Tetra-primer Amplification Refractory Mutation System for Screening T2D, *TCF7L2* Variant

Afira Waqar, Ikram ul Haq, Bushra Chaudhry

Abstract—Tetra-primer amplification refractory mutation system PCR (T-ARMS PCR) is a fast and economical means to analyze single nucleotide polymorphisms (SNPs). It requires only PCR amplification and electrophoresis afterwards for the determination of genotypes. Despite the reliability of the technique T-ARMS PCR, only fewer studies involving this have been published to date. This is because of the amplicons number which increases per reaction which is tough at times to separate on gel electrophoresis and also it is difficult to find such primers with similar annealing temperature. Considering expenditure of high throughput genotyping in the developing world, this study was designed to optimize an efficient technique, T-ARMSPCR for studying type 2 diabetes (T2D) susceptible gene using *TCF7L2* SNP. A pair of non-allele-specific outer forward, reverse primers (OF, OR) and two pairs of allele-specific inner primers (IF1, IF2, IR1, IR2) were designed and optimized in separate reactions after troubleshooting of PCR conditions particularly, annealing temperature. Total four primers, a pair of outer and inner T-ARMS-PCR primers (OF, OR, IF1 and IR1), were pooled together eventually, to amplify the two target alleles of a specific SNP, rs12255372 (G>T). A second pair of inner primers (IF2 and IR2) was optimized to recheck the results of genotyping. Optimization of the T-ARMS PCR method resulted in likelihood of genotyping the two alleles of the *TCF7L2* SNP rs12255372, G and T in a single, rapid and cost-effective PCR reaction. In this study T-ARMS PCR protocol was successively and thoroughly optimized in terms of PCR cycling conditions and reaction components. However, these results highlight the advantages of T-ARMS PCR in SNP genotyping, and have led to the incorporation of this method in the routine molecular diagnostic workup.

Index Terms— Allele, Genotyping, Screening, Single nucleotide polymorphism, Tetra-primer ARMS PCR, *TCF7L2*, T2DM

1 INTRODUCTION

T2DM is known as multifactorial disease that results from an increased permutation of various factors like environmental, genetic and shared cultural. Thirty different genes have been proposed as candidate genes for T2D but only few genes have been convincingly associated in several studies [1], [2], [3], [4]. According to GWAS, among these 30 genes of T2D, transcription factor 7-like 2 (*TCF7L2*) is one of the most susceptible genes with the highest effect on the disease [5], [6], [7]. The most common T2D associated single nucleotide polymorphisms (SNPs) are rs7903146 and rs12255372, present in the TCF7L2 gene [8], [9], [10], [11].

The high rate of recurrence of single nucleotide polymorphisms (SNPs) in the human genome and its association with diverse genetic diseases make SNP genotyping a valuable and constructive tool in molecular diagnostics. Most of these SNP genotyping techniques depend on the amplification of target DNA sequence by polymerase chain reaction (PCR) which subsequently followed allelic discrimination by way of post-PCR manipulation [12].

In view of the developing countries for which it is very hard and costly to study by PCR and post-PCR manipulation on a large number of samples for analyses. To curb these outcomes, reliable genotyping method which is sufficiently simple, fast and cost effective must be continuously developed [13], [14].

Tetra-primer amplification refractory mutation system (ARMS) PCR employs astutely designed primer sets based on two outer, non-allele-specific primers and two inner, allele-specific primers in opposite orientation to each other. T-ARMS PCR amplifies both wild type and rare allele together with a control fragment in a single reaction, a distinguishing feature from conventional ARMS PCR that amplifies bi-alleles in two separate reactions. In a single step reaction, the outer primers amplify a large fragment of the target gene, irrespective of its genotype although each inner primer combines with the particular opposite outer primer to generate smaller allele-specific amplicons, which are of different sizes and can easily be discriminated on gel electrophoresis either as homozygous or heterozygous. To improve allele specificity, a deliberate mismatch can be incorporated into the inner primers at position -2 or -3 from the 3' terminal end [15], 16].

By considering high throughput and expenditure of genotyping in the developing countries, this study was aimed to optimize an efficient technique; T-ARMS PCR for studying T2D associated *TCF7L2* SNP, rs12255372.

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2 METHODOLOGY

2.1 SAMPLES

Genomic DNA samples of individuals deposited previously in our laboratory's bio-bank were used for the optimization of analysis. These samples were collected after taking an informed consent of individuals and ethically approved procedure of sample recruitment from the AKU ethical review committee. Genomic DNA was isolated from blood samples according to a standard Promega kit protocol. Extracted DNA was dissolved in 50 ml of TE buffer (20 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at -20 °C until the genotype analysis was performed.

