

# K-*ras* Point Mutation Detection in Lung Cancer: Comparison of Two Approaches to Somatic Mutation Detection Using ARMS Allele-specific Amplification

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**Background:** The use of sensitive molecular techniques to detect rare cells in a population is of increasing interest to the molecular pathologist, but detection limits often are poorly defined in any given molecular assay. We combined the approaches of real-time quantitative PCR with ARMS<sup>TM</sup> allele-specific amplification in a novel assay for detecting mutant *K-ras* sequences in clinical samples.

**Methods:** ARMS reactions were used to detect seven commonly occurring mutations in the *K-ras* oncogene. These mutations produce amino acid changes in codon 12 (Gly to Ala, Arg, Asp, Cys, Ser, or Val) and codon 13 (Gly to Asp). A control reaction was used to measure the total amount of amplifiable *K-ras* sequence in a sample so that the ratio of mutant to wild-type sequence could be measured. Quantitative data were confirmed for a selection of samples by an independent cloning and sequencing method. The assay was used to analyze 82 lung tumor DNA samples.

**Results:** The assay detected *K-ras* mutations in 44% of adenocarcinomas, which is equivalent to frequencies

reported in the literature using ultrasensitive techniques. Forty-six percent of squamous carcinomas were also positive. The ratio of mutant sequence in the tumor DNA samples was 0.04–100%.

**Conclusions:** The assay is homogeneous, with addition of tumor DNA sample being the only step before results are generated. The quantitative nature of the assay can potentially be used to define the analytical sensitivity necessary for any specified diagnostic application of *K-ras* (or other) point mutation detection.

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Detection of acquired nucleic acid changes in the management of cancer is a challenging and controversial area (1). Despite routine tumor assessments (including stage and grade) and therapeutic interventions in all cancer patients, survival from the disease generally is poor [see, for example, Ref. (2)]. This means that efforts to find molecular cancer management tools are likely to continue, although progress has been perceived by some as disappointing (3).

A vast increase in knowledge of the molecular processes underlying malignant change and progression has occurred, which will be useful in the development of new therapies and in areas such as diagnosis of occult disease (4–6), monitoring of disease progress (7, 8), presymptomatic disease detection (screening), and therapy selection/optimization (9–11). In addition, there is increasing excitement about the future potential of individualized therapy (12).

The use of molecular markers in the early detection and management of lung cancer patients has been reviewed (13, 14). One such marker, mutations of the *K-ras* oncogene, has been reported in lung tumors (15) and a variety of other cancers (16). Clinical applications of *K-ras*

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mutation analysis in lung disease include its use as a diagnostic marker of malignancy (of greater sensitivity than cytology alone) in sputum and bronchoalveolar lavage (BAL)<sup>6</sup> samples (17) and its use as a marker of prognosis in lung cancer patients (15, 18, 19). The presence of *K-ras* mutations in lung tumors has been linked with shorter disease-free and overall survival (20–23). Some studies have also specified which codon 12 and 13 changes have prognostic value and which have less clinical relevance (24, 25). Improved stratification of lung tumors using molecular markers such as *K-ras* may lead to the identification of a patient subgroup who require a more aggressive therapy.

Techniques for the detection of *K-ras* mutations in clinical samples have been reviewed (26). Because the detection limits of currently used methods vary, different assays used for the same clinical application lead to different results. It is therefore vital for any novel technique to be fully validated so that the sensitivity and specificity of mutation detection in the relevant clinical sample can be determined. The limit of detection then needs to be taken into account when comparing data obtained by use of different molecular assays to answer the same clinical question (27). We investigated two approaches to *K-ras* mutation detection in samples from lung cancer patients. Both methods used allele-specific amplification (ARMS<sup>TM</sup>) for mutant sequence discrimination. The first method, the Elucigene K-RAS7 method (AstraZeneca Diagnostics, Abingdon, UK), is similar to a previously reported assay (28). The second, a real-time ARMS assay, provides quantitative data for samples positive for *K-ras* mutations. The real-time quantitative approach, with its sensitive detection and mutant sequence quantification, allows the determination of the true detection limit in any clinical application.

### Materials and Methods

#### DNA EXTRACTION

Primary lung tumor samples were obtained from patients undergoing surgery in the Cardiothoracic Centre of Broadgreen Hospital, Liverpool. DNA was extracted from formalin-fixed, paraffin-embedded or frozen tissue sections using a solution of 400 mmol/L Tris-HCl, 150 mmol/L NaCl, 60 mmol/L EDTA, 10 g/L sodium dodecyl sulfate, and 0.2 g/L proteinase K (Sigma-Aldrich) for 2 h at 60 °C. Additional proteinase K was added to a final concentration of 0.3 g/L, and incubation was continued for 16 h at 40 °C. Samples were deproteinized by the addition of NaClO<sub>4</sub> to 1.1 mol/L, followed by chloroform extraction. DNA was precipitated with isopropanol at –20 °C, washed with 700 mL/L ethanol, and redissolved in 20–50 μL of distilled water.

#### ALLELE-SPECIFIC AMPLIFICATION

Two systems that detect the seven most commonly reported *K-ras* mutations in codons 12 and 13 of exon 1 were used in this study. The prototype of a commercially available method (Elucigene K-RAS7) has been described previously (29).

#### ELUCIGENE K-RAS7 METHOD

The Elucigene K-RAS7 method was used in exact compliance with the manufacturer's instructions. Briefly, 5 μL of each DNA sample was added to each reaction mixture and thermocycled in a 9600 machine (PE Applied Biosystems). AmpliTaq Gold DNA polymerase activation was carried out at 94 °C for 20 min, followed by 36 cycles of 94 °C for 45 s (denaturation), 63 °C for 45 s (annealing); and 72 °C for 45 s (extension), with a final extension step of 72 °C for 10 min. Amplification products were analyzed by gel electrophoresis in accordance with the manufacturer's recommendations.

