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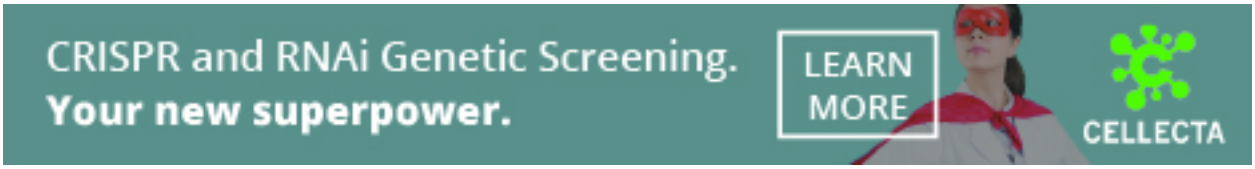

Mismatch Amplification Mutation Assay (MAMA): Application to the c-H-*ras* Gene

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We have found that under appropriate conditions, an allele-specific polymerase chain reaction (PCR) can achieve a sensitivity suitable for measuring specific, infrequent mutations in single cell systems or in animal tissues. Using the 12th codon GC-to-AT mutation in the rat c-Ha-*ras* gene as a model system, we have defined conditions that allow for measurement of mutations present at frequencies as low as one in 10^5 gene copies. Our approach involved the use of PCR primers that created a single mismatch with the mutated allele (GAA) but created a double mismatch with the wild-type allele (GGA). Five out of the six such double-mismatch primers we tested permitted amplification of the mutant allele (GAA) with a high degree of specificity. The specificity of the assay was further enhanced by using a two-step PCR cycle consisting of a denaturation step (1 min incubation at 94°C) and an annealing/extension step (1 min incubation at 50°C) in the presence of 10% (vol/vol) glycerol. Reconstruction experiments using genomic DNA demonstrate that this procedure can measure the presence of 30 copies of the transforming *ras* allele present amongst 3×10^6 copies of the wild-type allele.

Allele-specific PCR is a modification of the polymerase chain reaction that permits specific amplification of sequences differing by as little as a single base pair.[1–5]. The technique utilizes primers with specific mismatches at or near the 3′ end to permit preferential amplification of one allele relative to another. Allele-specific PCR offers several advantages over other molecular techniques, such as restriction fragment length polymorphism (RFLP)[6] or differential oligonucleotide hybridization,[7] that are routinely used for measuring known point mutations. The technique is rapid and simple and can readily be applied to analyze nanogram quantities of genomic DNA.[8,9] However, one drawback of the currently available allele-specific PCR protocols is their lack of sensitivity. For instance, whereas RFLP in combination with PCR can detect a point mutation in genomic DNA samples occurring at a frequency of 1 in 10,000 or less,[10,11] the previous estimate of the sensitivity of the allele-specific PCR (determined by ethidium bromide staining) was about 1 in 40.[8] This low sensitivity is due to the fact that primers with mismatches at or near the 3′ end are still able to extend to some degree under most PCR conditions. Kwok et al.[12] estimated the efficiency of a single-base mismatch primer extension to be between 15% and 50% per cycle of the efficiency for a perfect match primer extension, depending on the nature of mismatches.

The sensitivity of mutation assays is not critical when analyzing a relatively homogeneous population of cells where a mutation is shared by a significant fraction of the population. However, the study of a heterogeneous cell population where only a small fraction of the cells ($\leq 10^{-4}$) are expected to contain a mutation necessitates the use of an assay with a high level of sensitivity. In our attempt to improve the sensitivity of the technique, we optimized the procedure by varying (1) the number and kinds of mismatches in the primers, (2) the temperature and duration of the primer annealing and extension step, and (3) the solvent condition of the reaction mixture. As an example, we utilized one of the transforming alleles of rat c-H-*ras* gene (G-to-A transition at the 12th codon) as the specific sequence to be amplified. In this paper, we describe the modified allele-specific PCR protocol, which we have designated as the mismatch amplification mutation assay (MAMA), and we demonstrate that the technique is sensitive enough to detect 30 copies of the transforming allele present amongst 3×10^6 copies of wild-type allele.

MATERIALS AND METHODS

Development of MAMA

Development of this protocol involved four variables: (1) mismatch primer sequence, (2) temperature of primer extension, (3) time permitted for extension, and (4) concentration of glycerol. We tested and optimized each of these variables as described below.

