

# Guidelines for the Tetra-Primer ARMS–PCR Technique Development

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**Abstract** The tetra-primer amplification refractory mutation system–polymerase chain (ARMS–PCR) reaction is a simple and economical method to genotype single-nucleotide polymorphisms (SNPs). It uses four primers in a single PCR and is followed just by gel electrophoresis. However, the optimization step can be very hardworking and time-consuming. Hence, we propose to demonstrate and discuss critical steps for its development, in a way to provide useful information. Two SNPs that provided different amplification conditions were selected. DNA extraction methods, annealing temperatures, PCR cycles protocols, reagents, and primers concentration were also analyzed. The use of tetra-primer ARMS–PCR could be impaired for SNPs in DNA regions rich in cytosine and guanine and for samples with DNA not purified. The melting temperature was considered the factor of greater interference. However, small changes in the reagents concentration significantly affect the PCR, especially  $MgCl_2$ . Balancing the inner primers band is also a key step. So, in order to balance the inner primers band, intensity is important to observe which one has the weakest band and promote its band by increasing its concentration. The use of tetra-primer ARMS–PCR attends the expectations of modern genomic research and allows the study of SNPs in a fast, reliable, and low-cost way.

**Keywords** Polymorphism · SNP · Genotyping · Optimization · Tetra-primer ARMS–PCR

## Introduction

Since the completion of the Human Genome Project in 2003 [1] and the International HapMap Project in 2005 [2] up to recent studies, the single-nucleotide polymorphisms (SNPs) are considered as being one of the most important genetic markers due to its abundance in the genome and relatively easy analysis [3].

An SNP is the simplest form of polymorphism between two randomly selected genomes. It's basically a substitution of one nucleotide for another one at a specific location, which by definition is found in more than 1 % of the population [4]. It occurs at a frequency of approximately every 800 base pairs (bp) all over the genome, which has more than 9 millions SNPs [5].

Over the last years, genome-wide association studies have made possible to measure the associations between mapped SNPs and their presence in common complex conditions of large patient's groups, revolutionizing the study of many diseases [6].

Nowadays, considering the development of techniques for high-throughput genotyping, the genetic and genomic research has entered into a new age [7]. Researchers are now more than ever taking advantage of this technology to increase the number of well-validated gene-disease associations and produce genomic information every week [8]. The main methods of high-throughput genotyping are as follows: the Taqman technology [9], DNA microarray [10], MALDI-TOF mass spectrometry [11], and pyrosequencing [12].

However, all those methods require expensive tools and reagents, whose high cost is a secondary issue to be taken into account. In order to validate the association of a SNP to a

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determinate disease, a large number of patients must be studied to eliminate false results, thus, making the use of those methods unfeasible to laboratories with limited resources and to future routine diagnostic applications [13].

Polymerase chain reaction (PCR) is the most common technique used for low- and medium-throughput SNP genotyping [14]. Several PCR methods are available and selecting the most suitable one for each research is a critical step for successful study. Type of polymorphism, accuracy of genotyping, number of samples, and available PCR equipments are factors to be taken into account when making that choice [15].

A simple and economical SNP genotyping method which only involves a single PCR followed by gel electrophoresis is named tetra-primer amplification refractory mutation system-PCR (ARMS-PCR) [16]. It is based on the principles of the tetra-primer PCR and the ARMS techniques. Nevertheless, both methods are derived from the mismatch strategy to produce an allele-specific reaction. This allele-specific approach relies on the use of allele-specific primers containing a mismatch in their 3' terminus, making this primer specific to only one allele of the SNP and refractory to the other allele. Consequently, the DNA polymerase will only be able to extend a primer when its 3' end is perfectly complementary to the template. When this condition happens, a PCR amplicon is produced. By determining whether an amplicon is produced or not, the target DNA can be genotyped [17].

The difference between the tetra-primer and the ARMS techniques is the mismatch location. For the tetra-primer, the mismatch is located in the middle of the allele-specific primers and four primers are utilized in the reaction. However, in the ARMS system, the mismatches are located in the 3' terminus of the allele-specific primer and five primers are used. Another difference is that the tetra-primer genotyping is done in a single reaction, with two different annealing temperatures ( $T_A$ ), one higher in the first cycles and lower in the remaining cycles [16]. However, only one  $T_A$  is used in the ARMS, but the genotyping is made on two different reactions, amplifying on each reaction one of the alleles [18].

In the tetra-primer ARMS-PCR, the high specificity of the reaction does not rely only on the 3' terminus mismatch, but also on a deliberate mismatch at position-2 (second to the terminal) from 3' terminus of the same allele-specific primer. This extra mismatch destabilizes the base pairing between the primers and their corresponding non-targets templates and have been found to increase the specificity of the reaction by eliminating false-positives results [16].