#### REAL-TIME ARMS *K-ras* MUTATION ANALYSIS

The second system combined the approach described above with real-time fluorescent detection of amplification products, thus introducing quantification into the basic ARMS format (Fig. 1). Amplification products were detected by molecular beacons (30). Each reaction contained 50 μmol/L ARMS and common primers, 100 μmol/L beacon, and 60 nmol/L ROX internal reference (PE Applied Biosystems) together with the standard reaction components described previously (29). 2'-O-Methyl RNA beacons complementary to the sense and antisense strands of *K-ras* exon 1 were designed: 5'-FAM-CGCGGUGCCUUGACGAUACAGCUAAUU-CAGAACGCG-MR-3' and 5'-FAM-CGCGGUGCU-GAAAUGACUGAAUAUAAACUUGUGGACGCG-MR-3', respectively. Both probes were labeled with a 5' 6-carboxyfluorescein (FAM) fluorophore and a 3' methyl red (MR) monomer, which acted as a nonfluorogenic (dark) quencher (Oswel DNA Service). 2'-O-methyl RNA and not DNA was used here to avoid fluorescent signal generation from the 5'-3' exonuclease activity of *Taq* polymerase. The cycling conditions were as follows: 94 °C for 20 min to activate the AmpliTaq Gold (PE Applied Biosystems), followed by 50 cycles of a 94 °C denaturation step and a 60 °C combined annealing and extension step. An additional reaction, designed to amplify all *K-ras* exon 1 sequences, was carried out under the same conditions as for the mutant-specific reactions (Fig. 2). When amplification occurred, relative fluorescence increased exponentially in relation to cycle number, and the point at which it passed a fixed threshold above baseline was defined as the threshold cycle number, or C<sub>t</sub> (in accordance with PE Applied Biosystems).

With real-time ARMS *K-ras* mutation detection as described above, a reference data set was generated to establish background signals in the absence of mutations, using wild-type genomic DNA extracted from the periph-

<sup>6</sup> Nonstandard abbreviations: BAL, bronchoalveolar lavage; FAM, carboxyfluorescein; MR, methyl red; C<sub>t</sub>, threshold cycle; Y<sub>i</sub>, inauthentic product; and ASO, allele-specific oligonucleotide.

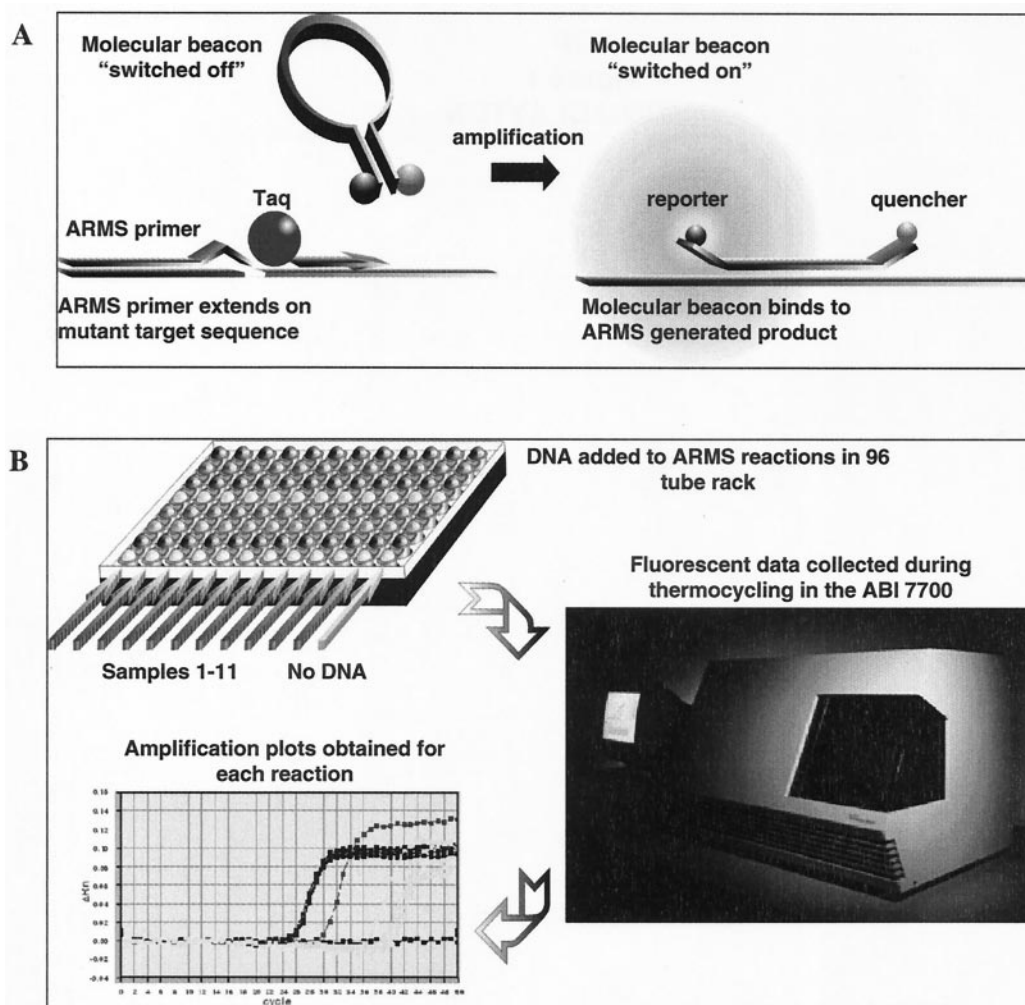


Fig. 1. ARMS allele-specific amplification assay for the real-time amplification of *K-ras* mutant DNA.

(A), when an ARMS primer binds to mutant target DNA, extension and amplification occurs. On the corresponding wild-type target, which may be present in great excess, the 3' nucleotide is not matched, and amplification can only occur at extremely low efficiency. Amplification products can be detected by sequence-specific probes that have been fluorescently labeled. In our assay, molecular beacons (30) have been used to detect amplification products. (B), 11 DNA samples are usually added to all eight ARMS reactions and a control reaction (for total DNA amount) in a 96-tube format. The reactions are thermocycled in the ABI 7700 (PE Applied Biosystems), which measures and records fluorescent signal during amplification.

eral blood of 12 healthy volunteers. This consisted of more than 2000 individual amplifications using DNA of a predetermined concentration. DNA was extracted using a DNA extraction method (Qiagen). DNA was quantified with care ( $A_{260}$  readings were taken from multiple dilutions of each stock DNA to ensure maximum accuracy of quantification). Initial template concentrations of  $1 \times 10^3$ ,  $1 \times 10^4$ , and  $1 \times 10^5$  diploid genome-equivalents (defined as 6–600 ng) of human DNA were selected as the dynamic range over which control data would be generated. Results of the eight real-time ARMS reactions ( $C_t$  values) were analyzed by plotting *K-ras* mutant ARMS reaction  $C_t$  against control  $C_t$ .

