Amplification-Refractory Mutation System (ARMS) Analysis of Point Mutations

The amplification-refractory mutation system (ARMS), also known as allele-specific polymerase chain reaction (ASPCR) or PCR amplification of specific alleles, is a simple, rapid, and reliable method for detecting any mutation involving single base changes or small deletions. ARMS is based on the use of sequence-specific PCR primers that allow amplification of test DNA only when the target allele is contained within the sample and will not amplify the nontarget allele. Following an ARMS reaction the presence or absence of a PCR product is diagnostic for the presence or absence of the target allele.

The protocols detailed here outline methods that can be used to analyze human genomic DNA for one or more mutations. The basic protocol describes the development and application of an ARMS test for a single mutation; the alternate protocol extends this to multiplex ARMS for the analysis of two or more mutations. The support protocol describes a rapid DNA extraction method from blood or mouthwash samples that yields DNA compatible with the type of tests described.

Optimized ARMS tests are extremely straightforward and reliable to use—the key to success lies in the selection of appropriate primer sequences and reaction conditions. This unit describes not only the use of ARMS tests but also the process of test development.

NOTE: High-quality water (e.g., tissue culture grade) should be used in all solutions. Sigma double-processed tissue culture water has been shown to work well.

CAUTION: Radioactive, biological, and chemical substances require special handling; see *APPENDIX 2* for guidelines.

STRATEGIC PLANNING

The ARMS technique is based upon the observation that oligonucleotides that are complementary to a given DNA sequence except for a mismatched 3′ terminus will not function as PCR primers under appropriate conditions. For a robust and reliable ARMS test it is necessary to determine a combination of primer sequence and reaction conditions that will generate a detectable ARMS product from the target allele while minimizing false priming at the nontarget allele.

The solution suggested here is empirical and is based upon the development of a large number of ARMS tests. The approach is to standardize as many variables as possible (e.g., thermal cycling parameters, enzyme concentration, etc.) and to optimize the reaction by altering just two parameters, namely ARMS primer sequence (discussed in the following paragraphs) and ARMS primer concentration (which is optimized based on results of a pilot experiment, as described in the basic protocol).

The following guidelines are used to generate ARMS primers that will detect point mutations when used in combination with the reaction conditions outlined in the basic protocol. An example is given in Figure 9.8.1.

1. The ARMS primers should be oligonucleotides of around 30 or more bases. Primers less than 28 bases long should be avoided. Longer primers (up to 60-mers) may be used.
2. For the mutant-specific primer (M), the 3′ terminal base of the ARMS primer should be complementary to the mutation; for the normal-specific primer (N), the 3′ terminal base should be complementary to the corresponding normal sequence.

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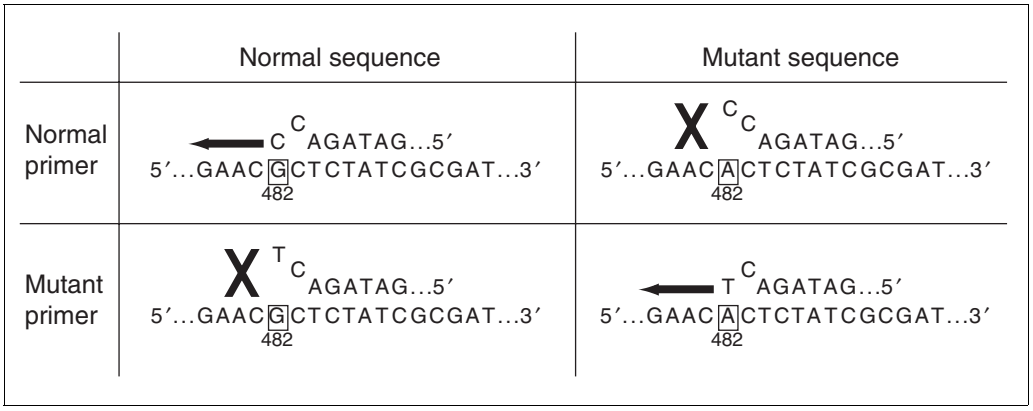

Figure 9.8.1 ARMS primer sequences for a single ARMS test. Sequences of the ARMS primers and target DNA sequences around the *R117H* mutation of the *CFTR* gene (G→A at position 482; Dean et al., 1990). The base that is altered is indicated in the normal and mutant DNA sequences by a box. The presence of an arrow indicates that primer/target combinations can be extended by *Taq* DNA polymerase; an "X" indicates extension does not occur. Bases in the ARMS primers that are not complementary to the target are shown displaced from the target sequence. A single mismatch (in this case a C/C) at the penultimate base is not sufficient to prevent extension whereas a primer with two adjacent mismatches, at the terminal and the penultimate base, is not extended.

