Effect of Dimethylsulfoxide and Betaine on duplex polymerase chain reaction of human beta-globin gene amplification

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الخلاصة:

يمكن استعمال العديد من الأضافات والمواد التي تزيد من انتاج سلاسل الحامض النووي منقوص الأوكسجين وزيادة خصوصية تضخيمها في تفاعل السلسلة المتبلمرة (PCR) , , جمعت عينات الدم من 35 شخص سليم مظهريا لغرض اجراء استخلاص الحامض النووي منقوص الأوكسجين ومن ثم تضخيم المنطقة من الجين المشمول بالدراسة باستخدام تفاعل السلسلة المتبلمرة طبقا لأربعة نماذج مختلفة من المواد المستخدمة بالتفاعل والتي شملت: 1-التضخيم باستعمال المواد القياسية (بدون اضافة محسنات التفاعل), 2- بأضافة 5% من ثنائي ميثيل سلفوكساید , 3- بأضافة 1 مولاري من مادة البيتايين , و4- بأضافة 5% من ثنائي ميثيل سلفوكساید مع 1 مولاري من مادة البيتايين. أظهرت النتائج ان استعمال المواد القياسية في تضخيم المناطق المدروسة من جين الكلوبين – بيتا البشري تكون كافية لأعطاء نتائج جيدة في عملية التضخيم , وان اضافة المحسنات المشمولة بالدراسة ساعدت على زيادة كمية الحامض النووي منقوص الأوكسجين في المناطق المضخمة وخصوصا عند استعمال أضافة 5% من ثنائي ميثيل سلفوكساید مع 1 مولاري من مادة البيتايين .

Abstracts:

A variety of additives and enhancing agents can be included in PCR amplifications to increase yield, specificity and consistency of PCR products. Blood samples were collected from (35) apparently healthy individual for DNA extraction and duplex PCR amplification according to the four different PCR component set up include: **1**- standard set up (without PCR additives), **2**- standard PCR set up plus 5% DMSO, **3**- standard PCR set up plus 1M betaine, and **4**- standard PCR set up plus 5% DMSO and 1M betaine. The results revealed that the standard optimization condition of duplex PCR amplification is enough to reveal a good PCR amplification of human beta-globin gene, and the studied PCR additives are useful in improvement of amplification in combination of 5 % DMSO and 1 M betaine.

Key words: Polymerase chain reaction; PCR; PCR additives; Dimethylsulfoxide; Betaine

Introduction:

Polymerase chain reaction (PCR) is one of the most widely used methods in molecular biology and genetics. Since it was first described in 1988 (1), PCR has been applied to a wide range of research in the life sciences, and there has been a continuous optimization of several approaches have been taken to improve the polymerization activity (2). However, there are instances in which a particular DNA region proves difficult to amplify by PCR. The amplification of targets rich in GC content or ones that can form secondary structure often result in little or no yield of expected product. Furthermore,amplification may result in products derived from regions other than the target DNA region, indicated by multiple bands on a stained agarose gel (3). .

Optimization of magnesium concentration, buffer pH, denaturing and annealing times and temperatures, and cycle number is useful in some, but not all cases. Targets that are refractory to amplification, despite optimization attempts, can often be amplified if the appropriate additive is included in the amplification mix (3,4). .

The GC-rich DNA sequences often require laborious work to optimize the amplification assay, several agents that facilitate product formation in PCR amplifications are now commercially available. These agents alter the melting characteristics of DNA. Their identities, however, are not revealed by the respective suppliers (5). .

A variety of additives and enhancing agents can be included in PCR amplifications to increase

yield, specificity and consistency. Agents include: dimethyl sulfoxide (DMSO), N,N,N-trimethylglycine (betaine), formamide, glycerol, nonionic detergents, bovine serum albumin , polyethylene glycol and tetra methyl ammonium chloride. These additives have beneficial effects on some PCR amplifications; however, it is not possible to predict which agents might be useful for a particular target (5,6, 7).

This study aimed to evaluate the effect and the activity of DMSO, betaine, and DMSO-betaine combination to improve the yield and specificity of target duplex PCR amplification of human beta-globin gene in comparison to the standard duplex PCR amplification.

Materials & Methods:

Blood Sampling:

2 ml of peripheral whole blood was collected by vein puncture from 35 individual , the collected blood samples in EDTA anticoagulant tubes were transmitted within 2-24 hours using cooling container for DNA extraction and PCR amplification(5).

Isolation of genomic DNA:

The genomic DNA isolated from the whole fresh blood collected in EDTA anticoagulant tubes for molecular studies was applied using Wizard genomic DNA purification kits using genomics isolation principles (5).