Twelve peripheral blood DNA samples were analyzed for each ARMS reaction in batches of eight replicates at three concentrations, by three independent operators. This gave a total of 288 results for each ARMS test and

2016 reactions in total. Inauthentic priming of mutant-specific ARMS primers occurs at extremely low efficiency on wild-type DNA. This gives rise to a yield of inauthentic (non-mutant-derived) product ( $Y_i$ ) during a 50-cycle amplification. Statistical analysis of product appearance (the  $C_t$  value at which amplification products are first detected when fluorescent signal increases above the threshold value) attributable to rare mispriming on wild-type DNA alone, from the 2016 separate reactions, was therefore performed. One-sided prediction intervals were calculated on linear regression analyses of the  $Y_i$  data for each ARMS reaction, and 98% confidence limits were then set for subsequent analysis of samples of unknown mutant status.  $C_t$  values from clinical samples of unknown *K-ras* mutant status containing the wild-type *K-ras* exon 1 sequence have only a 1% probability of falling below the 98% confidence limit.

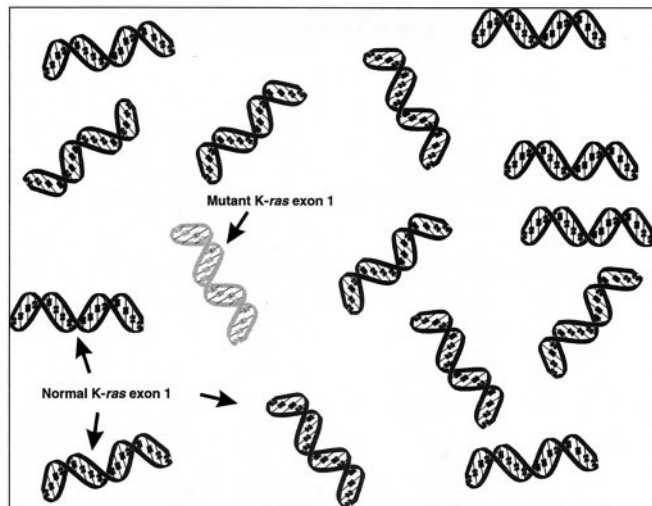


Fig. 2. Principle of underrepresented *K-ras* mutation detection.

The mutant ARMS primer discriminates between mutant and wild-type (*Normal*) genomic copies of *K-ras* exon 1 in any given clinical DNA sample. The control reaction amplifies all *K-ras* exon 1 sequences regardless of mutation status. The relationship between the appearance of amplification products from the ARMS and control reactions is a quantitative measure of the amounts of mutant and wild-type sequences in the clinical sample.

Mutant-only reactions were set up using cloned DNA sequences corresponding to each of the seven most prevalent *K-ras* mutations, as described previously (29). As a control, the wild-type cloned *K-ras* sequence was amplified in the real-time assay to ensure that there were no significant differences in the background signals generated when either genomic DNA or amplicon was used as template. Mutant and wild-type cloned DNA templates were amplified over a concentration range similar to that described above for the genomic template. All of the cloned and amplified *K-ras* sequences were verified by standard sequencing methodology (data not shown).

DNA derived from lung tumor samples of unknown *K-ras* mutant status was analyzed in relation to the data described above. A 5- $\mu$ L aliquot of each sample was added to the seven ARMS reactions and a control reaction. The reactions were thermocycled and analyzed in an ABI 7700 as described above. The ratio of mutant to wild-type *K-ras* exon 1 in the mutant-positive tumor samples was calculated from the data obtained using the cloned mutant *K-ras* sequence. This was done by interpolation from the equation of the regression analysis of results obtained using 100% mutant *K-ras* sequence. For example, in the codon 12 Gly-to-Arg reaction, the equation of the 100% mutant regression line was:  $y = 0.9955x - 0.5808$ , where  $y$  is the ARMS  $C_t$ , and  $x$  is the control  $C_t$ . Therefore, a sample with a control  $C_t$  of 32 cycles would have an ARMS  $C_t$  of 31.3 cycles if it contained 100% mutant (Gly-to-Arg) *K-ras* alone. However, if the actual ARMS  $C_t$  value was 32.3 cycles or 33.3 cycles, then the sample contained 50% or 25% mutant DNA, respectively. This calculation assumes a doubling in amplification product amount after each cycle (31). To

control for this mathematical calculation, samples with established mutant-to-wild-type ratios, from cloning and sequencing experiments (29), were analyzed empirically, and the ratio of mutant to wild-type sequence was compared.

#### MUTATION DETECTION BY PCR-ALLELE-SPECIFIC OLIGONUCLEOTIDE HYBRIDIZATION

Lung tumor DNA samples, analyzed in this study by the real-time assay, were independently analyzed by PCR-allele-specific oligonucleotide (ASO) hybridization. Briefly, samples were amplified in two rounds of PCR, each consisting of 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. PCR products were purified using the Wizard reagent set (Promega), and their concentrations were estimated by agarose gel electrophoresis using molecular weight markers (Life Technologies). Approximately 40 ng of PCR product was transferred to Hybond N+ membrane (Amersham Pharmacia Biotech). Labeled ASO probe (40 ng) was hybridized to the membrane at 40 °C, filters were washed using 3 mol/L tetramethylammonium chloride (Sigma) at 56 °C, and probe was detected as described in the ECL method (Amersham Pharmacia Biotech). The sensitivity of the ASO approach was established using carefully prepared mixtures of mutant and wild-type genomic DNA (50%, 20%, 10%, 5%, and 1% mutant) as described previously (29). A selection of lung tumor DNA samples, positive for a mutation by real-time analysis, were analyzed by ASO hybridization. Tumor DNA samples that were found to contain only wild-type *K-ras* by real-time analysis were analyzed by ASO as a control.

#### Results

The presence of *K-ras* mutations, as detected by ARMS (Table 4), was confirmed by two alternative methods. In the first method, an ASO-based assay was used to verify the results for a selection of mutation-positive lung tumor samples. Fig. 3 shows the ASO hybridization results for the codon 12 GGT-to-CGT (Gly-to-Arg) change. The signals for 50%, 20%, 10%, 5%, and 1% mutant controls (mutation-positive cell line/wild-type DNA admixtures) were higher than signals observed for controls representing the six other *K-ras* mutations or tumor samples known to contain mutations other than Gly-to-Arg. Sample 42, however, was positive for the Gly-to-Arg mutation by real-time analysis and had a signal above background by ASO. Signals from lung tumor samples containing high concentrations of codon 12 Gly-to-Cys mutant DNA were higher than the background wild-type signal (Fig. 3). For example, sample 38 contained 100% mutant Cys DNA by real-time analysis, and this cross-reacted with the ASO probe for the codon 12 Gly-to-Arg mutation. The probe, intended to be specific for Arg, contains a guanosine at the first position of codon 12, complementary to the cytosine in mutant (Gly-to-Arg) *K-ras* exon 1. This interacts very weakly with the guanosine at the same position

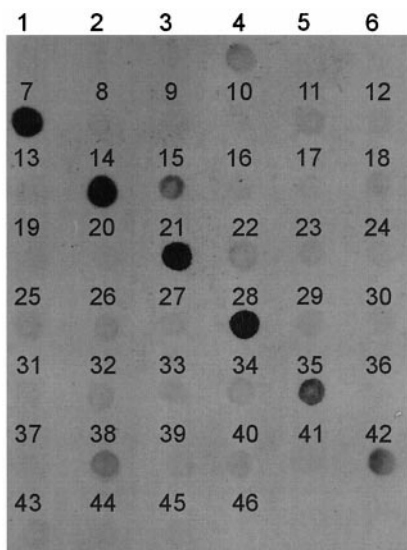


Fig. 3. PCR-ASO confirmation of samples positive for codon 12 Gly-to-Arg mutations by ARMS analysis.

