(RESEARCH ARTICLE)

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Modified Tetra-Primer ARMS PCR optimization for detection of ADIPOQ rs267729 SNP (C>G) in type 2 diabetes patients in San Luis, Argentina population

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Abstract

The human Adiponectin (ADIPOQ) is an important adipocytokine that is secreted by adipocytes and plays a key role in the inflammatory response that is associated with insulin-resistant states and T2DM. The transversion of C to G (rs267729) in the promoter region of ADIPOQ gene could affect its transcription and secretion. Development of a new Modified Tetra Primer Amplification Refractory Mutation System - Polymerase Chain Reaction (MTPA-PCR) for detection of rs267729 (C>G) in the human promoter of ADIPOQ gene was sought. A MTPA-PCR for rs267729 polymorphism in a single-step PCR was carried out, and the results were confirmed by ASO-PCR technique in 25 type 2 diabetes mellitus patients and 25 controls. We have developed a sensitive single tube MTPA-PCR assay to detect rs267729 (C>G) in the human promoter of ADIPOQ gene, which allowed us to distinguish homozygous and heterozygous forms of this polymorphism successfully. Full accordance between ASO-PCR and MTPA-PCR methods for genotyping of rs267729 (C>G) polymorphism was found. MTPA-PCR could be a reliable, accurate and simple technique for genotyping SNP and different mutations. This work describes a rapid, relatively cheap, high throughput detection of C>G polymorphism in ADIPOQ that can be used in large scale clinical studies. So far, no study was done on optimization methods for genotyping rs267729 (C>G) polymorphism by MTPA-PCR. Here, we successfully adjusted and enhanced this method for recognizing rs267729 (C>G) polymorphism in the promoter of ADIPOQ gene in Argentinian population.

Keywords: Adipocytes; Inflammatory response; Insulin-Resistant; Genotyping

1. Introduction

Ninety-nine percent of human DNA sequences are identical. Polymorphisms are the basis of genetic heterogeneity among individuals. Single-nucleotide polymorphisms (SNPs) are alterations in DNA at the single base level that are the most frequent variations in human genome [1, 2]. The high density and distribution of SNPs make them suitable for association studies, population genetics, and indirect diagnosis [3, 4].

Many techniques for genotyping had been designed based on polymerization such as allele-specific primers and tetra primer amplification refractory mutation system (ARMS) polymerase chain reaction (PCR) [5, 6]. Ye et al. [6] introduced tetra primer ARMS PCR as a simple, effective, and economical SNP genotyping method, which uses four primers in one PCR, followed by gel electrophoresis. This method was derived from a tetra-primer PCR method and the ARMS [7, 8]. In this method, two smaller and allele-specific fragments are amplified by inner and outer primers. In

