PCR-based Testing for Therapy-related *EGFR* Mutations in Patients with Non-small Cell Lung Cancer

REGINE DAHSE[1], ALEXANDER BERNDT[2] and HARTWIG KOSMEHL[1]

[1]*HELIOS Clinic Erfurt, Institute of Pathology, Erfurt;*
[2]*Friedrich Schiller University Jena, Institute of Pathology, Jena, Germany*

Abstract. *Background: In patients with non-small cell lung cancer (NSCLC), mutations in the epidermal growth factor receptor gene (EGFR) have been associated with improved response to tyrosine kinase inhibitors. Two hotspot mutations located in exon 19 and exon 21 account for about 90% of all EGFR mutations. Materials and Methods: We designed a Bi-PASA (bidirectional PCR amplification of specific alleles) assay to detect the most common exon 19 deletion (codons 746-750) and an allele-specific PCR for the L858R mutation in exon 21. To validate the assays for use in clinical diagnostics, 35 lung adenocarcinoma samples were analyzed. Results: Both assays provided the predicted amplification pattern for normal and mutant genotypes with high specificity and sensitivity. In serial dilution experiments, the mutant alleles were detectable in mixed samples with an at least 6-fold excess of normal DNA. Three exon 19 deletions were identified in the tumor samples. Conclusion: Both assays are fast and easy to perform in any routine PCR laboratory with no special equipment other than thermocyclers. They provide sensitive and costeffective initial EGFR testing for identifying lung cancer patients who might clinically benefit from tyrosine kinase inhibitors.*

Non-small cell lung cancer (NSCLC) is one of the most common types of cancer worldwide. Relapses are frequent after therapy and often evolve into lethal metastatic disease. Recent studies (1-3) have identified a subset of NSCLC patients with rapid and durable clinical responses to EGFR tyrosine kinase inhibitors (*i.e.* gefitinib, erlotinib) and found an underlying association between mutations in the *EGFR* tyrosine kinase domain and drug-responsive NSCLC. These heterozygous *EGFR* tyrosine kinase mutations, clustered within the ATP-binding pocket (4), cause ligand-independent activation of EGFR, while simultaneously increasing sensitivity to tyrosine kinase inhibitors. Two mutations account for approximately 90% of all *EGFR* mutations reported to date in lung adenocarcinoma (5-6). An in-frame deletion of 9-24 nucleotides in exon 19 centered around codons 746-750 makes up about 50% of mutations. The second most common mutation, found in up to 45% of cases with *EGFR* mutations, is a point mutation at nucleotide 2573 (C**T**G to C**G**G) in exon 21 that results in substitution of leucine by arginine at codon 858 (L858R). For current clinical practice in the care of lung cancer patients, simple screening assays for molecular predictors of response to EGFR tyrosine kinase inhibitors are needed. The gold standard for mutation detection is the genomic sequencing of DNA amplified from tumor tissues, however, this method is labor-intensive and of suboptimal sensitivity because clinical tumor specimens may contain a high percentage of nonneoplastic cells. Over the past three years, many methods have been proposed for the improved detection of *EGFR* mutations [reviewed in (7)]. Most of them involve complex assays or require expensive equipment or reagents and cannot be easily adapted for use in routine diagnostics. The aim of this study was to develop two simple and sensitive assays for the initial screening of the two most common therapy-related *EGFR* mutations in clinical tumor samples based on allele-specific PCR.

Correspondence to: Dr. Regine Dahse, HELIOS Klinikum Erfurt, Institute of Pathology, Nordhäuser Str. 74, D-99089 Erfurt, Germany. Tel: +493617812770, Fax: +493617812760, e-mail: rdahse@erfurt.helios-kliniken.de

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Materials and Methods

Samples and DNA isolation. Genomic DNA was extracted and pooled from oral mucosa samples of five healthy individuals. This pooled DNA was used as a normal DNA control for the development of the PCR assays. Heterozygous mutant control DNA was extracted from lung cancer cell lines NCI-H-1650 and NCI-H-1975 (American Type Culture Collection, LGC Promochem, Wesel, Germany) which contain the heterozygous exon 19 deletion (H-1650) and the exon 21 L858R mutation (H-1975) (4).