The autoradiograph shows results from the codon 12 Gly-to-Arg-specific ASO hybridization. Samples were analyzed blind by an independent operator. Cell line DNA representing each of seven *K-ras* mutations was mixed in decreasing amounts (50%, 20%, 10%, 5%, and 1%) with wild-type genomic DNA. PCR products in samples 1, 8, 15, 22, and 29 correspond to 50%, 20%, 10%, 5%, and 1% codon 12 Gly-to-Ser mutant DNA, respectively. The same positive control amounts were analyzed for each *K-ras* mutation: samples 2, 9, 16, 23, and 30, codon 12 Gly-to-Val; samples 3, 10, 17, 24, and 31, codon 12 Gly-to-Asp; samples 4, 11, 18, 25, and 32, codon 12 Gly-to-Cys; samples 5, 12, 19, 26, and 33, codon 13 Gly-to-Asp; samples 6, 13, 20, 27, and 34, codon 12 Gly-to-Ala; samples 7, 14, 21, 28, and 35, codon 12 Gly-to-Arg. Samples 36 and 37 are products from wild-type control DNA samples extracted from peripheral blood. Six lung tumor samples were included that were positive for codon 12 Gly-to-Cys (sample 38), codon 12 Gly-to-Val (sample 39), codon 12 Gly-to-Ser (sample 40), codon 12 Gly-to-Ala (sample 41), codon 12 Gly-to-Arg (sample 42), and codon 12 Gly-to-Asp (sample 43). Two PCR-negative controls (samples 44 and 45) and one water control (sample 46) were also used.

in wild-type DNA. However, the codon 12 Gly-to-Cys mutation, resulting from a guanosine-to-thymidine change at this position, means that the ASO probe binds

with a higher affinity and so gives a higher signal than other codons (G-T mismatches are weakly destabilizing compared with G-G mismatches) (32).

A second approach was used to confirm the mutation status derived from the ARMS assays. Colorectal tumor DNA samples, in which *K-ras* mutant sequences were quantified previously by cloning and sequencing (29), were tested in the new ARMS assay. These samples were analyzed using the real-time assay, and mutant/wild-type quantities were compared. The results of these experiments are summarized in Table 1. The reported frequencies obtained by the two approaches were largely in agreement, and discrepancies may have been attributable to sampling issues because different aliquots of the tumor DNA samples were analyzed at two different geographic sites with a long time interval between measurements (3 years). These two approaches confirmed both the qualitative and quantitative aspects of the real-time ARMS assay, within the sensitivity range of the ASO procedure ( $\geq 5\%$  mutant target).

An example of the Elucigene K-RAS7 assay results are shown in Fig. 4. Tumor DNA was extracted from both paraffin-embedded and frozen tissue samples. The paraffin-embedded tissue samples in general showed either partial or complete loss of the upper control product (360 bp). This is characteristic of amplified DNA obtained from this sample source in which genomic DNA is generally degraded. The lower control product (111 bp) usually is present even when the upper control is missing. The ARMS products were 158–190 bp in length, and were therefore largely unaffected by the DNA degradation observed for paraffin-embedded samples. However, in samples where the upper control product was missing and the lower control was present (although absence of an ARMS product is probably attributable to the fact that the sample is wild type), DNA fragmentation may have

Table 1. Comparison of *K-ras* mutation quantities in colorectal tumor samples quantified previously by an alternative method.

Tumor no.	<i>K-ras</i> mutation	Real-Time, <sup>a</sup> %	Alternative method		Real-Time/Alternative method, %
			% <sup>b</sup>	No. clones <sup>c</sup>	
546	Codon 12 GAT	11	8	39	1.4
565	Codon 12 GAT	5	6	34	0.9
302	Codon 13 GAC	12.5	10	41	1.3
596	Codon 13 GAC	14	21	24	0.7
500	Codon 12 TGT	33	30	33	1.1
530	Codon 12 TGT	11	6	34	1.8
188	Codon 12 GTT	33	21	39	1.6
556	Codon 12 GTT	25	32	37	0.8
777	Codon 12 GTT	20	11	38	1.8
1210	Codon 12 GTT	2	7	42	0.3
1289	Codon 12 GTT	20	26	38	0.8

<sup>a</sup> Amount of mutant *K-ras* exon 1 calculated using the real-time assay.

<sup>b</sup> Amount of mutant *K-ras* exon 1 calculated using the cloning and confirmatory sequencing method described by Fox et al. (29).

<sup>c</sup> Number of clones analyzed to obtain the quantitative data in Fox et al. (29).

