Journal of Clinical Laboratory Analysis 27: 487–493 (2013)

Optimization of PCR Conditions for Amplification of GC-Rich *EGFR* Promoter Sequence

Jasmina Obradovic,[1,2] Vladimir Jurisic,[1] Natasa Tosic,[3] Jasminka Mrdjanovic,[4] Branislav Perin,[5] Sonja Pavlovic,[3] and Natasa Djordjevic[1]*

[1]*Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia*
[2]*Faculty of Science, Institute of Biology and Ecology, University of Kragujevac, Kragujevac, Serbia*
[3]*Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia*
[4]*Oncology Institute of Vojvodina, Faculty of Medicine, University of Novi Sad, Sremska Kamenica, Serbia*
[5]*Institute for Pulmonary Diseases of Vojvodina, Clinic for Pulmonary Oncology, Faculty of Medicine, University of Novi Sad, Sremska Kamenica, Serbia*

Background: Polymerase chain reaction (PCR) is an extremely sensitive method that often demands optimization, especially when difficult templates need to be amplified. The aim of the present study was to optimize the PCR conditions for amplification of the epidermal growth factor receptor (*EGFR*) promoter sequence featuring an extremely high guanine-cytosine (GC) content in order to detect single nucleotide polymorphisms -216G>T and -191C>A. *Methods:* Genomic DNA used for amplification was extracted from formalin-fixed paraffin-embedded lung tumor tissue and PCR products were detected by agarose gel electrophoresis. *Results:* Results showed that addition of 5% dimethyl sulfoxide (DMSO), as well as DNA concentration in PCR reaction of at least 2 μg/ml, were necessary for successful amplification. Due to high GC content, optimal annealing temperature was 7°C higher than calculated, while adequate $MgCl_2$ concentration ranged from 1.5 to 2.0 mM. *Conclusion:* In conclusion, *EGFR* promoter region is a difficult PCR target, but it could be amplified after optimization of $MgCl_2$ concentration and annealing temperature in the presence of DMSO and the DNA template of acceptable concentration. J. Clin. Lab. Anal. 27:487–493, 2013.

Key words: EGFR; GC-rich; genotyping; optimization; PCR

INTRODUCTION

Polymerase chain reaction (PCR) is an enzymatic in vitro method for exponential amplification of specific DNA target sequence, affordable and suitable for both basic research and various clinical applications (1). However, the method is extremely sensitive, thus, it could be a considerable challenge to optimize the conditions of the reaction in order to obtain the desired results, especially when difficult templates, such as GC-rich regions, need to be amplified. Namely, GC-rich regions, due to formation of stable and complex secondary structures within a DNA template, could block DNA polymerase during PCR reaction and lead to an ineffective amplification (2–6). PCR technique parameters that could affect its accuracy and efficacy are numerous, including concentration of DNA template, concentration of magnesium ions, PCR thermal cycling conditions, as well as addition and concentration of PCR additives (7, 8). If there is a scientific or clinical need for specific and efficient amplification of GC-rich DNA template, tuning the PCR reaction could be highly demanding, yet, critically important.

Epidermal growth factor receptor (EGFR) expressed in several epithelial cancers, including lung, breast,

This work was supported by the grant No. 175056 of the Ministry of Science and Technology of the Republic of Serbia.

*Correspondence to: Natasa Djordjevic, Department of Pharmacology and Toxicology, Faculty of Medical Sciences, University of Kragujevac, Svetozara Markovica 69,34000 Kragujevac, Serbia. E-mail: natashadj2002@yahoo.com

Received 13 March 2013; Accepted 7 May 2013
DOI 10.1002/jcla.21632
Published online in Wiley Online Library (wileyonlinelibrary.com).

bladder, prostate, and colorectal, plays an important role not only in carcinogenesis, but also in the cancer treatment involving tyrosine kinase inhibitors (TKIs) (9–11). A number of mutations within the *EGFR* coding gene has been identified, including well-known nonsynonimous deletion/insertion of exon 19 and point mutations L858R (c.2573T>G, rs121434568) and T790M (c.2369C>T, rs121434569) in exon 21 (10–13). Due to their established clinical significance, EGFR is recognized as a biomarker for the development and implementation of targeted cancer therapies with EGFR-TKI, such as erlotinib or gefitinib (14, 15).

