FLAG assay as a novel method for real-time signal generation during PCR: application to detection and genotyping of KRAS codon 12 mutations

Giulia Amicarelli[1], Erlet Shehi[1], G. Mike Makrigiorgos[2] and Daniel Adlerstein[1,*]

[1]DiaSorin SpA, via Crescentino snc, 13040 Saluggia (VC), Italy and [2]Dana Farber-Brigham and Women's Cancer Center, Harvard Medical School, Boston, MA, USA

ABSTRACT

Real-time signal generation methods for detection and characterization of low-abundance mutations in genomic DNA are powerful tools for cancer diagnosis and prognosis. Mutations in codon 12 of the oncogene KRAS, for example, are frequently found in several types of human cancers. We have developed a novel real-time PCR technology, FLAG (FLuorescent Amplicon Generation) and adapted it for simultaneously (i) amplifying mutated codon 12 KRAS sequences, (ii) monitoring in real-time the amplification and (iii) genotyping the exact nucleotide alteration. FLAG utilizes the exceptionally thermostable endonuclease PspGI for real-time signal generation by cleavage of quenched fluorophores from the 5'-end of the PCR products and, concurrently, for selecting KRAS mutations over wild type. By including peptide-nucleic-acid probes in the reaction, simultaneous genotyping is achieved that circumvents the requirement for sequencing. FLAG enables high-throughput, closed-tube KRAS mutation detection down to ~0.1% mutant-to-wild type. The assay was validated on model systems and compared with allele-specific PCR sequencing for screening 27 cancer specimens. Diverse applications of FLAG for real-time PCR or genotyping applications in cancer, virology or infectious diseases are envisioned.

INTRODUCTION

Diagnostic methods for the detection of specific gene mutations associated with diseases play an increasingly important role for early cancer diagnosis and prognosis in clinical practice. For example, KRAS codon 12 mutations are a predictor of resistance to tyrosine kinase inhibitors in lung cancer (1) and of non-response to drugs like cetuximab in colon cancer (2). One of the first described methods for detection of known mutations includes PCR-RFLP (3). Subsequently, several other approaches for discriminating specific sequence variations were described including hybridization with allele-specific probes (4,5), allele-specific PCR (6,7), dye terminator incorporation (8–12), oligonucleotide ligation (13), Flap-Endonuclease digestion (14) isothermal amplification assay (15) and others, reviewed by Parsons *et al.* (16). These methods can be convenient, but require an additional step for end point detection. Analytical methods that reduce sample processing to a single-step procedure were also developed, including nick-translation PCR (17), hybridization with FRET probes (18), melting curves analysis (19–22), fluorogenic allele-specific PCR (23), universal FRET reagents in a single-step Invader assay (24), universal TaqMan probes (25) and others. However, several of these methods cannot deal with detection of a low concentration of mutant target in the presence of a large excess of wild-type DNA that is often found in clinical specimens. The excess wild-type DNA can mask the mutant signal during the detection step of the assay. For these reasons, mutation-selection technologies such as PCR-clamping (26) and restriction endonuclease-mediated selection (REMS)-PCR (27) apply a preliminary step of selective suppression of the wild-type sequences during PCR followed by a mutation detection step. However, the additional detection step increases the risk for contamination and false positives. Furthermore, most methods allow the detection of the presence of a mutation but not characterization of the exact nucleotide change that took place, and sequencing is required as an extra step for genotyping the mutation.

Here we describe a novel real-time PCR signal generation technology (FLAG, FLuorescent Amplicon Generation) that is adapted to the selective amplification of DNA sequences with defined KRAS mutations within a large excess of wild-type DNA, and simultaneously monitors the amplification in real time. In addition, FLAG can genotype the exact nucleotide alteration, thus

*To whom correspondence should be addressed. Tel: +0039 0331581473; Fax: +0039 03311547; Email: daniel.adlerstein@diasorin.it

circumventing the need for sequencing. We evaluate the specificity and sensitivity of this new assay, and validate its use for real-time KRAS mutation detection and genotyping on cell lines and clinical tumor samples.