Mismatch Primers

Because a single G-to-A transition was to be assayed, we created a series of primers that contained an A in the appropriate position and varied the base 3′ or 5′ to

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this position. This series of six potentially useful double-mismatch primers contained one mismatch within the 3′-CTT-5′ sequence of the mutated allele but two mismatches with the 3′-CCT-5′ sequence of the wild-type allele (Table 1). To test the report that a single 3′ ultimate or penultimate mismatch would not yield a preferential amplification,[1,12] two primers with these single mismatch configurations (PA and PA-1) were also prepared, as were the two primers carrying triple mismatches to the wild-type sequence (Table 1). All the primers tested are 20 nucleotides long and synthesized by Research Genetics (Huntsville, AL).

Temperature and Duration of Primer Extension

Instead of using a standard three-step PCR cycle consisting of a denaturation step, a primer annealing step, and an extension step,[13,14] we tested two-step PCR cycles consisting of a denaturation step and a primer annealing/extension step. The first step was kept constant at 94°C for 1 min, while the second step was varied. Temperature was varied between 48°C and 57°C, and the extension was permitted to proceed for either 1 min or 5 sec.

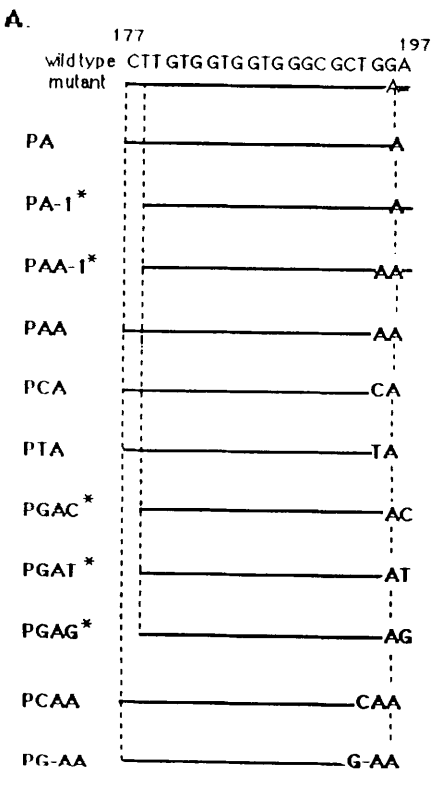
Concentration of Glycerol

To test whether solvent condition would affect the specificity of allele-specific amplification, we modified the PCR reaction mixture by an addition of either 5% or 10% (vol/vol) glycerol.

DNA Preparation

Wild-type genomic DNA was obtained

TABLE 1 The Mismatch Primers Tested for Allele-specific Amplification



	Wild type allele		Transforming allele	
B.	5'- GCT GGA -3' 3'- CGA CCT -5		5'- GCT GAA -3' 3'- CGA CTT -5'	
	Mismatches	**$Y_{o(wt)}$****	**Mismatches**	**$Y_{o(t)}$****
PA	5' - CTG**A** - 3' 3' - GAC**C**T -5'	0.70	5'- CTGA -3' 3'- GACTT -5'	0.70
PA-1*	5'- CTG**A**A -3' 3'- GAC**C**T -5'	0.70	5'- CTGAA -3' 3'- GACTT -5'	0.70
PAA-1*	5'- CT**A**AA -3' 3'- GA**C**CT -5	<0.007#	5'- CT**A**AA -3' 3'- GA**C**TT -5'	0.70
PAA	5'- CT**A**A -3' 3'- GA**C**CT -5'	≤0.0002##	5'- CT**A**A -3' 3'- GA**C**TT -5'	0.70
PCA	5'- CT**C**A -3' 3'- GA**C**CT -5'	<0.007	5'- CT**C**A -3' 3'- GA**C**TT -5'	0.70
PTA	5'- CT**T**A -3' 3'- GA**C**CT -5'	<0.007	5'- CT**T**A -3' 3'- GA**C**TT -5'	0.70
PGAC*	5'- CTG**AC** -3' 3'- GAC**CT** -5'	<0.007	5'- CTGA**C** -3' 3'- GACT**T** -5'	0.70
PGAT*	5'- CTG**AT** -3' 3'- GAC**CT** -5'	<0.007	5'- CTGA**T** -3' 3'- GACT**T** -5'	0.70
PGAG*	5'- CTG**AG** -3' 3'- GAC**CT**- 5'	0.70	5'- CTGA**G** -3' 3'- GACT**T** -5'	0.70
PCAA	5'- C**CAA** -3' 3'- G**ACC**T -5'	<0.007	5'- C**CA**A -3' 3'- G**AC**TT -5'	<0.007
PG-AA	5'- **GTAA** -3' 3'- **GACC**T -5'	<0.007	5'- **GTA**A -3' 3'- **GAC**TT -5'	0.58