Previous studies reported several single nucleotide polymorphisms (SNPs) in the transcriptional start site region of the *EGFR* gene promoter, including -216G>T at the Sp1 transcription factor recognition site, and -191C>A, located 4 bp upstream of one of the transcriptional start sites (16, 17). Due to their location in a region essential for transcription, these polymorphisms were investigated both in vitro and in vivo for their suggested role in modification of promoter activity and response to EGFR-TKI therapy. In 2005, Liu et al. (16), employing transient transfection in human cancer and primary cell lines, observed a significantly higher promoter activity and *EGFR* expression in -216T compared to -216G allele. In two prospective clinical studies of cancer patients treated with erlotinib (17) or gefitinib (12), -216G>T and -191C>A were associated with higher frequency of adverse drug reactions, such as rash or diarrhea. Nevertheless, carriers of -216T allele had an improved progression-free survival on gefitinib (12). Similar results were reported by Jung et al. (18), as a higher response rate to gefitinib or erlotinib treatment and longer progression-free survival corresponded to -216G/T compared to G/G genotype. Based on these data, it would not be surprising if the observed potential to predict efficacy and safety of the cancer treatment nominates these two polymorphisms for possible pharmacogenetic biomarkers for EGFR-TKI activity. However, *EGFR* promoter region has an extremely high GC content of up to 88% (19), which makes it difficult target for PCR amplification, especially in the clinical setting. The aim of the present study was to optimize the PCR conditions for amplification of the *EGFR* promoter sequence comprising two SNPs of interest, namely, -216G>T and -191C>A.

MATERIALS AND METHODS

Samples and DNA Isolation

DNA from formalin-fixed paraffin-embedded (FFPE) lung tumor tissue was extracted using the PureLink™ Genomic DNA Kits (Invitrogen/Life Technologies, Carlsbad, CA), according to the manufacturer's recommendations. DNA concentration was measured using Qubit® Fluorometer (Invitrogen/Life Technologies).

Bioinformatic Sequence Analysis

Melting temperature of the primers was calculated as $Tm = 4 \times (G + C) + 2 \times (A + T)$ (7), and the annealing temperature was determined as $Ta = 0.3 \times (Tm \text{ of primer}) + 0.7 \times (Tm \text{ of product}) - 25$ (20). GC content and CpG nucleotide composition of the template DNA were determined and presented using the bioinformatic tool "EMBOSS CpGPlot /CpGReport/Isochore" program (http://www.ebi.ac.uk/Tools/emboss/cpgplot/), with a sliding window of 100 nucleotides, shifted one nucleotide at a time.

Genotyping Method

Genotyping for -216G>T/-191C>A EGFR polymorphisms was carried out using the polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) method according to Liu et al. (12), but with modifications due to necessity of protocol optimization. In brief, using the primers described in the article, the part of the EGFR promoter region spanning both SNPs was amplified in the PCR reaction on Techne Genius Thermocycler (Techne Ltd, Cambridge, UK).

PCR reactions were run in a final volume of 25 μl. The reaction mix consisted of 1 μl genomic DNA, 0.2 μM of each primer, 0.25 mM of each of the dNTPs, and 0.625 U of TaqDNA polymerase, and it was carried out in 1× PCR buffer. Concentrations of $MgCl_2$ and dimethyl sulfoxide (DMSO) ranged from 0.5 to 2.5 mM, and from 1% to 5%, respectively. The initial denaturation was performed at 94°C for 3 min; followed by 45 cycles of denaturation at 94°C for 30 sec, gradient annealing at 61°C/63°C/65°C/67°C/69°C for 20 sec, and extension at 72°C for 60 sec; and with a final extension at 72°C for 7 min. All reagents used for PCR amplification were purchased from Invitrogen.

PCR products of 197 bp were detected by gel electrophoresis on a 2% agarose gel stained with SYBR® Safe DNA Gel Stain (Invitrogen/Life Technologies) and visualized under blue light on E-Gel® Safe Imager™ Real-time Transilluminator (Invitrogen/Life Technologies). To detect -216G>T or -191C>A, PCR products were later subjected to the restriction enzymes BseRI (New England Biolabs, Ipswich, MA) or Cfr42I (Fermentas/Thermo Fisher Scientific, Vilnius, Lithuania), respectively (12) (data not shown).