Table 9.8.1 ARMS Primer Additional Mismatch Selection[a]

Terminal mismatch	Coding strand nucleotide corresponding to penultimate nucleotide in the primer			
	A	G	C	T
AA	A	G	A	G
AG	C	T	A	G
AC	G	A	C	T
TT	C	T	A	G
TG	G	A	T	C or T
TC	C	T	A	G
CC	C	T	A	G
GG	A	G	A	G

[a]The table indicates which base to include at the penultimate position of the ARMS primer. The left-hand column lists the 3′ terminal mismatches specified (i.e., the mutation in the coding strand with the normal base in the anti-coding strand) and the top row indicates the target base in the coding strand corresponding to the penultimate base in the primer.

3. Additional deliberate mismatches should normally be introduced at the penultimate base of the ARMS primer to increase the specificity of the ARMS reaction. Because different mismatches have been found to have different destabilizing effects, it is necessary to consider both terminal and penultimate mismatches together. If the mutation-induced terminal mismatch is strong, a weak additional mismatch should be selected, and vice versa, as indicated in Table 9.8.1.
4. The remainder of the ARMS primer should be complementary to the target sequence.
5. The design of the common primer is straightforward. The primer should be 30 bases long; selected to have ~50% G + C content, no 3′ complementarity with the ARMS primer or the internal control primers, and no repeated or unusual sequences (e.g., runs of a single base or palindromes); and, for use with the control PCR reactions suggested in this unit, should give a 150- to 250-bp PCR product.

ANALYSIS OF SINGLE MUTATIONS BY ARMS TESTS

A typical single ARMS test is comprised of two complementary reactions each conducted using the same substrate DNA. The first reaction contains an ARMS primer specific for the normal DNA sequence and cannot amplify mutant DNA at a given locus. Similarly, the second reaction contains a mutant-specific primer and cannot amplify normal DNA. Both reactions contain the same common PCR primer and also a pair of internal control primers that coamplify a different region of the genome (see Fig. 9.8.1).

The protocol describes a two-stage approach to designing ARMS tests. Primer sequences selected on the basis of the guidelines provided (see Strategic Planning) are used in ARMS reactions with known DNA samples. Based on the outcome of these pilot reactions, the tests are optimized by modifying the ARMS primer sequence and concentration.

Materials

*For recipes, see **Reagents and solutions** in this unit (or cross-referenced unit); for common stock solutions, see **APPENDIX 2;** for suppliers, see **SUPPLIERS APPENDIX.***

50 μM normal ARMS primer
50 μM mutant ARMS primer
50 μM common primer
50 μM each control primer A and B (or other appropriate pair; see recipe)
1 mM 4dNTP mix (*APPENDIX 2*)
10× PCR amplification buffer containing 12 mM $MgCl_2$ (*APPENDIX 2*)
Control DNA samples of known genotype (10 to 50 ng/μl in H_2O)
Light mineral oil
5 U/μl *Taq* DNA polymerase (Perkin-Elmer Cetus AmpliTaq or equivalent)
Nusieve 3:1 agarose (FMC Bioproducts)
1× TBE buffer (*APPENDIX 2*) containing 0.5 μg/ml ethidium bromide
Loading buffer (see recipe)
DNA molecular size markers
Test DNA samples (10 to 50 ng/μl in H_2O; support protocols)
Perkin-Elmer Cetus thermal cycler (480 or TC) and suitable reaction tubes

Additional reagents and equipment for agarose gel electrophoresis (*UNIT 2.7*)

Perform pilot ARMS reactions

1. Follow the ARMS primer guidelines outlined above (see Strategic Planning) to design normal, mutant, and common ARMS primers suitable for the mutation of interest. Select an appropriate control primer pair (see recipe). Obtain all primers and prepare 50 μM solutions of each.

   In general, use control primers A and B. If it is necessary to amplify a >300-bp ARMS product, use primer pair C and D. If the ARMS product has a high G+C content (>65%) and is >300 bp in length, use primer pair E and F.