A. The solid line represents the nucleotides that are identical to the wild-type allele. All of the mismatch primers tested carry an A at position 196 and are 20 nucleotides long, encompassing regions between positions 177 and 196 or 178 and 197 (denoted by *).

B. The nature and the position of mismatches within the wild-type allele and the transforming allele. Mispaired bases are denoted by underlined bold letters.

**Y_0 is the estimated efficiency of the mismatch extension during the first cycle of PCR (see Materials and Methods). The numbers are calculated based on the amount of specific PCR products generated following 30 cycles of PCR under the conditions specified in Figs. 2 and 3.

#The value <0.007 was calculated based on the observation that there was no detectable amplification product (10^{10} copies of 74-bp fragment) following 30 cycles of PCR from 3×10^5 copies of template DNA. The calculated value for Y_0 using the equation $Y_0(1 + Y)^{n-1}$ = fold amplification is 0.007, indicating that under the conditions tested, less than 2100 molecules of the primers were extended from 3×10^5 copies of template DNA during the first cycle.

##The value 0.0002 was calculated based on the observation that approximately 10^{12} copies of 74-bp fragment were generated from 3×10^5 copies of wild-type DNA following 45 cycles of PCR under the conditions specified in Fig. 2 (with the assumption that PCR was at the exponential phase of DNA synthesis throughout the 45 cycles; see Results).

from Fischer 344 rat liver by phenol/chloroform extractions.[15] The source of the transforming allele of c-H-*ras* gene was a rat mammary tumor induced by nitrosomethylurea (NMU, Sigma). The nature of alteration (G-to-A transition at the 12th codon) was identified by denaturing gradient gel electrophoresis (DGGE)[16,17] and confirmed by Sanger sequencing (data not shown). DGGE analysis indicated that 25% of the genomic DNA isolated from tumor tissue was the mutated *ras* allele. Tumor tissue was comprised of about 50% cancerous cells, with the rest of the tissue being comprised of normal stromal cells which surrounded the malignant cells (histological analysis by Dr. Robert Cardiff, pers. comm.). Thus, a mutant fraction of 25% indicated that one allele of the c-Ha-*ras* gene was mutated in the cancerous cells. The quantity and purity of DNA were determined by spectrophotometric measurement.[15]

PCR Procedures

For each PCR reaction, 1 μg (approximately 3×10^5 copies of *ras* gene; see ref. 18) of genomic DNA was mixed with 300 nM each of P2 and P1 or one of the test primers, 37 μM of each 2′-deoxynucleoside 5′-triphosphates (dNTPs, Pharmacia), and 1 μl of [α-^{32}P]ATP (3000 ci/mmole, New England Nuclear) in 100 μl of PCR buffer (10 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.25 mM $MgCl_2$). Approximately 1.7 units of *Taq* polymerase (Perkin-Elmer Cetus) were added and the mixture was subjected to a number of different PCR conditions as described in the figure legends.

Efficiency

The efficiency of amplification during the exponential phase of DNA synthesis was estimated by analyzing a 10-μl aliquot of the reaction mixture on a 7% polyacrylamide gel (acrylamide/bis-acrylamide = 38/2). The PCR products of the correct size were visualized and quantitated under ultraviolet light following ethidium bromide staining by comparing them to the molecular size standard. For the perfect match primers and one mismatch primer, the efficiencies of amplification were estimated according to the equation $(1+Y)^n$ = fold amplification, where Y is the efficiency per cycle during the exponential phase of DNA synthesis and n is the number of cycles.[18] For the primers carrying two or more mismatches, we estimated the efficiency of mismatch extension during the first cycle of PCR (Y_0) using the equation, $Y_0(1+Y)^{n-1}$ = fold amplification, where Y is the efficiency of perfect match extension. Since any extension from mismatch primers carries the sequence of the primer (confirmed by sequencing, data not shown), they are the perfect match templates during subsequent cycles of PCR. The equation assumes that the number of templates extended during the first cycle would be amplified at the same efficiency as the perfect match primers during cycles to follow.