MATERIALS AND METHODS

Source and extraction of genomic DNA

Control cell lines. Genomic DNA from cultured cell lines SW480 (KRAS codon 12 homozygous GTT), PL45 (heterozygous GAT/GGT) and CALU 1 (heterozygous TGT/GGT) was obtained from the American Type Culture Collection, (Manassas, California, USA) and extracted by the NucleoSpin^{TM} Tissue kit (Macherey-Nagel, Duren, Germany).

Clinical samples. Tissue specimens from sporadic colorectal cancers (SCRCs) were obtained with informed consent from previously untreated patients who underwent surgical resection at the Istituto Nazionale per lo Studio e la Cura dei Tumori of Milan between 1998 and 2000. Tumor specimens and its surrounding normal mucosa were selected by an experienced pathologist from formalin-fixed, paraffin-embedded tissue. Genomic DNA were extracted using QIAamp Tissue Extraction kit (Qiagen, Hilden, Germany) in the Lab of Dr Milo Frattini, Department of Experimental Oncology and Unit of Experimental Molecular Pathology, Istituto Cantonale di Patologia (Locarno, Switzerland).

Selective PNA-mediated FLAG on DNA from cell lines with KRAS mutations and clinical samples

We designed and synthesized four PNA (peptide nucleic acids) probes (Eurogentec, Liege, Belgium) specific for the KRAS codon 12 mutations GTT, GAT, TGT and GCT (PNA_1 NH$_2$-GGACCTGTTGGCGTA-CON$_2$H, PNA_2 NH$_2$-GGACCTGATGGCGTA-CON$_2$H, PNA_3 NH$_2$-GGACCTTGTGGCGTA-CON$_2$H, PNA_4 NH$_2$-GGACCTGCTGGCGTA-CON$_2$H, respectively). Concentration of forward (GA112:/5IAbFQ/TTTCCTGGTTT/iFluorT/AGATTTACCTCTATTGTTGGATCATAT) and reverse (GA111: 5'TTTCCTGGTTTGAATATAAACTTGTGGTAGTTGGACCT 3') primers was 300 nM (IDT Inc, Coralville, IA, USA). The forward primer carries a C/C mismatch, which allows the creation of a PspGI recognition site only in the presence of wild-type templates.

In each reaction, 68 ng of genomic DNA extracted from clinical samples or from wild-type K562, mutant SW480 (homozygous GTT/GTT), PL45 (heterozygous GAT/GGT) and CALU1 (heterozygous TGT/GGT) cell lines were used as a template. The reaction was performed for 35 cycles (on DNA from cell lines) or 38 cycles (on DNA from clinical samples) as follows: denaturation step at 94°C for 10 s, primers annealing at 56°C for 30 s, primers extension and PNA annealing at 72°C for 60 s in a Chromo 4^{TM} Detector Real Time PCR machine (MJ research, Waltham, MA, USA) or an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The reaction mix contained 0.1× BD

Titanium^{TM} Taq polymerase, 1× PCR buffer (BD Biosciences, Franklin Lakes, NJ, USA), 200 μM dNTPs, 1.5 mM MgCl$_2$ in 20 μl total volume. The reaction mix was split into six reactions, performed in parallel and compared to each other: the first reaction is conducted without restriction endonuclease or PNA, while the others are in presence of PspGI (New England Biolabs, Ipswich, MA, USA), 0.5 or 1 U/μl for analysis of DNA extracted from cell lines and clinical samples, respectively.

The third, fourth, fifth and sixth reactions contain also one of the four different PNA probes: 300 nM PNA_1 specific for codon 12 GTT, 200 nM PNA_2 specific for codon 12 GAT, 200 nM PNA_3 complementary to the TGT mutation and 200 nM PNA_4 specific for GCT targets, respectively.