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addition, outer primers amplify common fragment that is larger than the former products and contains the selected SNP. Moreover, inner primers have mismatches at 3' ends and the - 2 position (third position from the 3' end). In detail, mismatch at the 3´ end causes allele specificity, and mismatch at - 2 positions was designed for enhancing the specificity of allele-based polymerization (Figure 1). As a result, DNA polymerase would be able to commence polymerization, if the 3´ terminus of inner primer was complimentary to the template. Whether the allele-specific fragment is amplified or not, the sample is genotyped [9].
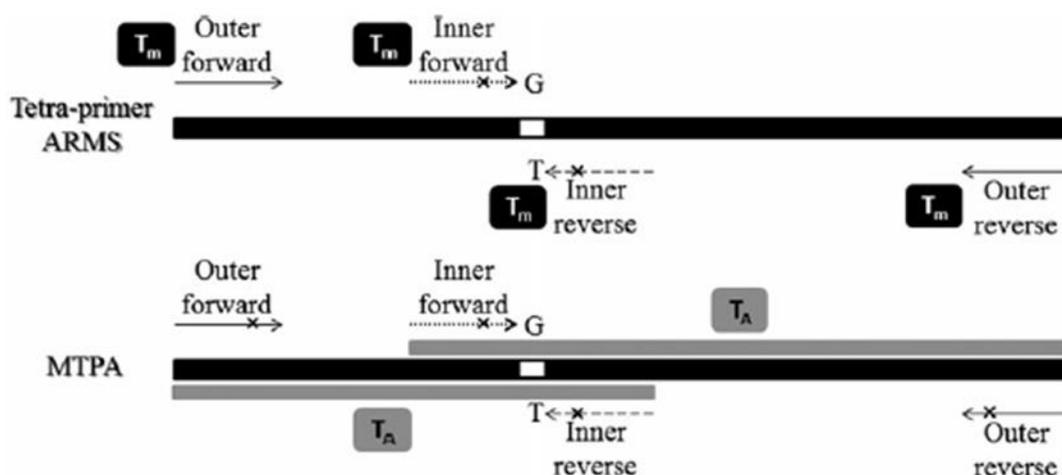
Figure 1 Schematic representation of the tetra-primer amplification refractory mutation system (ARMS) polymerase chain reaction (PCR) technique and modified tetra-primer ARMS (MTPA) PCR. Four primers were involved in one reaction: the two outer primers amplify a common fragment that contains a single-nucleotide polymorphism (SNP; white box) and two specific fragments. The inner primers amplify the two allelic specific fragments, which have additional mismatch at -2 positions. The MTPA PCR method has two facilities compared to casual tetra-primer ARMS PCR. First, additional mismatch at - 2 position of outer primers is improvised to equalize the strength of primers. Second feature is considering same annealing temperature for allelic fragments more than same melting temperature for primers. X, Tm, and TA represent additional mismatch at – 2 position, melting temperature, and annealing temperature of primers, respectively [9].

Designed mismatch at the - 2 position follows rules that are represented in Table 1 [10]. To facilitate these instructions, the accessible web program (http://primer1.soton.ac.uk/primer1.html) is available.

Table 1 IUPAC codes and mismatches at -2 positions.

IUPAC codes	Alleles	Additional mismatch at – 2 position
M	A/C	G/T, A/C
R	A/G	G/A, T/C
W	A/T	C/C, G/G, A/A, T/T
S	C/G	C/C, G/G, A/A, T/T
Y	C/T	G/A, T/C
K	G/T	G/T, A/C

A "strong" mismatch (G/A or C/T mismatches) at the 3' end of allele-specific primers would be completed with a "weak" second mismatch (C/A or G/T) at - 2 position and vice versa, whereas a "medium" mismatch (A/A, C/C, G/G, or T/T) at the 3' end would be completed with a "medium" second mismatch. IUPAC, International Union of Pure and Applied Chemistry.

However, the tetra-primer ARMS PCR has not only a difficult procedure for optimization but also fails to distinguish the target allele in SNP genotyping on some occasions [6, 11].

The tetra-primer ARMS PCR technique was modified and introduced as a new method to facilitate the evaluation of SNPs, which may not be accurately identified by the traditional technique [12]. The introduced technique was named MTPA PCR (modified tetra-primer ARMS PCR). The new MTPA PCR primer designer is introduced elsewhere (http://sci.ui.ac.ir/*rahgozar). This program performs the new rules automatically and is prepared for free download.

Type 2 diabetes mellitus (T2DM) is one of the most common progressive metabolic diseases and poses a substantial burden on health-care systems globally. The International Diabetes Federation (IDF) has estimated that the prevalence of diabetes was 366 million worldwide, and is expected to increase up to 552 million by 2030 [13]. However, the mechanisms associated with T2DM remain uncertain. It is widely accepted that T2DM is a complex disease and both environmental and genetic factors can contribute to disease initiation as well as its evolution.