2. Prepare normal (N) and mutant (M) ARMS reaction premixes according to the guidelines presented in Table 9.8.2. Dispense 40-μl aliquots of the premix into reaction tubes suitable for use in the thermal cycler.

   The amounts of reagents shown in the table are sufficient for ten ARMS reactions; they can be scaled up for larger numbers of reactions.

   The use of different-colored reaction tubes for the normal and mutant reaction mixes eliminates the need for labeling the premix aliquots. Use a fresh pipet tip for each successive transfer step.

   ARMS reaction mixes are stable at this stage and can be stored several days at room temperature or several months at −20°C.

Table 9.8.2 ARMS Reaction Premixes

Reagent	Normal ARMS reaction (N)	Mutant ARMS reaction (M)
50 µM common primer	10 µl	10 µl
50 µM normal ARMS primer	10 µl	—
50 µM mutant ARMS primer	—	10 µl
50 µM control primer A	10 µl	10 µl
50 µM control primer B	10 µl	10 µl
1 mM 4dNTP mix	50 µl	50 µl
10× PCR amplification buffer	50 µl	50 µl
H_2O	260 µl	260 µl

3. Add 5 µl of 10 to 50 ng/ml control DNA to each tube of an N and M premix pair at room temperature. If possible set up separate reactions containing DNA from normal, heterozygote, and homozygote individuals. Include a negative control test in which no DNA is added to the N and M tubes.

4. Add one drop mineral oil to each reaction tube and cap firmly. Microcentrifuge 10 sec at high speed.

5. Dilute *Taq* DNA polymerase by adding 12.5 µl of 10× PCR amplification buffer and 5 µl *Taq* DNA polymerase (1 U/5 µl final) to 107.5 µl sterile water. Mix by pipetting.

   This amount is sufficient for ten tests.

   Because the specificity of an ARMS reaction involves the nonextension of a 3′ mismatched nucleotide, it is essential that a DNA polymerase without 3′ to 5′ proofreading activity is used. Therefore, Taq DNA polymerase is suitable for ARMS reactions, but other DNA polymerases such as Vent and Pfu cannot be used (see CPMB UNIT 7.4A for more extensive discussion of the properties of different DNA polymerases).

6. Place tubes in thermal cycler block. Run 94°C hold program. After 5 min at 94°C, remove one tube from the block and add 5 µl *Taq* DNA polymerase dilution to the lower (aqueous) phase through the mineral oil layer. Return tube to 94°C block and repeat process until enzyme has been added to all tubes.

   This 94°C "hot start" for the PCR amplification helps eliminate nonspecific annealing of the primer to the template. Minimize the time each tube is out of the block at the enzyme addition stage to ensure that the temperature of the reaction mix does not drop significantly.

   Care should be taken to avoid depositing the enzyme into the oil layer.

7. Stop the 94°C hold program and immediately run the following amplification program:

35 cycles:	1 min	94°C	(denaturation)
	1 min	60°C	(annealing)
	1 min	72°C	(extension)
1 cycle:	10 min	72°C	(extension).

   If a rapid thermal cycler such as the Perkin-Elmer Cetus 9600 is used, follow the manufacturer's recommendations to alter the cycling times to suit the characteristics of this type of machine.

   At the end of the amplification program, it is advisable to leave the reaction tubes until the block temperature has reached 50°C to prevent the formation of artifact products.

   The amplified samples are stable up to 1 week at room temperature.

Visualize the reaction products

8. Prepare a 3% agarose gel using 4.5 g Nusieve 3:1 agarose and 150 ml of 1× TBE buffer with 0.5 µg/ml ethidium bromide.

   A 150-ml gel is suitable for the analysis of at least ten tests. Larger gels can be used if required. This gel results in good separation of the ARMS products. Other agaroses that give good separation in the product size-range may be substituted.

   CAUTION: *TBE buffer and gel containing ethidium bromide should not be allowed to come into contact with skin.*

9. Label a 0.5-ml microcentrifuge tube for each reaction. Add 10 µl loading buffer to each tube.

10. Transfer 25 µl ARMS reaction from beneath the oil layer into the corresponding labeled tube. Mix by pipetting.

    To avoid contaminating the laboratory with ARMS-product aerosols, it is preferable to uncap the reaction tubes in a fume hood or a separate laboratory.