By the comparison between the six parallel reactions, the mutant samples are detected and genotyped.

Detection and genotyping of mutant clinical samples by allele-specific PCR followed by sequencing

Mutations of codon 12 of the KRAS oncogene were detected in clinical samples by allele-specific PCR, and characterized by automatic sequencing in the laboratory of Dr M. Frattini, Department of Experimental Oncology and Unit of Experimental Molecular Pathology, Istituto Cantonale di Patologia (Locarno, Switzerland).

Codon 12 sequence of the KRAS gene was examined as follows: 200 ng of genomic DNA was amplified in 30 μl of final volume reaction with 1× PCR Buffer II, 1.5 mM MgCl$_2$, 0.2 mM dNTPs, 0.5 μM appropriate primer and 2 U of AmpliTaq Gold Polymerase (all from Applied Biosystems, Foster City, CA, USA). PCR amplifications were performed as follows: initial denaturation step of 96°C for 3 min, 45 cycles of 96°C for 30 s, 50°C for 1 min, 72°C for 1 min, and a final elongation step of 72°C for 4 min. Samples were run on 4% polyacrylamide gels and then purified using the Purification Kit Qiagen (Hilden, Germany). Purified products were sequenced in a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

RESULTS

The FLAG assay

FLAG is a novel method for real-time PCR signal generation that utilizes thermostable restriction enzymes (Figure 1). The signal generation strategy consists of amplification of the gene region of interest using a specific primer pair designed to carry an 11-base sequence-tag at the 5'-end, containing the recognition site for an exceptionally thermostable restriction endonuclease, PspGI. The forward primer is doubly labeled at the tag region with a quencher and a fluorophore in 5' and 3' positions, respectively. During sequence extension by the polymerase, the sequence-tag region becomes double stranded, resulting to recognition and cleavage by PspGI. Separation of the fluorophore and the quencher leads to generation of fluorescence signal due to loss of quenching. The unusually thermostable PspGI [activity half-life of 2 h at 95°C (28)] resists deactivation during
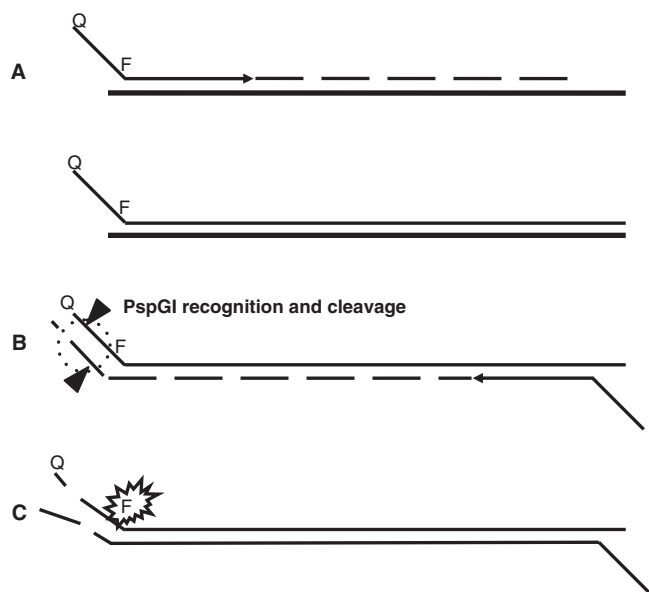
Figure 1. FLAG principle for real-time PCR signal generation. The FLAG assay utilizes a primer pair carrying a tag sequence at the 5′ end that contains the recognition site for the highly thermostable enzyme PspGI. (**A**) One of the primers is doubly labeled at the tag region with a quencher Q and a fluorophore F. (**B**) During the primer extension, the primer tag becomes double stranded, becoming available for recognition and cleavage by the endonuclease. (**C**) The digestion leads to generation of fluorescence due to loss of quenching.

thermal cycling of the reaction, allowing the continuous generation of fluorescent signal during the entire course of PCR amplification. As a result, the exponential build-up of double-stranded PCR product is monitored in real time.

