Research Report

Fast and Reliable Screening of Mutations in Human Tumors: Use of Multiple Fluorescence-Based Long Linker Arm Nucleotides Assay (mf-LLA)

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ABSTRACT

*Human tumor samples were screened for point mutations by adapting a mobility-shift assay to automated DNA sizing. This screen identifies the type of point mutation and relative amount of mutated DNA sequences present in a sample. Test samples having known hypoxanthine-guanine phosphoribosyl transferase (*hprt*)/exon-3 sequence mutations were characterized by:* (i) *PCR amplification,* (ii) *fluorescent dye-primer extension with 36-atom linker derived deoxycytosine or deoxyuridine triphosphate and the remaining three natural nucleotides and* (iii) *sizing of the resulting fluorescently labeled modified strands, using an automated DNA sequencer. Routinely, a range of sizes is observed among the sequence variants of a single DNA target sequence. This is because nucleotide analogs are incorporated into DNA strands in a sequence-dependent manner, resulting in composition-dependent electrophoretic mobility. Thus, point mutations are identified as shifts in mobility between the fluorescently labeled modified strands of the control and test samples. The twenty different* hprt*/exon-3 single-base substitution mutations tested were easily identified, even at fourfold dilution with control DNA.*

INTRODUCTION

The mobility-shift assay coupled with polymerase chain reaction (PCR) amplification reported by Kornher and Livak (10) and Livak et al. (13) has shown to be a simple and efficient method in detecting point mutations and polymorphisms in different genes, such as in the human insulin receptor gene (10), in the β-hexosaminidase α gene, in the human cytochrome *P450* debrisoquine *4*-hydroxylase gene and in the human Apopolipoprotein E gene (13).

Here, we describe the use of multiple-color, fluorescence-based detection combined with the mobility-shift assay, designated by us as the multiple fluorescence-based long linker arms assay (mf-LLA). Multiple color fluorescence-based detection, using the Model 373A DNA Sequencer and GENESCAN™ 672 computer software, allows for the simultaneous screening of many samples, because DNA fragments with different sizes and labeled with different colored fluorescent tags can be run in the same gel lane (18). We tested if the mf-LLA assay could be a fast, sensitive and reliable method for the routine detection of mutations.

MATERIALS AND METHODS

Samples

Twenty different Chinese hamster ovary (CHO) point mutants were previously identified as differing from the wild-type by single-base substitutions in exon 3 of the hypoxanthine-guanine phosphoribosyl transferase (*hprt*) gene (12,14,15) (Figure 1).

Surgical discard samples of gastric and lung tumors, and the matching non-tumorous tissues, were generously provided by the pathology units of the Hospitals Pulido Valente, São José and Santa Cruz in Lisbon. All patients voluntarily gave informed consent. Samples were immediately fixed in 10% formalin and embedded in paraffin.

Materials

Nucleotide analogs of cytosine (biotin-36-dCTP) and uracil (biotin-36-dUTP) containing a biotin molecule at the end of a 36 atom chain, which is linked to the carbon 5 of the purine ring (nucleotide analogs with long linker arms), were synthesized as described (13).

Oligonucleotides used in this experiment were synthesized as previously described (5). After synthesis, each primer was labeled at the 5′ end with a fluorescent dye (FAM, JOE or TAMRA, which are blue, green or yellow color dyes, respectively) (PE Biosystems, Foster City, CA, USA) or left unlabeled and HPLC purified (5). Table 1 gives the sequence of the primers used in PCR amplification and the mf-LLA assay for mutation detection in different DNA target sequences.

DNA Extraction from Human Tumor Biopsies

DNA isolation was performed by an adaptation of the method described by Impraim et al. (8). Briefly, DNA was isolated from scalpel-minced tissues by

Table 1. Sequence of Oligonucleotides Used as Primers in PCR Amplification of a mf-LLA Assay for Mutation Analysis in Different DNA Target Sequences

DNA Sequence	Primers	Primers Sequence	Base Pair Position	Fluorescent-Dye Label	Reference
CHO *hprt*	hprt 1	(Forward) 5′ ACCTCACCGCTTTCTCGTGC 3′	-88 – -69	not labeled	16[a]
	hprt 2	(Reverse) 5′ TTCGTCTACCGACGTCTTGA 3′	731 – 750		
CHO *hprt*	hprt 3	(Forward) 5′ CAT GGA GTG ATT ATG GAC AG 3′	115 – 134	blue (FAM)	16[a]
(exon-3)	hprt 4	(Reverse) 5′ ATG TCC CCT GTT GAC TGA TC 3′	322 – 341	green (JOE)	
Human *HRAS*	hHras1	(Forward) 5′ GGA GAC CCT GTA GGA GGA CC 3′	1619 – 1638	blue (FAM)	this work[b]
(intron-1 and	hHras2	(Reverse) 5′ AGC TGC TGG CAC CTG GAC 3′	1792 – 1809	yellow	
exon-1)				(TAMRA)	
Human *KRAS*	hKras1	(Forward) 5′ GAC TGA ATA TAA ACT TGT GG 3′	98 – 117	blue (FAM)	20[c]
(exon-1)	hKras2	(Reverse) 5′ CTA TTG TTG GAT CAT ATT CG 3′	185 – 204	green (JOE)	

[a]The base pair positions given are from the CHO *hprt* cDNA (16).
[b]Primers were designed using the PRIMER® software (PREMIER Biosoft International, Palo Alto, CA, USA) on the HSRAS1 sequence, GenBank® Accession No. V00574.
[c]The base pair positions are from the HSRASK22 sequence, GenBank Accession No. L00045 (20).

lysis with proteinase K (500 mg/mL) in 1% sodium dodecyl sulfate (SDS) and TEN buffer (100 mM Tris-HCl, 40 mM EDTA, 10 M NaCl, pH 8.0) for 16–40 h at 50°C, followed by phenol/chloroform/isoamyl-alcohol extractions and ethanol-precipitation. The DNA pellet was resuspended in deionized water.