2.2 Primer Design

The tetra-primer ARMS PCR procedure was used to genotype the T2D associated TCF7L2 SNP, rs12255372. Primer designing is a very critical step and all pairs of primer were designed by using web-based software made by Ye et al. in 2001 available at http://cedar.genetics.soton.ac.uk/public_html/primer1.html [16]. We used the 'BLAST' program at http://www.ncbi.nlm.nih.gov/blast to check for the specificity of the primers. The primers used in this study are listed in Table I.

TABLE I
List of Primers Used

Gene, SNP	Primers	Primer Sequences 5'-3'	Tm [a]	Length (bp)	Primer Conc. (Pmol)	[b]Ann. Temp., °C	Expected Amplicons (bp)
TCF7L2, rs12255372 G→T	outer forward, OF	GGGCAATAGATACATTTTAAGA	53	22	25	53.2	760, non specific
	outer reverse, OR	GAGATAGATGATAGGCTGTT	48	20	25		494, G-allele
	inner forward IF1, G-allele	GGAATATCCAGGCAAGAATG	57	20	25		310, T-allele)
	inner reverse IR1, T-allele	CCTGAGTAATTATCAGAATATGGTA	54	25	25		
	inner forward IF2, G-allele	GGAATATCCAGGCAAGAATT	56	20	25		
	inner reverse IR2, T-allele	CCTGAGTAATTATCAGAATATGGTC	55	25	25		

[a]Melting Temperature; [b]Annealing Temperature

2.3 PCR METHOD

The gradient PCR was carried out on the eppendorf mastercycler to determine the best annealing temperature for each primer set. PCR was performed in a total volume of 10 ul containing approximately 150 ng genomic DNA, 1X PCR buffer, 3.0 mM MgCl2, 1.0 mM dNTPs, 25 pmol (optimized concentration) of each outer and allele-specific primers and 1.5 U of Taq polymerase (Promega, USA). Genomic DNA was placed at -55 °C for 15 minutes before adding it into the reaction mixture.

For optimization, PCR program was as followed as : an initial denaturation step at 95 °C for 7 min, final denaturation step at 95 °C for 45 sec, 1 min at annealing temperature (53 °C) with Gradient ± 2, 1 min at 72 °C for extension followed by 35 repeats and a final extension step of 10 min at 72 °C. A total of 10 ul from the PCR product were electrophoresed on 1% standard agarose gels at 80 V for 40 min. The fragments were visualized by ethidium bromide on a UV transilluminator (Gel Doc, BioRad).

First, individual PCR reactions were performed to optimize each pair of primers thereafter, primer sets with the closest optimal annealing temperatures were then incorporated into single mixes (OF, OR, IF1, IR1; OF, OR, IF2, IR2) and gradient PCR was repeated, in an attempt to optimize the techniques in tetra-primer ARMS PCR that came out with satisfactory results. The second pair of inner primers (IF2 and IR2) was optimized to recheck the results of genotyping. The optimized techniques with common annealing temperature (53.2 °C) were then validated on study samples for genotyping of T2D associated *TCF7L2* SNPs.

3 RESULTS

In the present study, we have developed a simple, economical, reliable and rapid tetra-primer ARMS PCR assay for the mutation genotyping. The assay was developed and tested for a group of commonly investigated T2D associated *TCF7L2* SNP; rs12255372 G>T. An illustrative assay of T-ARMS PCR is shown in Fig. 1.

Several factors, including concentration of outer and inner primers and PCR cycling conditions particularly annealing temperatures which can affect PCR specificity and efficiency were optimized (Table 1). First, we tested annealing temperatures for each pair of primers including OR with OF and IF1/ IF2 ranging from 50.1 °C to 58 °C and OF with IR1/IR2 from 52.9 °C to 65.8 °C subsequently that determined the best results for each assay (Fig. 2). Moreover, closest and the common annealing temperatures of each primer pair were utilized into single mixes (OF, OR, IF1/IF2, IR1/IR2) and gradient PCR was repeated in order to optimize the technique, T-ARMS PCR that came out with appropriate results. The second pair of inner primers (IF2 and IR2) was optimized to recheck the results of genotyping. The illustrative view of T-ARMS PCR is shown in fig. 3.