Formalin-fixed tumor samples were obtained from 35 patients treated at the HELIOS Clinical Center Erfurt, Germany, with the

Table I. *Primer sequences and PCR parameters.*

	Primer	Primer sequences (5'-3')	Optimal primer concentration (μmol/l)	Amplicon size (bp)
Bi-PASA exon 19	19 P	GTAACATCCACCCAGATCACTG	0.25	PQ: 444
	19Q	GTGTCAAGAAACTAGTGCTGGG	0.25	AQ: 325
	19A	CCCGTCGCTATCAAGGAATTAA	0.25	PB: 134
	19B	GTTGGCTTTCGGAGATGTTTTGATAG	0.25	
Allele-specific PCR exon 21	21P	AGGGTCTTCTCTGTTTCAGGGCAT	0.1	
	21A	TTCCGCACCCAGCAGTTTGGCTA	0.1	PA: 137
	21B	CGC ACC CAG CAG TTT GGTTC	0.1	PB: 134

histopathological diagnosis of lung adenocarcinoma. The study cohort consisted of 19 males and 16 females with a median age at diagnosis of 68.8 years. Informed consent was obtained in writing from all patients. This study was conducted in accordance with the principles of the Declaration of Helsinki, as adopted by the 29th World Medical Assembly, Helsinki, Finland, and revised at the 48th World Medical Assembly in Somerset West, Republic of South Africa, 1996. DNA was isolated using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

Exon 19 E746_A750del deletion assay. For detecting the exon 19 deletion, a so-called bidirectional PCR amplification of specific alleles procedure (8) was designed. Bi-PASA contains four primers: i) two allele-specific primers complementary to the sequence variation in a bi-directional orientation such that both primers terminate at the variation site and ii) two so-called outer primers that anneal at different distances from the sequence variation to differentiate the downstream and upstream reactions on an agarose gel. In this way, the assay has an inherent control, since at least one allele specific fragment is amplified per reaction The two outer primers (P and Q) were placed on the opposing strands at pre-defined bases, resulting in an amplicon of 444 bp as internal standard. The 3' end of allele-specific inner primer A matches a part of the deletion sequence, *i.e.* the AQ fragment is only amplified in the case of the wild-type sequence. The inner primer B matches the mutant DNA sequence containing the deletion, *i.e.* PB can be amplified only in homozygous mutant DNA. A heterozygous genotype (N/M) is characterized by both a 325 bp AQ- and a 134 bp PB amplification fragment (Figure 1). The allele-specific primers are designed to detect both possible nucleotide deletions (c.2235_2249del15 and c.2236_2250del15) that give rise to a E746_A750del protein change. Parameters important for designing the four Bi-PASA primers were the melting temperature (with Tm values of A and B lower than that of the PQ segment) and the annealing temperature (relatively high to avoid hybridization between the multiple PCR products).

Exon 21 L 858R point mutation assay. The basis for discrimination using allele-specific PCR is that a PCR primer mismatched at its 3' end with the DNA template will react less efficiently than one that is entirely complementary. Allele-specific primers, A and B, were designed with mismatches at their 3' end – A complements the normal allele (2573T) while B complements the mutant allele (2573G). At the allele-specific primer's 3' end, one (21A) or two (21B) further mismatches were added in order to weaken hydrogen bonding between primer and template and therefore increase the likelihood of discrimination. A consensus primer, P, was designed in the sense direction within exon 21 overlapping the intron 20-21/exon 21 boundary. The exon 21 PCR assay involves two reactions: i) the 'T-reaction', containing the 5' consensus primer P, and the primer A complementary to the normal *EGFR* allele; ii) the 'G-reaction' containing P, and the primer B complementary to the mutant *EGFR* allele (Figure 2). For testing and proving the specificity of the allele-specific PCR primers A and B, a homozygous mutant control sample was artificially created: 2 primers, 21F (5'-TGATGATCT GTCCCTCACAGCA-3') and 21R (5'-ACCCAGCAGTTTGGCC CGC-3') were designed with 21R complementary to the mutant allele (2573 G) at its 3' end. DNA of the cell line NCI-H-1975 was amplified with these primers, resulting in a 160 bp PCR fragment containing the 2573G point mutation. All PCR primers were designed with the aid of OLIGO software (Software IDT SciTools, Integrated DNA Technologies, Coralville, USA). Primer sequences and PCR parameters are listed in Table I.