As different mismatches have different destabilizing effects, both the terminal and penultimate mismatches have to be considered together when designing the primer [19]. As a result, these rules must be considered when adding the second mismatch: a strong mismatch (G/A or C/T

mismatches) at the 3' terminus of an allele-specific will require a weak second mismatch (C/A or C/T) and vice versa, whereas a medium mismatch (A/A, C/C, G/G or T/T) at the 3' terminus will likely require a medium second mismatch [16]. This combination when applied correctly and in conjunct with the right PCR reagents concentrations destabilizes unspecific amplifications in a way if the primers bind to the template this bond can only occur due to the perfect complementarity of the 3' terminus with the target nucleotide [20].

The tetra-primer ARMS-PCR uses four primers in a single PCR to determine the genotype. In the beginning of the reaction, two non-allele-specific primers amplify the region that comprises the SNP. They are named outer primers, then. As the outer primers fragment is produced, it serves as a template to the two allele-specific primers (inner primers) which will produce the allele-specific fragments [21]. By placing the outer primers at different distances from the polymorphic nucleotide, the two allele-specific fragments can be distinguished by their different sizes in an agarose gel [16] (Fig. 1).

The number of publications using the tetra-primer ARMS-PCR is growing, and novel variations of allele-specific methods are still being created, such as the simple allele discriminating PCR (SAP PCR) [7]. However, the optimization of these methods types can be very hard-working and time-consuming. Therefore, we propose to demonstrate and discuss the critical steps in the development of the tetra-primer ARMS-PCR technique in a way to provide useful information on its optimization stage. This kind of information lacks in the literature and is very helpful and time-saving to new users and could be easily applied for other allele-specific PCR.

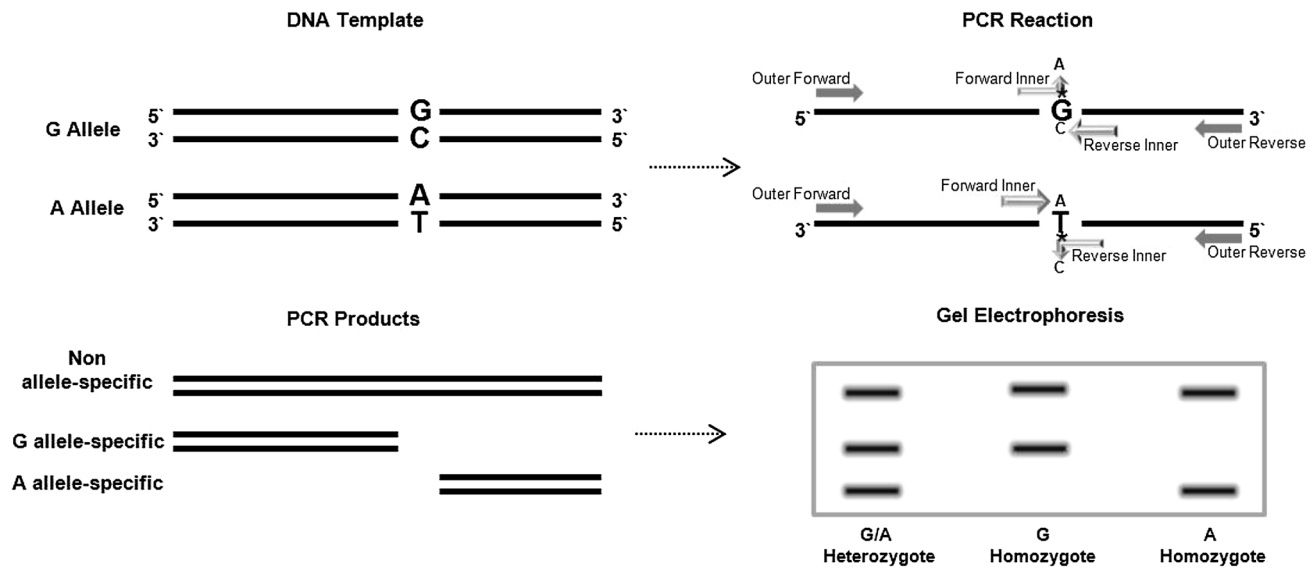
Two SNPs that would provide different amplification conditions were selected; one is located in a genome region with average percentage of cytosine and guanidine (CG) and the other one in a CG high-percentage region. The effects of DNA extraction methods, annealing temperatures, variations of PCR cycles protocols, reagents and primers concentration were also analyzed.

## Materials and Methods

### SNP Information

#### *SNP rs9550621*

It is located in the promoter of the connexin 26 gene (GJB2), at the relative position-3663 and has a polymorphism of A/G. It is in a high percentage of CG DNA region and near several CG boxes.



**Fig. 1** Schematic illustration of the tetra-primer ARMS-PCR assay for SNP genotyping. The C/T substitution of a heterozygote individual is presented as an example. *Upper* Two allele-specific amplicons are generated using two pairs of primers, one pair (*Outer Forward* and *Reverse Inner*) producing an amplicon representing the C allele and the other pair (*Forward Inner* and *Outer Reverse*) producing an amplicon representing the T allele. Specificity of the

inner primers is conferred by two mismatches, one between the 3'terminal base of an inner primer and the template, and the second at position-2 from the 3'terminus (indicated by an *asterisk*). *Lower* By positioning the two outer primers at different distances from the polymorphic nucleotide, the two allele-specific amplicons differ in length, allowing them to be discriminated by gel electrophoresis (Adapted from Ye et al. [16])

#### SNP rs3751385

It is situated in a DNA region with an average percentage of CG, in the intron 2 of the connexin 26 gene (GJB2), at the relative position 764 and has a polymorphism of C/T.