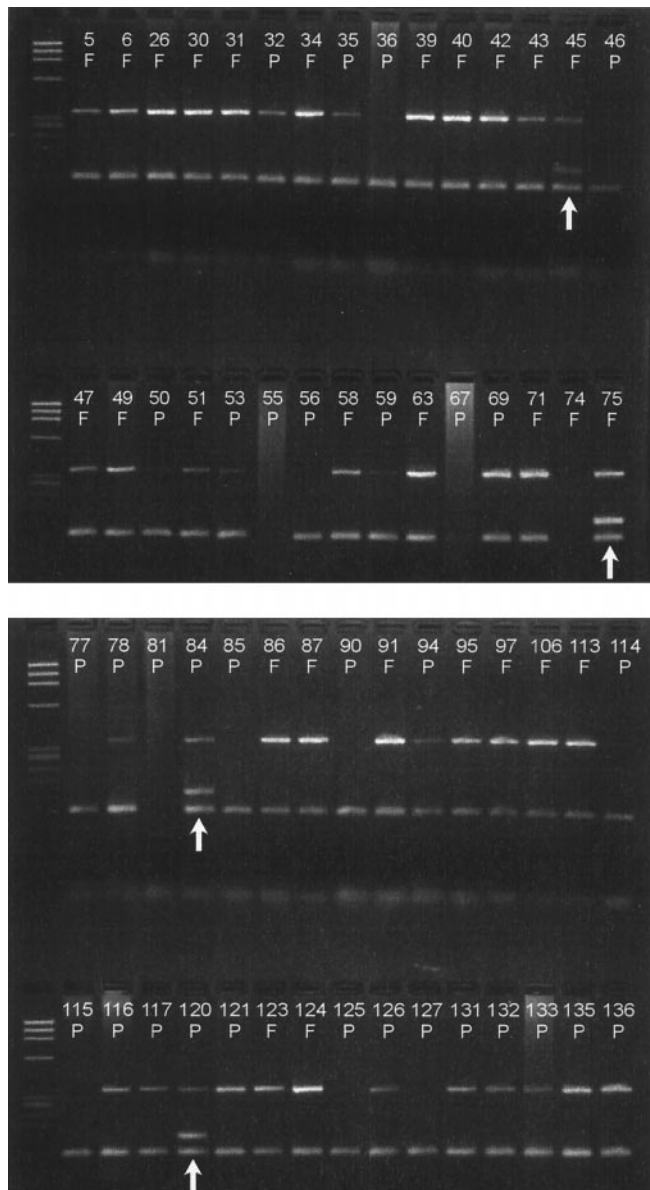


Fig. 4. Results of the Elucigene K-RAS7 codon 12 GGT-to-GTT (Gly-to-Val) reaction.

The photographs of agarose gels show amplification products from 60 lung tumor DNA samples. Presence of an ARMS product indicates tumors with a mutation (white arrow). Tissue sources were a paraffin-embedded (P) and a frozen (F) section.

occurred, i.e., fragmentation of target mutant genomic DNA above 111 bp (lower control product size) in length. These events were recorded as wild type in the results summarized in Table 2.

Tumor samples 45, 75, 84, and 120 were positive for codon 12 GGT-to-GAT (Gly-to-Val) mutations, as indicated by the presence of ARMS products on the gel (Fig. 4). DNA concentrations after extraction from the lung tissue were not standardized before amplification, and this most likely accounted for the visible differences in overall product yields among the samples. One advantage

of the real-time assay is that initial DNA concentrations do not require standardization.

*K-ras* mutations were detected in 15% (9 of 62) lung tumor DNA samples by Elucigene KRAS-7 ARMS analysis (Table 2.). Nineteen percent of adenocarcinomas and 10% of squamous cell carcinomas were found to have mutations. No *K-ras* mutations were detected for the other lung tumors analyzed. The frequencies of the mutant sequences in *K-ras*-positive lung tumor samples were calculated using real-time analysis, by interpolation from the equation of the regression analysis for 100% mutant DNA for each ARMS reaction, and were expressed as percentage of total *K-ras* exon 1 in the samples (Table 3). All mutations detected by the Elucigene K-RAS7 method were also detected by real-time analysis. Twenty additional squamous cell carcinomas were analyzed by the real-time assay to further characterize *K-ras* mutations occurring in this tumor type. The results of the real-time analyses are summarized in Table 4. The ratios of mutant to wild-type sequences in the positive samples were expressed as percentages of the total number of *K-ras* exon 1 sequences (Table 5).

The nature of ARMS mispriming on non-mutant DNA is determined by the 3' nucleotide species that mispairs with the wild-type nucleotide in the target sequence. These mismatches are not absolute and produce rare priming events, occurring at extremely low efficiencies, that depend on the strength of the mismatched base pair interaction (32). These rare mispriming events were controlled for in our assay, thus allowing the reliable analysis of clinical samples of unknown *K-ras* status. The strength of the mismatched base pair interaction is reflected in the  $Y_i$  data for each test (see Fig. 5). For example, in the codon 12 Gly-to-Ala test (Fig. 5B), the ARMS primer specific for the GGT-to-GCT change has a 3' terminal guanosine that is strongly destabilized from the guanosine at position 2 in codon 12 in wild-type *K-ras*. This means that  $Y_i$  resulting from inauthentic priming events in this assay is rarely seen. In the codon 12 Gly-to-Asp test (Fig. 5A), the ARMS primer has a 3' terminal thymidine mismatched with the guanosine in wild-type *K-ras* exon 1. This interaction is less destabilizing, and so  $Y_i$  appears earlier in the amplification process.

Negligible variations in  $Y_i$  data were observed between operators or between DNA samples of equivalent DNA concentration (results not shown). Fig. 5 shows the real-time data for all lung tumor samples in all seven ARMS tests. As described in *Materials and Methods*, tumor samples were analyzed in relation to  $Y_i$  data to determine mutational status. There was a 1% chance of a sample appearing below the lower limit of the 98% confidence interval if it contained wild-type sequence alone, and if such a case was observed, it was considered positive for the mutation interrogated by that specific ARMS primer. Duplicate analysis of each sample markedly reduced the chance of a sample appearing as mutation-positive twice when it contained wild-type sequence alone. The quantity

**Table 2. Distribution of K-ras mutations found with the Elucigene K-RAS7 method by histological subtype.**

Histology	Codon 12						Codon 13	Total	Positive samples <sup>a</sup>
	GCT	CGT	GAT	TGT	AGT	GTT	GAC		
Adenocarcinoma	0	1	2	1	0	3	0	7	19% (7/36)
Adenosquamous	0	0	0	0	0	0	0	0	0% (0/2)
Atypical carcinoid	0	0	0	0	0	0	0	0	0% (0/1)
Carcinosaroid	0	0	0	0	0	0	0	0	0% (0/1)
Large cell carcinoma	0	0	0	0	0	0	0	0	0% (0/2)
Non-small cell carcinoma	0	0	0	0	0	0	0	0	0% (0/1)
Squamous	0	0	0	1	0	1	0	2	10% (2/19)
Total	0	1	2	2	0	4	0	9	15% (9/62)

<sup>a</sup> Number of K-ras mutation-positive samples as a percentage of each subtype (actual numbers in parentheses).

of mutant sequence in these samples was calculated by interpolation using the 100% mutant data (not shown). Fifty cycles is the endpoint of the assay, and samples were ascribed a  $C_t$  of 50 cycles if they had not yielded a detectable ARMS product by this point in the amplification process. The majority of tumor samples were negative for K-ras mutations in each test. This provided the opportunity to observe the nature of mispriming events for individual ARMS primers on the wild-type K-ras exon 1 sequence. At high concentrations of starting genomic target DNA ( $>1 \times 10^4$  copies), mispriming events did occur, and  $Y_i$  appeared relatively early and with limited variability. At lower starting template concentrations, however, mispriming events were rarer and amplification varied stochastically, with detection of  $Y_i$  dramatically reduced to the extent that samples often gave no detectable product up to 50 cycles. Addition experiments conducted with low ratios of mutant to wild-type DNA added to a K-ras mutant-negative tumor sample showed that this type of sample still yielded ARMS products below the background cutoff for wild-type DNA if mutant sequence was present (data not shown).