The originally described REMS-PCR approach (26) consists of using a mutagenic forward primer together with the enzyme BstNI that suppresses amplification of wild-type sequences and to selectively amplify mutant alleles. PspGI is a highly thermostable isoschizomer of BstNI, a restriction endonuclease commonly used in REMS protocols for selective detection of KRAS codon 12 mutation (26,29). The primer-introduced variation creates a recognition site (5′-CCTGG-3′) for PspGI only when the codon 12 sequence is wild type (GGT) (Figure 2A). PspGI is present during the amplification reaction and digests newly formed amplicons, suppressing their amplification. On the other hand, the mutant alleles amplify exponentially during PCR since they carry a nucleotide variation in codon 12. Consequently, the use of PspGI in FLAG assay enables the concurrent generation of signal and the selective amplification of mutant codon 12 KRAS sequences during real-time PCR.

Since identification of specific genotypes of KRAS codon 12 mutations is useful for disease prognosis and for differentiation of benign from malignant tissues (30,31), we decided to genotype mutant amplification products obtained via FLAG, by employing PNAs in a second FLAG reaction (Figure 2B). PNAs are non-extendable oligonucleotides where the ribose-phosphate backbone is replaced by (2-aminoethyl)-glycine units linked by amide bonds (32). These synthetic oligomers form hybrids

PNA/DNA with higher thermal stability than DNA/DNA, but which are significantly destabilized in the case of a single mismatch (33). A PNA probe fully complementary to the sequence of a specific KRAS codon 12 mutant prevents annealing and extension of the forward primer, suppressing amplification. In presence of a single mismatch, PNA does not inhibit primer hybridization, which leads to amplification and consequent fluorescence signal generation. Thus, four distinct FLAG reactions are conducted for each mutation-containing sample, each one containing a different PNA probe specific for the four most common KRAS codon 12 mutations (GTT, GAT, GCT, TGT) (Figure 2B). By observing the relative signal intensity from each of these four reactions, the identity of the mutant sequence is inferred, as the resulting signal is weak or non-measurable for the mutation that is fully complementary to the mutation of the target DNA analyzed in the presence of PNA. In summary, in the strategy depicted in Figure 2, the first FLAG reaction identifies and monitors in real time the mutant KRAS DNA sequences, and if the sample is mutated a second set of real-time FLAG assays homogenously genotypes the exact nucleotide alteration, thereby circumventing the need for sequencing.

As an alternative, the 5 FLAG reactions depicted in Figure 2 can be routinely carried out in parallel, thereby obtaining both the mutation and genotyping the information. This results to a single-step procedure at the expense of increasing the overall number of real-time PCR reactions required.
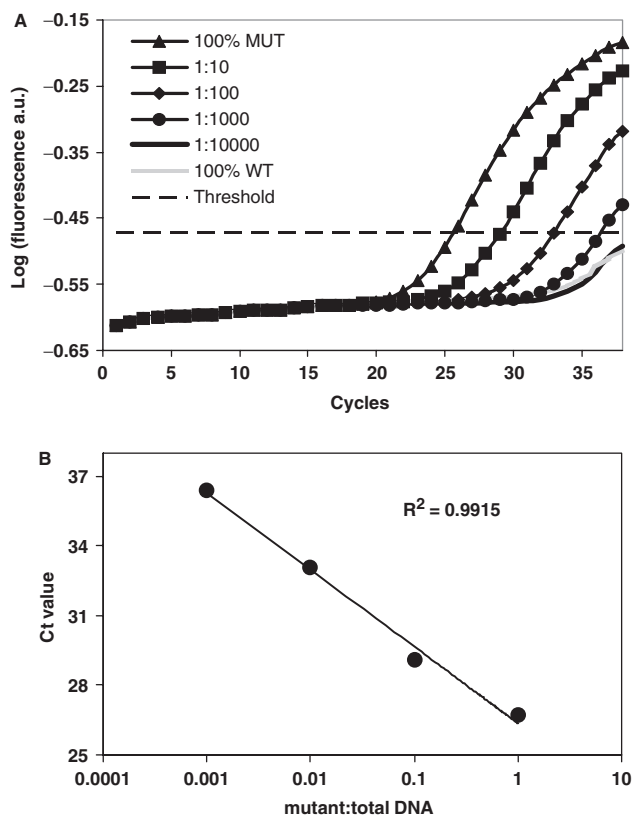
Selectivity of FLAG-KRAS assay