RESULTS

Specificity of the Allele-specific Primers

The transforming allele of rat c-H-*ras* gene used in the current study differs from the wild-type allele by a single nucleotide substitution at the second nucleotide of the 12th codon (Fig. 1 and Table 1). Since the allele to be amplified (the transforming allele) carries an A instead of a G at the second nucleotide of the 12th codon, all the allele-specific primers were designed to carry an A at this position. In addition to varying the number of mismatches from one to three (Table 1), we also varied the position of the allele-specific adenosine residue within the primer between the 3′ ultimate or penultimate position. As shown in Figure 2, the allele-specific primers carrying a single mismatch had no effect on the overall PCR yield, amplifying the target sequence at a calculated efficiency of 70% per cycle during the exponential phase of DNA synthesis. On the other hand, primers carrying two or more consecutive mismatches at the 3′ end (Fig. 2A, lanes 3, 4, 5, 6, and B, lane 5) failed to generate any detectable amplification products during the first 30 cycles of PCR. Under the condition tested, extension efficiency (Y_0) for these primers containing two or more mismatches was less than 0.007, indicating that less than 2100 copies of the 3×10^5 template copies were extended during the first cycle of PCR (Table 1; also see Materials and Methods). For the purpose of specifically amplifying only the transforming allele of the c-H-*ras* gene but not the wild-type allele, we therefore chose to use the double-mismatch primers that generate two mismatches and one mismatch at their 3′ terminus when hybridized to wild-type and transforming alleles, respectively.

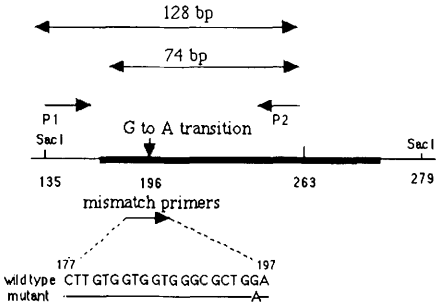


FIGURE 1 Structure of rat c-H-*ras* exon 1 and the primers used for PCR. The solid bar represents the coding region of c-H-*ras* exon 1. The transforming allele has a G-to-A transition at the second nucleotide of the 12th codon (position 196). Primer P1 is a distal primer that does not have any mismatches, and is used as a control PCR primer. For the allele-specific amplification, one of the mismatch primers and P2 were used. The expected size PCR products using P1 and P2, and P(mismatch) and P2 are 128 and 74 bp, respectively. The primary sequence of primers P1 and P2 are 5′-CCTGGTTTGGCAACCCCTGT-3′ and 5′-ACTCGTCCACAAAATGGTTC-3′ respectively.

For the G-to-A mutation at the 12th codon of the c-H-*ras* gene, there are 12 double-mismatch primers with the characteristic of having two mismatches with the wild-type allele and one mismatch with the transforming allele. Among the six double-mismatch primers tested (Table 1: PAA, PCA, PTA, PGAC, PGAT, PGAG), five permitted amplification of the mutant allele with a high degree of specificity, suggesting that for the G-to-A transition of the *ras* gene the nature of mismatches may not be crucial for the allele-specific amplification (Fig. 3, Table 1). A notable exception was the PGAG primer, which amplified the wild-type allele with two mismatched bases at the 3′ (AG/CT) as efficiently as the transforming allele that had one base mismatch (A/C) (Fig. 3g, $Y_{O(wt)} = Y_{O(t)} = 0.7$).