### Discussion

In the real-time assay, 44% (16 of 36) of adenocarcinomas were positive for K-ras mutations, whereas 19% (7 of 36)

were positive in the Elucigene method. The frequency of positives obtained using the real-time assay was higher than some published data, which reflects differences in assay sensitivity. Mutation frequencies of 29% (17 of 58) (24), 26% (47 of 184) (25), and 28% (19 of 69) (15) have been reported for ASO-based studies. Frequencies of 38% (6 of 16) (22) and 11% (10 of 88) (21) have been reported for a single-strand conformation polymorphism study, with confirmatory sequencing of samples that were positive by single-strand conformation polymorphism analysis. A restriction fragment length polymorphism assay (23), with a sensitivity of 10%, found 8% of adenocarcinomas positive for K-ras mutations. Using ultrasensitive assays that use restriction enzymes to cut wild-type amplicon and thus enrich for mutant K-ras amplicon during additional rounds of amplification, investigators found 46% (13 of 28) of adenocarcinomas positive for K-ras mutations (33). This is similar to the frequency observed in the real-time ARMS assay in this study (44%). The real-time assay is a more convenient and highly reproducible method by comparison. Any laboratory routinely using sensitive assays involving multiple rounds of amplification and manipulation of PCR products may be prone to contamination problems even with the strictest control measures in place. The real-time assay has the advantages of being homogeneous and requiring no manipulation of amplification products. In addition, data were obtained over a dynamic range of DNA concentrations to facilitate direct analysis of DNA routinely extracted from clinical samples without the need to standardize DNA input amount.

There are two major reasons for the increased sensitivity of the real-time assay over the Elucigene method and other assay systems. The assay was designed to identify mutation-positive samples as "not wild type" by comparison to a data set obtained using wild-type DNA alone. This maximizes sensitivity compared with alternative methods that specify a cutoff at which the "signal" is considered positive in relation to a positive control containing a low mutant fraction. In addition, the behavior of the ARMS primers on wild-type DNA was established over a dynamic concentration range (100-fold) in a 50-

**Table 3. K-ras mutation frequencies for samples positive with the Elucigene K-RAS7 method.**

Tumor no.	K-ras mutation <sup>a</sup>	Mutant:Wild-type sequence, <sup>b</sup> %
5	Codon 12 TGT (Cys)	50
35	Codon 12 TGT (Cys)	100
45	Codon 12 GTT (Val)	14
47	Codon 12 GAT (Asp)	50
75	Codon 12 GTT (Val)	100
84	Codon 12 GTT (Val)	33
120	Codon 12 GTT (Val)	33
155	Codon 12 GAT (Asp)	20
156	Codon 12 CGT (Arg)	50

<sup>a</sup> Resultant amino acid changes are shown in parentheses.

<sup>b</sup> Amount of mutant K-ras exon 1 calculated by analysis of the sample using the real-time assay.

**Table 4. Distribution of K-ras mutations detected with the real-time assay by histological subtype.**

Histology	Codon 12						Codon 13	Total	Positive samples <sup>a</sup>
	GCT	CGT	GAT	TGT	AGT	GTT	GAC		
Adenocarcinoma	3	3 <sup>b</sup>	3 <sup>b</sup>	3	0	6 <sup>b</sup>	0	16	44% (16/36)
Adenosquamous	0	0	0	0	0	0	0	0	0% (0/2)
Atypical carcinoid	0	0	0	0	0	0	0	0	0% (0/1)
Carcinosarroid	0	0	0	1	0	0	0	1	100% (1/1)
Large cell carcinoma	0	1	0	0	0	0	0	1	50% (1/2)
Non-small cell carcinoma	0	0	0	0	0	0	0	0	0% (0/1)
Squamous	4 <sup>b</sup>	2	4 <sup>b</sup>	7 <sup>b</sup>	1	7 <sup>b</sup>	3 <sup>b</sup>	18	46% (18/39)
Total	7	6	7	11	1	13	03	36	44% (36/82)

<sup>a</sup> Number of K-ras mutation-positive samples as a percentage of each subtype (actual numbers in parentheses).

<sup>b</sup> Includes samples that have more than one K-ras mutation.

cycle amplification. This range was chosen by reference to DNA yields routinely obtained from various clinical samples entering our laboratory (tissue and cell suspensions in body fluids). Therefore, unknown clinical samples containing different DNA amounts in the fixed assay volume could all be analyzed in the same 50-cycle amplification. Measurement and standardization of the DNA concentration before any molecular assay can be unrealistic in the clinical setting when overall yields are low and the sample is limited. If an assay involves PCR with an early fixed endpoint (to maintain specificity), then unknown samples with low DNA concentrations and K-ras mutations will be reported as false negatives.

It is worth pointing out that a high percentage of squamous cancers were positive for K-ras mutations in this study. Mutations in the K-ras oncogene are not restricted to adenocarcinomas, and other investigators have demonstrated this (24, 34) or referred to it (28). There may be several reasons for differences in perception concerning the occurrence of K-ras mutations and whether they are restricted to the adenocarcinoma tumor type. These may include differences in the molecular assays used and heterogeneity of the tumor tissue. Multiple sections from a tumor sample may indicate either adenocarcinoma or squamous histological types, and this gives rise to the possibility that a histological definition of tumor type may not reflect the true nature of the cell

population analyzed for K-ras mutations. There were no differences in the amounts of K-ras mutant sequence between the adenocarcinoma and squamous carcinoma types in this study.

The clinical significance of the quantity of K-ras mutation in a positive clinical sample is yet to be established. One study has separated K-ras results into low-fraction and high-fraction positives. The authors reported an increased stage of disease and decreased survival linked to the high-fraction positives, although statistical significance was not reached with the limited number of samples investigated (34). Mutation of the K-ras oncogene is considered an early event in lung cancer development (35), although not necessarily the first (36), and measurement of the mutation fraction may simply reflect disease stage. Larger studies using quantitative assays may establish the clinical relevance of molecular markers in relation to treatment or survival endpoints. Quantitative data may also be important when looking for malignant cells distant to the site of a tumor, such as in the screening or early diagnosis setting, where less-invasive clinical procedures are used. Quantitative data could be used in establishing the required assay sensitivity for a particular clinical application. For example, in lung cancer this would involve the analysis of sputum and BAL samples to potentially detect clinically occult tumors. Matched K-ras mutations have been demonstrated in adenocarcinomas of the lung and corresponding sputum (37) and BAL (38) samples in lung cancer patients. To date, we have used our assay on 108 bronchial lavage samples (58 from patients with a confirmed malignancy and 50 from non-malignant cases). In five mutation-positive BAL samples, cytology reported no malignant cells present despite malignancy being confirmed (data not shown). Three of eight BALs from confirmed adenocarcinomas were positive in the assay. One of 50 non-malignant cases was positive, with the patient reported as having heart disease.

