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Author: Vladimir Jurišić Jasmina Obradović Natasa Tošić Sonja Pavlović Milan Kulić Nataša Djordjević

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Effects of DMSO, glycerol, betaine and their combinations in detecting single nucleotide polymorphisms of epidermal growth factor receptor (EGFR) gene promoter sequence in non-small-cell lung cancer (NSCL) patients

Vladimir Jurišić[1], Jasmina Obradović [2], Natasa Tošić[3], Sonja Pavlović[3], Milan Kulić [4], Nataša Djordjević[1]

[1]Faculty of Medical Sciences, University of Kragujevac, Svetozara Markovica 69, Kragujevac, Serbia

[2]Institute of Biology and Ecology, Faculty of Science, University of Kragujevac, Radoja Domanovica 12, Kragujevac, Serbia

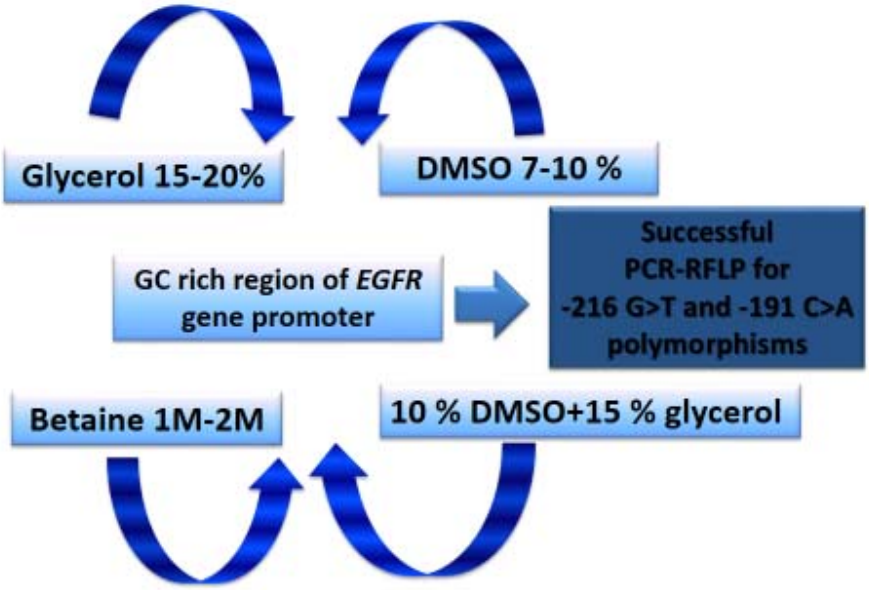
[3]Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Vojvode Stepe 444a, Belgrade, Serbia

[4] University of East Sarajevo, Faculty of Medicine, Foca, Republic of Srpska, Bosnia and Hercegovina

Correspondence

Prof. Vladimir Jurisic
University of Kragujevac
Faculty of Medical Sciences
P.O. Box 124
34 000 Kragujevac,
Serbia
e-mail: vdvd@lycos.com

Graphical abstract



Highlights

- Diverse additives for specificity of PCR reaction for EGFR promoter region were tested.
- DMSO, Glycerol and betaine as single detergents significantly enhanced yield and specificity of PCR reaction for EGFR promotor.
- DMSO in combination with glycerol also have positive effects on amplification EGFR promotor region, but in the high concentration block the amplification.

Abstract

The aim of the study was to examine the effects of frequently used polymerase chain reaction (PCR) additives DMSO, glycerol and betaine on amplification of GC-rich epidermal growth factor receptor (EGFR) gene promoter region, in order to detect the presence of -216G>T and -

191C>A gene variations in non-small-cell lung cancer (NSCLC) patients. PCR products and restriction fragments were detected by electrophoresis on 8% polyacrylamide gel and 3% agarose gel. Our analysis shows that single used additives including DMSO in concentration of 7% and 10%, glycerol in concentration of 10%, 15% and 20%, as well as betaine in concentration of 1 M, 1.5 M and 2 M significantly enhanced the yield and specificity of PCR reaction. In addition, the combination of 10% DMSO with 15% glycerol has shown positive effects, whereas other analyzed combinations of additives failed to amplify the *EGFR* promoter region.

Key worlds: EGFR, PCR, optimization, additives, DMSO, glycerol, betaine

1. Introduction

Non-small-cell lung cancer (NSCLC), as the most frequent lung cancer type and the leading cause of cancer death worldwide [1, 2], has been thoroughly researched over the years from different perspectives, but with a common aim: to improve efficiency and safety of disease treatment. Studies revealed epidermal growth factor receptor (*EGFR*) to be an important regulator of tumor growth and metastasis in NSCLC patients [3]. *EGFR* was thus recruited as a promising drug target, and two specific *EGFR* tyrosine kinase inhibitors (gefitinib and erlotinib) have been approved and widely prescribed for this indication [4, 5]. However, due to specific mutations or polymorphisms in *EGFR* gene, not all NSCLC patients respond equally to this therapy [6, 7]. Therefore, personalization of drug therapy in NSCLC patients requires genotyping of the *EGFR* gene, and the genotyping method needs to be reliable and easily applicable in everyday practice.