Optimization of the PCR Conditions for the Allele-specific Amplification

For the analysis of the various mismatch primers described above, we utilized a three-step *Taq* PCR which consisted of

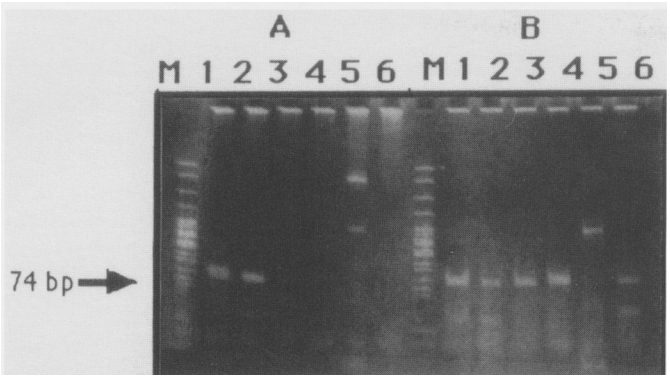


FIGURE 2 Specificity of mismatch primers. One-microgram aliquots of genomic DNA (~3×10^5 copies of *ras* gene) from rat liver or NMU-induced rat mammary tumor carrying the 12th codon G-to-A alteration was subjected to 30 cycles of PCR using primers P2 and one of the mismatch primers (Fig. 1). Each cycle consisted of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Following the completion of mismatch PCR, one-tenth of the product was analyzed on a polyacrylamide gel. The arrow indicates the position of the 74-bp transforming allele-specific amplification band. Lane *M* contained 250 ng of pBR322 digested with *Msp*I as molecular size marker. (*A*) Amplification products from wild-type rat genomic DNA. (*B*) Allele-specific PCR product from the rat mammary tumor genomic DNA. The mismatch primers tested are: (lane *1*) PA-1: (lane *2*) PA; (lane *3*) PAA-1; (lane *4*) PAA; (lane *5*) PCAA; (lane *6*) PG-AA.

94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Following 30 cycles of PCR under such conditions, no detectable amplification product was generated from wild-type DNA using the double-mismatch primer PAA (Fig. 2A, lanes 3 and 4). However, when an additional 15 cycles of amplification were carried out, about 10^{12} copies of the 74-bp fragment were generated from the wild-type DNA (data not shown). Assuming that the PCR was in the exponential phase of DNA synthesis during the 45 cycles, the estimated efficiency of double-mismatch extension (Y_0) is about 0.0002, indicating that approximately 70 copies of PAA were extended from the 3×10^5 copies of wild-type DNA during the first cycle (Table 1; also see Materials and Methods). To reduce the extension from the double-mismatch primer even further, we first tried placing an additional third mismatch in the primer. This primer failed to amplify the transforming allele (Fig. 2B, lane 5), and therefore could not be used for the purpose of selectively amplifying the mutant sequence.

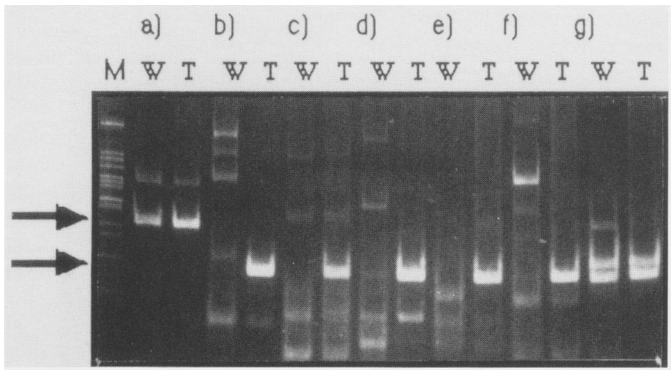


FIGURE 3 Specificity of double-mismatch primers. One-microgram aliquots of genomic DNA (~3×10^5 copies of *ras* gene) from either rat liver (W) or a NMU-induced rat mammary tumor (T) carrying the 12th codon G-to-A alteration were subjected to 30 cycles of PCR using primers P2 and each of the six different double-mismatch primers (lanes *b–g*). Each cycle consisted of incubation at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Following the completion of mismatch PCR, one-tenth of the product is analyzed on a polyacrylamide gel. Lane *M* contained 250 ng of pBR322 digested with *Msp*I as molecular size marker. The 74-bp band represents the mutant-specific amplification band and the 128-bp band is the control amplification product using P1 and P2 (Fig. 1). The primers tested are: (lane *a*) the control primer P1; (lane *b*) PAA; (lane *c*) PCA; (lane *d*) PTA; (lane *e*) PGAC; (lane *f*) PGAT; (lane *g*) PGAG.