The real-time assay used here offers a unique opportunity to assess the clinical significance of K-ras mutations in lung and other cancers. The sensitive nature of the

**Table 5. Amount of K-ras mutant sequence in the positive samples detected by the real-time assay.**

K-ras mutation <sup>a</sup>	Total	Mutant:Wild-type sequence, <sup>b</sup> %
Codon 12 GCT (Ala)	7	0.04–100
Codon 12 CGT (Arg)	6	0.05–50
Codon 12 GAT (Asp)	7	0.5–50
Codon 12 TGT (Cys)	10	8–100
Codon 12 AGT (Ser)	2	3–25
Codon 12 GTT (Val)	11	0.4–100
Codon 13 GAC (Asp)	3	3–33

<sup>a</sup> Resultant amino acid changes shown in parentheses.

<sup>b</sup> Range of mutant K-ras exon 1 amounts are shown for each mutation.

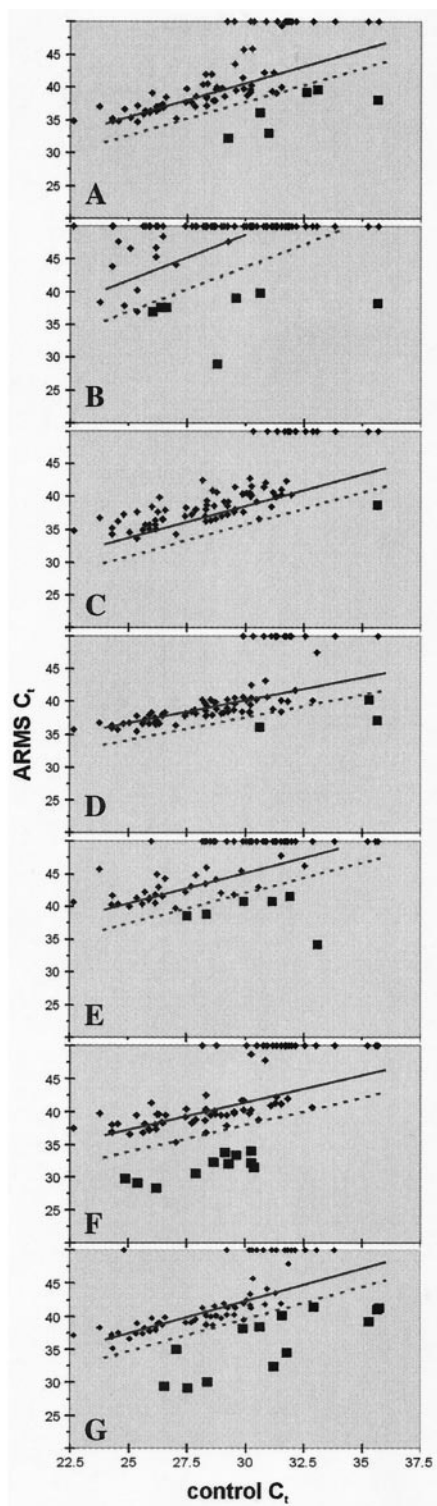


Fig. 5. Real-time ARMS analysis of 84 lung tumor DNA samples for *K-ras* mutations.

Appearance of products in the ARMS and control reactions ( $C_t$ ) for each tumor sample were plotted against the wild-type data. DNA samples were analyzed in each *K-ras* test: (A), codon 12 GGT-to-GAT (Gly-to-Asp); (B), codon 12 GGT-to-GCT (Gly-to-Ala); (C), codon 12 GGT-to-AGT (Gly-to-Ser); (D), codon 13 GGC-to-GAC (Gly-to-Asp); (E), codon 12 GGT-to-CGT (Gly-to-Arg); (F), codon 12 GGT-to-TGT (Gly-to-Cys); and (G), codon 12 GGT-to-GTT (Gly-to-Val). Tumor DNA samples are labeled as either mutation-positive (■) or -negative (◆). The two regression lines represent mean  $Y_i$  (—) and the 99% confidence limit for  $Y_i$  data (---).

assay provides the opportunity to screen large numbers of clinical samples for malignant cells in the early detection setting. The utility of *K-ras* mutation detection in BAL samples as a precursor to lung cancer diagnosis has been demonstrated previously (37). At present, we are using this method to investigate the utility of *K-ras* mutation detection for pancreatic and colorectal disease management. The assay has been designed to facilitate high-throughput sample analysis after routine nucleic acid extraction procedures for a range of clinical materials.