Adiponectin encoded by ADIPOQ (OMIM: 605441), also known as APM1, ACRP30 or GBP28, is a kind of adipose tissue specific cytokine secreted mainly by white adipose tissue, which plays an important role in regulating insulin sensitivity, glucose homeostasis and lipid metabolism [14, 15]. Moreover, ADIPOQ plays a key role in the inflammatory response that is associated with insulin-resistant states and T2DM [16, 17, 18]. The human ADIPOQ gene is mapped to chromosome 3q27 [19], displays a few polymorphisms in the promoter region (e.g., −11426A>G (rs16861194), −11391G>A (rs17300539), −11377C>G (rs267729) or in the exon 2 (+45T>G, rs16861194) or in the intron 2 (+276G>T, rs1501299), which could affect ADIPOQ gene transcription and its secretion [20]. In recent years, the associations of single nucleotide polymorphisms (SNPs) of the ADIPOQ gene with T2DM have been reported [21, 22, 23, 24, 25, 26]. However, the results of these studies are still controversial, and show strong racial and regional variations.

Therefore, we have developed a sensitive single tube Modified tetra-primer ARMS PCR assay to evaluate the genetic risk of the rs267729 (C>G) polymorphism in the human promoter of ADIPOQ gene for T2DM.

2. Methods

2.1. DNA extraction

Genomic DNA for this study was obtained by venous blood draws from adult volunteers. The blood samples were collected in tubes containing EDTA. DNA from 50 subjects (25 T2DM patients and 25 controls) was used for the validation of the technique. Leukocyte genomic DNA was extracted using High Pure PCR Template Preparation Kit (Roché), quantified using Nanodrop-1000 Spectrophotometer (Thermo Scientific) and run in 1% gel agarose electrophoresis. Genomics DNA were stored in -20 °C after determining their relevant concentrations and analyses on gels electrophoresis.

2.2. Primer design

Based on the GenBank sequence of human ADIPOQ gene (accession number: NT_005612.16), we designed two special set of primers for MTPA PCR (modified tetra-primer ARMS PCR) using web-based software accessible from the website (http://sci.ui.ac.ir/*rahgozar). The software showed several pairs of primers, whose specificities were analyzed with the Primer-BLAST program (http://www.ncbi.nlm.nih.gov/blast) and *OligoAnalyzer 3.1* IDT (https://www.idtdna.com/calc/analyzer).

The set of primers that joined in the specific target sequence were selected, with an adequate melting temperature (Tm) and the lowest probability of formation of secondary structures. The primers used for MTPA PCR for detection of -11.377C>G (rs266729) are provided in Table 2**.**

Table 2 MTPA PCR Primers and Concentration of Reagents

Primer	Primer sequence (5' –3')	Tm	Ta	Fragment size	Concentration
Forward Inner (G allele)	CAAGAACCGGCTCAGATCCTCCG	61.6 °C		239 bp	0.2 mM
Reverse Inner (C allele)	TGGCACGCTCATGTTTTGTTTTTGATGG	61.8 °C		333 bp	0.2 mM
Forward Outer	CATGTCTCTCATCTCAACGGCCAAA	59.7 °C	61.3 ° C	522 bp (from two outer primers)	0.2 mM
Reverse Outer	TGTGTGTGTGTTTGTGAGAGAGACAG	59.5 °C			0.2 mM

Tm, melting temperature; the mismatches of the primers were emphasized in bold. Ta, annealing temperature. MTPA-PCR, modified tetra-primer ARMS-PCR.

2.3. Optimization steps of MTPA-PCR

The determining factors for the optimization of MTPA PCR method were: the correct concentration of the PCR reagent, the ratio of outer and inner primers, as well as the annealing temperature. Those factors were important to increase the PCR product specificity.

2.3.1. Amplification of outer primers region

The reaction was performed in a total volume of 20 μl, containing 125 ng of template DNA, 0.25 mM of each outer primer, 0.2 mM dNTPs (Invitrogen, CA, USA), 1.5 mM MgCl2, 1xPCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), and 1 U of Taq DNA polymerase (Productos Biológicos, Argentina).