Sequencing Analysis

In order to confirm the specificity of PCR amplification, direct sequencing analysis of the obtained PCR

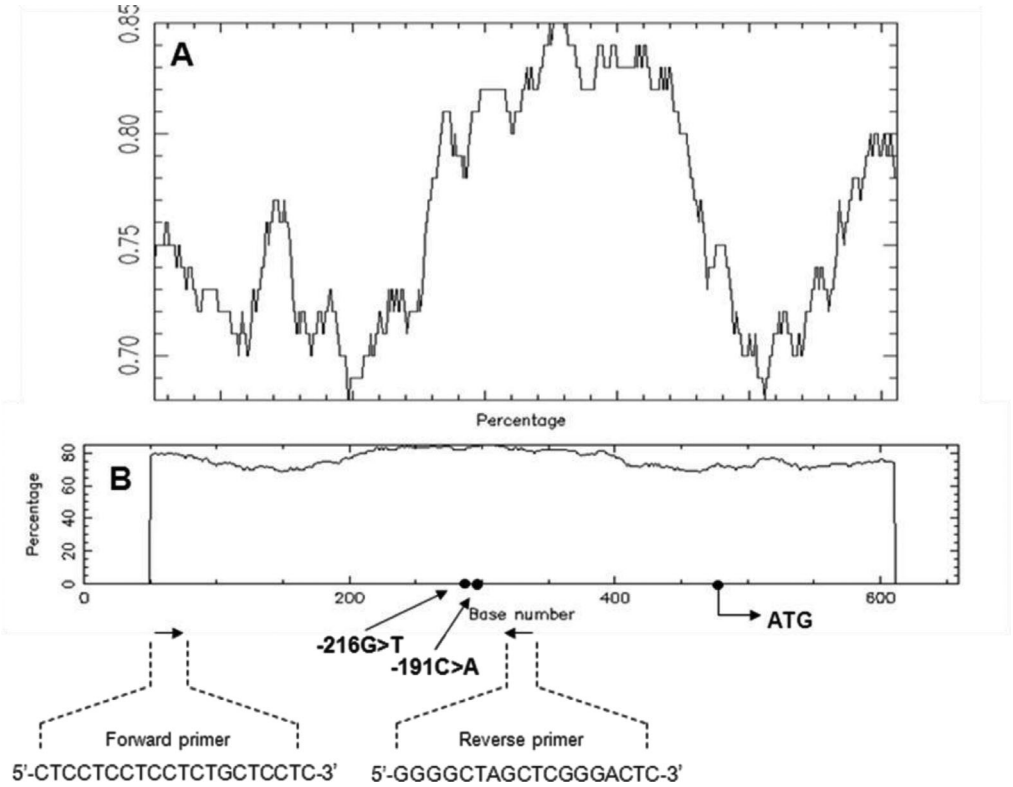

Fig. 1. GC content (A) and CpG nucleotide composition (B) of 660 bp epidermal growth factor receptor (EGFR) promoter region. Sequences and positions of primers used, as well as single nucleotide polymorphisms (SNPs) positions and translation start site ATG, are indicated in the graph.

products was performed. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Germany) and directly sequenced on ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, California). Sequencing was conducted using ABI PRISM® BigDyeTM Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) in both forward and reverse direction, using the same primers that was used for the PCR amplification. Comparison of the obtained sequence with the reference sequence of *EGFR* promoter region (http://www.ncbi.nlm.nih.gov; GenBank reference: M11234.1) revealed that the PCR amplification was highly specific.

RESULTS

Sequence analysis of the template DNA (Fig. 1) showed that the region is extremely GC rich, with 75.45% G + C content in a sequence of 660 bp (sum C + G = 421). The examined region contains a CpG island region spanning 558 bp (−450/+108 from translation start site), with an observed-to-expected ratio of CpG 0.97.

To determine the optimal concentration of DMSO, which proved to be necessary for successful amplification, separate PCR reactions were setup with addition of 1%, 3%, and 5% of DMSO. Final concentration of 5% DMSO was the only one to provide the desired amplicon yield without nonspecific amplification (Fig. 2).