PCR. PCR reactions were run at a final volume of 25 μl. Reactions consisted of: 80-100 ng genomic DNA; 200 μmol/l dNTP; 0.1-0.25 μmol/l of primers; 1.5 mmol/l $MgCl_2$; 0.5 U *Taq* polymerase (Roche Diagnostics, Penzberg, Germany). Cycling conditions were as follows: 5 min of denaturing at 94˚C and 30 cycles of 94˚C for 0.30 min, annealing 58˚C (exon 19) or 60˚C (exon 21) for 45 s and 72˚C for 1 min. A volume of 10 μl of the PCR products was electrophoresed on a standard 2% agarose gel stained with SYBR-Green I for visualization under UV light.

Sequencing. The *EGFR* mutations were analyzed by DNA sequencing of the relevant regions of exon 19 and 21. PCR products were purified with the MinElute PCR Purification Kit (Qiagen GmbH). Genomic sequencing (MWG Biotech, Penzberg, Germany) was performed using the appropriate downstream PCR primer.

Results

Identification of the exon 19 E746_A750del deletion and the exon 21 nt2573 point mutation in control samples. Before testing clinical samples, both allele-specific PCR assays were validated in known genomic samples (normal wild-type control DNA, heterozygous mutant control DNA from cell lines NCI-H-1650 and NCI-H-1975 and an nt2573 homozygous mutant PCR fragment). The predicted amplification pattern for normal and mutant genotypes was

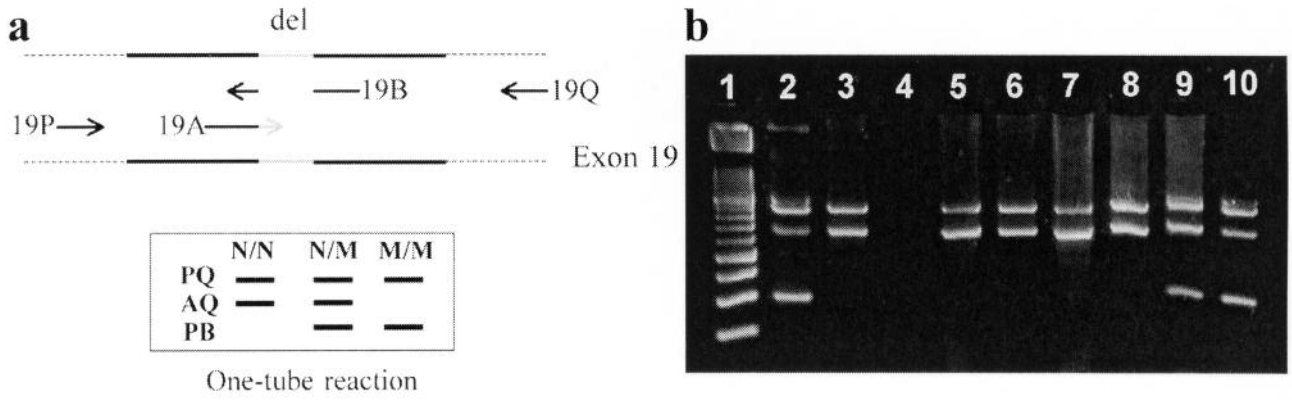

Figure 1. *Exon 19 Bi-PASA assay. a) Scheme of the Bi-PASA assay. The deletion sequence variation in exon 19 is indicated by a light gray line. The four primers, two outer primers P and Q, and two allele-specific inner primers A and B, are represented by arrows. P and Q anneal at different distances from the deletion. The PQ fragment is always amplified and serves as an internal reaction control. The inner primer A matches a part of the deletion sequence at its 3'end. The AQ fragment can be amplified only with the wild-type sequence (N/N). The inner primer B matches the mutant DNA sequence containing the 15 bp deletion, i.e. PB can be amplified only in mutant DNA (M/M). A heterozygous genotype (N/M) is characterized by both an AQ and PB amplification fragment. b) Mutation screening. Lane 2: heterozygous mutant control (NCI-H-1650), lane 3: homozygous wild-type control, lanes 5-10: analysis of tumor samples. The samples in lanes 5-9 have the exon 19 wild-type sequence. The samples in lanes 9 and 10 contain the heterozygous exon 19 deletion. Lane 1: molecular weight marker, lane 4: no template PCR control (water).*