A scheme of the tetra-primer ARMS-PCR and the SNP location is represented in Fig. 4a.

#### Primer Design

The program developed by Ye [16] was used to design the primers, following their specifications and limiting the fragment sizes to the range of 200–700 bp with a ratio of allelic bands to 1,5. Default settings were used for other parameters (Table 1).

Computer software to design primer for tetra-primers ARMS-PCR is available at: <http://primer1.soton.ac.uk/primer1.html>.

#### Genomic DNA Extraction

The human genomic DNA was extracted from peripheral blood using the reagent DNAzol<sup>®</sup> (Invitrogen, CA, USA) and standard phenol–chloroform method. The study was approved by the Ethics Committee of UNIARARAS (protocol 744/2010).

#### Optimization and Development Strategy

The development of the tetra-primer ARMS-PCR was divided into three stages, because as it was originally described [16] the outer primers fragment is used for the inner primers as a template in order to produce the allele-specific fragments. So, we optimized in the first stage the right formation of the outer primers amplicon just using the outer primers.

The second stage was used the restriction fragment length polymorphism-PCR (RFLP-PCR) to obtain control samples. The genotype scored by this technique was used to validate the genotype obtained by the tetra-primers ARMS-PCR.

The third stage was to add to the reaction the inner primers and elaborate protocol changes till the genotype obtained by the tetra-primers ARMS-PCR is the same as the one obtained by the PCR-RFLP in all the control samples. This strategy planning is a good way to ensure a correct formation of all the fragments, since the number of unspecific amplifications is bigger when all the four primers are been used.

#### SNP rs9550621 PCR

The outer primers fragment of the SNP rs9550621 were amplified in a total volume of 30  $\mu$ l, containing 30 ng of DNA template, 0.33 pmol of each outer primer (Table 1),

**Table 1** Primers used in the tetra-primer ARMS-PCR technique

SNP	System	Primer sequence (5'–3')*	Allele	T <sub>m</sub> (°C)	Amplicon (bp)
rs9550621	Forward inner primer	CGACTCTCAGCCGCCCGGATG	G	75	258
	Reverse inner primer	CCTTGGCGGCCCGGGGT	A	75	361
	Forward outer primer	AAGCGGGACTTCGCCGCA		75	579
	Reverse outer primer	TTACGGAGGGGGCGGCC		75	
rs3751385	Forward inner primer	GCTCAGCTGTCAAGGCTCAGTCTCT	T	66	285
	Reverse inner primer	TCAGAACTTTGTGTTGGGAAATGATG	C	66	392
	Forward outer primer	TGGACCTACACAAGCAGCATCTTCTT		66	625
	Reverse outer primer	TTGTCCTCAGAGAAAGAAACAAATG		66	

T<sub>m</sub> Temperature of melting

\* The mismatches of the allele-specific primers are emphasized in bold

166 μM dNTP (Invitrogen, CA, USA), 1.3 mM MgCl<sub>2</sub>, 0.01 % BSA and 0.03 U of *Taq* DNA polymerase (Biotools, Madrid, Spain). The procedure consisted of denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 1 min, 70 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The fragment of 579 bp was visualized in 1.5 % agarose gel stained with ethidium bromide (Fig. 2a).

#### SNP rs3751385 PCR

The reaction used to amplify the region of outer primers was performed in a total volume of 30 μl, containing 30 ng de DNA extracted by the phenol–chloroform method, 0.33 pmol of each outer primer, 330 μM dNTP (Invitrogen, CA, USA), 2.5 mM MgCl<sub>2</sub>, 0.01 % BSA, and 0.08 U of *Taq* DNA polymerase (Biotools, Madrid, Spain). Initial denaturation at 95 °C for 5 min was followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 62 °C for 1 min and extension at 72 °C for 1 min. Final extension step was at 72 °C for 10 min. For genotyping the polymorphism of SNP rs3751385, the PCR products were digested with the restriction enzyme *Nhe* I (Fermentas®), according to the product's instructions. After electrophoresis in 1.5 % agarose gel and staining with ethidium bromide, the genotypes were determined. *Nhe* I digestion cleaved the 625-bp PCR products into two fragments of 364 bp and 261 bp when the T allele was present (Fig. 4b, c).