Amplification Conditions

All PCR amplifications contained 200 µM of each deoxynucleotide

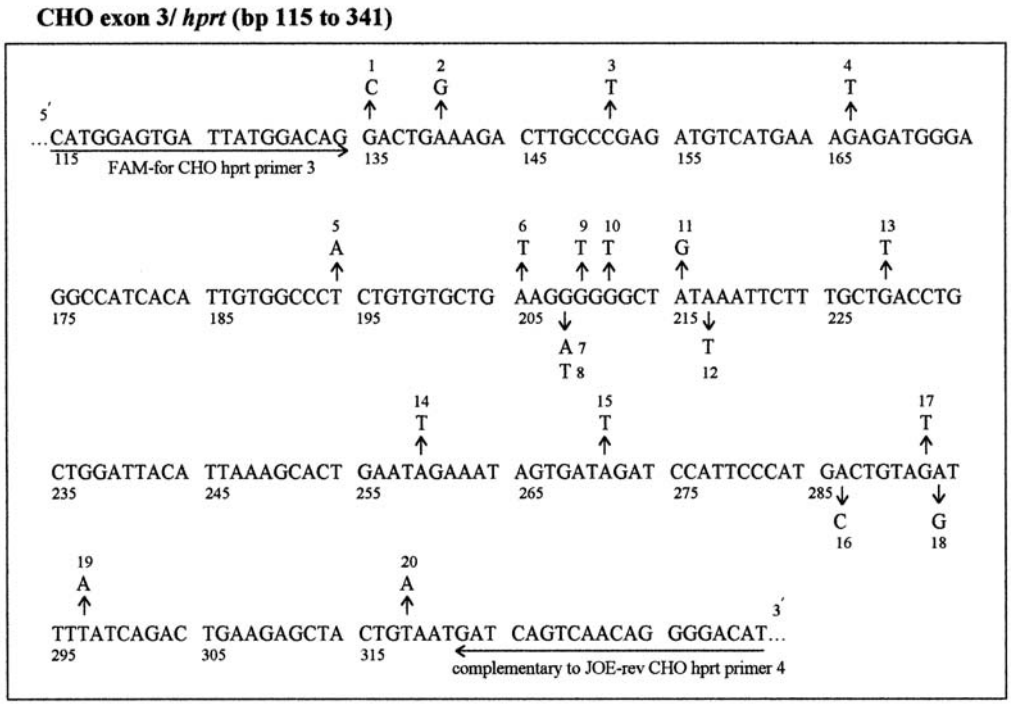


Figure 1. *hprt*/exon-3 sequence (bp 115–341; Reference 9) of CHO cells used as target sequence for studies of mutation detection efficiency. Shown are the previously identified type and position of each mutant carrying one single-base substitution (12,14,15). Sequences recognized by DNA primers used for the mf-LLA assay are underlined.

Table 2. PCR Amplification of Different DNA Target Sequences

	Reaction Mixtures				DNA Thermal Cycler Conditions				
DNA Sequences	Primers[a] (μM)	Buffer	Enzyme (U)	DNA (μg)	Denaturation Step	Annealing Step	Extension Step	Cycle No.	Reference
Chinese hamster ovary *hprt*	0.15 hprt 1 and hprt 2	1× *Taq*[b]	1.25 *Taq* pol	0.1–0.5 cDNA	94°C, 1 min	60°C, 2 min	72°C, 3 min	30	16
Chinese hamster ovary *hprt*/exon-3	0.6 hprt 3 and hprt 4	1× *Taq*[b]	2 *Taq* pol	0.05 PCR product[e]	94°C, 1 min	55°C, 30 s	72°C, 1 min	30	16
Human *HRAS*	0.2 hHras1 and hHras2	1× *Taq*[c] Gold	1.25 *Taq* pol[d] Gold	1 genomic DNA	95°C, 30 s	64°C, 30 s	72°C, 1 min	37	this work
Human *KRAS*	0.2 hKras1 and hKras2	1× *Taq*[c] Gold	1.25 *Taq* pol[d] Gold	1 genomic DNA	95°C, 20 s	55°C, 20 s	72°C, 30 s	37	this work

[a]See Table 1 for information on primer sequence and position.
[b]1× AmpliTaq buffer [10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% (wt/vol) gelatin (Perkin-Elmer)].
[c]1× AmpliTaq Gold buffer [10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% (wt/vol) gelatin (Perkin-Elmer)].
[d]The use of Ampli*Taq* Gold DNA polymerase (*Taq* pol Gold) instead of AmpliTaq DNA polymerase (*Taq* pol) helped to significantly reduce nonspecific PCR products for specific sequences only.
[e]A PCR product of 833 bp amplified from total CHO cDNA (16) was used as a template for PCR amplification with the nested primers hprt 1 and hprt 2.

triphosphate (dNTP) and were performed in a final volume of 50 μL in a Model 480 or 9600 DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA). Table 2 shows the PCR amplification conditions specific of each target DNA sequence. Before cycling, reaction mixtures containing either AmpliTaq® DNA Polymerase or AmpliTaq Gold™ Polymerase (both from Perkin-Elmer) were incubated for 5 or 10 min at 94°C, respectively.