2.3.2. MTPA-PCR reaction

It was performed in a total volume of 25 μl containing 50 ng of template DNA, 0.2 mM of both inner primers and both outer primers, 0.16 mM dNTPs (Invitrogen, CA, USA), 1.2 mM $MgCl_2$, 0.8× PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), and 1 U Taq polymerase (Productos Biológicos, Argentina).

2.3.3. Validation assay: Aso-PCR reaction

Allele A amplification: It was performed in a total volume of 25 μl containing 50 ng of template DNA, 0.25 mM of forward outer and reverse inner primers, 0.16 mM dNTPs (Invitrogen, CA, USA), 2.5 mM $MgCl_2$, 1x PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), and 1 U Taq polymerase (Productos Biológicos, Argentina). Allele G amplification: a total volume of 25 μl containing 100 ng of template DNA, 0.25 mM of reverse outer primer, 0.25 mM of forward inner primer, 0.16 mM dNTPs (Invitrogen, CA, USA), 1.2 mM $MgCl_2$, 1× PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), and 1 U Taq polymerase (Productos Biológicos, Argentina).

2.3.4. Termocycler program

The samples were incubated for 3 min at 94 ˚C, followed by 30 cycles of 1 min denaturation (95 ˚C), 1 min annealing (61.3 ˚C), and 1 min extension (72 ˚C), and an additional 5 min extension at 72 ˚C at the end of the 30 cycles.

2.4. Gel electrophoresis

After completion of PCR reactions, the products were run on 2 % agarose gels alongside a 100 bp marker, and dying with GelRed. After electrophoresis, bands were observed using a UV Gel Doc apparatus.

3. Results and discussion

The MTPA-PCR is a rapid and reliable method for analysis of point mutations or small deletions [12]. Mesrian Tanha et al. [12] were the first to describe MTPA-PCR for which allele-specific amplification is achieved in a single PCR reaction using four primers. The MTPA-PCR method has two facilities compared to casual tetra-primer ARMS PCR. First, additional mismatch at - 2 position of outer primers is improvised to equalize the strength of primers. Second feature is considering same annealing temperature for allelic fragments more than same melting temperature for primers.

MTPA-PCR primer pairs were designed using Primer software (http://sci.ui.ac.ir/*rahgozar).The inner MTPA-PCR primer pair was designed with a deliberate mismatch at - 2 positions for enhancing the specificity of allele-based polymerization and the mismatches at -2 positions of outer primers were contrived.

The program follows the Little [10] rules for selecting the additional mismatch base. Under this scheme "strong" mismatches at the 3' terminus (A/G or C/T mismatches) are optimally paired with "weak" second mismatches (C/A or G/T) and vice versa with "medium, medium" strength combinations (A/A, C/C, G/G or T/T) as an alternative. Tm is calculated using the nearest neighbor parameters [27] and the formula given by Rychlik et al. [28].The MTPA-PCR method was successfully applied to classify rs267729 (C>G). The optimal PCR conditions and fragment sizes of the C and G alleles for the ADIPOQ gene are shown in Table 2.

The allele-specific segments differed sufficiently in size to be distinguished by 2 % agarose gel electrophoresis. For that reason was possible to differentiate between the G and C alleles of the ADIPOQ gene by means of the MTPA-PCR methodology. The interactions between the inner and outer primers affect both the yield and the specificity of the reaction. Therefore the primer design is a critical step for the success of the T-ARMS PCR method. The inner primers were developed with length of 25-28 bases with a deliberate mismatch at position 2 from the 3'-end. The mismatched bases are shown in bold letter in Table 2.

The development of the MTPA-PCR was divided into three stages. In the first stage we optimized the right amplification of the outer primers amplicon. This fragment was used as a template for the inner primers, in order to produce the allele specific fragments in MTPA-PCR. We successfully obtained the fragment of 522 pb (data not shown).The second stage was to evaluate de optimal composition of each MTPA- PCR reaction and elaborate protocol changes for genotyping different samples. The objective of this step was to obtain the expected theoretical products by electrophoresis separation: three bands in heterozygous or two bands in homozygous.