To assess the selectivity of FLAG-KRAS assay, genomic DNA from the homozygous GTT mutant cell line SW480 was serially diluted into wild-type KRAS codon 12 DNA from K562 cells to obtain decreasing ratios of mutant-to-wild type sequences. Samples where mutant alleles represented respectively 100, 10, 1, 0.1 and 0.01% of the total DNA were subjected to FLAG-KRAS in the presence of PspGI. Real-time PCR signals (Figure 3, panel A) show that mutant alleles are detectable down to 1:1000 mutant-to-wild type DNA ratio and that there is a linear relation between the threshold cycle (Ct) and the logarithm-dilution factor of the mutant DNA in each sample (Figure 3, panel B).

The high level of selectivity of FLAG-KRAS is enabled by the use of PspGI, which is much more thermostable than the isoschizomer restriction endonuclease BstNI commonly used in REMS protocols (13,15,34). To demonstrate this point, we performed the FLAG-KRAS experiment using $1\,U/\mu l$ PspGI or $1\,U/\mu l$ BstNI, in parallel. PspGI digested the wild-type codon 12 sequences during all 38 thermal cycles of the FLAG assay, while BstNI lost its activity, resulting in insufficient suppression of the wild-type component of the samples analyzed, thus producing signal indiscriminately from mutant or wild-type samples (Figure 4A and B, respectively). For BstNI to reach a selectivity of 1:100 mutant-to-wild type ratio following 38 PCR cycles, an addition of $10\,U$ of BstNI
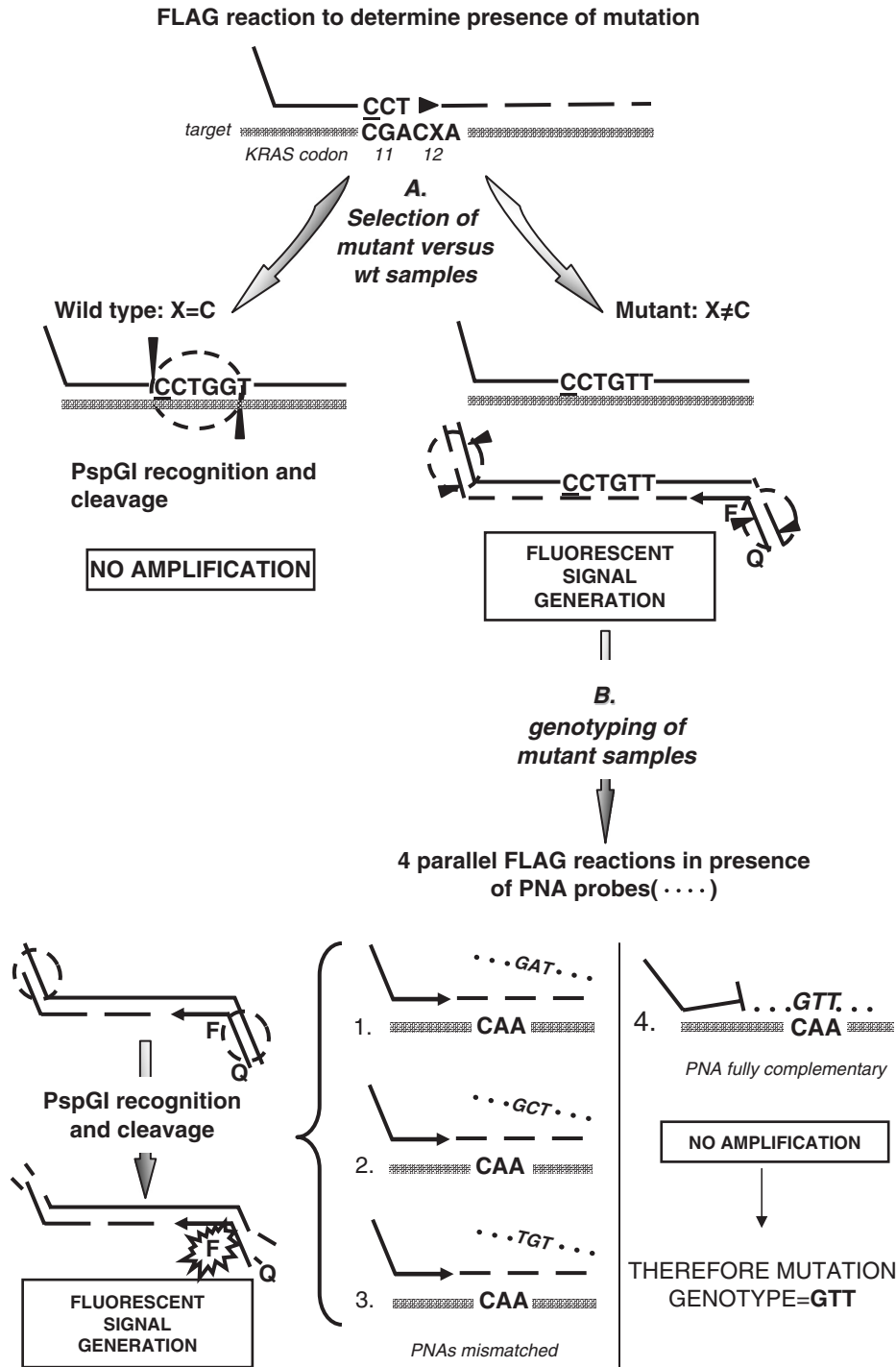
Figure 2. PNA-mediated FLAG –KRAS assay. FLAG-KRAS assay leads to selective amplification of mutant codon 12 KRAS sequences during the real-time fluorescent reaction through a REMS-PCR approach. (**A**) a mutagenic (underlined base) primer introduces a variation that creates the recognition site for PspGI (circle) in the wild-type codon 12 (X = C, ACC) target sequence, thereby suppressing the exponential amplification of the wild-type allele. The mutant alleles escape digestion and this enables their exponential amplification. (**B**) to assess the exact nucleotide alteration of the target amplified, the FLAG-KRAS assay is repeated in four parallel reactions in the