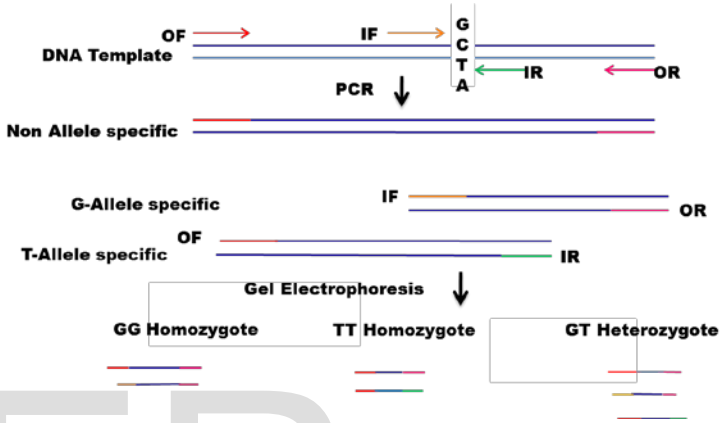

Fig. 1. This figure illustrates the schematic presentation of the most rapid and cost effective technique, tetra-primer ARMS PCR by taking an example of a SNP, G to T substitution. Two pairs of primers are used to generate two allele-specific with common non allele-specific amplicons by PCR. One pair (showed by yellow and pink arrows) producing an amplicon representing the G-allele whereas the other pair (showed by dark red and green) producing an amplicon representing the T-allele. By positioning the two outer primers at different distances from the polymorphic nucleotide, the two allele-specific amplicons differ in length that allows them to be discriminated from gel electrophoresis.

Initially we observed inhibited or less amplification efficiency and non-specific bands by using the relative high concentration of outer primers that has been conventionally used in various studies. Okayama *et al* (2004) suggested such problems can be resolved in T-ARMS PCR by reducing the ratio of inner primers, but unlikely we used equal and high concentrations of both outer and inner primers to get efficient and specific PCR amplification (Table I).

As for any T-ARMS PCR, we recommend that users should optimize the conditions, principally the primer concentrations for each set, in their laboratory. Although more than 3 mM MgCl2 concentration increased the intensity of our desired bands and also resulted in nonspecific backgrounds. The optimum amount of MgCl2 was found to be 2.5 to 3.0 mM to ensure the specificity of the T-ARMS PCR assay.

A range of Taq polymerase concentration from 0.5 to 2.0 U was also tested but PCR specificity was found to be highest with 1.5 U of Taq polymerase. To eliminate the long nonspecific amplification products, 72°C extension time was settled to 1min. Genomic DNA was placed at 55°C prior to use for better efficacy.

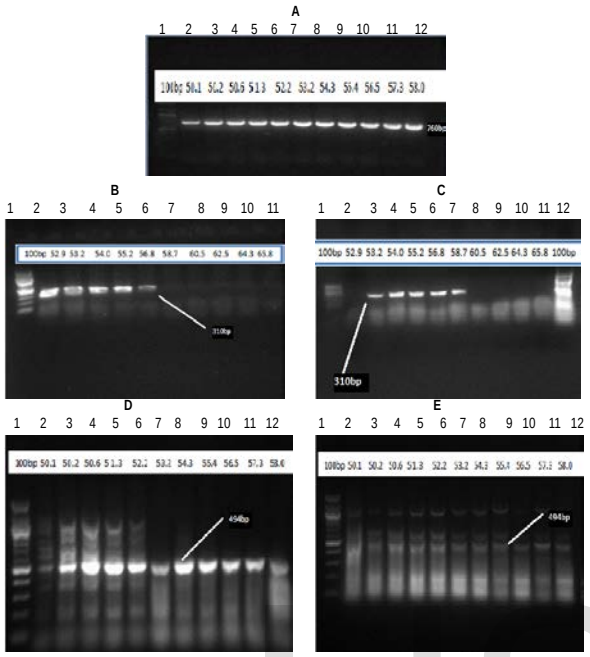

Fig. 2. Optimized Temperature Gradient of individual pair of primers: "A" represents the optimized temperature gradient at G 54 ± 4°C of the outer forward (OF) and outer reverse (OR) primers on 1% agarose gel. Lane 1 is showing the 100bp DNA ladder and lane 2 to 12 are showing samples optimized at different temperatures from 50.1 to 58.0°. The fragment of DNA is of 760bp; "B" represents the optimized temperature gradient at G 60 ± 7°C of the outer forward (OF) and inner reverse 1 (IR1) primers on 1% agarose gel. Lane 1 and 12 are showing the 100bp DNA ladder and lane 2 to 11 are showing samples optimized at different temperatures but results found at temperature from 53.2 to 58.7°C. The fragment of DNA is of 310bp; "C" represents the optimized temperature gradient at G 60 ± 7°C of the outer forward (OF) and inner reverse 2 (IR2) primers on 1% agarose gel. Lane 1 is showing the 100bp DNA ladder and lane 2 to 11 are showing samples optimized at different temperatures but results found at temperature from 52.9 to 56.8°. The fragment of DNA is of 310bp;"D" represents the optimized temperature gradient at G 54 ± 4°C of the inner forward 1 (IF1) and outer reverse (OR) primers on 1% agarose gel. Lane 1 is showing the 100bp DNA ladder and lane 2 to 12 are showing samples optimized at different temperatures from 50.1 to 58.0°. The fragment of DNA is of 494bp;"E" represents the optimized temperature gradient at G 54 ± 4° of the inner forward 2 (IF2) and outer reverse (OR) primers on 1% agarose gel. Lane 1 is showing the 100bp DNA ladder and lane 2 to 12 are showing samples optimized at different temperatures from 50.1 to 58.0°. The fragment of DNA is of 494bp.