Polymerase chain reaction (PCR) is a simple genotyping technique that is used worldwide in both research and practice. Yet, it depends on a number of various parameters, which makes the optimization of the method sensitive, laborious, time and cost consuming, especially if the products should undergo further analysis, such is endonuclease cleavage in PCR-restriction fragment length polymorphism (PCR–RFLP) method [8]. Indeed, there is no single set of conditions that is optimal for all PCRs [9], especially not for DNA templates difficult to amplificate, such as GC-rich regions of *EGFR* [10]. Usual strategy in such case is adjustment of cycling conditions or components concentration, as well as utilization of additives that could enable amplification of desired target sequence [9]. The list of most important additives, reported

to enhance yield and specificity of PCR, includes glycerol, betaine, formamide, dimethyl sulfoxide (DMSO), non-ionic detergents Tween 20 and Triton X-100, bovine serum albumin (BSA), and polyethylene glycol (PEG) [11,12, 13, 14, 15, 16, 17, 18, 19, 20, 21].

Previously, we performed and reported optimization of conditions for amplification of the GC-rich *EGFR* promoter sequence in NSCLC patients, including the influence of MgCl2 concentration, temperature and DMSO as single additive [22]. Here, we further focused on the research of the effects of three frequently used additives, i.e. DMSO, glycerol and betaine, in an attempt to amplify the same difficult template for genotyping of -216G>T (rs712829) and -191C>A (rs712830) single nucleotide polymorphisms (SNPs) of *EGFR* gene in NSCLC patients, with the aim of achieving optimal results. In addition, we tested combinations of our additives with the aim of witnessing their synergistic effects on amplification.

2. Materials and methods

2.1 Sample preparation

DNA used for PCR optimisation was extracted from formalin-fixed paraffin-embedded (FFPE) lung tumor tissue obtained from non–small-cell lung cancer patients using the PureLink™ Genomic DNA Kits (Invitrogen/ Life Technologies, Carlsbad, CA).

2.2. PCR technique

EGFR polymorphisms -216G>T and -191C>A were genotyped using the PCR-RFLP method, previously reported [22]. PCR reaction was carried out in the total volume of 25 μl, with 1 μl genomic DNA, 0.4 μl of each primer, 0.2 mM dNTPs and 1U of KAPA Taq DNA polymerase (Kapabiosystems, Boston, Massachusetts, USA) in 1x PCR Buffer A (with 1.5 mM $MgCl_2$).

Each of the separately tested additives was included in the reaction at different concentrations, i.e. DMSO (51%, 7% and 10%), glycerol (5%, 10%, 15%, 20% and 25%), betaine (0.5 M, 1.0 M, 1.5 M, 2.0 M and 2.5 M). Synergistic effects were determined by using combinations of additives at their determined optimal concentrations, including: DMSO with glycerol or betaine; combination of betaine and glycerol, as well as all three additives together at two different concentrations. For confirmation of the observed effects of additives, each PCR was repeated at least three times. Detection of 197bp PCR products was performed by electrophoresis on 3% agarose gel stained with ethidium bromide. To detect -216G>T and -191C>A polymorphisms, restriction with enzymes BseRI and Cfr42I enzymes, respectively, was used. Restriction fragments corresponding to -216G/T (-216G: 159bp and 32bp; -216T: 86bp, 73bp and 32bp) and -191C/A (-191C: 165bp and 32bp; -191A: 197bp) polymorphisms were detected by electrophoresis on 8% polyacrylamide gel and 3% agarose gel, respectively.

2.3 Reagents

Primers and restriction enzymes used in our research for PCR were identical to the ones previously described by the present authors [22]. All additives were purchased from Sigma-Aldrich Inc (St. Louis, MO, USA).

2.4 Sequencing

Our results were confirmed by direct sequencing of PCR products on ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, USA), using ABI PRISM® BigDyeTM Terminator v 3.1 Cycle Sequencing Kit and the primers used for PCR amplification.

3. Results

DMSO at concentrations of 7% and 10 % gave positive reaction, with lower concentrations (range from 1.5% and 7%) resulting in unspecific yield (Fig. 1a). Glycerol gave desired PCR products at any concentration between 5% and 25% (Fig. 1b), but the highest concentration resulted in lower yield, and at lower unspecific smaller fragments were detected, resolving gradually with increasing concentration. Positive effects of betaine on PCR product amplification were shown at concentrations ranging from 1 M to 2 M (Fig. 1c), without interfering with BseRI (Fig. 2c) and Cfr42I (Fig. 2d) enzyme restriction of PCR products.

The comparison of DMSO, glycerol and betaine effects as single mediators showed influence of 10% DMSO to be stronger than that of glycerol and betaine at their optimal concentrations (Fig. 1d). On the other hand, combining of additives showed that 10% DMSO with 15% glycerol had positive effects on amplification of 197 bp long DNA sequence (Figure 1d), while other mixtures failed to show any positive effect.