presence of PNA probes (dotted lines) specific for the most common mutations of codon 12. When the reaction is conducted in the presence of the fully complementary PNA (4), no exponential amplification is allowed. In presence of a mismatched PNA (1–3) the probe is destabilized and the primer can consequently anneal and extend causing an exponential amplification and generation of FLAG fluorescent signal as explained in Figure 1.
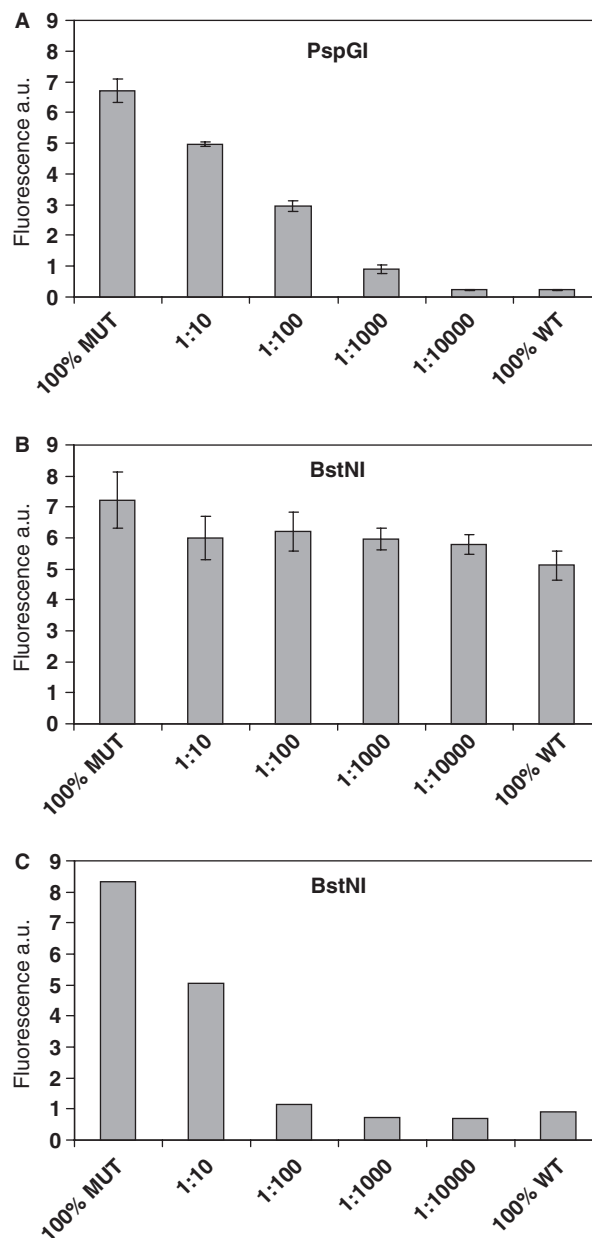
Figure 3. Selectivity of Selective FLAG-KRAS assay on cell lines. Genomic DNA from the homozygous GTT mutant cell line SW480 was serially diluted into wild-type KRAS codon 12 DNA from K562 cells to obtain decreasing ratios of mutant-to-total DNA of 1:1, 1:10, 1:100, 1:1000 and 1:10000. The fluorescent signals, monitored in real time, are distinguishable from those obtained for wild-type samples down to 1:1000 mutant-to-total DNA ratio (**A**). A linear change of the PCR threshold cycle versus the logarithm concentration of the mutant DNA was observed down to 0.1% mutant-to-total DNA ratio (**B**).