In the thirst stage we validated the results by ASO-PCR, placing the primers pair for the specific allele amplification separately (Forward outer/Reverse inner and Forward inner / Reverse outer). Thereby we could design a simple and economical method to validate MTPA-PCR. Thereby we obtained control samples of known genotype to optimize the experimental condition for a certain analysis of the samples by MTPA -PCR. One of the key points for the success of the method is that oligonucleotides with a mismatched 3' residue do not function as PCR primers under appropriate conditions. Thus, for the allele specific primers, the normal primer is refractory to PCR on mutant DNA and the mutant specific primer is similarly non-functional on normal DNA. Newton et al. [7] noted that, in some cases, having only the single 3' mismatched base was insufficient to prevent undesired amplification so they deliberately introduced additional mismatched bases near the 3' end.

MTPA-PCR results require a validation assay to confirm the specificity of the assay. The results obtained by T-ARMS PCR were confirmed with ASO-PCR (Figure 2).
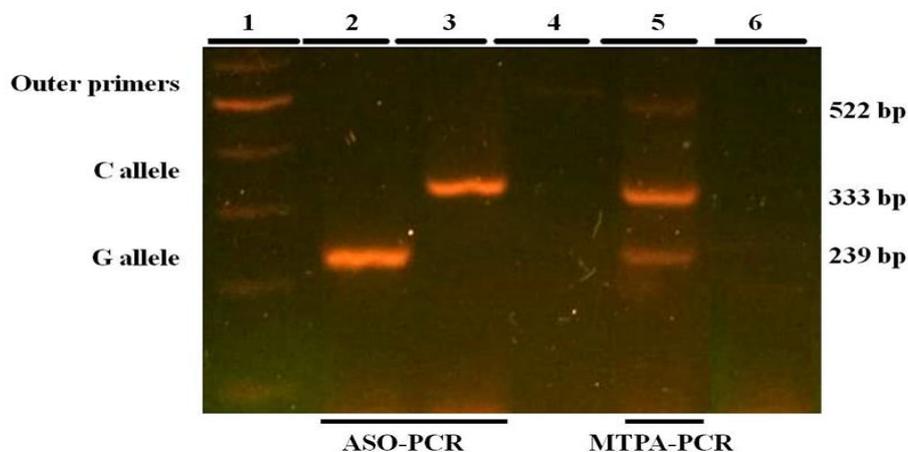
Figure 2 Validation assay to confirm MTPA-PCR result by ASO-PCR. Lane 1 molecular weight marker; lane 2 (G allele amplification); lane 3 (C allele amplification); lane 4 (outer primer amplification); lane 5 MTPA- PCR and lane 6 negative control.

The melting temperature (Tm) should be considered one of the most important factors in order to achieve an allele-specific amplification [29]. The main difficulty to amplify the correct product of primers with GC-rich sequences was needed high temperatures to break up the strong secondary structure, formed by hydrogen bonds between the cytosine and guanine. During the PCR melting step, the DNA strands begin to separate in a lower Tm, eventually the primers that have a high Tm will not be completely disnatured and the DNA template will be partially open inhibiting the ability of the Taq DNA polymerase to act in those regions [30]. McDowell et al. [31] demonstrated that GC-rich sequences, in conjunct with high Tm, leads to the formation of stable secondary structures in primers, which can reduce the PCR efficiency by serving as termination or stop sites.

