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Providing an ARMS-PCR method for detecting of known SNPs

Ismaeil Gheshmzadeh[1]

[1] Bachelor of Molecular Cell Biology, Microbiology, Islamic Azad University (IAU), Qom Branch, Qom, Iran

Abstract

Pistachio dieback occurs in the main pistachio growing areas of Australia. Xanthomonas strains belonging to the translucens group have been identified as the causal agent of the disease and two distinct groups, A and B, have been recognised within the pathogen population. In this study, specific primers for amplification of DNA of the pathogen were developed by sequencing the Internal Transcribed Spacer (ITS) region of rDNA from strains representing groups A and B, as well as from X. translucens isolated from wheat in Australia and one Xanthomonas translucens strain from orchard floor grasses. Primers were designed for amplification of DNA sequences specific to each group and a multiplex PCR test was developed that identified and differentiated strains of each group in a single PCR assay. To determine the specificity of the primers, PCR was carried out with DNA from 65 strains of the pistachio pathogen, 31 type and reference strains of Xanthomonas, and from 191 phytobacteria commonly found in and around pistachio orchards. In the multiplex PCR, a 331 bp fragment was amplified from all strains belonging to group A and a 120 bp fragment from all strains in group B. No PCR products were obtained from the other bacteria tested except for the type strain of X. translucens pv. cerealis, which has not been found in Australia. The assay was used to detect strains from both groups of the pathogen in pistachio plant material.

Keywords: ARMS-PCR method, DNA, Internal Transcribed Spacer (ITS), SNPs

Introduction:

The ARMS PCR technique, fully known as Amplification Refractory Mutation System, is a reliable and rapid method for detecting spot mutations and polymorphisms and small deletions [1]. This technique can be used to isolate alleles of a gene that are even up to one pair, as well as to determine the genotype and to separate homozygous and heterozygous individuals for a gene locus. In the RFLP technique, it was said that sometimes there is no suitable restriction enzyme for the mutation and polymorphism, so we must use another technique, one of the most important and easiest of which is ARMS_PCR, but this technique also has its own limitations, especially from In terms of design, it has a special primer, which is the most basic element of this technique [2]. These specific primers only reproduce when the allele we want is present in the sample, for example, if a mutation has occurred and the allele is mutant and the primer designed for the allele is normal, our primer will no longer be able to replicate the sample. For this technique, we have to design 3 specific primers, which in fact, we design 2 Reverse primers or 2 Forward primers in such a way that the end of 3 of them is a mutant allele and the end of the other 3 primers is a normal allele, ie mutation. It is not found and now suppose we have designed the end of 3 Forward primers specifically, so we have to design a Reverse primer with a suitable distance from Forward primers and it depends on which of the F or R primers we design specifically for the allele [3]. Which side of the primer design conditions is more appropriate?

Since DNA polymerases such as Taq polymerase do not have exonuclease properties of '3 to' 5, they are suitable for this technique because if the mismatch at the end of '3 is between the primer and DNA template, the amplification efficiency is greatly reduced, so high poly fidelity DNA polymerases cannot be used [4]. Used like pfu and vent, but in addition adding another mismatch near the '3' end position with about 5 nucleotides remaining at the '3' end makes the technique work properly and increases the specificity of the reaction. For this purpose, we use 2 tubes for each sample, in both tubes we use a DNA sample, but in the first tube we use a primer that is specific to the normal allele, and in the second tube we use a primer that is specific to the mutant allele [5]. Depending on what allele the DNA contains, a replication reaction takes place in one of the tubes. This indicates that the person is homozygous, and if replication occurs in both tubes, the person has both normal and mutant alleles. Therefore, in this way, individuals can be genotyped according to a gene locus [6].