Our next approach was to alter the PCR reaction conditions. First, we shortened the PCR cycle to a two-step reaction consisting of a template denaturation step and a primer annealing/extension step. We kept the first step constant at 94°C for 1 min and varied the second step. We tested temperatures ranging from 48°C to 57°C, and the samples were incubated at the specified temperature for either 1 min or 5 sec (Fig. 4). Under all the conditions tested, no detectable amplification product was generated from 1 μg of wild-type DNA following 30 cycles of PCR, while all the mutant DNA samples were efficiently amplified. When 15 additional cycles of amplification were carried out, however, a difference in the overall amplification yield from the wild-type allele became apparent (Fig. 4). Surprisingly, the signal corresponding to the 74-bp transforming allele-specific fragment generated from wild-type DNA sample was minimal when the temperature was lowered to 50°C or 48°C, a condition usually regarded as being less stringent/specific. Among the conditions tested, 94°C for 1 min followed by 50°C for 1 min generated the minimum amount of the specific 74-bp fragment from the wild-type DNA sample (Fig. 4).

Reconstruction Experiment

The limit of sensitivity the MAMA procedure was estimated by a reconstruction experiment. Approximately 3×10^6 copies of (10 μg) of wild-type genomic DNA were mixed with 0, 30, 300, and 3000 copies of transforming allele from tumor genomic DNA. Each mixture was subjected to the two-step PCR using PAA as the allele-specific primer. At the same time, 30, 300, and 3000 copies of transforming allele were amplified in the absence of wild-type DNA as a set of controls (Fig. 5a). Under the current reaction conditions, the two-step PCR using PAA as the allele-specific primer displayed a sensitivity of 10^{-4}. The signal from 3×10^6 copies of the wild-type allele was stronger than that from 30 copies of the mutant allele alone (Fig. 5a), suggesting that the limit of sensitivity was due to the generation of the 74-bp PCR product from the vast excess of wild-type allele present. In an attempt to reduce the

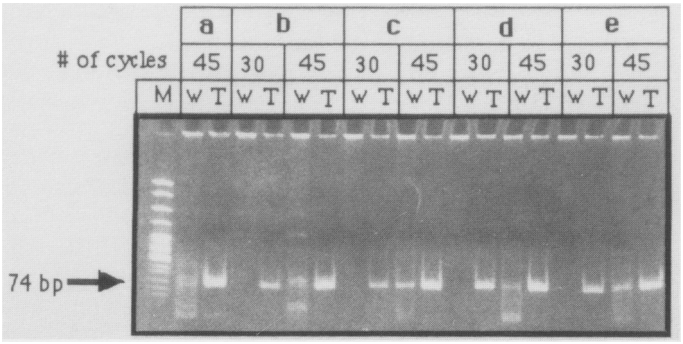


FIGURE 4 Effect of modifying PCR cycles on the specificity of PAA. One microgram (~3 × 10^5 copies of *ras* gene) of genomic DNA from rat liver (W) or NMU-induced rat mammary tumor (T) with the 12th codon G-to-A alteration is subjected to two-step PCR using primers PAA and P2. Each reaction mixture was subjected to a different PCR procedure. (*a*) 94°C for 1 min and 48°C for 1 min. (*b*) 94°C for 1 min and 50°C for 1 min. (*c*) 94°C for 1 min and 55°C for 1 min. (*d*) 94°C for 1 min and 55°C for 5 sec. (*e*) 94°C for 1 min and 57°C for 1 min. One-tenth of the reaction time mixture was taken following 30 and 45 cycles of PCR and analyzed on a polyacrylamide gel. The arrow indicates the 74-bp transforming allele-specific amplification product. Lane *M* contained 250 ng of pBR322 digested with *Msp*I as molecular size marker.