The tetra-primer ARMS-PCR was carried out in a final volume of 30 μl, containing 30 ng of the DNA extracted by the phenol–chloroform method, 0.33 pmol of each outer primer, 0.5 pmol of the forward inner primer, 0.83 pmol of the reverse inner primer, 166 μM dNTP (Invitrogen, CA,

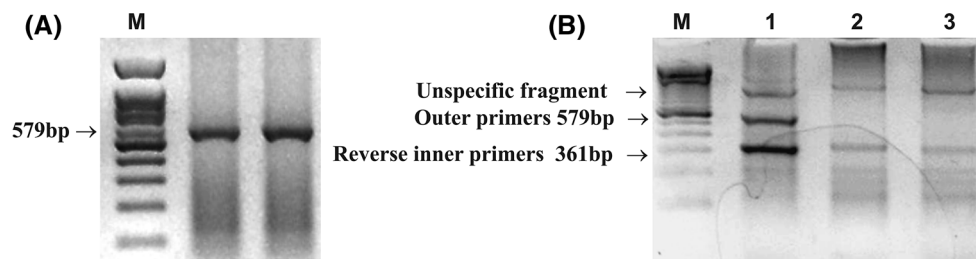
USA), 1.33 mM MgCl<sub>2</sub>, 0.01 % BSA, and 0.03 U of *Taq* DNA polymerase (Biotools, Madrid, Spain). The PCR cycle consisted 95 °C for 5 min, 35 cycles of 95 °C for 1 min, 60 °C of 1 min, 72 °C of 1 min, respectively, following 72 °C for 10 min. Products were analyzed by 1.5 % agarose gel electrophoresis, in TBE 1X buffer, containing ethidium bromide.

## Results

#### Development of the Tetra-Primer ARMS-PCR to Genotype the SNP rs9550621

The tetra-primer ARMS-PCR to genotype the SNP rs9550621 had one major difficulty; a persistent formation of unspecific bands. To overcome these problem were tested the PCR touchdown, hot start protocols, and the adjuvant betaine. However, all these strategies failed to improve the reaction and in some cases inhibited the amplification the outers primers fragment (Fig. 2b).

Among the PCR reagents, the major interference was the MgCl<sub>2</sub> concentration, because just by slightly lowering its concentration the unspecific bands tend to disappear, but the forward inner primer did not amplify as well. After several attempts, it was noticed that reagents modifications could not improve more the reaction and that it was very sensitive to the annealing temperatures variations. The forward inner primer, reverse primer, and outer primers have the same T<sub>m</sub> and were designed to amplify with a similar T<sub>A</sub>. Strangely, a common T<sub>A</sub> that could correctly amplify all the fragments was never found, and even the same T<sub>A</sub> that guaranteed the correct outer primer amplification was not compatible with the inner primers band formation. Therefore, the use the



**Fig. 2** **a** Amplification of the outer primers fragment of the SNP rs9550621. *M* 100-bp molecular weight marker. **b** SNP rs9550621 tetra-primer ARMS-PCR optimization. *1* Normal reaction; *2* Touchdown + hot start; *3* 1.5 M of betaine

tetra-primer ARMS-PCR technique to genotype SNPs in a high CG percentage region may not be appropriate, since a favorable combination of all the PCR reagents and cycles seems to be unworkable.

#### Tetra-Primer ARMS-PCR Development to Genotype the SNP rs3751385

When starting to optimize the tetra-primer ARMS-PCR, a set of factors have to be taken into account. The first one is the DNA extraction method, since it determinates the DNA quality and influences the reagents concentration. We have tested the tetra-primer ARMS-PCR using two different types of DNA qualities successfully; one was extracted with a simple and fast method, the reagent DNAzol<sup>®</sup> (Invitrogen), and the other using the standard phenol-chloroform method. For DNA samples extracted with DNAzol<sup>®</sup> reagent, although its protocol was optimized, the reaction lacked in reproducibility, because more than one assay was needed to genotype most of the samples. Likewise, the same protocol used for the DNAzol<sup>®</sup> did not work in the DNA samples extracted with phenol-chloroform, not showing any fragment on the gel. The main reason was a higher concentration of dNTP (332  $\mu$ M) and *Taq* DNA polymerase (0.06 U) in the DNAzol protocol compared with the phenol-chloroform. So, by lowering half of the dNTP (166  $\mu$ M) and *Taq* DNA polymerase (0.03 U) concentrations, the PCR bands could be visualized again in the phenol-chloroform samples.

The next thing that should be made after seeing the outer primer and inner primer band is to evaluate the annealing temperature. Therefore, the present  $T_A$  of 62 °C was raised and decreased 2 °C. Analyzing the effect of this change is a critical step, because even though with the  $T_A$  of 64 °C the outer primer fragment had a better amplification, there was no allelic discrimination. However, in the  $T_A$  of 60 °C even without the outer primers amplification, not only there was allelic discrimination, but the genotype scored was correct (Fig. 3).