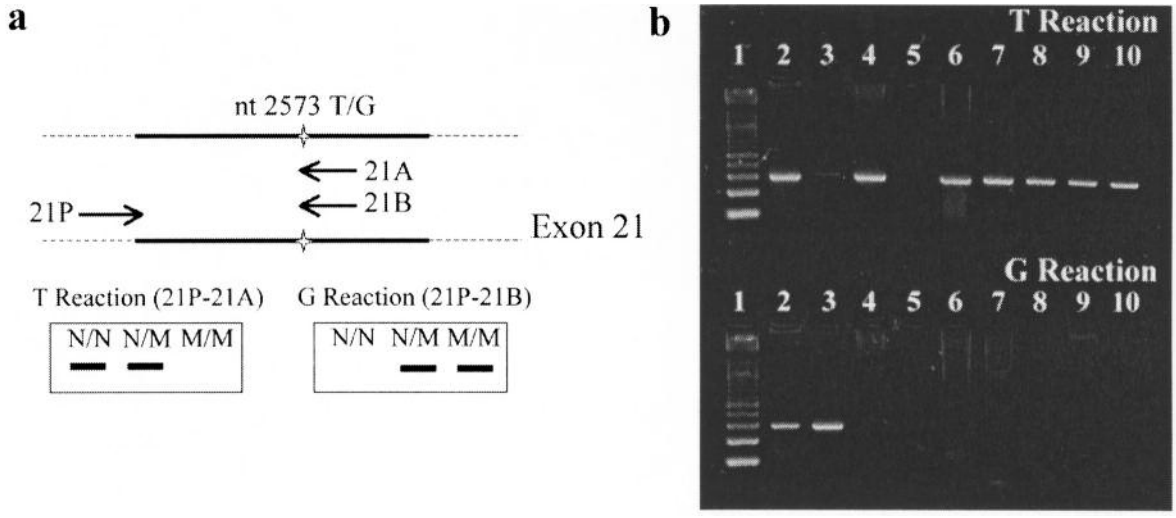

Figure 2. *Exon 21 L858R allele-specific PCR assay. a) Scheme of the allele-specific PCR assay. Exon 21 and the adjacent exon/intron boundary is depicted. The location of the nt 2573 T>G point mutation is indicated by an asterisk. The forward orientation of the consensus primer (21 P) and the reverse position of each of the sequence specific primers (21A and 21B) are illustrated by arrows. PCR is performed in a two-tube-reaction. The 'T- reaction' contains the consensus primer P and the primer 21A complementary to the normal EGFR exon 21 allele. Amplification occurs only with the normal allele as template. In the 'G- reaction', which contains primer 21P and primer 21B complementary to the mutant allele, amplification occurs only with the mutant allele as template. A heterozygous genotype (N/M) is characterized by an amplification fragment in both T- and G-reactions. b) Mutation screening. Lane 2: heterozygous mutant control (NCI-H-1975), lane 3: homozygous mutant control (PCR fragment), lane 4: homozygous wild-type control; lanes 6-10: analysis of tumor samples. All tumor samples have the exon 21 wild-type sequence. Lane 1: molecular weight marker, lane 5: no template PCR control (water).*

obtained with high specificity *i.e.* detection of only the normal or only the mutant allele, high sensitivity *i.e.* no spurious PCR fragments and an acceptable yield (Figure 1b and 2b, lanes 1-3).

Sensitivity of the PCR-based based assays in mixed cell populations. Because tumor specimens may contain a high percentage of nonneoplastic cells, screening assays must be able to detect mutations in heterogenous genomic samples.