The restriction of the amplicons with BseRI revealed that 25% glycerol, although optimal for PCR reaction, interfered with the BseRI enzymatic reaction (Fig. 2a). At the same time, restriction with Cfr42I was not affected by glycerol addition (Fig. 2b).

Finally, specificity of PCR reaction for our EGFR promoter region was confirmed by direct sequencing (Figure. 3).

4. Discussion

Within the *EGFR* gene, both somatic mutations and SNPs have been discovered, some of them already accepted as predictive pharmacogenomics biomarkers in the treatment of cancer [23]. Others, for which biological function has been proposed, include -216G>T and -191C>A, both located in the transcriptional start site region of the *EGFR* gene promoter. Due to their specific location, it was suggested that they could affect *EGFR* regulation and modify response to anti-EGFR therapy [24, 25]. However, genotyping for these two SNPs proved to be difficult to perform [22]. In order to enable both research and prospective routine pretreatment -216G>T and -191C>A genotyping, adjustment of PCR method is essential. In the present study, following our previous publication on optimizing PCR conditions [26], we tested the effect of several additives and their combinations on genotyping of -216G>T and -191C>A *EGFR* polymorphisms. The results showed that DMSO, glycerol and betaine could improve amplification of *EGFR* promoter region.

There are number of additives recommended in the literature that can facilitate PCR amplification [12, 13, 14, 15, 16, 17, 18, 19]. They are often included as components in commercially available PCR kits, however, the result is not guaranteed. In fact, it is extremely difficult to predict which enhancing additive and at which concentration will enable amplification of the desired target sequence [20, 21], so PCR often needs specific empirical adjustment.

EGFR promoter region is extremely GC-rich, thus prone to formation of inter- and intra-strand secondary structures [24, 25, 27, 28, 29, 30, 31, 32] which renders PCR amplification difficult

and additional optimization necessary. Several additives that have been recognized as able to improve yield and specificity of PCR have an ability to unwind the double-stranded DNA helix [31, 33, 34]. It has been observed that DMSO can increase both specificity and productivity of PCR, most probably by decreasing inter or intra strand re-annealing and formation of the problematic secondary structure [13]. Previously, we reported that 5% DMSO can provide the desired amplicon yield without non-specific amplification [22]. Here, we tested the same additive at higher concentrations, achieving good results with 7% and 10 % DMSO as well.

In addition to assisting denaturation of the template, glycerol has been suggested to increase the thermal stability of the polymerase and the specificity of primer annealing [8, 34]. Effects of glycerol on PCR in the presence of GC-rich templates have been reported in literature at concentrations ranging from 0.5% to 27%, with the best results observed at 10-20% [11, 15, 33].

In the present study, glycerol was added as a single additive at the concentrations ranging from 5% to 25%, with the 5% increment. Although all reactions resulted in desired amplicon production, lower glycerol concentrations led to additional unspecific fragments, most probably primer-dimers. Therefore, reactions containing 5% and 10% glycerol were considered unsatisfactory and excluded from further analysis. On the other hand, the highest glycerol concentration of 25% resulted in somewhat lower yield of the desired PCR product, and this phenomenon corresponds to already described inhibitory effect of high glycerol concentrations on Taq DNA polymerase [15]. PCR product obtained at 15-25% concentration glycerol were subsequently submitted to restriction, resulting in expected fragment size for lower concentrations, but inadequate restriction pattern for the highest (25%) glycerol concentration. It

could be argued that high glycerol concentrations, besides polymerases, can probably inhibit other enzymes as well. Therefore, for optimization of PCR, it would be advisable to use the lowest effective glycerol concentration, especially if post-PCR processing involves additional enzyme activity.

Betaine as non-ionic chemical has been used as a PCR facilitator for many years [15, 18]. It assists PCR via strand separation, lowering melting temperature and acting as an isostabilizing agent, equalizing the contribution of GC- and AT-base pairings to the stability of the DNA duplex [15, 18]. In addition, it has been observed that betaine successfully facilitates PCR with templates of high GC content regardless of the DNA polymerase type, eliminating the pauses in elongation process due to secondary structure formation of PCR template [15, 16, 17, 18]. In our study, effects of betaine at 0.5 M to 2.5 M concentration was investigated, and the detectable PCR amplification occurred after addition of either 1 M, 1.5 M or 2 M betaine. However, increasing betaine concentrations over 2 M inhibited PCR amplification, probably by inhibiting Taq polymerase [18].

DMSO in combination with other two investigated additives was also explored, and the positive effect on PCR amplification was shown only with glycerol. Glycerol is known to lower melting temperature of DNA and to reduce the inhibitory effect of DMSO on the polymerase, which could be the reason behind the success of this combination [13, 35]. The similar combination of additives used in our study was recommended by other authors for amplification of 5′ non-coding region of the Gas subunit of heterotrimeric G protein (GNAS1), located in GC-rich regions [36], which corresponds well with our observation related to *EGRF* gene. On the other

hand, the lack of effects of other additive combinations on PCR amplification could be explained most likely by synergistic inhibition of Taq polymerase or different chemical interactions or conflict [37].