mf-LLA Assay

Figure 2 shows an overview of the mf-LLA assay. After amplification of the desired sequence from genomic DNA, PCR products were purified in MicroSpin S-300HR Columns® (Amersham Pharmacia Biotech, Piscataway, NJ, USA) to remove excess dNTPs and residual unlabeled primer carried over from amplification. Purified PCR target sequences were used as templates for the mf-LLA assay in a 2-tubes reaction in the presence of either 1 mM biotin-36-dCTP or biotin-36-dUTP, 500 μM of the remaining three dNTPs, 10% dimethyl sulfoxide (DMSO), 0.2 U of

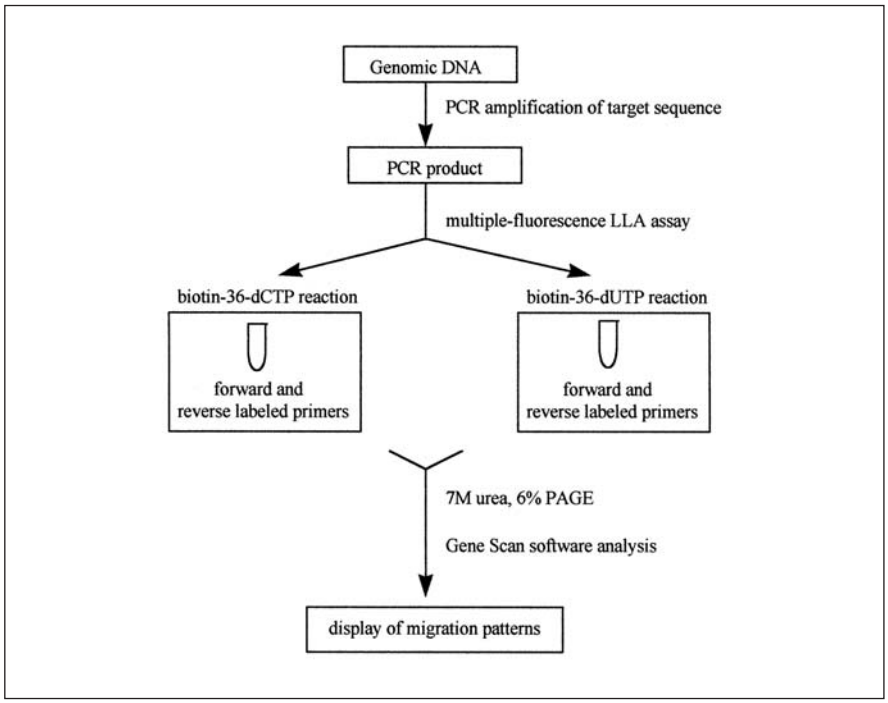


Figure 2. Flow diagram of the mf-LLA assay. Genomic DNA is amplified using target sequence-specific primers. PCR-amplified DNA fragments are used as templates for the mf-LLA assay using either biotin-36-dCTP or biotin-36-dUTP along with the remaining three natural nucleotides and both forward and reverse primers. mf-LLA products are electrophoresed on a 7 M urea, 6% polyacrylamide gel electrophoresis (PAGE), and GENESCAN 672 software determines the size of the DNA fragments based on the use of a DNA size marker, which is co-migrated with each sample.

Table 3. Effect of Sequence Context

hprt Mutant	Type of Mutation	FAM-Labeled Forward LLA Strand		JOE-Labeled Reverse LLA Strand	
		Biotin-36-dCTP	Biotin-36-dUTP	Biotin-36-dCTP	Biotin-36-dUTP
8	G→T	0.20 (0)	5.20 (+)	3.99 (-)	0.37 (0)
9	G→T	0.36 (0)	5.32 (+)	3.50 (-)	0.08 (0)
10	G→T	0.31 (0)	5.83 (+)	3.61 (-)	1.09 (0)
13	G→T	0.60 (0)	5.89 (+)	3.57 (-)	1.66 (0)
17	G→T	0.24 (0)	3.07 (+)	4.03 (-)	0.36 (0)
11	A→G	0.54 (0)	1.45 (0)	4.89 (+)	2.90 (-)
12	A→T	0.98 (0)	6.00 (+)	1.70 (0)	3.05 (-)

Mobility shifts displayed by different *hprt*/exon-3 CHO mutants after mf-LLA analysis. Mobility shifts (labeled as 0, + or -) correspond to the difference between the size of mutant- and wild-type-modified DNA strands, given in base pairs. The position of each mutant in the sequence is indicated in Figure 1.

Vent® DNA Polymerase without the 3′→5′ exonuclease activity (exo-) (New England Biolabs, Beverly, MA, USA), $Vent_R$ Polymerase buffer [20 mM Tris-HCl, pH 8.8, 10 mM KCl, 2 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Triton® X-100] (13) and primers labeled with different colored fluorescent tags (see Table 1). The reactions were subjected to 1 cycle of the following regimen: 95°C for 3 min; 55°C for 45 s and 75°C for 3 min. Each sample was then

