a fact continues to bother labs and researchers having PCR needs: the standard procedures are very hard to produce products when the template is rich with GC. The high GC content of human leucocyte antigen-B (HLA-B) (Robinson, 2003), ApoE (Jacobsen, 2002), p16 (Jung, 2002), prostate-specific membrane antigen (PSM) gene (Henke, 1997), even including HLA-A, C, etc., which is among 65% - 70% or higher, often leads to low yields of the target DNA sequence and the accompanying amplification of undesired nonspecific bands, and keeps to be a problem in PCR amplifications.

As GC base pair has 3 hydrogen bonds while AT pair has only 2 bonds, so GC pair exhibits stronger base-base interactions, leading to stable self-complementary superstructures such as hairpin-loop and dimer, producing regions with higher melting temperatures, and making the DNA strand harder to be opened and amplified (Benita, 2003; Weissensteiner, 1996). To tackle such problems, additives such as dimethylsulfoxide (DMSO), formamide, glycerol and betaine are often reported to be used in such amplifications due to their ability of reducing the effect of hydrogen bonding, destabilizing the secondary structure, and enhancing the specificity of the amplification reaction (Weissensteiner, 1996; Chakrabarti, 2002; Chakrabarti, 2001). However, until recently, no systematic

comparison study exists about the effect of each additive. Therefore, we performed an evaluation of the effect of these additives with HLA-B gene as a model and we were aimed at finding clues about which additive is more powerful than others.

Weissensteiner et al showed that there are two key factors in influencing the amplification outcomes when additives are used (Weissensteiner, 1996): one is the concentration of monovalent cations in the reaction mixture, such as K^+, NH_4^+, which will increase the stability of double-stranded DNA (dsDNA), thus make the GC-rich region more stable; the other is the concentration of additives like betaine, which will lower the stability of dsDNA and thus make the GC-rich region less stable, easier to be opened and more ready for the primers to bind and extend. So in our study we lowered the concentration of KCl to 0 mM to observe the full potential of each additive and to avoid any counteractions from the cations.

2 Materials and Methods

2.1 Materials

2.1.1 Blood samples

2 ml peripheral vein blood (from each of 20 paternity testing personnel, who belong to the Han nationality in the northeast China).

2.1.2 Reagents

dNTP (Takara Co., Shiga, Japan); rTaq DNA polymerase (Sino-American Biotechnology Company, Luoyang, China); betaine (Weifang Sunwin Chemicals, Shandong, China); DMSO (Sigma, USA); formamide (Sigma); glycerol (Shenyang Chemicals, Shenyang, China); primers pairs for HLA-B gene (Cao, 1999): forward 5′-GGG AGG AGC GAG GGG ACC G/CCA G-3′, reverse 5′-GGA GGC CAT CCC CGG CGA CCT AT-3′ (Sangon Company, Shanghai, China); primers pairs for CCR5 (chemokine C-C motif receptor 5) gene (Balotta, 1997): forward 5′-GAA GGT CTT CAT TAC ACC TG-3′, reverse 5′-AGA ATT CCT GGA AGG TGT TC-3′; φX174-*Hae* Ⅲ Digest DNA Marker (Takara).

2.1.3 Apparatus

UV-310 Ultraviolet Spectrophotometer (Pye-Unicam/Spectronic, UK); UNO Ⅱ thermocycler (Biometra, Germany); Mini-PROTEAN 3 Electrophoresis System (Bio-Rad, CA, USA); 377 ABI PRISM™ DNA Sequencer (Applied Biosystems, CA, USA).

2.2 Methods

2.2.1 DNA preparation

Genomic DNA was prepared by Phenol/Chloroform extraction and dissolved in 120 μl Tris/EDTA solution. The OD260:OD280 ratio was adjusted to near 1.8.