To ensure that no tubes amplify due to the absence of a complementary allele with a '3 primer' end, it is better to have a pair of internal control primers that attach to areas of DNA that do not have mutations and amplify a larger fragment [7]. In false negative conditions, none of the specific mutants and internal control primers are amplified and we must check the other conditions and materials used in the PCR reaction and ensure their accuracy. do . After PCR reaction in thermal cycler, the results are observed on polyacrylamide gel electrophoresis, mainly 3%, and genotype determination is possible. Finally, we realized that the reason for naming this technique Refractory is that the primer has a normal allele in the tube containing the mutant pattern DNA and the primer contains the mutant allele in the tube containing the pattern DNA with the normal allele [8].

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What is ARMS-PCR or allele-specific PCR?

The allele-specific PCR also called as an ARMS- PCR (amplification refractory mutation system) or PASA (PCR amplification of specific alleles) or AS-PCR used to detect the SNPs. More specifically, it is adopted to detect the known SNPs (single nucleotide polymorphism), however, we cannot identify new mutations by ARMS-PCR. *Kary Mullis* described the technique of *in vitro* amplification in the year 1983. After a few years of the discovery of the actual PCR technique, *C. R. Newton* and coworkers discovered the ARMS-PCR or allele-specific PCR technique [9].

ARMS PCR was first introduced in 1989 by Newton et al., Who studied defects in the α1 antitrypsin gene. This technique is able to distinguish heterozygous and homozygous individuals in terms of a gene locus. One of the advantages of this method is that it does not require the use of restriction enzymes. On the other hand, primer design for this technique is very accurate and professional [10]. As you know, the most important nucleotide in primers is the '3' end, where if it is not paired with the template strand, the polymerase enzyme will not be able to amplify the fragment and we will not see any PCR products. We use this principle in the design of primers for the ARMS technique [11]. Under the right conditions, oligonucleotides that do not complement the target sequence at the '3' end will not be able to bind to the target sequence, so we will not have an amplified product. Sometimes, even if the end of ‘3 is not complementary, the primer is still attached and the desired sequence is amplified [9]. To solve this problem, we add another mismatch in a position near the end of '3, which increases the specificity of the reaction. This extra mismatch is often in the penultimate nucleotide. If the SNP mismatch is strong, the extra open mismatch should be weak, and vice versa [12]. The technique is majorly used for the genotyping of the single nucleotide polymorphism with the help of the refractory primers. In this article, we will understand the allele-specific PCR/ ARMS-PCR and its importance in medical science. Further, we will explain the mechanism of how we can develop different primers for allele-specific PCR [13].

What is ARMS-PCR or allele – specific PCR? Before going to the actual process lets first understand the terms “Allele-specific PCR” and “ARMS-PCR. In the past, chemical methods were commonly used to produce nucleotide fragments, or to obtain multiple copies of a particular gene, the gene was inserted into a suitable vector and propagated into a bacterium. These methods were laborious and required a long time [14]. The Polymerase Chain Reaction (PCR) technique for amplifying a DNA fragment was proposed in 1984 by Cary Cary employee Kary Mullis. PCR had many advantages (including one-day examination of samples, relative cheapness, ease of performance, and highly specificity) and revolutionized clinical diagnosis of diseases, medicine, forensic science, microbiology, and many industries [15]. This technique solved all the previous problems in biomolecules that were caused by not having access to large amounts of the same DNA, and today it is a common task in almost all bimolecular laboratories and is done automatically by computer. Because of this discovery in 1993, the Nobel Prize was awarded to Kerry Mollies [16].

What is allele-specific PCR? The term suggests that the technique used in this type of PCR is specific to the particular type of allele [17]. An allele is the alternative form of a gene. If one

allele has an SNP and the other alternative form is normal, we can analyses both the alleles by designing specific primers for each allele [18]. PCR works like a photocopier, which can be used to duplicate as many pages of each creature's genome book as the original (albeit in some cases with minor errors) [19]. With this method, a gene can be amplified enough to be observed using methods such as electrophoresis. You can't see a single strand of hair from a distance of 6 meters, but a bunch of billions of hairs can be seen well together [20]. The basis of this method is very simple and is done like DNA replication reaction in living organisms by DNA polymerase enzyme [21]. In living organisms, a set of several proteins and enzymes are involved in the DNA replication process, while in the PCR reaction, only a specific type of heat-resistant DNA polymerase called Taq polymerase is used along with buffer, magnesium chloride, and nucleotides. In this technique, the desired DNA primrose is first designed and then, using PCR, the target is amplified and measured by electrophoresis against a control [22].