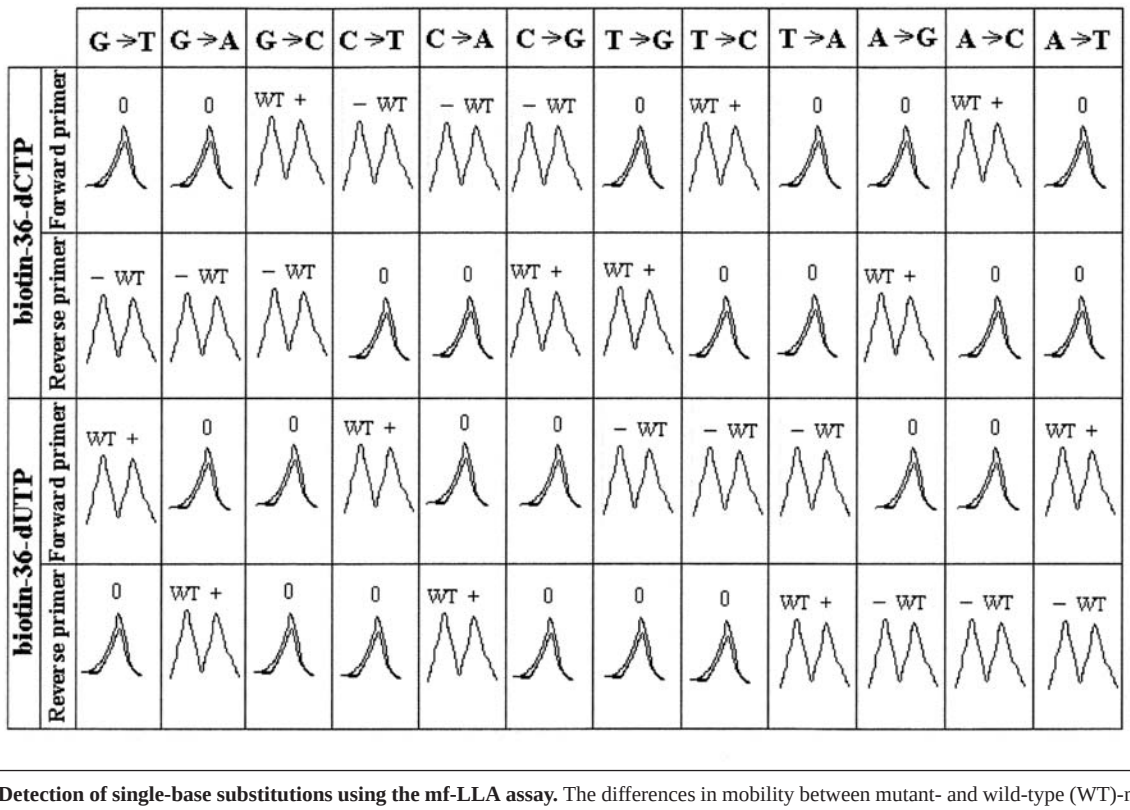


Figure 3. Detection of single-base substitutions using the mf-LLA assay. The differences in mobility between mutant- and wild-type (WT)-modified single strands can be used to predict the type of single-base substitution based on which analog was incorporated opposite the mutation (biotin-36-dCTP or biotin-36-dUTP), and on which primer was extended (forward or reverse). Each single-base substitution has a characteristic migration pattern, labeled as (0, + or -). A mutant-modified strand with a: *(i)* equal size, *(ii)* smaller size or *(iii)* bigger size than the wild-type-modified strand migrates: *(i)* the same, 0, *(ii)* faster, + or *(iii)* slower, -, than the wild-type, respectively.

lyophilized, resuspended in 4 µL of loading solution (95% deionized formamide, 12.5 mM EDTA, 0.05% blue dextran) and mixed with a commercial DNA size marker comprising *Alu*I digestion fragments of pBR322 labeled with the red color dye ROX (GENESCAN 2500-ROX) (PE Biosystems). Each sample was denatured (95°C for 3 min) and kept on ice before loading. LLA reaction products were subjected to electrophoresis in a 7 M urea/6% polyacrylamide gel (acrylamide/bisacrylamide 19:1) containing 1× MTB (0.14 M Tris-HCl, pH 9.5, 0.045 M boric acid, 2.3 mM EDTA) in both the gel and the running buffer (13). Electrophoresis was performed using a Model 373A Automated DNA Sequencer (PE Biosystems) at 45 V/cm for 7–12 h (depending on the expected size range of the fragments). Fluorescently labeled LLA products were detected as laser-excited fluorescence as they migrated through the fluorescence detection system. The sizes of the electrophoresed LLA products were then determined by GENESCAN 672 software (PE Biosystems), which uses the method of second-order regression to establish a curve of best fit generated from the DNA size marker in each lane. Electrophoresis of a size marker mixed with every sample reduces the errors in size estimation caused by lane-to-lane variability in migration rates. This allows comparison of shifts in mobility that mutant-modified strands show when compared to the corresponding wild-type-modified strands. Each of the twelve possible single-base substitutions has a characteristic set of mobility shifts (labeled as 0, + or -) depending on which nucleotide analog (biotin-36-dUTP or biotin-36-dCTP) was incorporated and on which primer (forward or reverse) was extended by $Vent_R$ DNA polymerase (Figure 3).

RESULTS

Efficiency of the mf-LLA Assay in Detecting Single-Base Substitutions

Effect of mutation type. To test the efficiency of the mf-LLA assay for detecting point mutations, we performed a blind analysis on twenty CHO *hprt* mutants (arbitrarily numbered 1–20) that had been identified by sequencing in an independent study (Figure 1).

All different mutations were easily detected by comparing the relative migration of both mutant and wild-type fluorescently labeled LLA products shown in Figure 4 with the predicted pattern of mobility shifts for any given point mutation, shown in Figure 3.

Furthermore, two different mutations at the same nucleotide position were studied; mutants *hprt* 7 (G:C to A:T) and *hprt* 8 (G:C to T:A) at bp 208. The mobility shifts observed for each mutant were different and dependent on the mutation type (Figure 4).