The PCR components and experimental design:

To evaluate the effect of PCR enhancers, a total volume of 25 µl of the PCR components were arranged in four setup patterns as in the following design shown in table (1):

Table (1): The components and PCR setup patterns of amplification of human beta-globin gene (5,6)

Components	Concentration	Amount (µl)	Setup 1 (standard)	Setup (2)	Setup (3)	Setup (4)
PCR buffer	10 X	2.5	+	+	+	+
$MgCl_2$	50 mM	2	+	+	+	+
$dNTP_s$	10 mM each	0.8	+	+	+	+
Primer 1 (F)	10 picomols/ µl	1	+	+	+	+
Primer1 (R)	10 picomols/ µl	1	+	+	+	+
Primer 2 (F)	10 picomols/ µl	1	+	+	+	+
Primer 2 (R)	10 picomols/ µl	1	+	+	+	+
DNA template	0.1µg/ µl	2	+	+	+	+
Taq DNA polymerase	5 unit/ µl	0.2	+	+	+	+
DMSO	5 %	3	-	+	-	+
Betaine	1 M	3	-	-	+	+
Deionized H_2O	----	Up to 25	+	+	+	+

Primers Selection and PCR Program:

Primer sets and PCR program which selected for PCR amplification of human beta-globin gene, were previously described(8) . Two primer sets were chooses for the amplification of two different region in the human beta-globin gene . The forward primer of first set was 5 ¯ CAA TGT ATC ATG CCT CTT TGC ACC and the reverse primer `was 5 ¯ GAG TCA AGG CTG AGA GAT GCA GGA , and the fragment size was 861 base pair. The second set were for forward primer was 5 ¯ ACC AGC AGC CTA AGG GTG GGA AAA TAC ACC and the reverse primer was 5 ¯ ACC TCA CCC TGT GGA GCC AC , and the fragment size was 419 base pair. The PCR program was optimized for 25 cycles as in the table (2):

Table (2): the PCR setup program for amplification of human beta-globin gene.

Step	Temperature (^{o}C)	Time (minutes)	No. of Cycles
Initial denaturation	94	2	1
First loop:			
Denaturation	94	1	25
Annealing	65	1	
Extension	72	1.5	
Final extension	72	3	1

PCR analysis:

The PCR products and the ladder marker were resolved by electrophoresis. 3 µl of loading blue dye (15% ficoll, 0.05% bromophenol blue) plus 10 µl of the product were mixed and loaded on 2 % agarose gel (2g agarose/100 ml 0.5X TBE buffer) and run at 100 volt for approximately one hour. The gel was stained with ethidium bromide solution (0.5 µg/ml) for 15-30 minutes; and bands were visualized by UV transiluminator (8).

Results:

The isolation of genomic DNA from the whole fresh blood was studied using Wizard genomic DNA purification kits of salting out method. Figure (1) showed the chromosomal DNA bands (lanes 1- 11) of eleven samples on 0.5 % agarose gel.