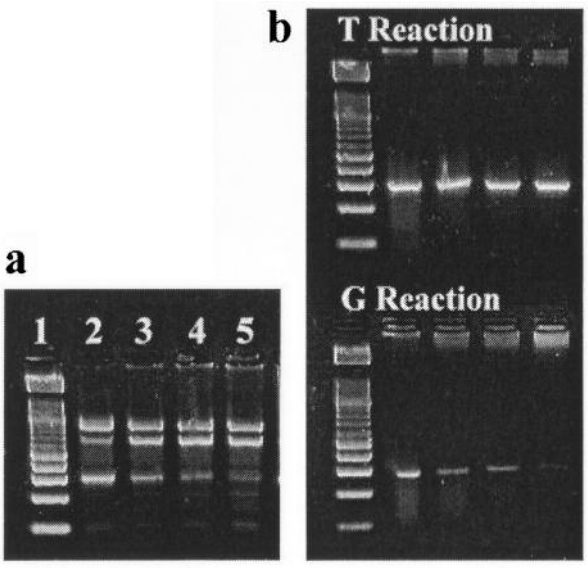

Figure 3. *Serial dilution experiment. a) Exon 19 assay. Serial dilution of the heterozygous mutant cell line DNA NCI-H-1650 with normal control DNA (lane 2: H-1650 undiluted; lane 3: 1:2 dilution; lane 4: 1:4; lane 5: 1:8). Lane 1: molecular weight marker. The PB fragment (arrow) is detectable in mixed samples in an up to 8-fold excess of normal DNA. b) Exon 21 assay. Serial dilution of the heterozygous mutant cell-line DNA NCI-H-1975 with normal control DNA (lane 2: H-1975 undiluted; lane 3: 1:2 dilution; lane 4: 1:4; lane 5: 1:8). Lane 1: molecular weight marker. The 'mutant' amplification fragment in the G-reaction (arrow) is detectable in mixed samples in a 4-fold excess of normal DNA.*

In order to provide defined heterogenous samples, serial dilutions were established by mixing DNA from the heterozygous mutant cell line NCI-H-1650 and NCI-H-1975 with wild-type normal DNA. The exon 19 Bi-PASA assay was able to detect the mutant allele in an 8-fold excess of normal DNA. The mutant exon 21 2573T allele was detectable in a mixed sample with a 4-fold excess of normal DNA (Figure 3).

Screening of patients samples. DNA was isolated from 35 formalin-fixed paraffin-embedded lung tumor samples (obtained by biopsy or surgical resection) and screened for the both most common *EGFR* mutations. The PQ fragment in the exon 19 assay was always amplified, indicating the integrity of the isolated DNA from clinical tissue samples. Three mutations were found of which all were deletions in exon 19. Genomic sequencing confirmed the exon 19 deletion to be the variant c.2235_2249del15 in all three samples. Both wild-type and mutant amplification curves were found in all three cases indicating that the samples were most likely heterozygous. Case 1 was a 79-year-old female patient with a subpleural lung adenocarcinoma metastasis (previously diagnosed with a moderately differentiated lung adenocarcinoma in 2003). Case 2 was a 71-year-old female patient with a moderately differentiated primary lung adenocarcinoma of the right middle lobe after having tuberculosis decades earlier. Case 3 was a 74-year-old female patient with a primary moderately differentiated lung adenocarcinoma.

Discussion

A major effort to improve the clinical management of lung cancer patients entails the use of molecular markers to provide accurate predictions of the outcome after cancer treatment. *EGFR* mutation testing in lung adenocarcinoma is already used to help guide treatment decisions and/or to enroll patients on specific arms of clinical trials (7). For clinical routine, mutation screening will need to rely on fast, inexpensive, simple and sensitive assays. Because of its universal availability as a standard methodology in molecular medicine, we designed *EGFR* mutation screening assays based on PCR. Our assays are able to detect the two most common EGFR mutations in lung adenocarcinoma simply by PCR amplification and discrimination of the allele-specific PCR fragments by agarose gel electrophoresis. Allele-specific PCR, also known as amplification refractory mutation system (ARMS) is a well-established method for discriminating between different alleles at specific loci resulting from single base mutations (9, 10). Compared to other proposed methods for the detection of EGFR mutations in lung cancer, such as SSCP (11), TaqMan PCR (12), MALDI-TOF (13), dHPLC (14-16), single molecule sequencing (17), or SMAP (18), our allele-specific PCR assays do not need special equipment other than thermocyclers, reaction steps are simple and allow troubleshooting, reagents are inexpensive and the methodology can be easily implemented at a clinical laboratory.