5. Conclusions

Our study provides evidence that PCR-RFLP method for genotyping of GC-rich *EGFR* gene promoter region in NSCLC patients for detection of -216G>T and -191C>A single nucleotide polymorphisms could benefit from addition of DMSO, glycerol and betaine.

Acknowledgements

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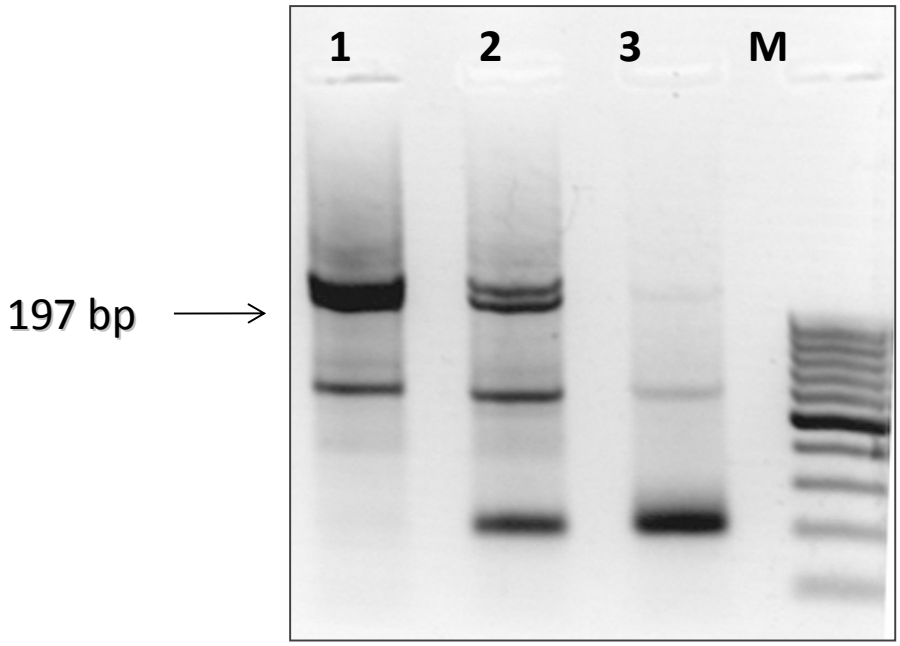

Fig. 1a. Effects of different concentrations of DMSO on amplification of 197bp long DNA sequence
Lane 1: 5% DMSO; lane 2: 7% DMSO; lane 3: 10% DMSO; lane M: 100 bp DNA ladder

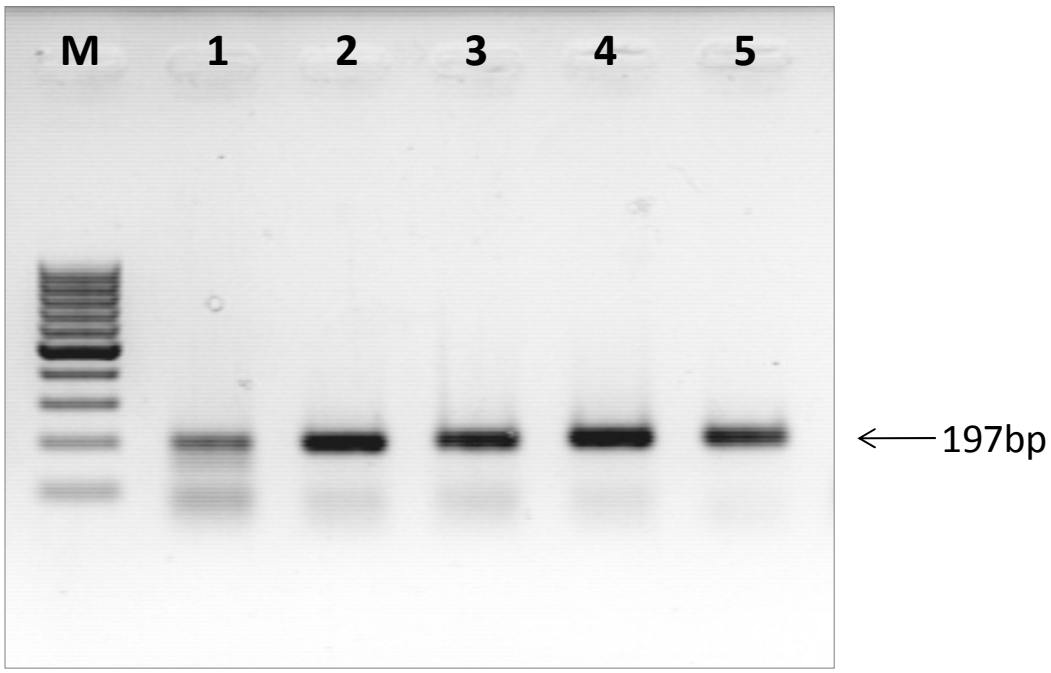

Fig. 1b. Effects of different concentrations of glycerol on amplification of 197bp long DNA sequence

Lane M: 100bp DNA ladder; lane 1: 5% glycerol; lane 2: 10% glycerol; lane 3: 15% glycerol; lane 4: 20% glycerol; lane 5: 25% glycerol