4 DISCUSSION

The most abundant form of genetic variation that takes place in a normal standard sequence are known to as mutation though, most usual ones are recognized as Single-nucleotide polymorphisms (SNPs) which are biallelic (wild or normal allele and lethal or rare allele). SNPs can be used as efficacious genetic markers in human gene studies for biological functions and also in association studies between genetic mutation and human diseases [12], [17]. Genetic testing is beneficial clinically and can provide dramatic prognosis.

At present, there is a growing need for clinical laboratories to provide high quality genotyping-based tests within the domain of clinical relevance however; this could not have been possible in the developing countries due to high expenditure. The reported genotyping based methods are on high-tech instrument techniques which are accurate but at the same time, they are costly and time consuming. Such high-tech techniques include MLPA assay, real-time PCR assays, capillary electrophoresis, MALDI-TOF mass spectrometry, DHPLC, probe array systems, and direct DNA sequencing [18], [19]. Because of their cost and complexity, these techniques are unable to use routinely in laboratories.

Due to these unfavorable features, the present study has developed and tested tetra-primer ARMS assay for genotyping of commonly investigated T2D associated mutations of *TCF7L2*. Most of the previous European studies used typical PCR-RFLP analysis, real time PCR and DNA sequencing. Etlik et al. validated on comparison with other assays that tetra-primer ARMS PCR assay could be more advantageous in terms of time, cost and applicability in a typical laboratory. Though, PCR-RFLP is comparable in cost but it requires step of incubation and electrophoresis [15].

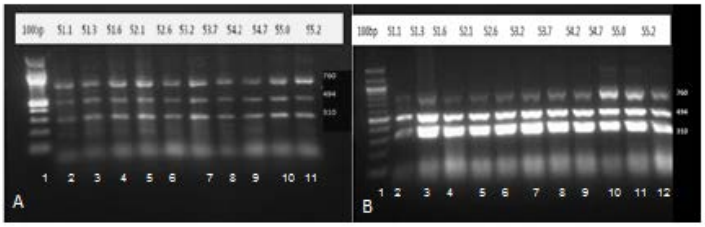

Fig. 3. Optimized Temperature Gradient of tetra primer ARMS PCR: This illustration represents the optimized temperature gradient at G 53 ±2° of the tetra primers, outer forward (OF), outer reverse (OR) inner forward 1 (IF1) and inner reverse 1 (IR1) [Figure 3a] outer forward (OF), outer reverse (OR), inner forward 2 (IF2) and inner reverse 2 (IR2) [Figure 3b] on 1% agarose gel. Lane 1 is showing the 100bp DNA ladder and lane 2 to 11 are showing samples optimized at different temperatures from 51.1 to 55.2°. The size of DNA fragments are represented as 760bp, 494and 310 base pairs.

For scientific analyses and diagnostic studies, it is an ample desire to use economical and fast assays that can be performed with standard in-house PCR. T-ARMS PCR technique is more valuable when compared with most commonly used in-house techniques like allele-specific PCR, ARMS, and RFLP because tetra-primer PCR allows the evaluation of the both wild type and lethal allele in a single reaction.

During the amplification reaction, a specific fragment of a gene is amplified to serve as an additional template for the subsequent allele-specific amplification. The additional templates improve and raise the amplification reaction from small amount of genomic DNA template. Additionally, this study suggests that T-ARMS PCR is reliable for retrospective studies in the developing world in terms of cost and time as this assay could be completed within 3 to 4 h after receipt of a specimen (around 60 min for DNA isolation, around 120 min for PCR reaction, and 40 min for electrophoresis), a reasonable time in the laboratory.

5 CONCLUSION

Despite the reliability of the technique T-ARMS PCR, only fewer studies involving this have been published to date. This is understandable as the number of amplicons increases per reaction which is tough at times to separate on gel electrophoresis and also it is difficult to find such primers with similar annealing temperature. Though, in this study T-ARMS PCR protocol was successively and thoroughly optimized in terms of PCR cycling conditions and reaction components. However, these results highlight the advantages of T-ARMS PCR in SNP genotyping, and have led to the incorporation of this method in the routine molecular diagnostic workup.

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