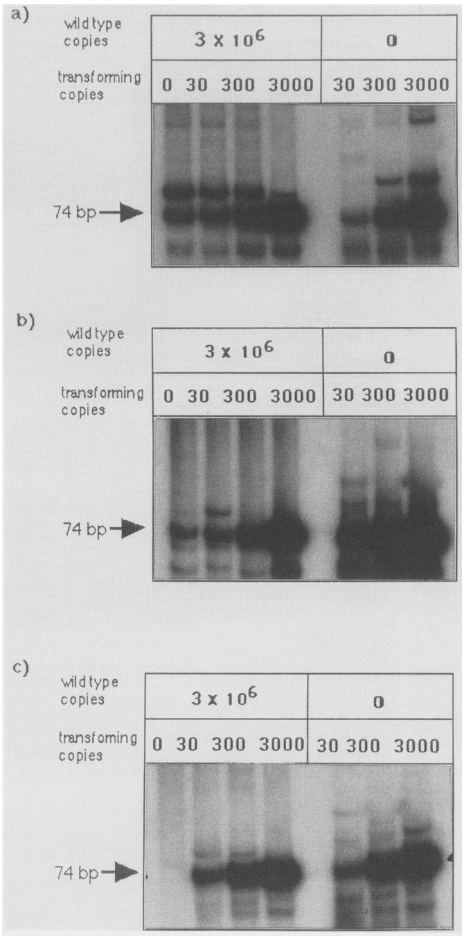


FIGURE 5 Effect of glycerol on the sensitivity of mismatch amplification mutation assay (MAMA). From the *left* to *right*, 10 μg (~3 × 10^6 copies of the *ras* gene) of genomic DNA from rat liver was mixed with various amounts of tumor genomic DNA representing approximately 30, 300, and 3000 copies of the mutant sequences. The next three lanes contain only the tumor genomic DNA. Samples were subjected to 35 cycles of two-step PCR consisting of 1 min of incubation at 94°C followed by 1 min of incubation at 50°C. The arrows indicate the position of 74-bp transforming allele-specific amplification band. Reaction mixture contained either 0% (*a*), 5% (*b*), or 10% (*c*) glycerol (vol/vol). For the reconstruction experiment, autoradiogram was utilized to visualize amplification products because 35 cycles of amplification from 30 copies of template at an efficiency of 70% per cycle would yield approximately 3 × 10^9 molecules of the 74-bp fragment, which is under the limit of detection by ethidium bromide staining.

overall yield from the wild-type DNA, we altered the solvent composition of the PCR reaction mixture by adding different amounts of glycerol.

Identical reaction mixtures were prepared with the addition of either 5% or 10% (vol/vol) of glycerol. These reaction mixtures were subjected to 35 cycles of two-step PCR protocol (Fig. 5b,c). As shown in Figure 5, the addition of glycerol enhances the specificity of the PCR reaction by reducing the amplification of the wild-type sequence with the allele-specific primer. As the concentration of glycerol increased, the signal generated from 3 × 10^6 copies of wild-type DNA decreased. With the addition of 10% glycerol in the PCR mixture, virtually no signal was detected from the sample containing 3 × 10^6 copies of wild-type allele alone while the identical mixture containing 30 molecules of transforming *ras* allele generated a clear signal, indicating that fewer than 30 molecules of PAA were extended in the presence of 3 × 10^6 copies wild-type allele during the first cycle of PCR (Fig. 5c).

DISCUSSION

In this study, we optimized the allele-specific PCR procedure with regard to its sensitivity utilizing one of the transforming alleles of rat c-H-*ras* gene (G-to-A transition at the 12th codon) as a model system. By exploring double-mismatch primers, altering the duration and the temperature of the primer annealing/extension step in PCR cycle, and modifying the solvent composition of the reaction mixture by adding glycerol, we could reproducibly carry out quantitative measurement of the transforming allele that was present at a frequency of 1 in 100,000. This improvement on the sensitivity of allele-specific amplification would greatly expand the general application of the procedure whose primary utility thus far had been limited to the detection of inherited genetic variations in human.

Ehlen and Dubeau have reported an assay for detection of GC-to-TA transversion at the 12th codon of human c-H-*ras* gene utilizing allele-specific mismatch primers.[5] These authors claimed a sensitivity of 10^{-7} based on a reconstruction experiment utilizing 1 μg of genomic DNA. However, 1 μg of mammalian genomic DNA contains only 3 × 10^5 copies of an autosomal gene, and therefore, it would be impossible to have detected a signal representing lower than about 10^{-5}. This apparent discrepancy may account for the fact that their approach has not been widely applied. On the other hand, the MAMA for the 12th codon G-to-A transition of the rat c-H-*ras* gene has already been found sufficient for measurement of c-H-*ras* mutations in untreated rats at frequency as low as 10^{-5} (R.S.C., W.G.T., and H.Z., in prep.). Moreover, an MNNG-induced mutational hotspot (Dr. Alexandra Kat, pers. comm.) and an X-ray–induced frameshift mutational hotspot (Dr. Rich Okinaka, pers. comm.) in human cells have also been successfully measured using our approach. Nevertheless, it should be pointed out that

whether or not the specific parameters optimized for the 12th codon G-to-A transition of the c-H-*ras* gene will be as effective for other sequences remains to be determined. Because numerous studies indicate that the specificity of allele-specific PCR is influenced by both the nature of mismatches and the sequence to be amplified,[1,12] it is possible that an optimal MAMA condition may need to be determined on a sequence-by-sequence basis.