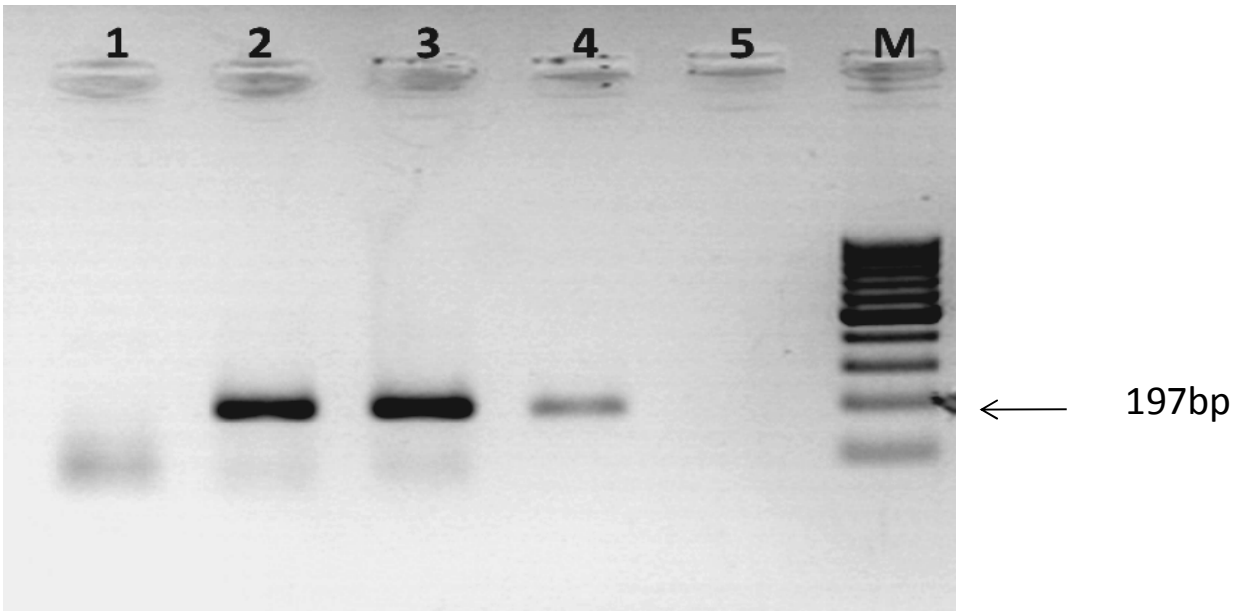

Fig. 1c. Effects of different concentrations of Betaine on amplification of 197bp long DNA sequence

Lane 1: 0,5 M betaine; lane 2: 1 M betaine; lane 3: 1,5 betaine; lane 4: 2 M betaine; lane 5: 2,5 M betaine; lane M: 100 bp DNA ladder