11. Load 20 µl of each reaction per lane and include a DNA molecular size marker in one lane of the gel.

12. Electrophorese 1 to 2 hr at 120 V and photograph under UV transillumination.

Analyze the pilot reactions

13. Analyze each lane for presence of the expected-size control fragment and ARMS fragment (control fragments should be 360 bp, 813 bp, or 825 bp for primer pairs A and B, C and D, or E and F respectively). For both the normal and the mutant ARMS reactions, there are three potential outcomes:

    Expected outcome. ARMS product when target allele is present in sample; no visible product when target allele is absent.

    Nonspecific outcome. ARMS product when target allele is present in sample; ARMS product still visible when target allele absent.

    Insensitive outcome. ARMS product absent or faint even when target is present; no visible product when target allele is absent.

    a. If the expected result is obtained for both the normal and mutant ARMS primers, then the optimization is complete and the ARMS test is ready for use—omit steps 14 and 15 and proceed to step 16.
    b. If one or both of the ARMS primers is nonspecific, proceed to step 14a.
    c. If one or both of the ARMS primers is insensitive, proceed to step 14b.

       It is only possible to test for nonspecificity of an ARMS primer if a sample that is homozygous for the nontarget allele is available. This is usually straightforward for the mutant ARMS primer, as the appropriate sample is wild-type. For rare mutant alleles, testing the specificity of the normal primer can be difficult due to the rarity of homozygous mutant individuals.

Optimize the ARMS reactions

For nonspecific ARMS primer(s):

14a. Increase the reaction specificity by repeating the procedure starting at step 2 with two sets of reactions. Reduce the amount of ARMS primer in the two reaction mixes by two- and five-fold respectively.

   The concentration of the common primer is not altered.

Table 9.8.3 ARMS Primer Mismatch Strength Table[a]

Destabilization strength	Mismatch pairing(s)
Maximum	GA, CT, TT
Strong	CC
Medium	AA, GG
Weak	CA, GT
None	AT, GC

[a]Mismatches are grouped according to destabilization strength. At least two mismatch strengths are available for each base.

15a. If nonspecific bands are still observed, increase the strength of the additional mismatch at the penultimate base. Repeat the procedure starting at step 1.

See Table 9.8.3 for guidelines about how to increase mismatch strength.

For insensitive ARMS primer(s):

14b. Increase the sensitivity by repeating the procedure starting at step 2 with two sets of reactions. Increase the amount of ARMS primer in the two reaction mixes by two- and four-fold respectively.

An alternative option is to reduce the concentration of the control primers by two- and four-fold. The concentration of the common primer is not altered.

15b. If bands for the expected ARMS products are still weak or absent, decrease the strength of the additional mismatch at the penultimate base. Repeat the procedure starting at step 1. Decrease the additional mismatch strength by following the guidelines shown in Table 9.8.3.

Changing the additional mismatch in an ARMS primer often causes a large alteration in the specificity and sensitivity of an ARMS reaction. Altering the primer concentration has a smaller effect and can be used to fine-tune the reaction.

Following optimization of the ARMS test using DNA samples of known genotype, it is recommended that a large batch of ARMS reactions be made and divided into aliquots. The individual ARMS reactions are stable at least 6 months at −20°C.

Perform optimized ARMS tests

16. Using the information obtained from the pilot experiments to optimize reaction conditions, analyze test DNA samples by following steps 2 through 13 but replacing the control DNA with the test samples to be analyzed.

It is recommended that both negative controls without DNA and positive control samples of known genotype be included.

ALTERNATE PROTOCOL

ANALYSIS OF MULTIPLE MUTATIONS BY MULTIPLEX ARMS

It is often necessary to analyze a particular gene for the presence or absence of several different point mutations. One option is to carry out a series of single ARMS tests; the alternative approach—described in this protocol—is to develop a multiplex ARMS test that will allow simultaneous analysis of several mutations.