The primers used for SNP rs267729 have a high concentration of CG, ranging from 39 % to 60 %, with a Tm ranging from 59.5 °C to 61.8 °C. To identify the optimal annealing temperature we ran 4 PCR reactions with a temperature gradient from 61.3 °C to 64.7 °C. The gradient reaction allowed us to observe the amplification of each primer in the set as they approach their optimal Tm. Therefore, to optimize the annealing temperature we preceded by running gradient PCR amplifications with DNA of a heterozygote sample. The gradient PCR experiment allowed a quick determination of the optimal annealing temperature for a given reaction as depicted in Figure 3. When the three expected PCR fragments from a heterozygote were observed in the range of several temperatures; one optimal annealing temperature was chosen. We consider the gradient range 62.5 °C - 61.3 °C to be optimal for differentiation of the PCR fragments from a heterozygote, hence the optimal annealing temperature chosen was 61.3°C (Table 1).
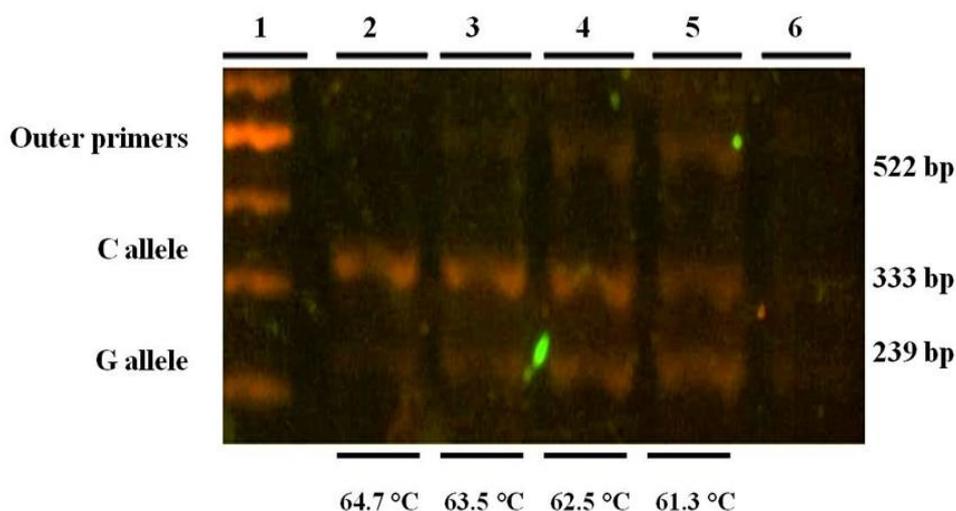
Figure 3 PCR product amplified from a temperature gradient profile utilized for optimizing the rs267729 (C>G). The same PCR reagents concentration was used in all samples. A heterozygote individual was amplified with a temperature gradient from 61.3 °C to 64.7 °C. Note that in lane 1 molecular weight marker; lanes 2 - 3 the allelic bands are weak. In lanes 4 - 5 the two allelic bands (239 and 333 bp) and common band (522 bp) are present and the profile is perfectly clear and lane 6 negative control. Results indicated that 61.3°C, in 25 µL PCR mixture, was optimal to amplify ADIPOQ rs267729 SNP (C>G) successfully.

Balancing the primer concentration was another important step to obtain specifics and sharp band, for a correct genotyping of the samples. The formation of outer primer fragment reacts strongly to small changes in its concentration and it is critical for the success of the reaction, since the inner primers binds on the outer primer amplicon [6].

Proceeding with the 61.3 °C Ta, the outer primers fragment was stabilized and the intensity of the two inner primer amplicons balanced. For that reason, a pattern among the intensity of the fragments and the each primers concentration could be designed. Optimization of MTPA-PCR was applied by using two different ratios of outer and inner primer concentration in 1:1 and 1:2 (Figure 4). We consider that the relation 1/1 to be optimal for differentiation of the PCR fragments from a heterozygote, hence the ratio chosen was 1/1. The final primer concentration for rs267729 detection was 0.2 mM for each primer (Table 1).
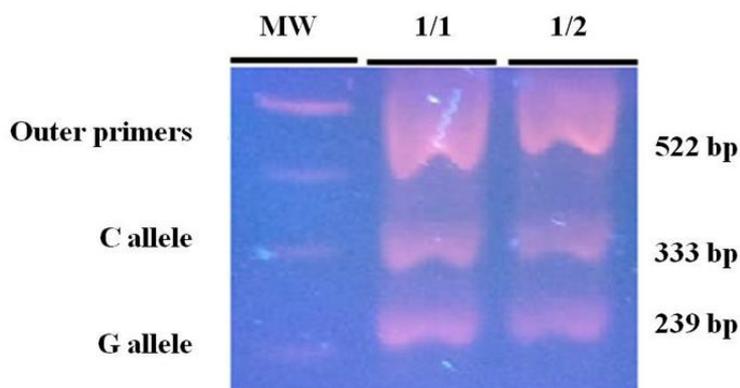
Figure 4 Presentation of MTPA PCR results two different ratios of outer and inner primer concentration in 1:1 and 1:2. MW molecular weight marker. Results indicated that a primer relation 1:1, in 25 µL PCR mixture, was optimal to amplify ADIPOQ rs267729 SNP (C>G) successfully.