The optimal annealing temperature was calculated to 56°C. Using gradient PCR method, five different annealing temperatures, ranging from 61°C to 69°C, were tested. The results revealed the optimal annealing at 63°C (Fig. 3). $MgCl_2$ concentrations ranging from 0.5 to 2.5 mM were tested, resulting in an optimum at 1.5 mM (Fig. 4).

DNA concentrations ranged from 0.25 to 28.20 μg/ml. Under the same conditions, which proved optimal for the templates with higher DNA quantity, samples with DNA concentration of less than 1.86 μg/ml gave no amplification results (Fig. 5).

DISCUSSION

Concentration of DNA Template

Recognized as "the golden standard" for sample preservation, formalin fixation and paraffin embedding of a tissue has been routinely used for over a century to enable long storage of samples for future investigations (21). These FFPE specimens have been successfully employed

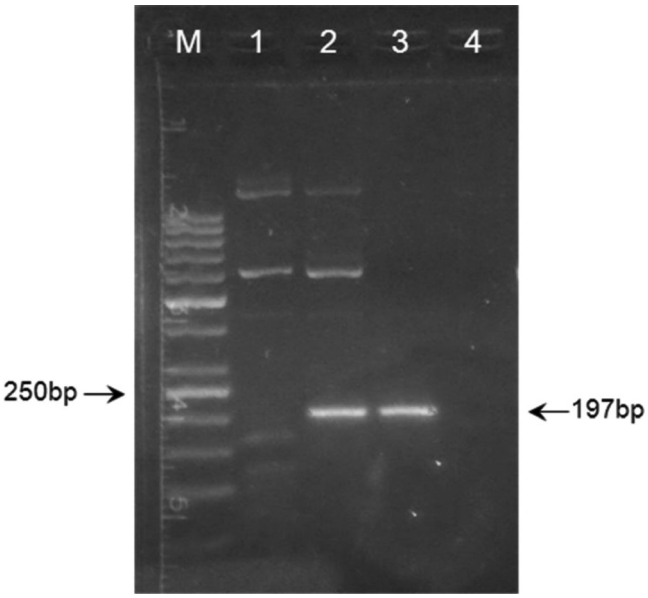

Fig. 2. Effects of dimethyl sulfoxide (DMSO) on the polymerase chain reaction (PCR) amplification. Lane M: 50 bp DNA ladder; lane 1: 1% DMSO; lane 2: 3% DMSO; lane 3: 5% DMSO; lane 4: nontemplate control.

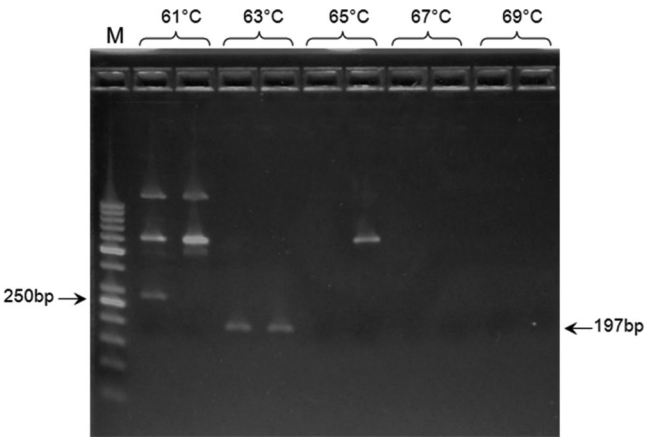

Fig. 3. Effects of annealing temperature on the polymerase chain reaction (PCR) amplification. Lane M: 50 bp DNA ladder.