For assay design, we had to solve the problem of having no homozygous mutant control DNA because *EGFR* mutations have been only found in a heterozygous status, both in patient samples and in the lung cancer cell lines NCI-H-1650 and NCI-H-1975. Consequently, these 'mutant' controls contain one wild-type allele and are not suitable to entirely control the allele-specificity of the primers, especially in the case of a point mutation in which it is demanded that one single base pair change is distinguished. There are several possible experimental approaches for creating artificially constructed mutation samples, including sample spiking, transfection, and genetic engineering, through such techniques as homologous recombination and site-directed mutagenesis (19). However, these techniques are time-consuming, labor-intensive and require some expertise with recombinant DNA techniques. Therefore, we created a homozygous mutant control fragment for the L858R point mutation artificially by PCR

and were able to validate the primer specificity and to establish appropriate PCR conditions. Creating a similar control fragment simply by PCR was technologically not possible in the case of the 15 bp deletion in exon 19. But our assay design as a Bi-PASA with two outer and two inner primers ensures the discrimination between the normal and the mutant allele because the allele-specific inner primers involve the deletion. In practice, in screening patients' samples, our exon 19 Bi-PASA was able to specifically detect the heterozygous *EGFR* mutation status in three patients. The PQ fragment that is always amplified serves not only as an internal control within the assay but also confirms the integrity of the isolated DNA from clinical tissue samples.

Approximately 10% of lung adenocarcinomas contain *EGFR* mutations (5), as also found in our study cohort. Of these, 90% are either nucleotide deletions around codons 746-750 in exon 19, or the exon 21 nt2573 point mutation (6). Both hot-spot mutations are detectable using the two PCR assays described herein. Among exon 19 mutations, few 9-, 12-, 18- and 24-bp deletions have been detected, and by far the most common deletion size was 15 bp (5). We decided to focus our assay design for lung cancer screening in clinical diagnostics on just the most common *EGFR* mutations related to chemotherapy sensitivity. Both PCR protocols should be appropriate for initial *EGFR* mutation analysis in patients with lung adenocarcinoma and provide a rapid, sensitive, and cost-effective screening method. Three other *EGFR* mutations, exon 19 D761Y, exon 20 T790M and exon 20 D770_N771insNPG, represent only about 10% of kinase domain mutations associated with drug resistance and should be analysed after an initial negative PCR test.

References

1 Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J and Haber DA: Activating mutations in the *epidermal growth factor receptor* underlying responsiveness of non-small cell lung cancer to gefitinib. N Engl J Med *350*: 2129-2139, 2004.

2 Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE and Meyerson M: *EGFR* mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science *304*: 1497-1500, 2004.

3 Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, Singh B, Heelan R, Rusch V, Fulton L, Mardis E, Kupfer D, Wilson R, Kris M and Varmus H: *EGF receptor* gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci USA *101*: 3306-3311, 2004.

4 Sordella R, Bell DW, Haber DA and Settleman J: Gefitinib-sensitizing *EGFR* mutations in lung cancer activate anti-apoptotic pathways. Science *305*: 1163-1167, 2004.

5 Pan Q, Pao W and Ladanyi M: Rapid polymerase chain reaction-based detection of *epidermal growth factor receptor* gene mutations in lung adenocarcinomas. J Mol Diagn *7*: 396-403, 2005.

6 EGFR mutation database: http://www.cityofhope.org/cmdl/egfr%5Fdb/

7 Pao W and Ladanyi M: *Epidermal growth factor receptor* mutation testing in lung cancer: searching for the ideal method. Clin Cancer Res *13*: 4954-4955, 2007.

8 Liu Q, Thorland EC, Heit JA and Sommer SS: Overlapping PCR for bidirectional PCR amplification of specific alleles: a rapid one-tube method for simultaneously differentiating homozygotes and heterozygotes. Genome Res *7*: 389-398, 1997.

9 Sarkar G, Cassady J, Bottema CD and Sommer SS: Characterization of polymerase chain reaction amplification of specific alleles. Anal Biochem *186*: 64-68, 1990.

10 Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, Smith JC and Markham AF: Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res *17*: 2503-2516, 1989.