Traditional tetra-primer ARMS PCR has a laborious process for optimization and the results do not seem stringent enough. Tetra-primer ARMS PCR is too sensitive to variations of annealing temperatures and MgCl2 concentrations [11]. Ye et al. [6] reported a SNP in the angiotensinogen gene that was failed in genotyping. The concluded problem is probably the unbalanced efficiency that appears between the outer and inner primers. Due to the designed mismatch, outer primers were more efficient than inner primers in the PCR. Consequently, 2–10 times more inner primers were used than outer primers, which may lead to decreasing specificity of genotyping. In the MTPA PCR technique, a new mismatch at - 2 positions of outer primers was designed to decrease the efficiency of the aforementioned primers. Moreover, to improve the strength of PCR, annealing temperatures of allelic fragments were calculated equally. Equal annealing temperature may facilitate the process of PCR.

In addition, some researches demonstrated that tetra primer ARMS-PCR may not be practical for studying SNPs in the CG-rich regions [29, 11]. In the present study, one of the inconvenient was the fact that the forward inner primer (specific for G allele) has GC-rich sequences near the refractory mutation, which could produce unspecific amplifications of G allele in all the samples, independently of the genotype. The beneficial usage of the novel method in this case showed that MTPA-PCR is more efficient than tetra primer ARMS PCR in a setup process and validity of results. As an advantage, short primers (22–28), which could not be used previously in tetra-primer ARMS PCR, now could be accomplished in the new method. Shorter primers have lower melting temperatures and fewer secondary structures that could be useful for PCR.

Another point considered was the optimization of Taq polymerase unit (U) in PCR mixtures. We consider the range of Taq units from 0.5 to 1 U. The optimal units of Taq polymerase, for differentiation of the PCR fragments from a heterozygote, were 1 U (Figure 5).
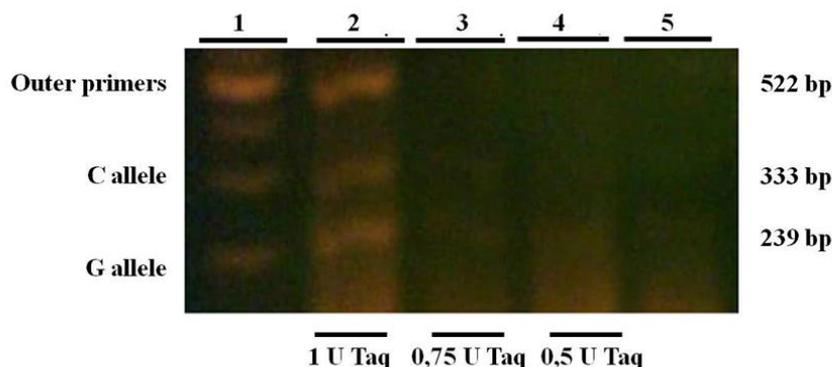
Figure 5 MTPA-PCR amplifications containing different Taq polymerase unit (U) in PCR mixtures. Lane 1 molecular weight marker; lane 2 to lane 4 corresponds to different Taq units of 1, 0.75 and 0.5 U, respectively; lane 5 negative control. The results indicated that 1 U Taq in 25 µL of PCR mix was optimal for amplifying ADIPOQ rs267729 SNP (C> G) successfully.