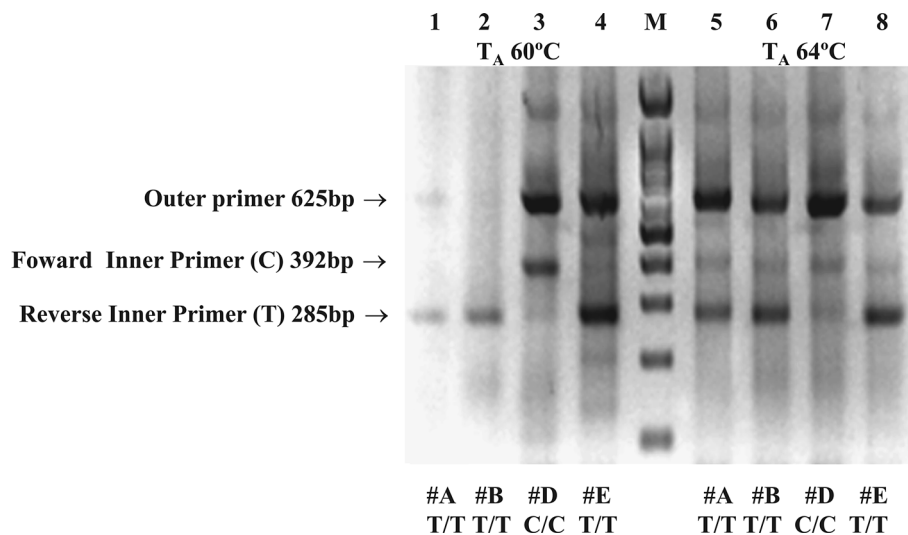
Proceeding with the 60 °C  $T_A$ , the outer primers fragment was stabilized and the intensity of the two inner

primers amplicons balanced. For that, changes in the PCR reagents will not work, because for now on the responsible for the success of technique is the delicate balance between the each primers concentration. A pattern among the intensity of the fragments and the each primers concentration could be designed. Changes of only 0.07 pmol of the outer primer are sufficient to modify in an expressive way its fragment intensity or to interfere on its formation. Modifications in the reverse inner primer (allele C) concentration slightly alter the intensity of its band; however, it directly affects the forward inner primer amplicon (allele T). In such case, when counterbalancing the concentrations of both inner primers, the fragment with the lower intensity (allele T) was favored, by keeping the reverse inner primer concentration and expressively raising the forward inner primer concentration. Therefore, the genotype scored by the tetra-primer ARMS-PCR was compared to the one obtained by the PCR-RFLP method and since there was no discordance in all control samples. The technique was considered as successfully validated (Fig. 4d).

#### Schematic Representation for the Tetra-Primer ARMS-PCR Technique Development

The information gathered during the development stage of the tetra-primer ARMS-PCR technique for identification of SNP rs9550621 and rs3751385 were collected and used to design a flowchart scheme that represents a guideline based on the success and failures encountered during stages of standardization of this analysis strategy. This diagram can be used as a guide, providing orientation in order to make the development easier and more practical, since it takes in consideration the conditions for the appropriate use of the tetra-primer ARMS-PCR method and also discuss possible problems faced during development, giving plausible solutions (Fig. 5).

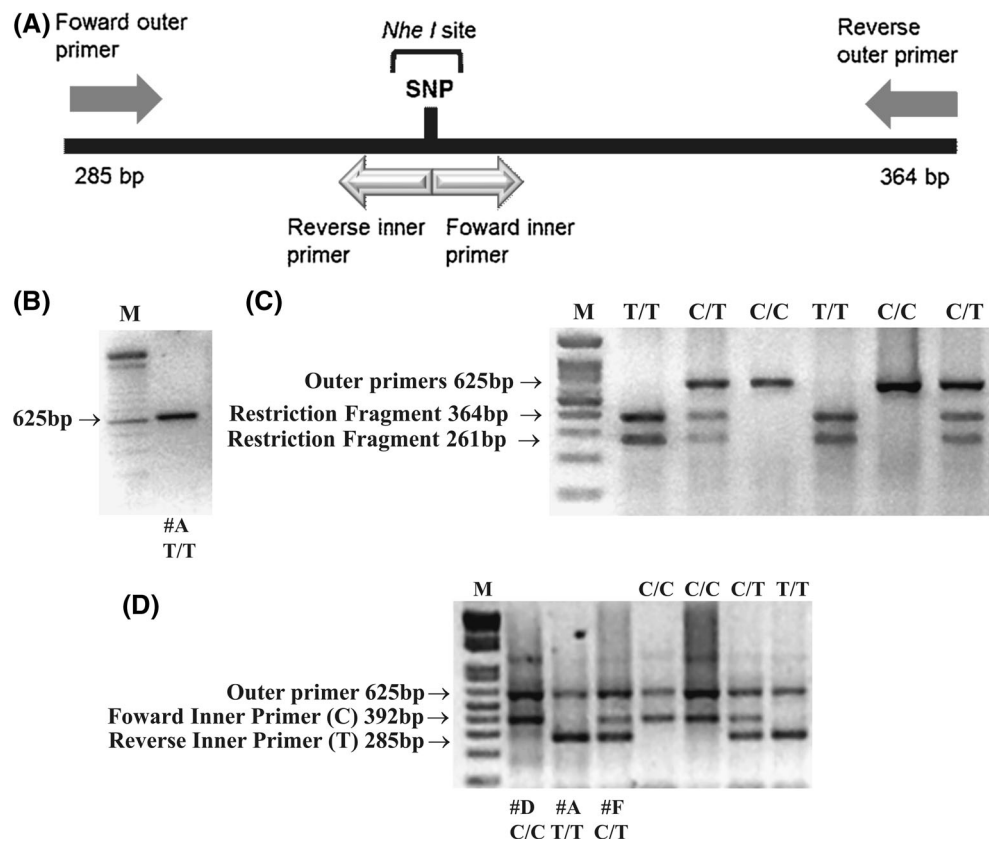
Nevertheless, it's worthy to note that this guide can be adapted. For example, the second step that consists in generating control samples with the PCR-RFLP may become the first one, or even skipped. Obtaining these control samples may be done first, specially, when the SNP