The approach is the same as that used for the single ARMS tests in that most of the variables are standardized and only primer sequences and concentrations are altered to achieve the desired result. The principle difference is that there are several primer combinations to be optimized simultaneously, which increases the complexity of the procedure. In view of this increased complexity it is not possible to outline a standard protocol for multiplex ARMS test development. The basic steps in the protocol are the same as for the single test development with the following differences:

1. In general, use less destabilizing mismatches in an ARMS test that is part of a multiplex than if it had been a single ARMS test. Use Table 9.8.1 (mismatch selection) followed by Table 9.8.3 (mismatch strength) to select less destabilizing mismatches.
2. The different ARMS products in the reaction are distinguished on the basis of size. It is best to plan the ARMS reactions so there is a ≥50-bp difference in the ARMS products' sizes. In some cases it will be necessary to use a control PCR amplification that gives a larger product; two alternatives are listed among the oligonucleotide primers (see Reagents and Solutions).
3. Combine several ARMS reactions into two multiplex reactions. Split the normal and mutant reactions so both multiplex reactions contain normal ARMS primers for some reactions and mutant ARMS primers for others. An example of a multiplex reaction containing two normal and two mutant primers is shown in Figure 9.8.2.
4. In testing for an autosomal recessive inherited disease gene, the PCR control reaction can be omitted if at least four mutations are analyzed simultaneously and both reaction tubes contain at least two normal specific ARMS reactions.
5. If the multiplex test is for several mutations in close proximity in the same exon, it is possible to use a single common primer in combination with a number of ARMS primers.
6. The multiplex protocol is essentially the same as for the single ARMS tests, except that it may be necessary to repeat the full procedure (steps 1 to 16) multiple times because there are several amplification reactions to optimize simultaneously.

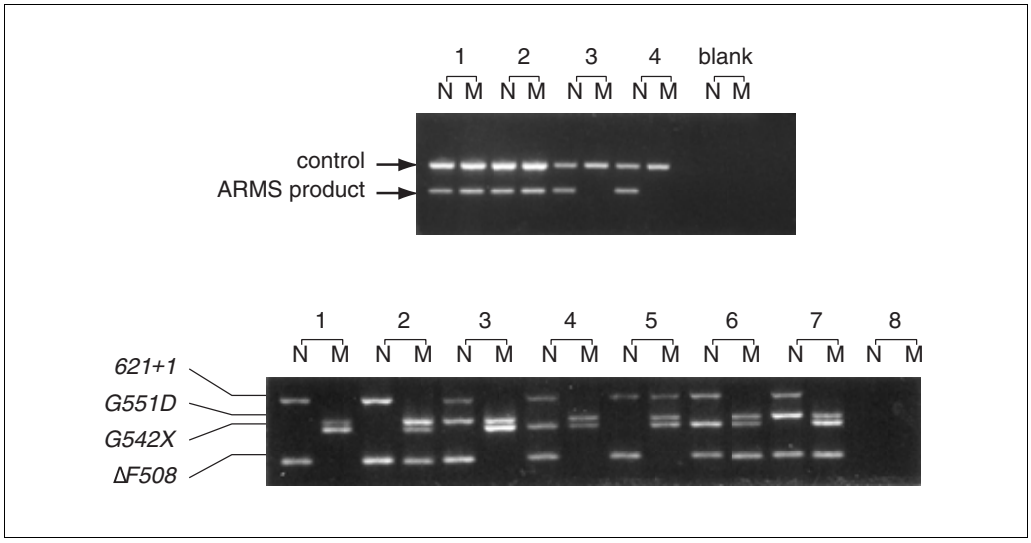

Figure 9.8.2 Single and multiplex ARMS tests. The upper panel shows the results from four individuals tested for the *R117H* mutation of the *CFTR* gene. Each ARMS test consists of two ARMS reactions specific for the normal (N) or mutant (M) sequences. The 3′ sequences of the ARMS primers are given in Figure 9.8.1. All sample lanes contain the 360-bp PCR control product amplified using control primer pair A and B. Samples 1 and 2 are from an individual heterozygous for the *R117H* mutation, whereas samples 3 and 4 are from normal individuals. The lower panel shows the results of seven individuals analyzed using a multiplex ARMS test for four common mutations of the *CFTR* gene (ΔF508, *G542X*, *G551D* and *621+1G→T*). There are two lanes for each sample. The first lane contains products of the normal ARMS primers for *621+1G→T* and ΔF508 and the mutant ARMS primers for *G551D* and *G542X*. The second lane contains the corresponding products for the *621+1G→T* and *508* mutant primers and the *G551D* and *G542X* normal primers. The location of the products of each primer set is indicated at the side of the figure. The genotypes of the seven samples are: 1, normal; 2, ΔF508 heterozygote; 3, *G551D* heterozygote; 4, *G542X* heterozygote; 5, *621+1G→T* heterozygote; 6, *G542X*, ΔF508 heterozygote; 7, *G551D*, ΔF508 heterozygote.