Successful amplification depends on DNA template quantity and quality. Higher DNA concentrations decrease amplicon specificity (i.e., extra bands are more likely), particularly when a large number of cycles are employed. The DNA quantity may need to be varied to determine the optimal amount. We use DNA template levels in the range of 50 ng -125 ng per reaction. The optimal DNA quantity, for differentiation of the PCR fragments from a heterozygote, was 50 ng (Figure 6).
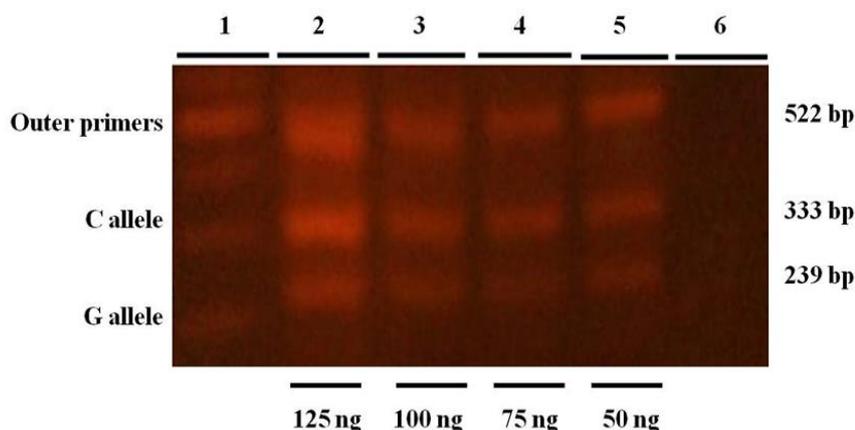
Figure 6 MTPA-PCR amplifications containing the indicated amounts of DNA were performed in PCR mixures. Lane 1 molecular weight marker; lane 2 to lane 5 corresponds to 125 ng, 100 ng, 75 ng and 50 ng of DNA, respectively and lane 6 negative control. Results indicated that 50 ng of DNA in 25 μL PCR mixture was optimal to amplify ADIPOQ rs267729 SNP (C>G) successfully**.**

Optimization of this assay is substantially easier than normal tetra-primer ARMS PCR. One sample of each identified genotype by this technique (GG, GC, and CC) was selected randomly and amplified by ASO-PCR. Results of ASO-PCR confirmed MTPA PCR data. Whenever, genotyping results were unclear, the samples were repeated again in duplicates until clear genotype was obtained. No genotyping error was observed during cross validation.

4. Conclusion

In this study, MTPA PCR protocol was successively and thoroughly optimized in terms of PCR cycling conditions and reaction components for SNP genotyping, with minimally equipped laboratories. We conclude that MTPA PCR is a robust, simple and relatively economic method for genotyping of ADIPOQ rs267729 SNP (C>G).

The SNP genotyping technique reported in this study contains additional advantages, according to our experience, compared to the normal tetra-primer ARMS PCR, including balanced strength between outer and inner primers and equal annealing temperature, which make MTPA PCR more efficient than tetra primer ARMS PCR in a setup process and validity of results.

Furthermore MTPA PCR is useful for large scale of SNP-based association study in different population to understand the genetics risk factor of Type 2 Diabetes Mellitus and metabolic disease.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare no conflict of interests with respect to the present paper**.**

Statement of ethical approval

This study has the approval of the ethics committee of the San Luis hospital. Informed consent was signed with all patients before sampling.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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How to cite this article

Orozco RAL, Diaz SB, Baigorria MES, Siewert SE and Vasquez GME. (2020). Modified Tetra-Primer ARMS PCR optimization for detection of ADIPOQ rs267729 SNP (C>G) in type 2 diabetes patients in San Luis, Argentina population. GSC Biological and Pharmaceutical Sciences, 10(2), 138-147.