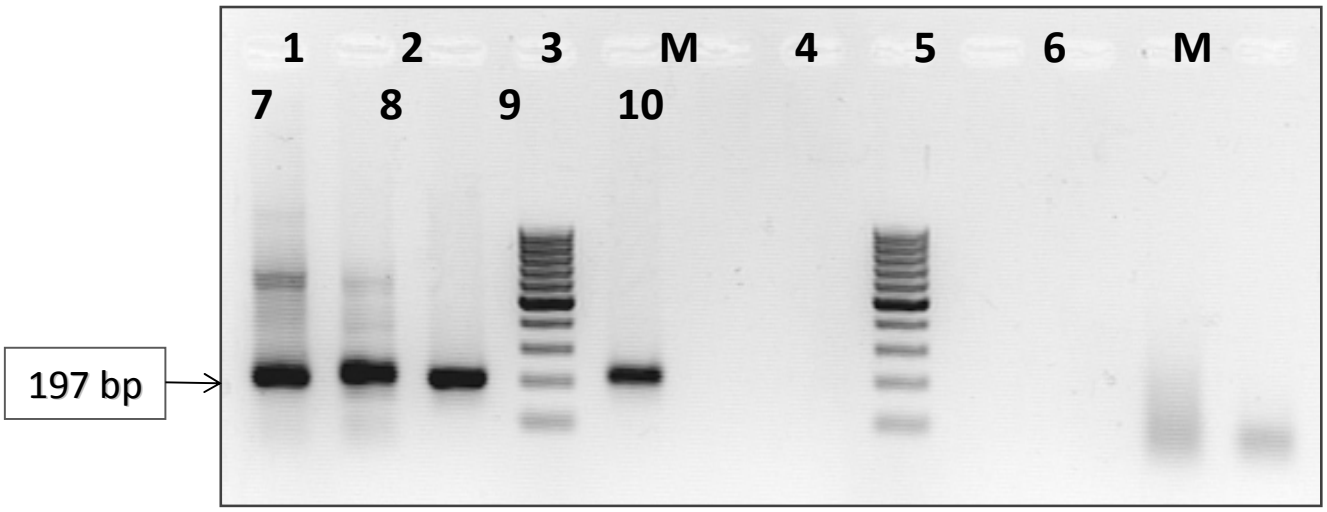

Fig.1d. Comparison effects of DMSO, glycerol, betaine and their combinations on amplification of 197bp long DNA sequence.
Lane 1: 10% DMSO; lane 2: 15% glycerol; lane 3: 1.5 M betaine; lane M: 100 bp DNA ladder; lane 4: 10% DMSO and 15% glycerol; lane 5: 10% DMSO and 1.5 M betaine; lane 6: 15% glycerol and 1.5 M betaine; lane M: 100 bp DNA ladder; lane 7: 10% DMSO, 1.5 M betaine and 15% glycerol; lane 8: 10% DMSO, 2 M betaine and 20% glycerol; lane 9: non template control; lane 10: non template control.

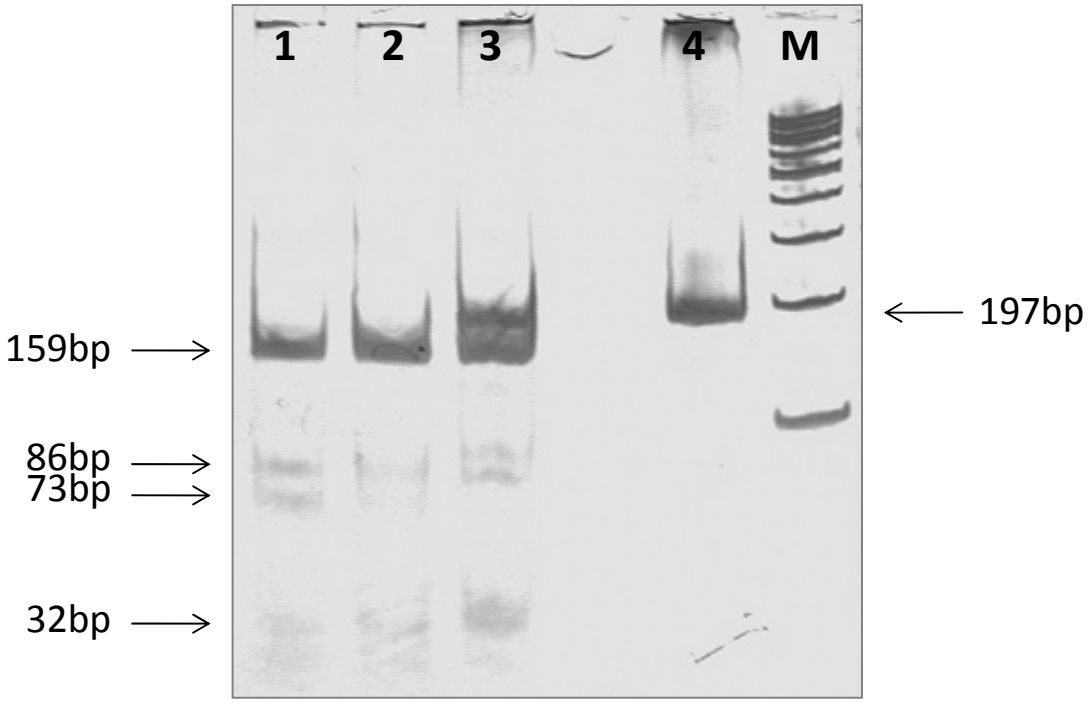

Fig. 2a. Effects of different concentrations of glycerol on BseRI enzyme restriction of PCR products

Lane 1: 15% glycerol; lane 2: 20% glycerol; lane 3: 25% glycerol; lane 4: not digested PCR product; lane M: 100bp DNA ladder