SUPPORT PROTOCOL

RAPID DNA EXTRACTION FROM MOUTHWASH AND BLOOD SAMPLES

Because of their ease of use, speed, and reliability, ARMS tests are often used to screen large numbers of DNA samples. The following protocol describes a rapid DNA extraction method for blood and mouthwash samples that provides DNA compatible with the ARMS reaction conditions described in this unit.

Materials

For common stock solutions, see ***APPENDIX 2.***

170 mM ammonium chloride (prepare fresh)
Blood sample, fresh or frozen
10 mM NaCl/10 mM EDTA
4% (w/v) sucrose in H_2O
50 mM sodium hydroxide
1 M Tris·Cl, pH 7.5 (*APPENDIX 2*)

1.5-ml screw-cap microcentrifuge tube
Rotator
25-ml plastic sample tube, sterile
Low-speed centrifuge
Boiling water bath

To extract DNA from blood:

1a. Add 800 µl freshly prepared 170 mM ammonium chloride to 200 µl blood in a 1.5-ml screw-cap microcentrifuge tube. Mix 20 min on a rotator.

The method is suitable for fresh or frozen blood with or without the addition of anticoagulating agents such as EDTA.

2a. Microcentrifuge 2 min at high speed to obtain a white cell pellet. Discard supernatant.

3a. Wash cell pellet with 300 µl of 10 mM NaCl/10 mM EDTA. Microcentrifuge 15 sec at high speed to pellet cells. Repeat wash three times to remove all visible hemoglobin.

To extract DNA from mouthwash samples:

1b. Vigorously agitate 10 ml of 4% sucrose in the mouth for 20 sec to produce a suspension of buccal epithelial cells. Collect suspension in a sterile 25-ml plastic sample tube.

To avoid any potential contamination problems, it is recommended that food and drink be avoided in the 30 min prior to sampling.

2b. Centrifuge 10 min at 1200 × *g*, room temperature, to collect cells. Discard supernatant.

3b. Resuspend cells in 500 µl of 10 mM NaCl/10 mM EDTA and transfer to screw-cap microcentrifuge tubes. Microcentrifuge 15 sec at high speed and discard supernatant.

4. Resuspend pellet in 500 µl of 50 mM sodium hydroxide and vortex 10 sec to resuspend white cell pellet. Incubate 20 min in boiling water bath.

5. Add 100 µl of 1 M Tris·Cl to neutralize and vortex 5 sec.

6. Microcentrifuge 15 sec at high speed to remove cell debris. Store supernatant at −20°C until use.

5 µl of DNA prepared in this way contains ~100 ng of DNA and is sufficient for a single ARMS reaction (2 × 5 µl per ARMS test). DNA can be stored at least 1 year at −20°C.

REAGENTS AND SOLUTIONS

Loading buffer

70% (v/v) 1× TBE buffer (*APPENDIX 2*)
30% (v/v) glycerol
0.1% (w/v) bromphenol blue

Oligonucleotide primers

Control primer A
5′-CCCACCTTCCCCTCTCTCCAGGCAAATGGG-3′
Control primer B
5′-GGGCCTCAGTCCCAACATGGCTAAGAGGTG-3′

These primers amplify a fragment of the ATT gene and generate a 360-bp PCR product.

Long control primer C
5′-CCAAGCCCAACCTTAAGAAGAAAATTGGAG-3′
Long control primer D
5′-CCAAACCCACGGTACGCATGGGAACACTGC-3′

These primers amplify a fragment of the APC gene and generate a 813-bp PCR product.

High G+C control primer E
5′-CTCACCTGCGTCAGGAGAGCACACACTTGC-3′
High G+C control F
5′-CATCGAGACCTCGGCCAAGACCCGGCAGG-3′

These primers amplify a fragment of the RAS gene and generate a 825-bp PCR product.

COMMENTARY

Background Information

Over the past few years there has been an exponential increase in the number of human genes cloned and sequenced coupled with an increase in the number of identified mutations and polymorphisms that are of clinical relevance. As a consequence there is now an increasing demand for analysis of human genetic variation in the fields of inherited disease testing, cancer diagnostics, tissue typing, and disease predisposition analysis.