2.2.2 PCR reaction

Both the commercial 10× reaction buffer and a self-made KCl-free 10× reaction buffer were being used, while the latter contains all the ingredients except KCl, e. g., including 100 mM Tris-HCl, 1.0% Triton X-100 and 15 mM $MgCl_2$. The PCR was carried out in a total volume of 20 μl on the UNO Ⅱ thermocycler. All PCR mixtures contained the following at a final concentration: 0.2 mM of each dNTP, 1 unit rTaq polymerase, 0.25 μM each of HLA-B-specific forward and reverse primers, approximately 1μg DNA template, and with or without additives. The forward primer was located in HLA-B intron 1 and the reverse primer was located in intron 3. Amplification parameters consisted of denaturation for 5 minutes at 96℃, followed by 35 cycles of 22 seconds at 94℃, 50 seconds at 65℃, 30 seconds at 72℃ and then a final extension of 10 minutes at 72℃ (Cao, 1999).

Additives and KCl were used with four combinations: KCl-contained, no additives; KCl-free, no additives; KCl-contained, with additives; and KCl-free, with additives. Each sample was amplified without additive and with each one of the 4 following additives: betaine, DMSO, formamide, glycerol. Glycerol was diluted to 50% to decrease the viscosity and facilitate the transfer. A concentration titration was used to determine which concentration is the optimal. The titration for betaine was 0.3 M, 0.6 M, 0.9 M, 1.5 M, while the gradient for DMSO, formamide and glycerol was 0%, 5%, 10% and 15%. Positive control was set up in a separate amplification with CCR5 gene, using the same dNTP, rTaq polymerase and DNA except two CCR5-specific primers. The cycling conditions were as follows: a 3-minute 94℃ pre-denaturation; 35 cycles of 30-second denaturation at 94℃, 30-second annealing at 56℃, 30-second extension at 72℃; and a final extension at 72℃ for 10 minutes (Balotta, 1997). Both KCl-contained buffer and KCl-free buffer were separately used. Negative control was set up with no template DNA added.

2.2.3 Polyacrylamide gel electrophoresis (PAGE) and silver staining

PCR products were analyzed by PAGE. Polyacrylamide gels ($C = 6\%$, $T = 5\%$) were prepared. After gel pre-run, the products as well as a lane of φX174-*Hae* Ⅲ Digest DNA Marker were loaded and a constant current was set to separate the PCR products. After electrophoresis, the gel was fixed with 10% acetic acid for 20 minutes, and then was put into 0.1% $AgNO_3$ solution (formaldehyde contained) for 30 minutes with gen-

tle shaking, and then the gel was developed in a 4% Na_2CO_3 solution (formaldehyde contained).

2.2.4 Products sequencing

Successful amplified products were sent to Takara Biotech Company and were sequenced with a 377 ABI PRISM™ DNA Sequencer.

2.2.5 Statistical analysis

A chi-square test was performed with SAS (Version 6.12) to find out whether there is a difference between these additives.

3 Results

3.1 Confirmation of successful amplification

Successful amplification of HLA-B gene will have a band at the 943 bp position. Successful amplification of the positive control, CCR5 gene, will produce a band at 276 bp. No PCR products were generated in our negative controls.

3.2 Amplification effects of KCl and additives

In all the 20 samples, CCR5 gene was amplified successfully with the KCl-contained or KCl-free PCR buffer, while the KCl-contained buffer generated more yields at 276 bp position as expected. Either with KCl-contained or KCl-free buffer, fewer yields were found when additives were used.

To the HLA-B amplifications, KCl-contained buffer produced only non-specific bands, KCl-free buffer with additives generated band at 943 bp position as expected. Either with or without additives, a KCl-contained buffer was able to produce more bands than a KCl-free buffer. But all the bands generated by the KCl-contained buffer were non-specific, not at the 943 bp position and usually smaller than 943 bp, while with the KCl-free buffer combining one of the additives, a single band of the expected size at 943 bp position was obtained. Different concentrations of each additive had different successful amplification rates (Figure 1). For details, see Table 1.