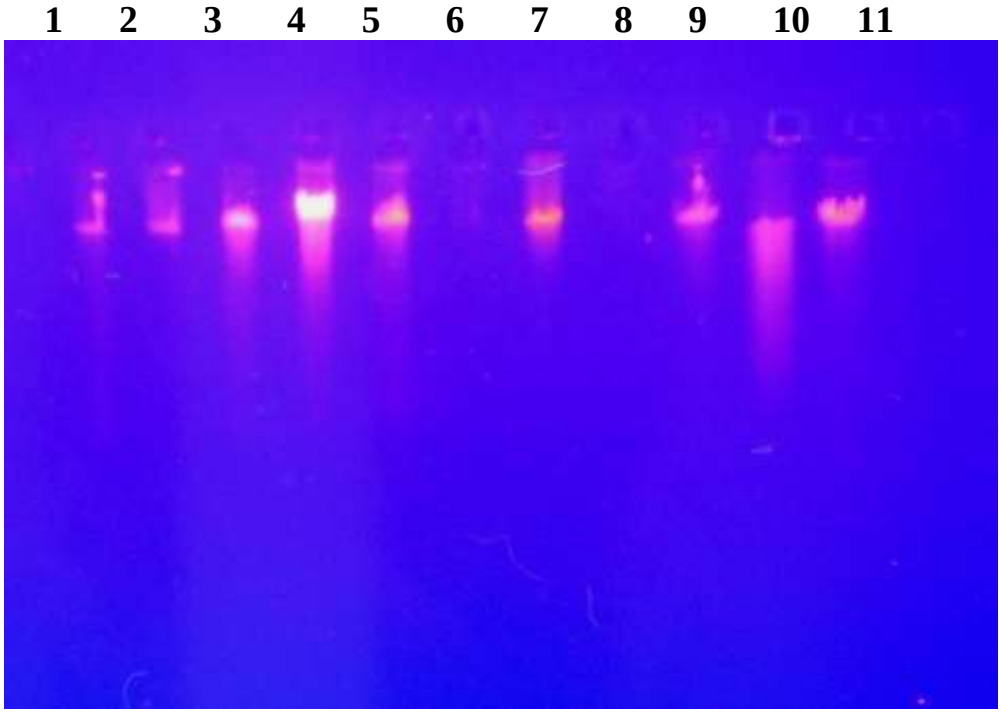

Figure (1): Chromosomal DNA bands on 0.5 % agarose gel at 100 voltages .

To study the effects of DMSO and betaine as PCR enhancers , the results of molecular amplification of target region of beta-globin gene revealed that there was a good amplification levels according to the presence and resolution of the bands for the first set of primers which amplify a 861 bp region in the human beta – globin gene in comparison to amplification levels of region covered by the second set of primers of 419 bp PCR products as shown in figure (2)

And to evaluate the effect of these PCR additives, the PCR products revealed that the results of amplification were produced different patterns of amplification depending upon the intensity of bands according to the type of additives as shown in figure (1) , these figure revealed that the lane 1 and 2 contain the PCR amplicon of standard PCR setup (without PCR additives), lane 3 and 4 showed the PCR amplicon of PCR setup in presence of DMSO , lane 5 and 6 showed the PCR amplicon of PCR setup in presence of betaine, and finally the lane 7 and 8 showed the PCR amplicon of PCR setup in presence of both DMSO and betaine.