**Fig. 3** Evaluating the annealing temperature during the SNP rs3751385 tetra-primer ARMS-PCR optimization. In this figure, the same PCR reagents concentration was used in all samples, the difference between them is the annealing temperature of 60 or 64 °C. *M* Molecular weight marker of 100 bp. [1, 2]—Samples without the

outer primers band. [3, 4] Samples with the outer primer and correct allelic differentiation [5–8]. Samples with the outer primers band, but with wrong allelic differentiation. #A T/T, #B T/T, and #E T/T—control sample homozygote's for the T allele. #D C/C—control sample homozygote for the C allele

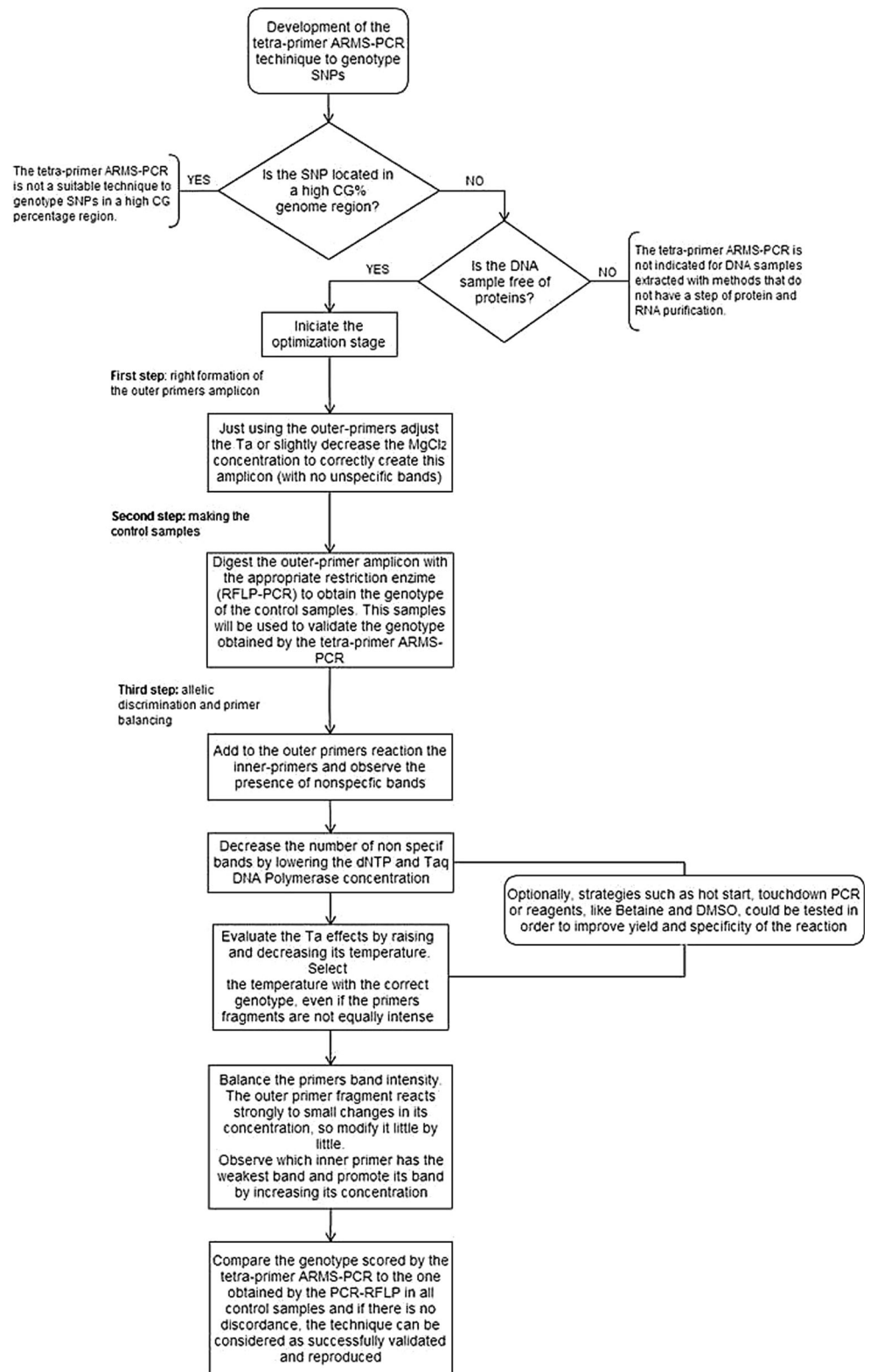
**Fig. 4** **a** Scheme of the tetra-primer ARMS-PCR and the SNP location. **b** Amplification of the outer primers fragment of the SNP rs3751385. *M* 100-bp molecular weight marker; #A T/T—control sample homozygote for the T allele. **c** SNP rs3751385 PCR-RFLP analysis with the enzyme *NheI*. T/T—Homozygote for the allele T; C/C—Homozygote for the allele C; C/T—Heterozygote for the alleles C and T. **d** Genotyping of the SNP rs3751385 by tetra-primer ARMS-PCR. #D C/C—control sample homozygote for the allele C. #A T/T—control sample homozygote for the allele T. #F C/T—control sample heterozygote for the alleles C and T. C/C—Homozygote for the allele C. T/T—Homozygote for the allele T. C/T—Heterozygote for the alleles C and T