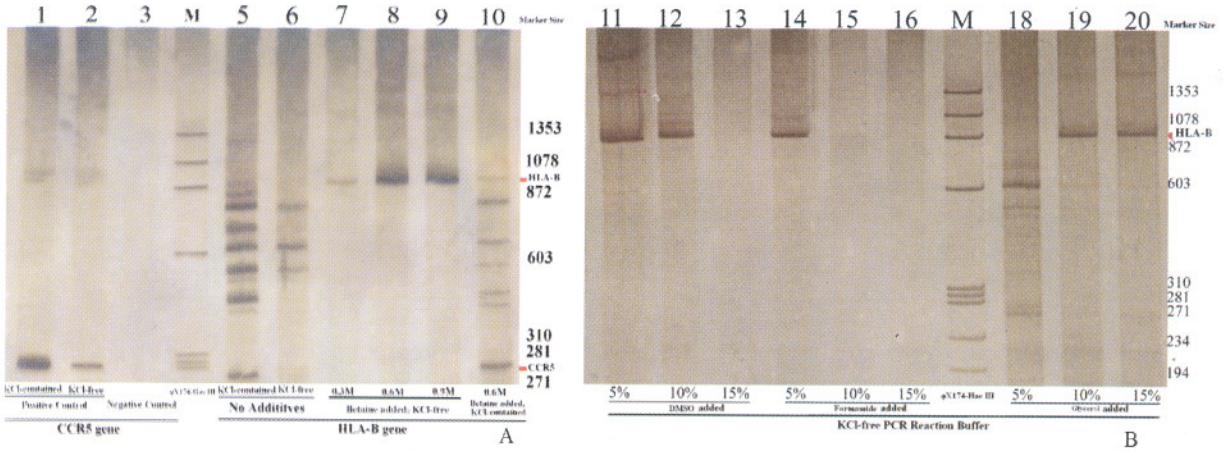

Figure 1. Amplification effects of KCl and the 4 additives on Sample 3. Lane 2 showed that without KCl in the PCR buffer, the amount of the CCR5 decreased greatly. Lanes 8, 9, 11, 12, 14, 19 and 20 showed that with a KCl-free buffer and a proper additive, HLA-B was amplified successfully.

3.3 Sequencing results

The products of successful amplification were reported to have HLA-B intron 1, exon 2, intron 2, exon 3 and partial intron 3 sequences, and were in accordance with the HLA-B sequences of GenBank (Takara sequencing No: SYS587).

3.4 Statistical analysis

Significant difference was observed among these 4 additives. When 5%, 10% and 15% concentrations were used, the overall successful rate of formamide was lower than that of DMSO or formanide or betaine.

4 Discussion

HLA typing is one of the most widely involved techniques in clinical applications such as liver transplantation, the diagnosis of axial spondyloarthritis (AS) (Rudwaleit, 2004) or insulin-dependent diabetes mellitus (IDDM) (Gillespie, 2004). Reliable HLA gene typing depends on the effective amplification of HLA genes. But the HLA-B gene sequence is rich with GC, which easily forms secondary structures such as hairpins, dimers. These will make the Taq polymerases (especially sequenases) falling off from the templates, causing premature termination of PCR or sequencing reactions (Baskaran, 1996) and leading to low yields insufficient for further analysis. So GC content is very critical in determining the amplification outcomes. Even more recently, Benita Y et al

found that the regionalized GC content is a good predictor of PCR success across multiple templates (Benita, 2003). However, this GC-content-caused problem is often overlooked when compared with those efforts put into primer design.

High GC content sequences widely exist among the whole genome. A common approach to the optimization of such GC-rich amplification reactions is the addition of small quantities of certain organic chemicals, such as DMSO, betaine, glycerol and formamide, etc., to the reaction mixture (Jung, 2002; Henke, 1997; Weissensteiner, 1996; Chakrabarti, 2002; Chakrabarti, 2001; Papp, 1996). These additives are minim but so effective that they break the GC hydrogen bonds, destabilize the GC-rich region, open the hairpin-loops, lower the melting temperature and greatly enhance the amplification yields and fidelity.