For that, we have to modify the single base at the 3’ end of the primer (one primer matches the normal allele and one primer matches the mutant allele). The PCR is performed simultaneously in a single reaction. If a mutant allele is present, then the PCR amplifies the mutant allele or if the normal allele is present, the normal allele will amplify

What is ARMS-PCR?

The *allele-specific PCR* is also called as the (amplification refractory mutation system) ARMS-PCR because of the use of two different primers for two different alleles. Here the word “refractory” is very important (Refractory= resistant to something) [23]. As we discussed, two sets of primers are designed, the mutant set of the primer is refractory (resistant) to the normal PCR and the normal set of the primers are refractory to the mutant PCR reaction.

That is why it is called an amplification refractory mutation system. The name ARMS-PCR is given by its actual developer *C. R. Newton.*

The principle of allele-specific ARMS-PCR:

The mechanism of the ARMS PCR is based on the modification of the primers for different alleles.

Here, the 3’ end of the primers is modified in such a way that one set of the primer can amplify the normal allele and others can amplify the mutant allele. The mismatch single base is introduced at the 3’ end of the primer. This mismatch allows the primer to amplify one single allele. **The concept of mismatch:**

Here the mismatch between the primer and the template DNA plays a crucial role in achieving the amplification.

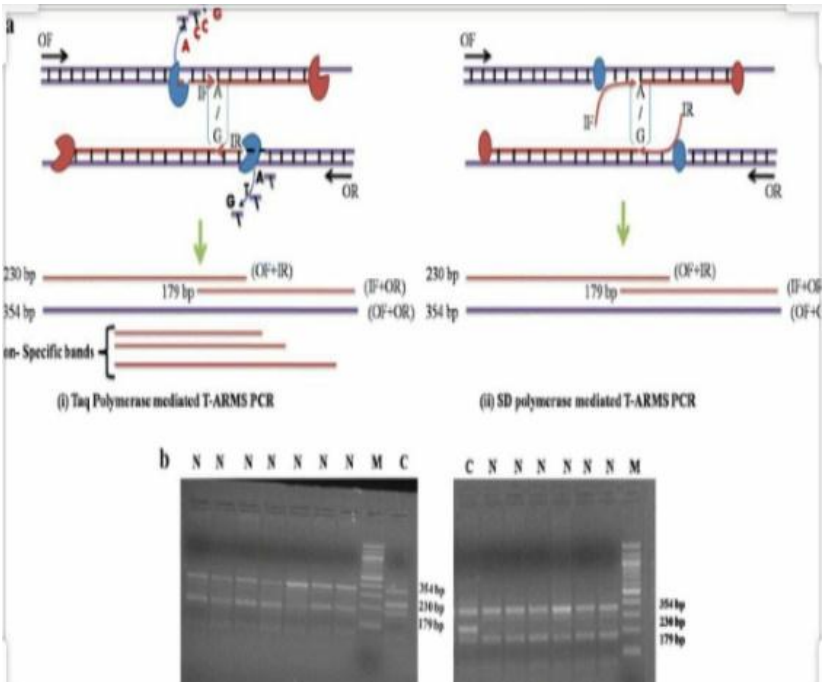
Introduction of a mismatch at the 3’ end of the primer alters the annealing temperature for that particular allele.

Strong mismatch: G/A, C/T, T/T

Medium mismatch: A/A, G/G, C/C,

Weak mismatch: C/A, G/T

What is ARMS-PCR or allele – specific PCR?