Effect of sequence context. To

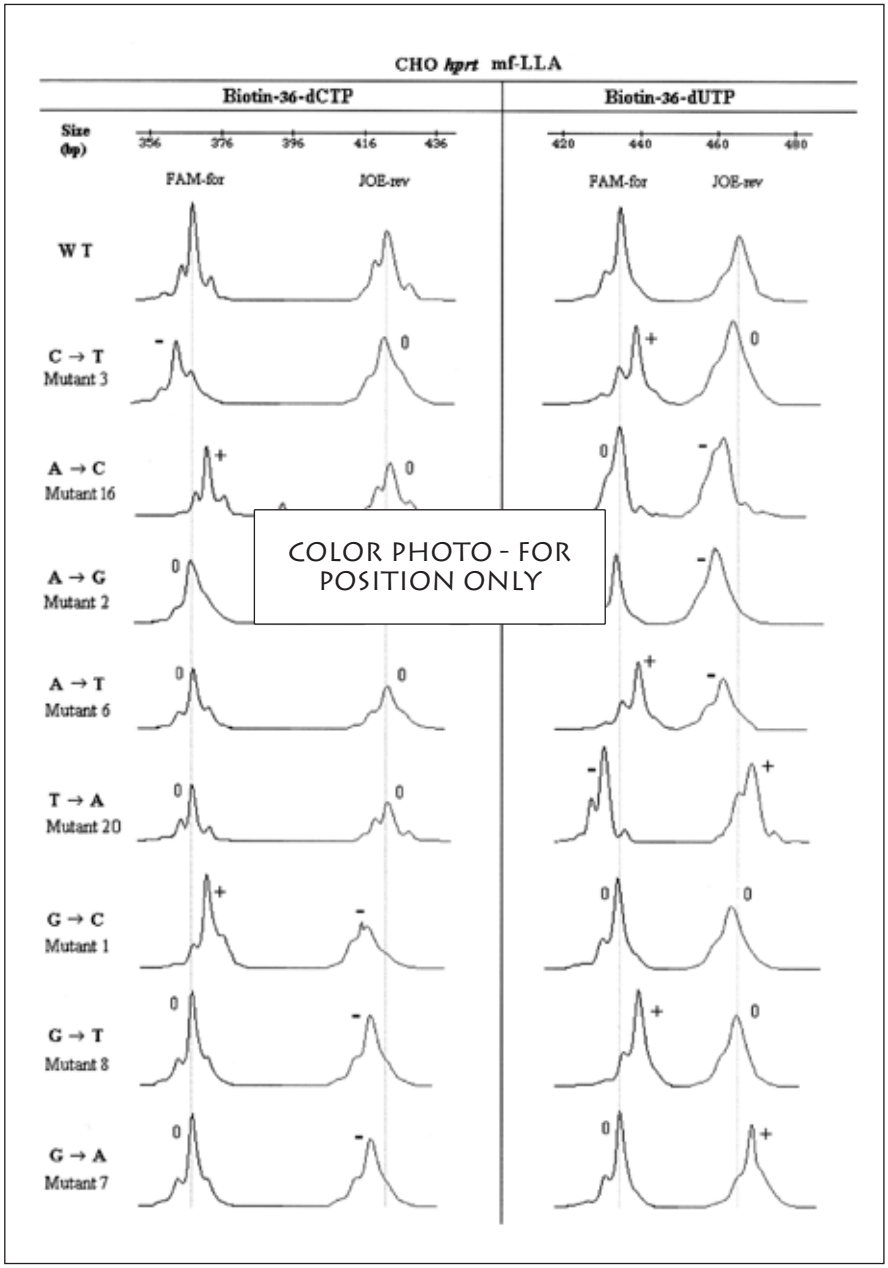


Figure 4. mf-LLA detection of different *hprt*-base substitutions. Electropherograms display FAM-labeled forward (FAM-for) and JOE-labeled reverse (JOE-rev) modified DNA strands with either biotin-36-dCTP or biotin-36-dUTP analogs incorporated. The type of mutation is determined based on a comparison of the relative mobility among mutant LLA products and the corresponding wild-type LLA products. Each type of mutation has a characteristic pattern of mobility shifts, labeled as (0, + or -). See Figure 1 for position of the mutant in the *hprt* target sequence.

evaluate the effect of sequence context in the sensitivity of mutation detection by mf-LLA, mutants carrying the same type of mutation (G:T to T:A) but in different positions in the sequence were analyzed by mf-LLA (Table 3). Of interest is the fact that three of the G:C to T:A mutants (*hprt* 8, 9 and 10) were present in a run of 6 guanines. This required $Vent_R$ (exo^-) DNA polymerase to incorporate six biotin-36-dCTP residues in a row, with no prematurely terminated strands due to the fall off of the enzyme (Table 3 and Figure 4). Furthermore, *hprt* mutants 11 (A:T to G:C, bp 215) and 12 (A:T to T:A, bp 217), present in a run of 7 adenines and thymines, show that bio-36-dUTP residues are also well accepted by $Vent_R$ DNA polymerase (Table 3).

Limit of Detection of mf-LLA Assay

The limit of detection of mf-LLA was determined by reconstruction experiments, in which two different PCR-amplified mutants were independently mixed with wild-type in different proportions and analyzed by mf-LLA (Figure 5). Both mutants tested were readily detectable by mf-LLA when present in 25% of the total DNA target sequences in the mixture. When the mutants were present in 20% of the mixture, their detection became ambiguous, and if any of the mutants were present in 10% of the mixture, it was no longer detectable. Therefore, by using mf-LLA, analysis of a mixed population of cells is feasible, provided that the mutant sequences represent at least 25% of the template mixture.

Use of the mf-LLA Assay to Detect Mutations in the *HRAS* and *KRAS* Genes of Human Tumor Biopsies

Thirty gastric tumor biopsies, along with corresponding non-tumorous biopsies, were screened by mf-LLA to assess point mutations in exon 1 of *HRAS* gene. Figure 6A shows a sample result. mf-LLA analysis identified the mutation as a C:G to T:A transition, and individuals were found heterozygous and homozygous for the mutation. Both normal and tumorous tissues of the same individual showed the same migration pattern, suggesting that the detected transition was not associated with stomach cancer. Sequencing of DNA samples revealed a C:G to T:A transition at bp 1744 (codon 27) of the *HRAS* gene (Figure 6B). A C:G to T:A transition was previously described as a sequencing variant (19) and later as a DNA polymorphism (1,6).