cannot be genotyped by PCR-RFLP or when sequencing is available. Besides that, finding samples for all genotypes may not be necessary for low frequency SNPs, in which a homozygote of the minor allele will be impractical to find. In that case, using just a heterozygote is acceptable.

Another observation would be during primer balancing. As noted in Fig. 4d, the intensity of the outer primers band can be different between samples, and that's alright. If both inner primers bands can be produced and non-specific bands do not interfere in the result, the genotype can be interpreted

**Fig. 5** Flowchart representation of critical steps during the tetra-primer ARMS-PCR technique development and considerations about its use



anyway, regardless of the outer primers band is visible or not. In some cases, when all four primers are been used, the shorter amplicons may compete with the outer primer larger fragment by using de genomic DNA as the template.

Redesigning primers is also a step that can be added to the guide as troubleshooting option. Trying different primers in different combinations, rather than change reactions conditions, can be done in parallel. Moreover,

optionally, strategies such as hot start, touchdown PCR or reagents, like Betaine and DMSO, could be tested in order to improve yield and specificity of the reaction.

## Discussion

During the development stages were seen as determining factors: the temperature of melting, the DNA quality, the correct concentration of the PCR reagents and primers.

### Temperature of Melting

The melting temperature can be considered as the most important factor in order to achieve an allele-specific amplification [22]. This statement was also confirmed by our results, since the tetra-primer ARMS-PCR was successfully developed for the SNP rs3751385 and not to the SNP rs9550621.

The primers used for SNP rs3751385 did not have a high concentration of CG, ranging from 40 % (outer reverse primer) to 56 % (forward inner primer), resulting in a  $T_m$  of 66 °C. However, the primers of the SNP rs9550621 have a high percentage of GC, which ranges from 70 % (forward outer primer) to 83 % (reverse inner primer) thereby, resulting in a  $T_m$  of 75 °C.

The main difficulty in correctly amplifying primers with high CG percentage 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  $T_m$ , eventually the primers that have a high  $T_m$  will not be completely disnaturated and the DNA template will be partially open inhibiting the ability of the *Taq* DNA polymerase to act in those regions [23]. In 1998, McDowell et al. [24] demonstrated that GC-rich sequences in conjunct with high  $T_m$  leads to a formation of stable secondary structures in the primers, which can reduce the PCR efficiency by serving as termination or stop sites and an erroneous incorporation of an extra base caused by the local sequence at the pause site would be highly resistant to further elongation as with ARMs style assays [18].

Even with those problems, Chiapparino et al. [22] reported that primers that had a strong secondary structure and a similar  $T_m$  showed better results than those who lacked a strong secondary structure. In our results, the primers that did not had a strong secondary structure were the ones that gave the best results.

Nevertheless, several strategies have been elaborated to ensure a right amplification in CG-rich regions. Loening et al. [25] demonstrated that the reagent Betaine is capable to eliminate the *Taq* DNA polymerase effect to create termination sites, reducing the appearance of the unspecific

bands. The betaine acts destabilizing the secondary structures formed by the CG, equaling the forces between the AT and CG bases [26]. Different concentrations of betaine [0.5 M a 2.0 M] were tested, but the use of betaine intensified the unspecific bands, not the outer primers band.

Hubé and collaborators [23] described other alternatives, such as the hot start combined with using touchdown protocol which together significantly the CG-rich primers amplification. The hot start protocol is the addition of *Taq* DNA polymerase during the first denaturation cycle, and the touchdown is the increase of the  $T_A$  in 5–10 °C above the  $T_m$ , trough the initial PCR cycles. But neither the use combined nor separate of these protocols showed almost any improvement in the reaction.

After evaluating all of these protocols, we believe that the use of tetra-primer ARMS-PCR technique to study SNPs in regions with high concentration of CGs may not be appropriate. Despite here we only report this single failed case, others works [16, 22, 26] also reported SNPs that the tetra-primer ARMS-PCR could not analyze. In these cases, the authors discussed that their main problem was a non-specific amplification or the non-appearance of the inner primers fragment. This amplification failure was attributed to the terminal 3' mismatch, which turns out to be refractory to the outer primers fragment. Thus, we suggest that in these cases the primers should be redesigned or another methodology be used.