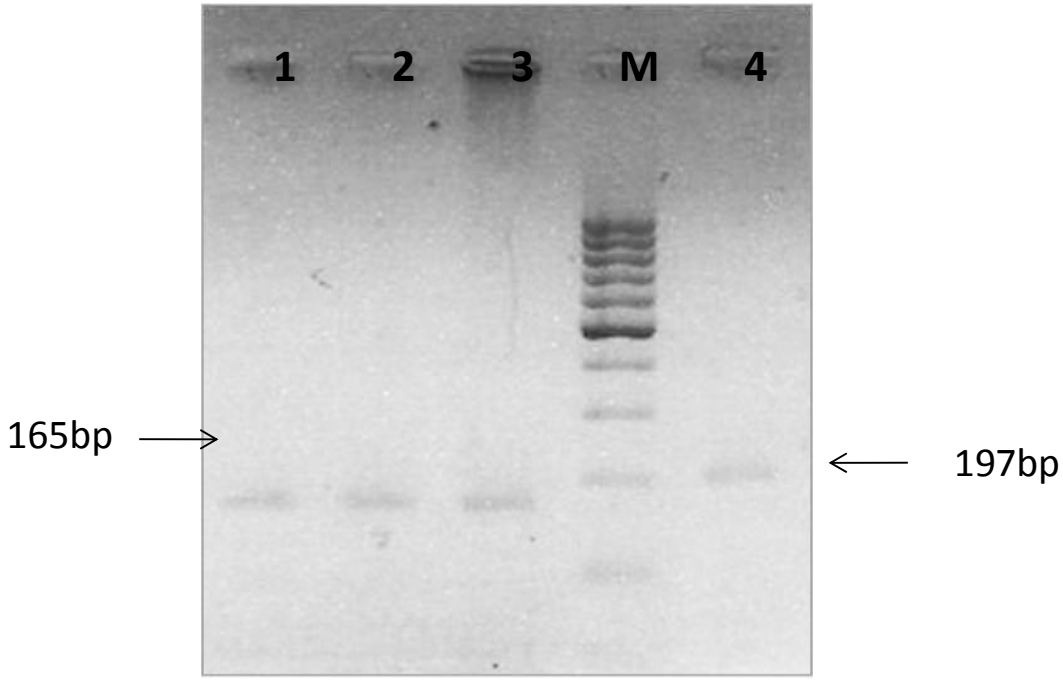

Fig. 2b. Effects of different concentrations of glycerol on Cfr42I enzyme restriction of PCR products

Lane 1: 15% glycerol; lane 2: 20% glycerol; lane 3: 25% glycerol; lane M: 100bp DNA ladder; lane 4: not digested PCR product

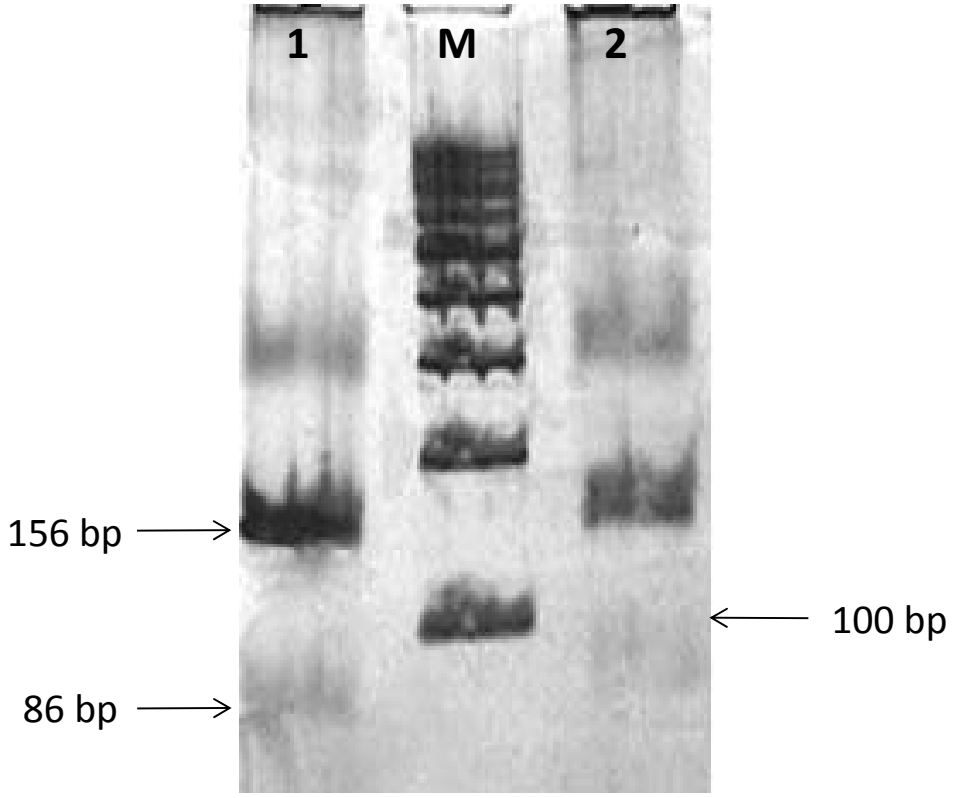

Fig 2c. Effects of different concentrations of betaine on BseRI enzyme restriction of PCR products

Lane 1 - PCR product with 1,5M betaine, DNA isolated from formalin fixed and paraffin embedded lung tumor tissue

Lane M - Ladder

Lane 4 - PCR product with 2M betaine, DNA isolated from formalin fixed and paraffin embedded lung tumor tissue