Twenty-one squamous cell carcinoma specimens and six adenocarcinoma specimens were then screened by the mf-LLA assay for mutations in a *KRAS* sequence containing part of exon 1. Figure 7 shows a sample result. A mobility shift was observed for the tumor tissue (sample 138 T) and not for the corresponding normal tissue (138 N) for only one individual. mf-LLA identified the mutation as a G:C to T:A transition, and it was confirmed by sequencing. Because the mutant DNA sequence was found in approximately 20%–30% of the tumor sample, to facilitate identification of the mutation by sequencing, single-strand conformation polymorphism (SSCP) was used as a preparative method to isolate the mutant bands. One of the two bands, observed only in sample 138 T (and not in its corresponding normal tissue sample), was excised from the SSCP gel, purified and subjected to mf-LLA to be used as a positive control (Figure 7A, band 3). Sequencing confirmed the G:C to A:T transition at the first base of codon 12 (bp 129). Randomly chosen samples, which had unaltered mobility patterns, were sequenced and shown to be the same as the wild-type in every case.

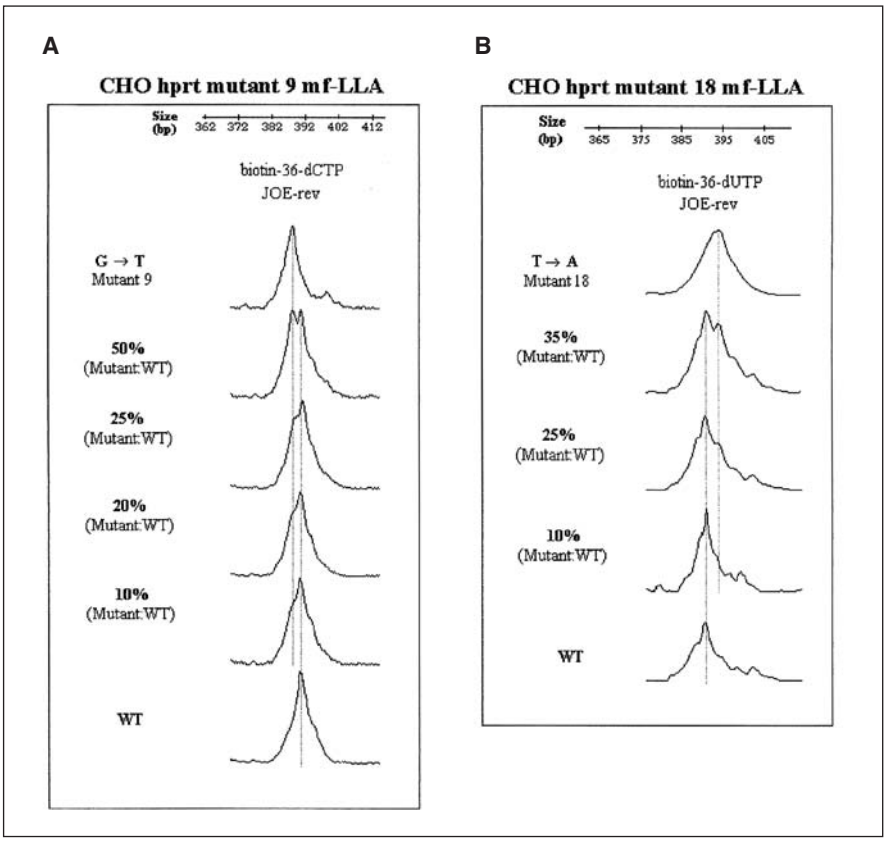
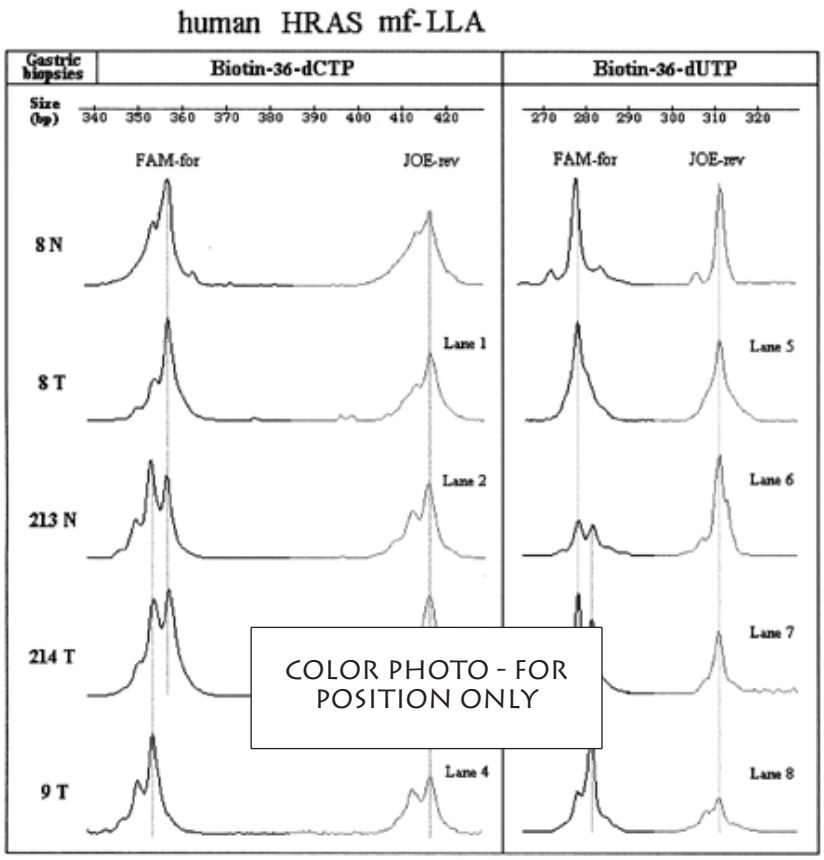