For clinical applications, the analysis technique must be simple and reliable and should allow analysis of several mutations simultaneously and be suitable for all point mutations. The amplification-refractory mutation system (ARMS) technique (Newton et al., 1989; see also Okayama et al., 1989; Wu et al., 1989; Sarkar et al., 1990) has a number of features that make it particularly suited for routine applications.

Reliability and test design. An ARMS test is specific when, using the detection system of choice (e.g., agarose gel electrophoresis), the yield of product from the target allele can be detected and the yield of product from the nontarget allele is not detectable. The process of ARMS test design and development essentially consists of the determination of reaction conditions that will allow this discrimination between target and nontarget alleles. The protocols outlined in this unit describe one approach to the development of ARMS tests based on the alteration of the ARMS primer sequence and concentration. Alternative strategies are also possible (Sommer et al., 1992).

The ARMS technique involves the extension or nonextension of an ARMS primer hybridized to target DNA. The yield of PCR product from a particular ARMS primer and target DNA combination is governed by two variables—the rate of hybridization of the ARMS primer to target DNA and the rate at which the bases at the 3′ end of the ARMS primer form a suitable substrate for *Taq* DNA polymerase (Wu et al., 1989). The approach in this unit is to use long (>28-mer) ARMS primers to minimize any differences in the stability of annealed primers between the target and nontarget allele. It is then the rate of suitable substrate formation that governs the overall rate of the reaction. The advantage of this approach is that the rate of hybridization is sensitive to small changes in ionic strength and temperature, whereas the rate of extension appears to be less affected by such changes. As a conse-

quence, the temperature window in which an ARMS reaction will give the correct result is larger than the corresponding window for techniques based solely on hybridization, such as allele-specific oligonucleotide (ASO) hybridization (*UNIT 9.4*). This makes ARMS a reliable technique that, once optimized, will perform in a reproducible fashion.

Applicability. There are many published examples of single and multiplex ARMS tests that include all possible combinations of transitions and transversions (Sommer et al., 1992). It seems likely that the ARMS technique is suitable for all point mutations. ARMS is particularly relevant where there is a need to analyze a large number of samples for one or more mutations.

An additional application of ARMS is based on the ability of the system to selectively amplify rare mutant sequences in a background of wild-type DNA. This makes ARMS testing suitable for cancer screening, where only a small fraction of the DNA being tested may carry a mutation (Sidransky et al., 1992).

Multiplex test development. Development of multiplex ARMS tests is more complex than for single tests, but once optimized the use of the tests is straightforward and their performance is reliable (Ferrie et al., 1992; Fortina et al., 1992). A particular advantage of the ARMS approach is that there is no need for the mutations to be located in the same exon, or even the same gene, because the method does not require initial amplification of a region of DNA for subsequent analysis.

Critical Parameters and Troubleshooting

Criteria for success. The key to developing a successful ARMS reaction is to ensure that the ARMS primer allows efficient amplification from the target allele while minimizing amplification from the nontarget allele. This unit outlines a reliable iterative approach to this problem. At present, the rules governing ARMS primer specificity are not completely understood and the available ARMS primer design guidelines are based on empirical observations. These guidelines generally work well, but it should be noted that the DNA sequences around the priming sites are also important. The combination of terminal and penultimate mismatches that gives an optimized ARMS test may not be that initially selected using Table 9.8.1.

Although the test optimization protocol is reliable, it is still possible that on rare occasions there may be problems of specificity or sensitivity with a particular reaction. The following are additional suggestions for these circumstances:

(1) Increase specificity by increasing the annealing temperature. This is not normally recommended as the resulting test will tend to be temperature-sensitive with an increased risk of producing a false-positive result.

(2) Alter the primer sequences by switching the direction of the ARMS reaction. There is no particular advantage in designing the ARMS primer to complement the sense rather than the antisense strand of a given gene. If a reaction is proving problematical to optimize, switching the direction of the ARMS primers allows a second attempt at test development.

There are a number of other parameters, some common to all PCR amplifications and some specific to ARMS reactions, that are important in test design and application.