Table 1. HLA-B amplification results of applying different PCR reaction buffer and different additives

PCR Buffer Types	Additives	Total Sample	Successful Amplifications	Rate
Standard buffer (KCl contained)	No	20	0 (except many non-specific bands)	0%
KCl-free buffer	No	20	0 (except few non-specific bands)	0%
KCl-free buffer	0.3 M Betaine	20	15	75%
	0.6 M Betaine	20	20	100%
	0.9 M Betaine	20	16	80%
	1.2 M Betaine	20	10	80%
KCl-free buffer	5% DMSO	20	17	85%
	10% DMSO	20	18	90%
	15% DMSO	20	0	0%
KCl-free buffer	5% Formamide	20	16	80%
	10% Formamide	20	7	35%
	15% Formamide	20	0	0%
KCl-free buffer	5% Glycerol	20	9	45%
	10% Glycerol	20	18	90%
	15% Glycerol	20	17	85%
	20% Glycerol	20	10	50%
KCl-contained buffer	Each additives	5	Non-specific: high amount; Specific: trace amount	0

But among those easy available ones as mentioned above, the optimal concentrations were reported to be in a wide range. For example, most researchers reported the effective concentration of formamide is between 5% – 15%, and that of glycerol is between 5% – 20%, while the wide range has made it very hard to choose a concrete concentration to begin. Moreover, so far, there has not been a systematic comparison once performed to demonstrate which additive has the most effective and consistent amplification-enhancing effect. So in our study, we used 4 easily accessible reagents to find out which concentration was optimal and which additive had the best enhancing effect. We collected 20 DNA samples and amplified them with four combinations: with or without KCl, combining with or without additives. Our results showed that 0.6 M for betaine, 10% for DMSO, 5% for formamide and 10% for glycerol were their optimal concentration, respectively. Our results demonstrated that formamide was less powerful than the other 3 additives. Our results also indicated that the use of 0.6 M betaine as a destabilizing additive yielded consistent amplification of HLA-B gene. In fact, betaine has more advantages over other additives: it is not as viscid as glycerol, which is hard to be transferred; it is not as inhibitive to Taq polymerase as formamide, which will require more enzymes when a higher concentration of formamide is applied. Furthermore, betaine is compatible with various downstream procedures such as product-purification, sequencing, etc. Other researchers demonstrated that betaine was able to amplify 84% GC sequences while other additives were not (Papp, 1996). Henke W et al also reported that betaine was able to amplify prostate-specific membrane antigen (PSM) mRNA (66% GC) while both DMSO and glycerol not (Henke, 1997).

Another feature of our research was the comparison of applying a KCl-contained and a KCl-free PCR reaction mixture except the addition of additives. We found that a KCl-contained buffer was able to improve the yields greatly when we were amplifying a medium GC content gene, CCR5. Even when KCl-contained buffer was used to amplify the high GC sequences, it still was able to

produce discernible bands. However, in the latter case, all the bands were non-specific, and there were no presence of the specific bands at 943 bp as expected, as shown in Figure 1. Only when the GC content was medium, the KCl-contained buffer was specific and effective. In contrast, the KCl-free buffer showed a synergistic effect with additives. As Weissensteiner T et al pointed out, without KCl, the dsDNA in the solution was much more unstable (Weissensteiner, 1996). A KCl-free buffer helped the additives destabilize the superstructure, strengthened the effects of additives, and thus enhanced specificity and improved yields. While there are many other attempts of combining two of the many enhancers to get better results, we suggest the combining use of betaine and the KCl-free reaction buffer should be more effective. The sequencing result demonstrated that such a strategy could be quite helpful in cases of difficult PCR amplification. We used HLA-B gene to demonstrate a series of conditions that may be used to optimize the amplification of other high-GC templates.

5 Conclusion

To find out the most optimal amplification conditions for high GC DNA template, we evaluated the effects of 4 additives Betaine, DMSO, formamide and glycerol on HLA-B amplification at different additive concentrations under the settings of KCl-contained or KCl-free reaction buffer. We found that 0.6 M for betaine, 10% for DMSO, 5% for formamide and 10% for glycerol were their optimal concentrations, respectively, and the 0.6 M Betaine had the highest successful rate. We also found that KCll greatly improved the amplification yields when the template GC content was moderate, while zero KCl could improve the yields when the template GC content was high. We thus recommended a 0.6 M Betaine and KCl-free buffer as the first choice in high GC amplifications.

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