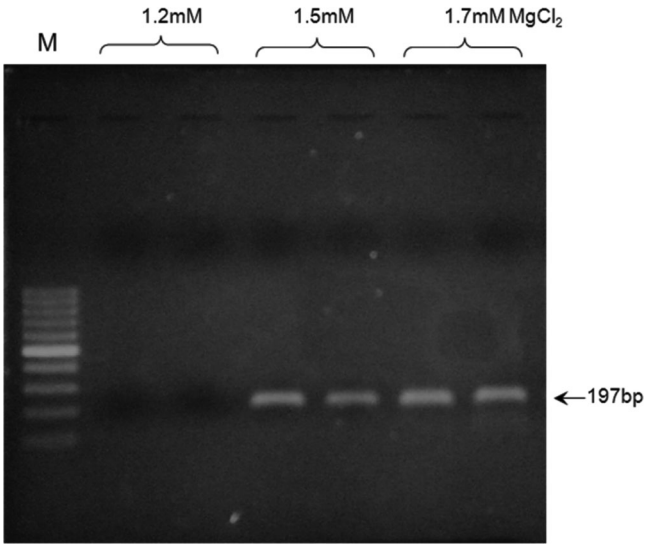

Fig. 4. Effects of $MgCl_2$ concentration on the polymerase chain reaction (PCR) amplification. Lane M: 100 bp DNA ladder.

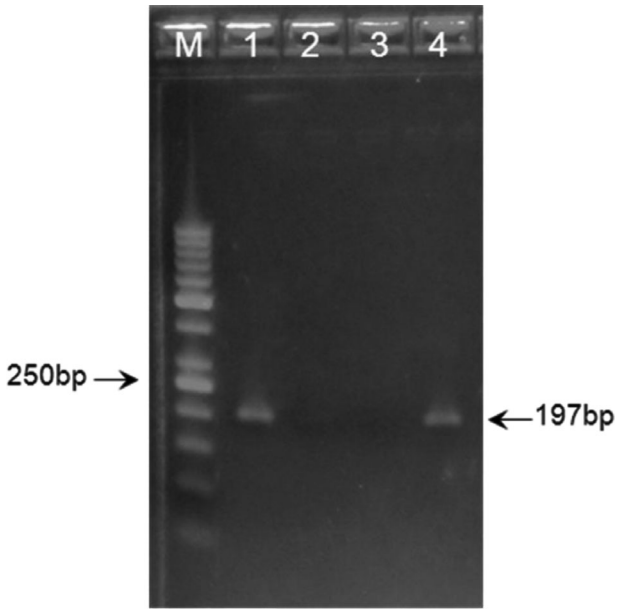

Fig. 5. Effects of template DNA concentration on the polymerase chain reaction (PCR) amplification. Lane M: 50 bp DNA ladder; lane 1: 11.3 μg/ml; lane 2: 1.0 μg/ml; lane 3: 1.4 μg/ml; lane 4: 5.7 μg/ml.

in numerous research techniques, including PCR method (22). However, PCR in general require high-quality DNA as a template, which turned out to be a challenge for extraction from FFPE tissue. Namely, formalin used for tissue fixation often reduce the amount and quality of available DNA by causing formation of nucleoprotein complexes, cross-linking of nucleic acids with histones, methylene bridging of neighboring amino groups of bases, and further nucleic acid fragmentation (23–25). Nevertheless, the method of isolation seems not to be of a crucial importance, as comparison of different techniques, including phenol–chloroform protocol, salting out method, and commercial kit application, revealed no significant difference in terms of yield, quality, and length of the extracted DNA (26).

In the present study, DNA was extracted using commercial kit, designed to efficiently isolate genomic DNA from FFPE specimens. The successful amplification was observed only with the DNA concentration of $\geq$1.86 μg/ml, which corresponded to 1.86 ng of genomic DNA per reaction, or approximately 0.07 μg/ml of the final DNA

concentration. The increase in the starting volume of the DNA template did not result in satisfactory amplification, most probably due to accompanying excess in the EDTA-containing elution buffer residue, which has a potential to inhibit the PCR reaction by chelation of magensium ions (27). In theory, even a single molecule of DNA could be successfully amplified, and the amount of genomic DNA appropriate for PCR has been determined to up to 1 μg (28, 29). Yet, here we dealt with a difficult DNA template, thus, good concentration DNA of at least 2 μg/ml proved to be a baseline condition for successful amplification.