Figure 5. mf-LLA limit of detection. Reconstruction experiments were performed in which (A) *hprt* 9 or (B) *hprt* 18 mutant sequences were independently mixed with wild-type (WT) sequences in different proportions and subjected to mf-LLA analysis. Electropherograms display JOE-labeled reverse (JOE-rev) modified DNA strands with either biotin-36-dCTP or biotin-36-dUTP analogs incorporated.

DISCUSSION

In this study, we present a modification of mobility-shift analysis by combining this method with multiple fluorescence-based detection, or mf-LLA. The efficiency of the mf-LLA assay in detecting mutations was evaluated by subjecting previously known mutants to

A



B

human HRAS sequence (bp 1611 to 1810)

5' ... GTGGGGCAGG AGACCCTGTA GGAGGACCCC GGGCCGCAGG CCCCTGAGGA
1611 1631 1641 1651
hHras primer 1

GCGATGACGG ATTATAAGCT GGTGGTGGTG GGCGCCGGCG GTGTGGGCAA
1661 1671 1681 1691 1701

GAGTGCGCTG ACCATCCAGC TGATCCAGAA CCATTTTGTG GACGAATACG
1711 1721 1731 codon 27 1751
C

ACCCCACTAT AGAGGTGAGC CTGGCGCCAC CGTCCAGGTG CCAGCAGCTG 3' ...
1761 1771 1781
hHras primer 2

Figure 6. *HRAS* mutation polymorphism detection in human gastric cancer specimens. (A) Depicted is a sample result from tumor (T) and corresponding normal (N) gastric biopsies of three individuals. Electropherograms display FAM-labeled forward (FAM-for) and JOE-labeled reverse (JOE-rev) modified DNA strands with either biotin-36-dCTP or biotin-36-dUTP analogs incorporated. (B) Human *HRAS* sequence (bp 1611–1810) used as target sequence in this study. The polymorphism C to T in codon 27 (bp 1744) is indicated. Sequences recognized by DNA primers used for mf-LLA assay are underlined.