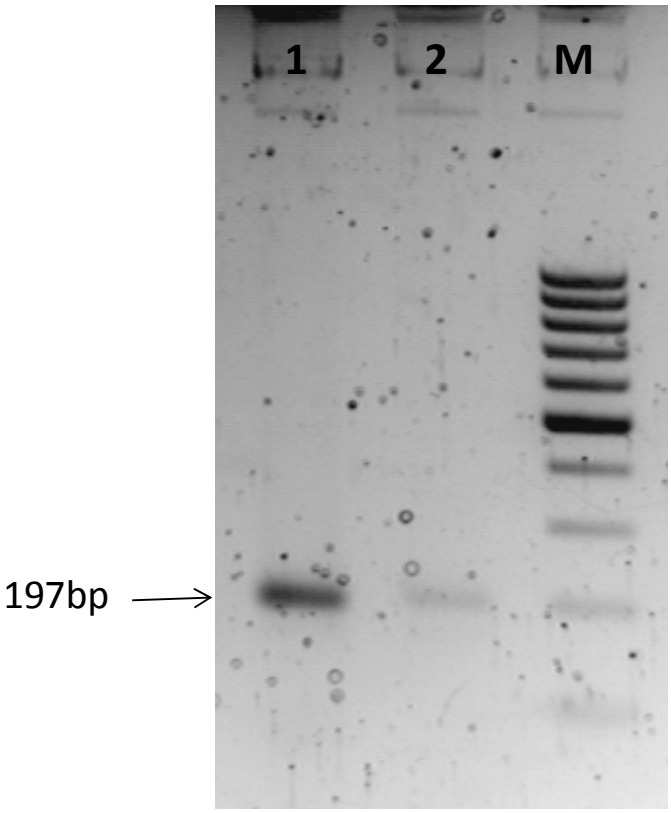

Fig 2d. Effects of different concentrations of betaine on Cfr42I enzyme restriction of PCR products

Lane 1: 1,5% betaine -FFPE DNA sample; lane 2: 2% betaine- FFPE DNA sample;

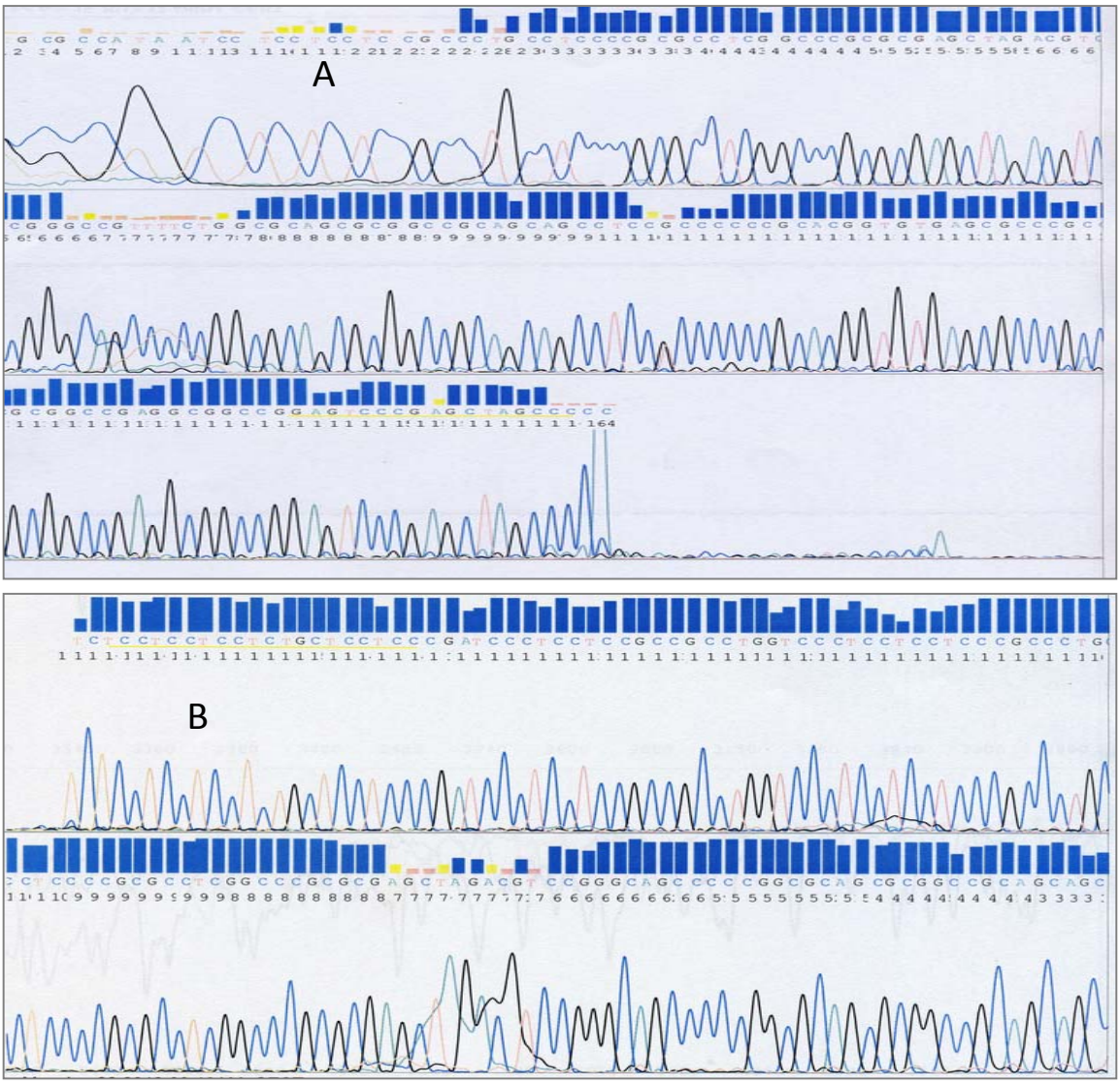

Figure 3. Results of direct sequencing of PCR products using forward (A) and reverse (B) PCR primers