11 Marchetti A, Martella C, Felicioni L, Barassi F, Salvatore S, Chella A, Camplese PP, Iarussi T, Mucilli F, Mezzetti A, Cuccurullo F, Sacco R and Buttitta F: *EGFR* mutations in non-small cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. J Clin Oncol *23*: 857-865, 2005.

12 Endo K, Konishi A, Sasaki H, Takada M, Tanaka H, Okumura M, Kawahara M, Sugiura H, Kuwabara Y, Fukai I, Matsumura A, Yano M, Kobayashi Y, Mizuno K, Haneda H, Suzuki E, Luchi K and Fujii Y: *Epidermal growth factor receptor* gene mutation in non-small cell lung cancer using highly sensitive and fast TaqMan PCR assay. Lung Cancer *50*: 375-384, 2005.

13 Thomas RK, Baker AC, Debiasi RM, Winckler W, Laframboise T, Lin WM, Wang M, Feng W, Zander T, MacConaill L, Lee JC, Nicoletti R, Hatton C, Goyette M, Girard L, Majmudar K, Ziaugra L, Wong KK, Gabriel S, Beroukhim R, Peyton M, Barretina J, Dutt A, Emery C, Greulich H, Shah K, Sasaki H, Gazdar A, Minna J, Armstrong SA, Mellinghoff IK, Hodi FS, Dranoff G, Mischel PS, Cloughesy TF, Nelson SF, Liau LM, Mertz K, Rubin MA, Moch H, Loda M, Catalona W, Fletcher J, Signoretti S, Kaye F, Anderson KC, Demetri GD, Dummer R, Wagner S, Herlyn M, Sellers WR, Meyerson M and Garraway LA: High-throughput oncogene mutation profiling in human cancer. Nat Genet *39*: 347-351, 2007.

14 Jänne PA, Borras AM, Kuang Y, Rogers AM, Joshi VA, Liyanage H, Lindeman N, Lee JC, Halmos B, Maher EA, Distel RJ, Meyerson M and Johnson BE: A rapid and sensitive enzymatic method for *epidermal growth factor receptor* mutation screening. Clin Cancer Res *12*: 751-758, 2006.

15 Chin TM, Anuar D, Soo R, Salto-Tellez M, Li WQ, Ahmad B, Lee SC, Goh BC, Kawakami K, Segal A, Iacopetta B and Soong R: Detection of *epidermal growth factor receptor* variations by partially denaturing HPLC. Clin Chem *53*: 62-70, 2007.

16 Cohen V, Agulnik JS, Jarry J, Batist G, Small D, Kreisman H, Tejada NA, Miller WHJr and Chong G: Evaluation of denaturing high-performance liquid chromatography as a rapid detection method for identification of *epidermal growth factor receptor* mutations in non-small cell lung cancer. Cancer *107*: 2858-2865, 2006.

17 Thomas RK, Nickerson E, Simons JF, Jänne PA, Tengs T, Yuza Y, Garraway LA,LaFramboise T, Lee JC, Shah K, O'Neill K, Sasaki H, Lindeman N, Wong KK, Borras AM, Gutmann EJ, Dragnev KH, DeBiasi R, Chen TH, Glatt KA, Greulich H, Desany B, Lubeski CK, Brockman W, Alvarez P, Hutchison SK, Leamon JH, Ronan MT, Turenchalk GS, Egholm M, Sellers WR, Rothberg JM and Meyerson M: Sensitive mutation detection in heterogeneous cancer specimens by massively parallel picoliter reactor sequencing. Nat Med *7*: 852-855, 2006.

18 Hoshi K, Takakura H, Mitani Y, Tatsumi K, Momiyama N, Ichikawa Y, Togo S, Miyagi T, Kawai Y, Kogo Y, Kikuchi T, Kato C, Arakawa T, Uno S, Cizdziel PE, Lezhava A, Ogawa N, Hayashizaki Y and Shimada H: Rapid detection of *epidermal growth factor receptor* mutations in lung cancer by the SMart-Amplification Process. Clin Cancer Res *13*: 4974-4983, 2007.

19 Jarvis M, Iyer RK, Williams LO, Noll WW, Thomas K, Telatar M and Grody WW: A novel method for creating artificial mutant samples for performance evaluation and quality control in clinical molecular genetics. J Mol Diagn *7*: 247-251, 2005.

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