## References

1. Sidransky D. Nucleic acid-based methods for the detection of cancer. *Science* 1997;278:1054–8.
2. Sikora K. Cancer. In: Marinker M, Peckham M, eds. *Clinical futures*. London: BMJ Publishing Group, 1998:74–95.
3. Jones D, Fletcher CD. How shall we apply the new biology to diagnostics in surgical pathology? *J Pathol* 1999;187:147–54.
4. Noguchi M, Tsugawa K, Bando E, Kawahara F, Miwa K, Yokoyama K, et al. Sentinel lymphadenectomy in breast cancer: identification of sentinel lymph node and detection of metastases. *Breast Cancer Res Treat* 1999;53:97–104.
5. Sturm PD, Hruban RH, Ramsoekh TB, Noorduy LA, Tytgat GN, Gouma DJ, et al. The potential diagnostic use of *K-ras* codon 12 and p53 alterations in brush cytology from the pancreatic head region. *J Pathol* 1998;186:247–53.
6. Sanchez-Cespedes M, Esteller M, Hibi K, Cope FO, Westra WH, Piantadosi S, et al. Molecular detection of neoplastic cells in lymph nodes of metastatic colorectal cancer patients predicts recurrence. *Clin Cancer Res* 1999;5:2450–4.
7. Steiner G, Schoenberg MP, Linn JF, Mao L, Sidransky D. Detection of bladder cancer recurrence by microsatellite analysis of urine. *Nat Med* 1997;3:621–4.
8. Hirsch-Ginsberg C. Detection of minimal residual disease: relevance for diagnosis and treatment of human malignancies. *Annu Rev Med* 1998;49:111–22.
9. Liefers GJ, Cleton-Jansen AM, van de Velde CJ, Hermans J, van Krieken JH, Comelisse CJ, et al. Micrometastases and survival in stage II colorectal cancer [see comments]. *N Engl J Med* 1998;339:223–8.
10. Leichman CG. Thymidylate synthase as a predictor of response. *Oncology* 1998;12:43–7.
11. Findlay MP, Cunningham D, Morgan G, Clinton S, Hardcastle A, Aherne GW. Lack of correlation between thymidylate synthase levels in primary colorectal tumours and subsequent response to chemotherapy. *Br J Cancer* 1997;75:903–9.
12. Evans WE, Relling MV. Pharmacogenomics: translating functional genomics into rational therapeutics. *Science* 1999;286:487–91.
13. Salgia R, Skarin AT. Molecular abnormalities in lung cancer. *J Clin Oncol* 1998;16:1207–17.
14. Field JK. Selection and validation of new lung cancer markers for the molecular-pathological assessment of individuals with a high risk of developing lung cancer. In: Brambilla C, Brambilla E, eds. *Lung tumours: fundamental biology and clinical management*, 2nd ed. New York: Marcel Dekker, 1999:287–302.
15. Slebos RJ, Kibbelaar RE, Dalesio O, Kooistra A, Stam J, Meijer CJ, et al. *K-ras* oncogene activation as a prognostic marker in adenocarcinoma of the lung. *N Engl J Med* 1990;323:561–5.
16. Bos JL. The *ras* gene family and human carcinogenesis. *Mutat Res* 1988;195:255–71.
17. Gazdar AF, Minna JD. Molecular detection of early lung cancer [Editorial]. *J Natl Cancer Inst* 1999;91:299–301.

18. Slebos RJ, Rodenhuis S. The *ras* gene family in human non-small-cell lung cancer. *J Natl Cancer Inst Monogr* 1992;23-9.
19. Huncharek M, Muscat J, Geschwind JF. *K-ras* oncogene mutation as a prognostic marker in non-small cell lung cancer: a combined analysis of 881 cases. *Carcinogenesis* 1999;20:1507-10.
20. Nemunaitis J, Klemow S, Tong A, Courtney A, Johnston W, Mack M, et al. Prognostic value of *K-ras* mutations, *ras* oncoprotein, and *c-erb B-2* oncoprotein expression in adenocarcinoma of the lung. *Am J Clin Oncol* 1998;21:155-60.
21. Huang CL, Taki T, Adachi M, Konishi T, Higashiyama M, Kinoshita M, et al. Mutations of *p53* and *K-ras* genes as prognostic factors for non-small cell lung cancer. *Int J Oncol* 1998;12:553-63.
22. Cho JY, Kim JH, Lee YH, Chung KY, Kim SK, Gong SJ, et al. Correlation between *K-ras* gene mutation and prognosis of patients with nonsmall cell lung carcinoma. *Cancer* 1997;79:462-7.
23. Fukuyama Y, Mitsudomi T, Sugio K, Ishida T, Akazawa K, Sugimachi K. *K-ras* and *p53* mutations are an independent unfavourable prognostic indicator in patients with non-small-cell lung cancer. *Br J Cancer* 1997;75:1125-30.
24. Rosell R, Monzo M, Molina F, Martinez E, Pifarre A, Moreno I, et al. *K-ras* genotypes and prognosis in non-small-cell lung cancer. *Ann Oncol* 1995;6:S15-20.
25. Gregorio LD, Manenti G, Incarbone M, Pilotti S, Pastorino U, Pierotti MA, et al. Prognostic value of loss of heterozygosity and *KRAS2* mutations in lung adenocarcinoma. *Int J Cancer* 1998;79:269-72.
26. Nollau P, Wagener C. Methods for detection of point mutations—performance and quality assessment [Review]. *Clin Chem* 1997;43:1114-28.
27. Gazdar AF, Virmani A. Sensitive methods for the detection of *ras* mutations in lung cancer: some answers, more questions [Editorial]. *Clin Chem* 1998;44:1376-8.
28. Neville EM, Ellison G, Kiaris H, Stewart M, Spandidos DA, Fox JC, et al. Detection of *K-ras* mutations in non-small cell lung carcinoma. *Int J Oncol* 1995;7:511-4.
29. Fox JC, England J, White P, Ellison G, Callaghan K, Charlesworth NR, et al. The detection of *K-ras* mutations in colorectal cancer using the amplification-refractory mutation system. *Br J Cancer* 1998;77:1267-74.
30. Tyagi S, Kramer FR. Molecular beacons—probes that fluoresce upon hybridization. *Nat Biotechnol* 1996;14:303-8.
31. Higuchi R, Fockler C, Dollinger G, Watson R. Kinetic PCR analysis. real-time monitoring of DNA amplification reactions. *Biotechnology* 1993;11:1026-30.
32. Huang MM, Arnheim N, Goodman MF. Extension of base mispairs by Taq DNA polymerase: implications for single nucleotide discrimination in PCR. *Nucleic Acids Res* 1992;20:4567-73.
33. Mills NE, Fishman CL, Rom WN, Dubin N, Jacobson DR. Increased prevalence of *K-ras* oncogene mutations in lung adenocarcinoma. *Cancer Res* 1995;55:1444-7.
34. Keohavong P, Zhu D, Melacrinis AC, Demichele AA, Weyant RJ, Luketich JD, et al. Detection of low-fraction *K-ras* mutations in primary lung tumors using a sensitive method. *Int J Cancer* 1997;74:162-70.
35. Sagawa M, Saito Y, Fujimura S, Linnoila RI. *K-ras* point mutation occurs in the early stage of carcinogenesis in lung cancer. *Br J Cancer* 1998;77:720-3.
36. Wistuba II, Lam S, Behrens C, Virmani AK, Fong KM, LeRiche J, et al. Molecular damage in the bronchial epithelium of current and former smokers. *J Natl Cancer Inst* 1997;89:1366-73.
37. Mao L, Hruban RH, Boyle JO, Tockman M, Sidransky D. Detection of oncogene mutations in sputum precedes diagnosis of lung cancer. *Cancer Res* 1994;54:1634-7.
38. Ahrendt SA, Chow JT, Xu LH, Yang SC, Eisenberger CF, Esteller M, et al. Molecular detection of tumor cells in bronchoalveolar lavage fluid from patients with early stage lung cancer [see comments]. *J Natl Cancer Inst* 1999;91:332-9.