Concentration of Magnesium Ions

$MgCl_2$ concentration has a significant influence on PCR amplification efficacy, serving as an essential cofactor that affects *Taq* DNA polymerase activity and fidelity, primer annealing, and melting temperatures, as well as the formation of artifacts (7, 28, 30, 31). $MgCl_2$ concentrations in PCR reactions usually range between 0.5 and 2.5 mM, depending on the concentration of both magnesium-binding reaction components, such as template DNA, primers, and dNTPs, and the residues of chelators, such as EDTA (7). If $MgCl_2$ concentration in the reaction is too high, reaction lacks specificity, while if it is too low, little or no amplification can be expected (7,32). Therefore, it is recommended that each PCR setup begins with the optimization of $MgCl_2$ concentration, by running several separate reactions of different magnesium molarities.

In the present study, the $MgCl_2$ concentration was tested within the range of 0.5 to 2.5 mM, with the optimum observed at 1.5 mM. Yet, up to 2.0 mM $MgCl_2$ also resulted in satisfactory amplification, so the criteria for the selection were based on the subsequent restriction fragment length polymorphism reactions, which gave best results when performed on PCR products obtained with 1.5 mM $MgCl_2$ concentration (data not shown). The observed range of acceptable molarities was broad most probably due to the presence of enhancer DMSO, which is known to improve the success of PCR reaction even at different $MgCl_2$ concentrations (30).

PCR Conditions: Temperature of Annealing

To optimize the PCR thermal cycling conditions, one has to determine the optimal temperature and length of each of the program segments, as well as the number of cycles. Of those, the most important parameter seems to be the temperature of primer annealing, as even the smallest deviation of 1°C or 2°C could make a difference between specific and nonspecific amplification (8, 29). The primer annealing temperature that is optimal for particular PCR reaction directly depends on the base composition of primers and their sequence length, and is usually around 5°C below the calculated primers melting temperature, defined as the dissociation temperature of the primer/template duplex (7, 33). In general, the annealing temperatures usually range between 55°C and 72°C. However, since the G–C pair is bound by three hydrogen bonds, while A–T pairs by only two, high GC content corresponds to higher melting temperature and requests higher temperature for primer annealing (30, 33).

In the present study, the annealing temperature was calculated to 56°C, but due to the high GC content of the template, the optimum was proposed to be at least 5°C higher. Gradient PCR reaction showed that the optimal annealing temperature for our PCR reaction was 61°C, which was even higher than predicted. As expected (20), annealing at the lower temperature allowed nonspecific amplification, while higher temperatures completely disabled annealing, thus, yielding no PCR products. It could be of interest to mention that "touchdown" approach, which represents the PCR modification that includes progressive lowering of annealing temperature throughout the cycles in order to increase both specificity and yield, was tested as well, but with no success (data not shown).

PCR Additives: DMSO

It has been shown that some PCR reactions, especially those involving GC-rich template, cannot be optimized solely by adjusting concentration of components or cycling conditions (7, 34, 35). In such cases, PCR additives or cosolvents, including DMSO, glycerol, formamide, and many others, could act as enhancers of amplification, and have been commonly used in research practice to increase yield and specificity of PCR reaction (8, 36–39). DMSO is a well-described cosolvent that increases both specificity and productivity of PCR reaction, most probably by decreasing inter- or intrastrand reannealing and formation of the problematic secondary structure (7, 37, 40, 41). Consequently, it reduces the melting temperature of the primers and facilitates the PCR product strand separation, providing more efficient amplification (6, 7, 34, 41). It should be taken into account that the concentration of DMSO in the reaction is of ultimate importance, due to its potential to reduce *Taq* DNA polymerase activity of up to 50% (7, 37).

In the present study, addition of DMSO to the reaction mixture turned out to be essential for successful amplification, and none of the previously described optimization strategies could have been implemented without its presence. Optimal concentration of 5% was determined after testing three different options, and was within the recommended range of 1–10% (7, 28).

CONCLUSION

In conclusion, *EGFR* promoter region, due to its high GC content, proved to be an extremely difficult PCR target. The optimization of the PCR conditions included determination of optimal $MgCl_2$ concentration and annealing temperature, which, in the presence of 5% DMSO and the DNA template of acceptable concentration, resulted in successful amplification.