Due to the absence of the exonuclease activity of *Taq* DNA polymerase, the mismatch cannot be repaired. Why high fidelity Taq DNA polymerase does not use in the ARMS PCR? we will answer this question in the last section of this article.

How to do ARMS-PCR?

The process of the ARMS PCR is simple and very effective. No harmful radiolabelling is involved in the ARMS allele-specific PCR.

The procedure of ARMS PCR is divided into 4 steps:

1. Primer designing
2. Amplification
3. Agarose gel electrophoresis
4. Results interpretations

1. Primer designing for ARMS PCR:

The primer must be allele-specific.

There are several points we have to understand while designing a primer for the ARMS-PCR.

Now carefully follow the figure above,

Suppose our DNA sequence has G-A point mutation viz, G in normal allele and A in place of G in the mutant allele.

We have to design a forward primer in such a manner that for normal allele the primer contains C (complementary to G) at 3' end and the mutant primer contains T in place of C.

The magic of the present technique has happened when we add a mismatch base near to our SNP at the 3' end.

Why we have to add mismatch?

The mismatch is the key factor in achieving the amplification. If the mismatch is weak the chance of amplification is higher. Add strong mismatch near the 3' end of the primer (at -2 position ideally) hence, in the non-complementary allele it can not bind to the DNA sequences and therefore terminates amplification.

C:T, G:A and A:G are the strong mismatch base pairs which reduce the amplification process up to 100 fold.

The reverse primer or another primer (which is not modified) generally remains the same. Furthermore, the primer must fulfil all the criteria for the ideal primer. Read the primer design guideline: The primer length of the allele-specific primer must be between 20 to 30 nucleotides.

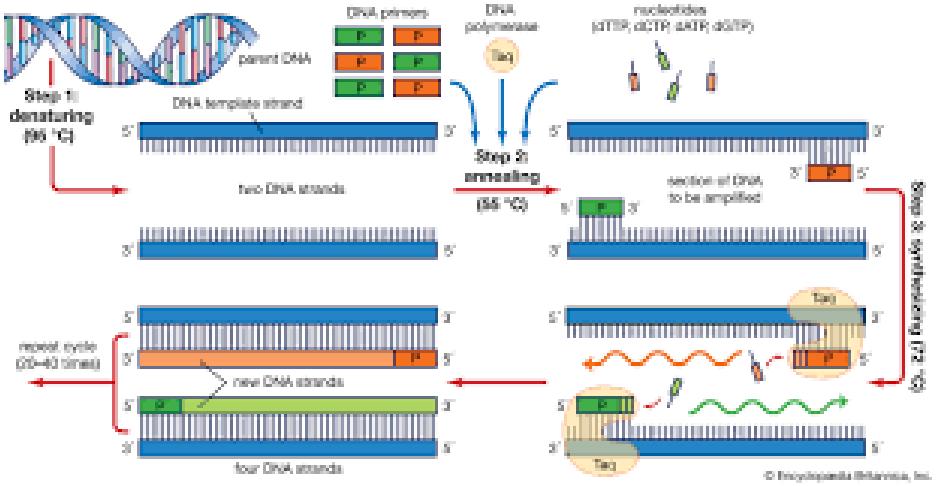
Once our primer is ready we have to modify the amplification conditions for achieving higher amplification.

2.Amplification conditions:

The annealing temperature should higher (do not compromise the annealing temperature).

The concentration of the PCR components is given into the table below.

The polymerase chain reaction:



Importantly, the PCR cycles for ARMS-PCR are lower than the normal PCR reaction. Set a PCR cycle between 22 to 25 but not 35. As the PCR cycles increase the chance of false-positive results increases

"An additional mismatch to the 3' end after the first mismatch increases the specificity of the ARMS-PCR reaction.

Here in the ARMS PCR, the internal control plays a crucial role, it facilitates additional accuracy in the allele-specific PCR by reducing the chance of the false-positive results.