ARMS primers. The ARMS primers must be of good quality. There will always be a small fraction of failure sequences in any oligonucleotide synthesis reaction, but because oligonucleotides are synthesized in a 3′→5′ direction, failures will not produce truncated primers that can allow mispriming. It is unnecessary to purify the ARMS primers.

PCR contamination. As with all PCR–based techniques, precautions must be taken to prevent contamination with the products of earlier reactions. This is particularly the case when an ARMS test is used regularly as part of a screening program. Because the tests are stable at least 6 months at −20°C, it is recommended that a large batch of test aliquots be prepared under clean conditions and stored for future use. It is also good practice to use separate laboratories for PCR setup and analysis, preferably under positive and negative air pressure respectively. See *APPENDIX 2A* for additional guidelines for preventing PCR contamination.

DNA samples. The least controllable variable in the ARMS test process is the test DNA sample. The support protocol outlines a rapid DNA extraction process that has been shown to work well with the reaction conditions described in this unit. One potential concern over the use of mouthwash samples is inhibition of the PCR by foodstuffs carried over in the sampling procedure. In a limited study of likely contaminants (snack foods, fruit, chocolate, coffee, etc.), no inhibition was observed. Nevertheless, as a precaution it is recommended that no food be con-

sumed for 30 min prior to sampling.

DNA prepared by other methods is also suitable for ARMS reactions. Any problems encountered are usually due to the presence of PCR inhibitors in the sample and can generally be solved by diluting the DNA sample 10- to 100-fold. If this fails, it may be helpful to boil the sample 5 min in 50 mM NaOH, then neutralize by adding $\frac{1}{10}$ vol of 1 M Tris·Cl, pH 7.5.

Other parameters. Other potential problems with ARMS are addressed in the troubleshooting guide in Table 9.8.4.

Table 9.8.4 Troubleshooting Guide

Problem	Possible cause	Solution
ARMS and control bands faint and/or absent in all reactions	Inactive enzyme	Use fresh batch of *Taq* DNA polymerase
	Component omitted from reaction	Repeat ARMS from reaction preparation
ARMS bands faint and/or absent in all reactions; control bands normal	Failure to denature high-G+C ARMS product	Increase denaturation temperature to 96°C
		Add 10% (v/v) glycerol to mix
	Insensitive ARMS test	Increase primer concentration, decrease mismatch strength
ARMS and control bands faint and/or absent in some reaction pairs	Sample-borne PCR inhibition	Dilute sample 1:100
	Insufficient DNA in affected samples	Add 10× more sample
ARMS bands absent in some reactions; control bands normal	Control reaction too efficient	Reduce concentration of control primers
Faint additional bands same size as ARMS products	Contamination	Check negative controls
	Excess enzyme	Check enzyme dilution
	Nonspecific ARMS test	Reduce primer concentration, increase mismatch strength
Faint additional bands, different size from ARMS products	Excess enzyme	Check enzyme dilution
	Cooling artifacts	Allow reaction tubes to cool to room temperature in PCR block
	Spurious priming elsewhere in genome	Identify specific primer(s) causing problem and redesign
Streakiness in some lanes	Excess DNA	Add less DNA
PCR products ~50-60 bp	Primer dimers	Ensure hot start is used Check 3′ ends of primers for complementarity
First lanes on gel give extra bands; later lanes give faint bands	Insufficient mixing of enzyme dilution	Increase mixing

Anticipated Results

DNA samples that contain the target allele will allow amplification of an ARMS product. The use of two separate ARMS reactions (normal and mutant) will allow discrimination of homozygotes and heterozygotes for a given allele. For some applications (e.g., genetic screening) such discrimination may not be required and the normal ARMS reaction may be omitted. Examples of typical results from a single ARMS test for the *R117H* mutation of the *CFTR* gene and a multiplex ARMS test for four additional mutations of the *CFTR* gene are given in Figure 9.8.2.

Time Considerations

Following optimization, the complete process from sample preparation to analysis can easily be performed in a single day. The thermal-cycling parameters have not been optimized for a rapid thermal cycler such as the Perkin-Elmer Cetus 9600; use of such an instrument could allow further reduction in the time needed to complete the protocol.

Any commercial inquiries regarding ARMS should be addressed to Cellmark Diagnostics. ARMS is the subject of European Patent No. 0332435 (ZENECA).

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Key Reference

Ferrie et al., 1992. See above.

Provides background information on ARMS and gives clinical data that support the reliability claims for the method.

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