REFERENCES

1. Coleman WB, Tsongalis GJ. The polymerase chain reaction. In: Coleman WB, Tsongalis GJ, editors. Molecular Diagnostics for the Clinical Laboratorian, edition, Totowa, NJ: Humana Press Inc; 2006. p 47–56.
2. Hube F, Reverdiau P, Iochmann S, Gruel Y. Improved PCR method for amplification of GC-rich DNA sequences. Mol Biotechnol 2005;31(1):81–84.
3. Woodford K, Weitzmann MN, Usdin K. The use of K(+)-free buffers eliminates a common cause of premature chain termination in PCR and PCR sequencing. Nucleic Acids Res 1995;23(3):539.
4. McDowell DG, Burns NA, Parkes HC. Localised sequence regions possessing high melting temperatures prevent the amplification of a DNA mimic in competitive PCR. Nucleic Acids Res 1998;26(14):3340–3347.
5. Usdin K, Woodford KJ. CGG repeats associated with DNA instability and chromosome fragility form structures that block DNA synthesis in vitro. Nucleic Acids Res 1995;23(20):4202–4209.
6. Kang J, Lee MS, Gorenstein DG. The enhancement of PCR amplification of a random sequence DNA library by DMSO and betaine: Application to in vitro combinatorial selection of aptamers. J Biochem Biophys Methods 2005;64(2):147–151.
7. Grunenwald H. Optimization of polymerase chain reactions. In: Bartlett JMS, Stirling D, editors. Methods in Molecular Biology. PCR Protocols, second edition, Totowa, NJ: Humana Press Inc; 2003.
8. Roux KH. Optimization and troubleshooting in PCR. Cold Spring Harb Protoc 2009;2009(4):pdb ip66.
9. Sweetman S, ed. Martindale: The Complete Drug Reference 36. London, UK: Pharmaceutical Press; 2009.
10. Liu W, Wu X, Zhang W, et al. Relationship of EGFR mutations, expression, amplification, and polymorphisms to epidermal growth factor receptor inhibitors in the NCI60 cell lines. Clin Cancer Res 2007;13(22 Pt 1):6788–6795.
11. Kotsakis A, Georgoulias V. Targeting epidermal growth factor receptor in the treatment of non-small-cell lung cancer. Expert Opin Pharmacother 2010;11(14):2363–2389.
12. Liu G, Gurubhagavatula S, Zhou W, et al. Epidermal growth factor receptor polymorphisms and clinical outcomes in non-small-cell lung cancer patients treated with gefitinib. Pharmacogenomics J 2008;8(2):129–138.
13. Han B, Zhou X, Zhang RX, et al. Mutations of the epidermal growth factor receptor gene in NSCLC patients. Oncol Lett 2011;2(6):1233–1237.
14. Keedy VL, Temin S, Somerfield MR, et al. American Society of Clinical Oncology provisional clinical opinion: Epidermal growth factor receptor (EGFR) mutation testing for patients with advanced non-small-cell lung cancer considering first-line EGFR tyrosine kinase inhibitor therapy. J Clin Oncol 2011;29(15): 2121–2127.
15. Table of Pharmacogenomic Biomarkers in Drug Labels [Internet]. The Food and Drug Administration [updated December 13, 2012]. Available from: http://www.fda.gov/drugs/scienceresearch/researchareas/pharmacogenetics/ucm083378.htm. Accessed on January 4, 2013.
16. Liu W, Innocenti F, Wu MH, et al. A functional common polymorphism in a Sp1 recognition site of the epidermal growth factor receptor gene promoter. Cancer Res 2005;65(1):46–53.
17. Rudin CM, Liu W, Desai A, et al. Pharmacogenomic and pharmacokinetic determinants of erlotinib toxicity. J Clin Oncol 2008;26(7):1119–1127.
18. Jung M, Cho BC, Lee CH, et al. EGFR polymorphism as a predictor of clinical outcome in advanced lung cancer patients treated with EGFR-TKI. Yonsei Med J 2012;53(6):1128–1135.
19. Ishii S, Xu YH, Stratton RH, et al. Characterization and sequence of the promoter region of the human epidermal growth factor receptor gene. Proc Natl Acad Sci USA 1985;82(15): 4920–4924.
20. Rychlik W, Spencer WJ, Rhoads RE. Optimization of the annealing temperature for DNA amplification in vitro. Nucleic Acids Res 1990;18(21):6409–6412.
21. Magdeldin S, Yamamoto T. Toward deciphering proteomes of formalin-fixed paraffin-embedded (FFPE) tissues. Proteomics 2012;12(7):1045–1058.
22. Ben-Ezra J, Johnson DA, Rossi J, et al. Effect of fixation on the amplification of nucleic acids from paraffin-embedded material by the polymerase chain reaction. J Histochem Cytochem 1991;39(3): 351–354.
23. Chaw YF, Crane LE, Lange P, Shapiro R. Isolation and identification of cross-links from formaldehyde-treated nucleic acids. Biochemistry 1980;19(24):5525–5531.
24. Brutlag D, Schlehuber C, Bonner J. Properties of formaldehyde-treated nucleohistone. Biochemistry 1969;8(8): 3214–3218.
25. Taga M, Eguchi H, Shinohara T, et al. Improved PCR amplification for molecular analysis using DNA from long-term preserved formalin-fixed, paraffin-embedded lung cancer tissue specimens. Int J Clin Exp Pathol 2013;6(1):76–79.
26. Mirmomeni MH, Sajjadi Majd S, Sisakhtnezhad S, Doranegard F. Comparison of the three methods for DNA extraction from paraffin-embedded tissues. Journal of Biological Sciences 2010;10:261–266.
27. Al-Soud WA, Radstrom P. Purification and characterization of PCR-inhibitory components in blood cells. J Clin Microbiol 2001;39(2):485–493.
28. Kolmodin LA, Birch DE. Polymerase chain reaction. Basic principles and routine practice. Methods Mol Biol 2002;192:3–18.
29. Lo YMD, Chan KCA. Introduction to the polymerase chain reaction. In: Lo YMD, Chiu RWK, Chan KCA, editors. Clinical Applications of PCR, second edition, Totowa, NJ: Humana Press; 2006.
30. Kramer MF, Coen DM. Enzymatic amplification of DNA by PCR: Standard procedures and optimization. Curr Protoc Mol Biol 2001; Chapter 15: Unit 15 11.
31. Innis MA, Gelfand D. Optimization of PCRs. In: Innes MA, Gelfand D, Sninsky JJ, White TJ, editors. PCR Protocols. A Guide to Methods and Application, edition, San Diego, CA: Academic Press; 1990. p 3–13.
32. Miller MC, Cunningham L. Introduction to polymerase chain reaction. Methods Mol Med 1999;22:1–25.
33. Mamedov TG, Pienaar E, Whitney SE, et al. A fundamental study of the PCR amplification of GC-rich DNA templates. Comput Biol Chem 2008;32(6):452–457.
34. Sahdev S, Saini S, Tiwari P, et al. Amplification of GC-rich genes by following a combination strategy of primer design, enhancers and modified PCR cycle conditions. Mol Cell Probes 2007;21(4): 303–307.