3. Agarose gel electrophoresis:

The beauty of the ARMS PCR is that it does not require any hybridization steps. The amplified fragments are directly loaded on the 2% agarose gel for getting the results. The samples are loaded sequentially on the agarose gel and run for 45 minutes.

4. Results and interpretation:

First, observe the internal control. If the internal controls bands are present in all the wells indicates that our reaction is completely fine, no false-positive results are present.

Now analyses each band in normal as well as in mutant allele. The graphical representation of the agarose gel electrophoresis results are shown in the figure below, Suppose we have three samples one normal, one heterozygous carrier and one homozygous dominant. We have prepared 2 tubes for each sample, a total of 6 tubes with one positive control and two negative

controls. As shown into the figure above, an only single band is observed in the homozygous normal sample, two bands (one for normal and one for mutant) are observed in the heterozygous carrier sample and a single band of a mutant allele is observed in the homozygous disease condition. The wells 7, 8 and 9 are the controls.

PCR steps

1- Denaturation step

This stage is the first part of regular thermal cycles and involves heating the solution for 20 to 30 seconds at a temperature of 94 to 98 degrees [24]. Denaturation time and temperature depend on the number of G and C. The shorter the Ramp time (the time it takes for the device's source temperature to reach the target temperature), the better the result and the shorter the response time at the unwanted temperature. At this stage, due to the rupture of hydrogen bonds (between nucleotides), the template DNA and primers are separated and single strands of DNA are obtained.

2- Annealing step

The temperature of the solution is reduced to 30 to 65 degrees for 20 to 40 seconds. At this temperature, the two strands of each molecule can be reunited, but this does not happen because the mixture contains more small DNA molecules called primers, which are usually made up of 18-25 organic bases and into single-stranded DNA [25]. O pattern are connected. The bonding temperature is about 3 to 5 degrees lower than the melting point of the primers. PCR is very similar in principle to DNA replication based on the famous Watson-Creek laws and is actually derived from it. A is always paired with T and G with C. Therefore, stable hydrogen bonds are formed only when the primer sequence and the pattern string complement each other, and the pattern string always determines the play sequence of the opposite string.

3- Extension / elongation step

After binding to the primer-template hybrid, the polymerase enzyme initiates DNA replication by adding nucleotide triphosphates in solution to the OH-3 agent. . The temperature of the solution at this stage should be commensurate with the type of DNA polymerase used [26]. The optimum temperature for polymerase enzyme is about 75 to 80 degrees, usually 72 degrees is chosen. The duration of this step should also be proportional to the type of DNA polymerase, the number of bases between the two primers, and the length of the DNA strand. At optimum temperature, DNA polymerase polymerizes one thousand bases per minute. In the elongation step, if there is sufficient substrate and optimal conditions are met, the amount of DNA in the PCR solution is doubled.

4- Continuation of PCR

After the first cycle after these 3 steps, the first cycle ends and the next cycles are the repetition of the first cycle. Because in the previous step the DNA strand was doubled in the desired region, in this step there are four pattern strands to replicate [27]. Once again the system heats up and here eight copies are created, and in the next step 16 copies and so on following several cycles of the desired DNA fragment increases exponentially and in proportion to n2, which is n the number of temperature cycles. A DNA molecule, for example, multiplies to 1,047,586 molecules after 20 cycles. The number of cycles is selected according to the amount of initial DNA, test conditions and the amount of product required. It

should be noted that short target products are strands that are enclosed between two primers exponentially and long target products are strands that are bounded on one side by a primer and from the other side is infinite and increases linearly.

5- Final elongation stage

This step, after the last PCR cycle, is performed for 5 to 15 minutes at 70 to 74 ° C to ensure that all single DNA strands are replicated. The temperature of the tubes and the time intervals are a function of various factors such as the melting point of the primers (Tm), the type of DNA polymerase, the concentration of divalent ions and the concentration of NTPs.