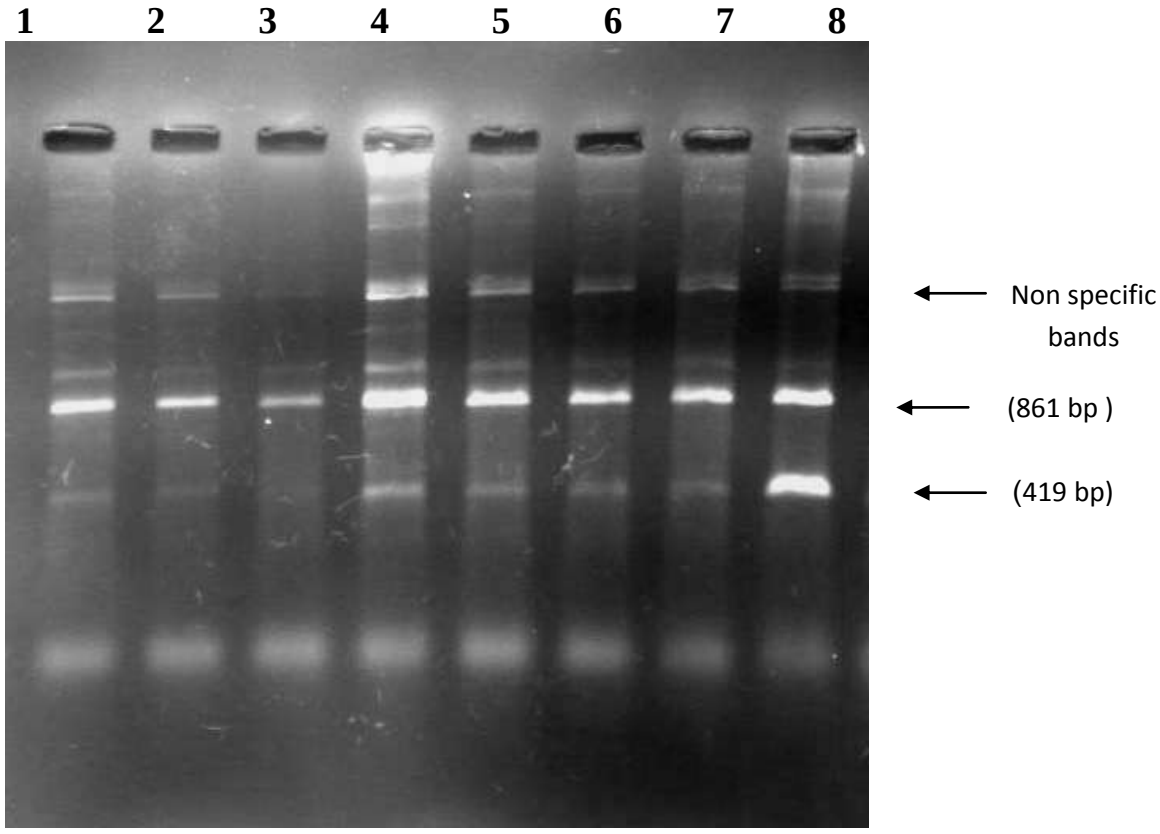

Figure 2: The duplex PCR amplification bands of human beta-globin gene on 2% agarose gel , lane 1 and 2 a standard PCR components set up (without PCR additives), lane 3 and 4 standard PCR set up plus 5% DMSO, lane 5 and 6 standard PCR set up plus 1M betaine, and lane 7 and 8 standard PCR set up plus 5% DMSO and 1M betaine.

Discussion:

Blood samples were subjected to DNA isolation procedure within 2-24 hours of aspiration. The band integrity and DNA concentration are found to be different according to the yielded amount of genomic DNA and its purity (figure 1) which depends on amount of WBCs in the blood samples. In addition, the use of fresh blood samples are found to be better, therefore, the DNA isolation should be applied as early as possible.

A variety of PCR additives and enhancing agents have been used to increase the yield, specificity and consistency of PCR reactions. Whilst these additives may have beneficial effects on some amplification it is impossible to predict which agents will be useful in a particular context and therefore they must be empirically tested for each combination of template and primers (7,9).

In this study, generally, the results of the usage of PCR additives revealed that the yield and consistency of PCR products of the first set of primer that involved in amplification of 681 bp were greater than the PCR amplicons which involved in the second set of primers that produce a 419 bp products, this may be reflect the positively effects of optimization conditions specially the annealing temperature for the first set of primers in comparison to the second set of primers, and the sequence of DNA region that complementary for the first set of primer which contain a high percentage of GC content (figure 2).

The results also revealed that the use of (5%) of DMSO as shown in the lane 3 and 4 produce a slight beneficial effects on the resulting PCR amplicon bands in comparison to the

standard amplicon bands which shown in lane 1 and 2 (figure 2) . Other studies (10,11,12) indicate that the DMSO at 2-10% may be necessary for amplification of some templates, however 10% DMSO can reduce *Taq* DNA polymerase activity by up to 50% , so it should not be used routinely. DMSO is thought to reduce secondary structure and is particularly useful for GC rich templates only.

Figure 2 also shown in lane 5 and 6 in the results of PCR amplification with 1M betaine additives that the PCR amplicon of both set of primers were slightly improved in comparison with the standard PCR amplification conditions in lane 1 and 2 .

A further example of the effectiveness of betaine for the PCR of GC-rich sequences, is the improvement of the amplification of c-jun prevented by a basic region containing 72% GC (1). For the amplification of the coding cDNA inserted in pBluescript ~2.5 M betaine is optimal. Thus, the optimal betaine concentration seems to be dependent on the composition of the amplified DNA sequence (13).

The results also revealed that the usage of both 5% DMSO and 1M betaine were significantly improved the PCR amplification as shown in lane 7 and 8 in comparison with the standard optimization conditions (lane 1 and 2) , with the additives of 5% DMSO (lane 3 and 4) and with the additives of 1M betaine (lane 5and 6)

Other studied indicate that the combination of betaine and DMSO: Enhancing agents for different PCR conditions and there are instances in which standard PCR amplification conditions do not produce acceptable results. In those cases there are a number of additives that can be used to increase yield and specificity of a reaction. With the information presently available it is not possible to predict which enhancing agent is best for any particular target, so it may be necessary to test several different additives (6,7).

Betaine and DMSO are two frequently used PCR additives that are effective separately or in combination (7). Both of these reagents are readily available from chemical supply companies. It should be noted that, although these agents can be useful for increasing efficiency and specificity of PCR amplifications, their effects on the melting temperature of DNA may alter the optimal annealing conditions for a particular reaction. Therefore, it may be necessary to empirically determine the optimal annealing temperature if adding one or both of these agents to a particular amplification reaction (7,13).

Conclusions:

1- The standard optimization condition of duplex PCR amplification is enough to reveal a good PCR amplification.
2- The PCR additives are useful in improvement of amplification in combination of 5 % DMSO and 1 M betaine.
3- The sequence of target DNA region (GC content) and the specificity of primers are affective factors in production of high yield PCR products and decrease the non-specific bands.

Recommendations:

1- Usage of different concentrations of DMSO and betaine to evaluate the most proper conditions of these additives in PCR amplification.
2- To improve the specificity of primers, we recommend the use of primer designing procedure to choose the more specific set of primers especially in duplex and multiplex PCR amplification and the proper optimization conditions.

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