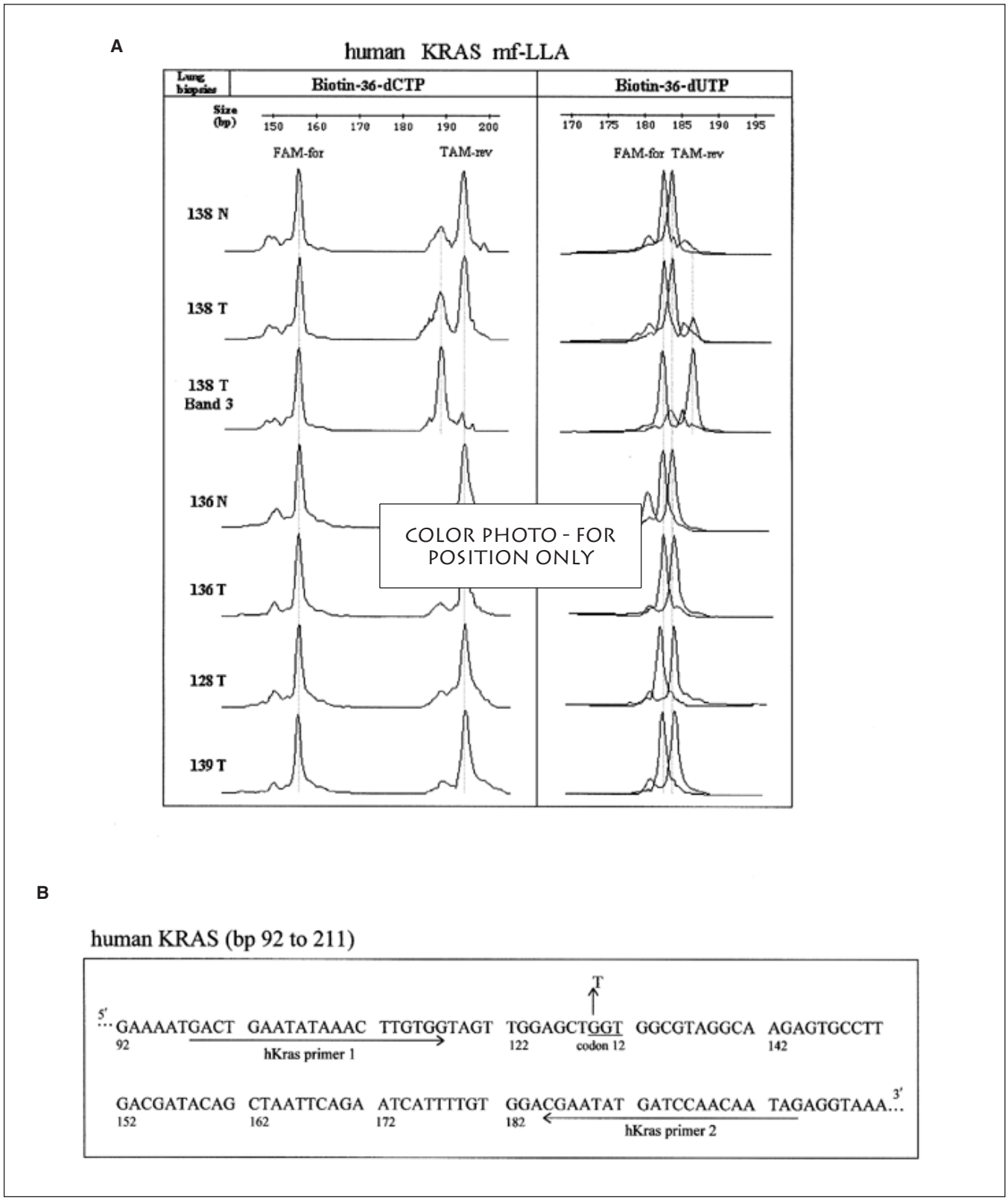


Figure 7. *KRAS* mutation detection in human lung cancer specimens. (A) A representative sample of tumor (T) and normal (N) lung biopsies of four individuals is shown. Band 3, used as a positive control, results from 138 T purified DNA, excised from a SSCP gel and subjected to mf-LLA. Electropherograms display FAM-labeled forward (FAM-for) and JOE-labeled reverse (JOE-rev) modified DNA strands with either biotin-36-dCTP or biotin-36-dUTP analogs incorporated. (B) Human *KRAS* sequence (bp 92–211) used as target sequence in this studies. The G:C to T:A transversion at codon 12 (bp 129) is indicated. Sequences recognized by DNA primers used for mf-LLA assay are underlined.

mf-LLA analysis. The sensitivity of the mf-LLA assay in detecting mutations in an excess of wild-type sequences was evaluated by performing reconstruction experiments. A prospective study of the utility of this technique to detect *HRAS* and *KRAS* mutations in human cancer specimens was also performed.

Efficiency of the mf-LLA Assay in Detecting Single-Base Substitutions

Twenty out of twenty previously known single-base substitutions (100%) were detected regardless of the type and position of the mutation in the sequence. The type of mutation was easily identified by comparing the characteristic set of mobility shifts of both mutant and wild-type DNA strands modified in the presence of either the cytosine or uracil analogs.

A given mutant-modified strand that shifted from the wild-type-modified strand due to the differences in the number of nucleotide analogs, was found to generate an average mobility shift of approximately 4.27 ± 0.33 [mean ±2 standard deviations (SD); sample of 38 mobility shifts; range of values (1.79–5.68); mode 4.06]. However, if a given mutant-modified strand did not differ in the number of nucleotide analogs from the wild-typemodified strand, the average mobility shift generated was 0.60 ± 0.21 [sample of 38 with no mobility shifts; range of values (0.05–2.16); mode 0.12]. The difference between the two means was found to be significant (P <0.002, Student's t-test). Thus, the easy detection of mobility shifts makes the identification of base substitutions by the mf-LLA assay a quick and simple procedure.

The mf-LLA assay was able to detect point mutations even at a fourfold dilution with control DNA (i.e., mutations present in at least 25% of the total template population). This level of sensitivity is similar to the 20% level shown for other methods, such as PCR-SSCP and direct sequencing (2), although detection of a mutant allele by SSCP present in 5% of the total DNA target sequences was reported (22). This level of sensitivity is sufficient for polymorphism detection and inherited-disease causing mutations and some mutations in tumors.

Use of the mf-LLA Assay to Detect Mutations in the *KRAS* and *HRAS* Genes of Human Tumor Biopsies

We further evaluated the use of the mf-LLA assay as a method for rapid screening of mutations in human tumor biopsies. We found no mutations in the *HRAS* gene for any of the gastric tumor biopsies, which is in agreement with previous reports showing that mutations in the *HRAS*, *KRAS* or *NRAS* oncogenes are infrequent in gastric cancer (4,11). However, a C:G to T:A transition, previously identified as a polymorphism (1,6), was easily identified even in individuals heterozygous for the mutation. Among 30 individuals studied, 19 (63%) were found heterozygous for both alleles, 2 (6.7%) were found homozygous for the C allele, and 9 (30%) were found homozygous for the T allele. These observations are in agreement with the results described by Hoban et al. (6), in which an observed heterozygote frequency of 46% was reported for a population of 60 unrelated individuals.

Analysis of twenty human lung carcinomas by mf-LLA showed one tumor biopsy (5%) to contain a *KRAS* gene with a G:C to T:A transversion, confirmed by sequencing to be in codon 12 (bp 129). These results are within the range of 5%–30% reported in independent studies that used SSCP coupled with PCR or direct sequencing after PCR amplification of the samples (3,7,20,21). Different studies (3,17) have shown that *KRAS* mutations, especially at codon 12, seem to be associated with a poor prognosis in patients with non-small cell lung carcinomas. The patients whose tumors had a mutation were less responsive to therapy and appeared to have a more aggressive disease and shortened survival. An assay such as mf-LLA analysis for population-based detection of mutations in the *KRAS* gene could be useful in predicting tumor behavior. However, because the limit of detection of the mf-LLA assay is approximately 25%, an assessment of the level of sensitivity required to detect *KRAS* mutations that might correlate with poor prognosis would have to be carried out.

The mf-LLA assay requires the use of an automated DNA sequencer, which is an expensive machine. However, when a large number of samples are analyzed, the running costs of the assay are considerably lower than when using radioisotopes. The number of samples screened in the same gel can be further increased, for example, by choosing target sequences with different sizes or with different colored fluorescent tags. Furthermore, the automated entry of data into a computer, which allows for the creation of databases, and immediate processing for analysis is much easier and less error-prone than manual reading of autoradiographs. Therefore, the use of the mf-LLA assay could facilitate the routine analysis of a large number of samples as a part of genetic diagnostics.

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