### DNA Quality

As observed in the third development stage of the SNP rs3751385, the optimized protocol for the samples extracted by the DNAzol<sup>®</sup> reagent had a very low reproducibility and did not work in the phenol–chloroform DNA samples. This problem occurred probably because in the DNAzol<sup>®</sup> extraction protocol, there is no purification process, thus hampering the amplification of fragments of interest. Accordingly, it was decided to use the standard phenol–chloroform method, which involves a purification of proteins and RNAs step and provides a DNA of high quality and purity. Furthermore, it could also be noticed that the PCR reagents concentration are in excess in unpurified DNA samples, since the concentration of *Taq* DNA polymerase and dNTP was almost 50 % higher in the protocol DNAzol than in the phenol–chloroform.

### Reagents Equilibrium

The PCR multiplex reactions are defined as reactions that amplify more than one fragment at the same time and that are employed more than one pair of primers. The main advantage is that once standardized they are very fast for laboratory routine and highly specific. But its development



requires a strategic planning, and many attempts to optimize reaction [28].

The usual difficulties during this optimization are the low sensitivity and specificity, and/or the amplification of unwanted products. The presence of more than one pair of primers increases the chances of getting non-specific products, because it can be formed dimers between the primers and the number of unwanted reactions increases exponentially during the PCR cycles [13].

From our optimization process, it was observed that the smallest changes in the reagents concentration significantly affect the quality of the tetra-primer ARMS-PCR. High concentrations of  $MgCl_2$ , favor the appearance of non-specific bands, and the concentrations of dNTP and *Taq* DNA polymerase are closely related. Since only with joint reduction of these two reagents, the outer primer amplification had a better reproducibility and the reaction was again visualized in the gel. Thus, the optimal concentration of  $MgCl_2$  will depend on the concentration of dNTP, the DNA quality, and composition [28]. Therefore, a way to increase the specificity and sensitivity of a multiplex and allele-specific technique, as the tetra-primer ARMS-PCR, is through the reduction in set of dNTP,  $MgCl_2$ , and *Taq* DNA polymerase.

### Primers Balancing

Before starting to balance the primers concentration, besides having optimized the PCR reagents concentration, the annealing temperature should already be defined as well. Balancing the concentration of primers should be done through a careful analysis of the fragments intensity, because each type of primer reacts differently to concentrations variations. 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 [16].

Garcés-Claver and collaborators [27] reported that the smaller fragment of the inner primers is disadvantaged by the formation of larger fragments, resulting in a weaker intensity of the band. Although our results indicate that the largest fragment of 395 bp (allele C) is inhibited by smallest fragment of 285 bp (allele T). The importance of this mechanism is to observe which inner primer has the weakest band and promote its band by increasing its concentration.

Ye et al. [16] proposed that the concentration (1:10) of the outer primer to the inner primers favored a specific amplification. But just like our study, Chiapparino et al. [22] also observed that this difference in concentration led to no amplification of the outer primers (data not shown). It

was also suggested by the authors [16, 27], the use of PCR touchdown protocol to strengthen the bands of the inner primers. But this protocol caused no improvement of the results (data not shown).

### Considerations About the Use of Tetra-Primer ARMS-PCR

Studies of SNPs in the promoter region as the SNP rs9550621 represents an important class of polymorphisms, since an SNP can eliminate a transcription factor binding site or even create a new site for a new transcription factor, modulating gene expression [29]. Many genotyping methods only detect 80 % of the mutations, given that polymorphisms in regions full of CG are not identified [4]. In a view of these facts, it was critical for us to evaluate the accuracy of the tetra-primer ARMS-PCR in genotyping an SNP in a CG-rich area.

As every method, the tetra-primer ARMS-PCR has its advantages and limitations. Its use is not indicated for SNPs in DNA regions rich in cytosine and guanine and for samples with DNA not purified. Though, it has been successfully used in several applications, for example, in the human diagnosis of spinal muscular atrophy simultaneously detecting the SMN1 and SMN2 deletion [30], to the evaluation of pork meat quality [31], and also to design markers for pepper cultivars [21].

Current techniques for SNP genotyping of medium and low cost are based on the polymerase chain reaction; nevertheless, many of them require post-PCR manipulations, such as radio-isotopes, restriction enzymes, or are required to two PCR rounds. The use of these reagents not just increase the methodology price, but also the time spent during its execution. The analysis of polymorphisms by the PCR-RFLP has proved to be extremely useful and created a massive amount of data in the literature, although it is relatively slow and limited, since its analysis is only possible if the polymorphisms creates or abolish a restriction enzyme site.

For research development and future clinical applications, the high-throughput techniques need to become more accessible. Newer, faster, and economic methods have to be developed and old methods improved. Therefore, the use of tetra-primer ARMS-PCR attends the expectations of modern genomic research, since it overcomes the limitations of many old methods and allows the study of SNPs in a fast, reliable, and low cost way.

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**Conflict of interests** The authors declare that there are no conflicts of interest.

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