The development of allele-specific PCR utilizing 3′ terminal mismatch(es) was based on observations that correctly paired bases were much more readily extended by DNA polymerases than mispaired bases.[19,20] By measuring the extension efficiencies of different mismatch primers hybridized to M13 template, investigators reported that perfectly matched bases were 10^2–10^6 times more efficiently extended than mismatched bases, depending on DNA polymerases and the nature of mismatches.[19,20] For the 12th codon G-to-A transition of the c-H-*ras* gene, we find that primers containing one mismatch at the ultimate or penultimate position of the 3′ end of the primer extend as efficiently as a perfect match primer with a calculated efficieny of 70% per cycle. When an additional mismatch was placed, about 100-fold reduction (from 0.7 to 0.007, Table 1) in the efficiency of extension was observed. Under the condition optimized for the transforming allele-specific amplification, the probability of a double-mismatch primer extension was less than 10^{-5} (Fig. 5; see Results). Undoubtedly, this probability determines the limit of sensitivity, and any modification of the procedure that results in the reduction of this probability would improve the sensitivity.

Numerous studies on fidelity of in vitro DNA polymerization and mispaired base extension have demonstrated that the extent of such discrimination by DNA polymerases is influenced by the number and nature of mismatches,[1,12] and many factors in the reaction mixture such as pH,[12,21,23] the concentration of primers,[8] dNTPs,[12,21] and magnesium ions (mg^{2+}).[12,23,24] To explore the possibility that altering these parameters would further enhance the sensitivity of the MAMA by lowering the likelihood of the double-mismatch extension, we modified the reaction buffer by varying the pH between 8.4 (original condition) and 5.0, the concentrations of $MgCl_2$ between 2.25 mM (original condition) and 0.1 mM, and the concentrations of dNTPs between 37 μM (original condition) and 3.7 μM. We chose the lower values for these three variables from the results of previous studies indicating that higher fidelity of in vitro polymerization is achieved when these values are lowered.[8,23,24] Under the various PCR conditions tested, we observed that the overall efficiency of amplification for both the wild-type allele and the transforming allele decreased. The extent of the reduction in the efficiencies was comparable between the two alleles, and no significant enhancement on the sensitivity was observed (data not shown). It should also be noted that no detectable amount of amplification product was generated from either the wild-type allele or the transforming allele when pH was lower than 7.0 or the concentrations of $MgCl_2$ were below 0.5 mM.

The mutation we have utilized as a model system in this study, the G-to-A transition at the second nucleotide of the 12th codon of *ras* gene, is one of the most frequently observed alterations in many types of tumors of both human and animal origins.[25] Consequently, tumors are screened routinely for the presence of this mutation. Utilizing MAMA for detecting the transforming *ras* allele in tumors would provide many advantages over other molecular techniques currently used, such as RFLP and differential oligonucleotide hybridization. In addition to its simplicity, speed, and sensitivity, the MAMA generates quantitative output (Fig. 5c), providing an opportunity to measure the actual fraction of the sample DNA carrying the 12th codon alteration. Assuming that tumors are clonal in origin, such analysis, in turn, would make it feasible to assess the timing of *ras* gene activation. In addition, with its present ability to detect 30 copies of transforming *ras* alleles present amongst 3×10^6 copies of wild-type allele, MAMA may be utilized to analyze quantitatively the molecular events that occur during the earlier stages of chemically induced tumorigenesis in a target organ where the number of cells with a mutated oncogene is believed to be very small. Finally, it should be pointed out that because there is a restriction enzyme (*Mnl*I) that selectively cleaves the wild-type c-H-*ras* allele, the current sensitivity of the MAMA for the G-to-A transition of the c-H-*ras* gene can be further enhanced by a factor of 10 or more by digesting genomic DNA samples prior to MAMA.

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