6- Final hold

At this stage, the final solution can be stored for a short time at a temperature of 4 to 15 degrees.

7- PCR product detection

Finally, PCR can be validated by agrees or polyacrylamide gel electrophoresis, hybridization of the PCR product with labeled oligonucleotides (probes), electrophoresis with a molecular weight marker (DNA ladder containing DNA fragments of specified sizes), and Or examined ethidium bromide staining.

Key tips:

- Primer length should be 25 to 28 nucleotide
- Incorporate one mismatch at -2 positions at the 3’ end
- The second mismatch must be a weak mismatch
- The concentration of ARMS primer should be 10 pM and internal control primers concentration should around 1 to 3pM.
- PCR program must be short.
- The concetration of MgCl2 in PCR reaction is > 1.5mM

Importance of ARMS-PCR in the diagnosis of genetic disease:

The ARMS PCR is one of the important tools in the genetic disease diagnosis in recent days. The restriction digestion method is not 100% accurate and also, not all the sequences have the restriction site.

Therefore, restriction digestion is not applicable in all types of mutation or polymorphism.

The allele-specific PCR is an accurate method for single-gene disorders having the SNPs. It is also a gold standard method for Sickle cell anaemia and thalassemia like inherited disorder.

Also, it is applicable in mutation detection of *JAK2* and HIV. Commercial kits are now available for the different range of disorders such as HbS and JAK2 gene SNPs detection kits.

S.Kwok and coworkers used ARMS-PCR in HIV type 1 module studies. Click here for the research paper.

The pioneers C.R.Newton and coworkers used this technique for the ATT gene deficiency SNP analysis. Click here for the research paper.

The advantage of ARMS PCR:

- The technique is exclusively for the SNP genotyping.
- Further, homozygous and heterozygous can be detected by this technique

- It is widely applicable in the single gene point mutation detection such as sickle cell anaemia and thalassemia.
- It is fast, reliable accurate and rapid**.**

Limitation of ARMS PCR:

- Deletion/other major duplication and chromosomal abnormalities cannot be detected.
- Only known SNPs are detected by ARMS-PCR.
- Internal control is required because of the chance of the false-negative results.
- It is temperature-sensitive. A minute fluctuation in the temperature leads to false-positive results.
- Thousands of SNPs cannot be detected in a single assay.

Now answer to our question, Why high fidelity Taq DNA polymerase does not use in the ARMS PCR?

The high fidelity Taq DNA polymerase has the 3′ to 5′ exonuclease activity which removes the mismatch bases. that is why it is not used in the ARMS-PCR.

Conclusion:

The ARMS-PCR method is popular for the detection of known SNPs, however, it is a very tedious and time-consuming process to encounter more SNPs at once. Because it is allele-specific, the accuracy of ARMS-PCR is higher. Some of the deletions can also be screened using ARMS-PCR method. Using the PCR method and examining the presence or absence of a gene, the presence of mycobacteria, anaerobic bacteria and viruses can be examined and, for example, whether the AIDS virus is present in the body or No? Suppose your patient has an unknown disease and you think it may be caused by a virus. The virus cannot be easily cultured in the laboratory. By PCR method, the possibility of this virus can be controlled within 1 hour. That is, we give the patient a sample of the patient's body, as well as a fragment of the virus gene called a primer. If there is even just one of these viruses in a patient sample, the machine will make billions of copies of its gene. This large volume of gene can then be seen on a fluorescent film screen from electrophoresis. If DNA bands are found on the gene, we can be sure that the patient is a carrier of the virus. Another use of PCR is in the diagnosis of tuberculosis. Mycobacterium tuberculosis is a very slow-growing bacterium and its normal growth in the laboratory takes about 20 days. In tuberculosis PCR, a sputum sample is used along with primers that are complementary to the specific sequence of mycobacteria. After PCR, the obtained DNA fragments are placed in the presence of specific markers for different strains of Mycobacterium and thus, its species and strain is identified.

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