35. Musso M, Bocciardi R, Parodi S, et al. Betaine, dimethyl sulfoxide, and 7-deaza-dGTP, a powerful mixture for amplification of GC-rich DNA sequences. J Mol Diagn 2006;8(5):544–550.
36. Sarkar G, Kapelner S, Sommer SS. Formamide can dramatically improve the specificity of PCR. Nucleic Acids Res 1990;18(24):7465.
37. Hung T, Mak K, Fong K. A specificity enhancer for polymerase chain reaction. Nucleic Acids Res 1990;18(16):4953.
38. Bachmann B, Luke W, Hunsmann G. Improvement of PCR amplified DNA sequencing with the aid of detergents. Nucleic Acids Res 1990;18(5):1309.
39. Farell EM, Alexandre G. Bovine serum albumin further enhances the effects of organic solvents on increased yield of polymerase chain reaction of GC-rich templates. BMC Res Notes 2012;5: 257. doi: 10.1186/1756-0500-5-257.
40. Bookstein R, Lai CC, To H, Lee WH. PCR-based detection of a polymorphic BamHI site in intron 1 of the human retinoblastoma (RB) gene. Nucleic Acids Res 1990;18(6): 1666.
41. Jensen MA, Fukushima M, Davis RW. DMSO and betaine greatly improve amplification of GC-rich constructs in de novo synthesis. PLOS ONE 2010;5(6):e11024. doi: 10.1371/journal.pone.0011024.