to the solution was needed every 10 cycles of reaction (Figure 4C).

### FLAG-KRAS combined with PNA for genotyping

FLAG-KRAS was combined with PNA, as described in Figure 2, for genotype determination via suppression of mutant alleles carrying specific mutations. To demonstrate the principle, we performed PNA-mediated FLAG-KRAS reactions in parallel for DNA extracted from wild-type K562 and mutant SW480 (homozygous GTT/GTT), PL45 (heterozygous GGT/GAT) and CALU1 (heterozygous GGT/TGT) cell lines and the PCR products were run on an ethidium-stained agarose gel. As shown in Figure 5, all cell lines generate PCR product in the absence of PspGI (first panel), while no product from K562 sample is visible in the presence of PspGI (second panel). When the reaction is conducted in the presence of PNA probe specific for GTT codon 12 sequence, no amplification product is obtained from SW480 DNA (Figure 5, panel 3). Similarly, no product from PL45 DNA was obtained when PNA specific for GAT was used (Figure 5, panel 4), and no product for CALU1 DNA was obtained when PNA specific for TGT was used (Figure 5, panel 5).

Figure 4. Selectivity of FLAG-KRAS assay: PspGI versus BstNI. The FLAG-KRAS assay was performed on genomic DNA from the homozygous GTT mutant cell line SW480 serially diluted into wild-type DNA from K562 cells to obtain decreasing ratios of mutant-to-total DNA of 1:1, 1:10, 1:100, 1:1000 and 1:10000. The reaction was performed for 38 cycles in presence of PspGI (**panel A**) or BstNI, (**panel B**). PspGI retained its activity during 38 cycles, while BstNI lost its activity thus enabling wild type sequences to amplify together with mutant sequences. To reach a level of selectivity of about 1:100 mutant-to-wild type ratio using BstNI, an addition of 10 U of enzyme every 10 cycles of reaction was needed (**panel C**). Panel A and Panel B show data from three independent experiments. The error bars represent the standard deviation values.
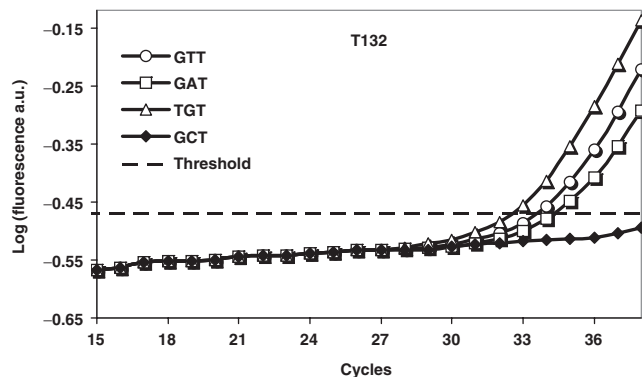
Analysis of clinical samples by PNA-mediated FLAG-KRAS assay

The PNA-mediated FLAG-KRAS assay was applied on 27 clinical samples from patients diagnosed with sporadic colorectal cancers, previously analyzed for codon 12
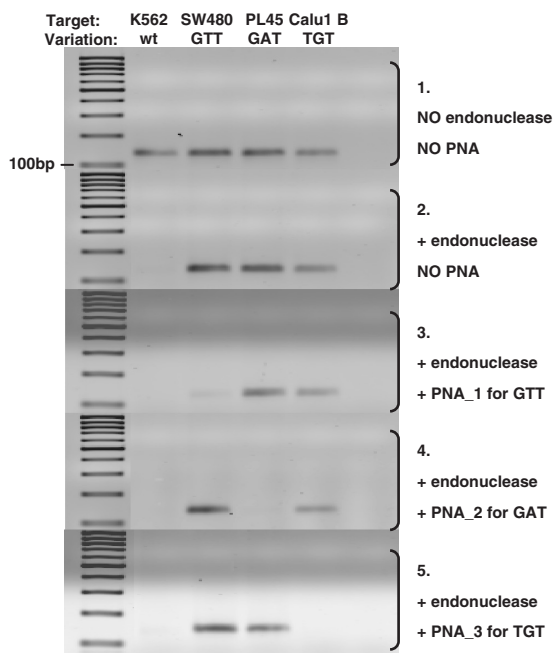
Figure 5. Selective PNA-mediated FLAG on cell lines. The FLAG assay was performed on cell lines K562 (GGT/GGT), SW480 (GTT/GTT), PL45 (GGT/GAT) and CALU1 (GGT/TGT), or in absence of DNA target, B. **Panel 1:** FLAG assay without PspGI restriction endonuclease or PNA (positive controls). An amplification product is produced from all the reactions performed. **Panel 2:** FLAG assay in presence of PspGI to detect mutant versus wild-type samples. The wild -type sample K562 was digested by the enzyme, while the cell lines containing a mutation are exponentially amplified. **Panels 3–5:** FLAG assay with addition of PspGI and PNA specific for GTT, GAT or TGT. No amplification product is obtained from wild-type sample K562 (digested by PspGI) and from the cell lines that are suppressed by the fully complementary PNA present in the reaction.

KRAS mutations via allele-specific PCR sequencing (ASP-S). To simultaneously detect and genotype codon 12 sequence variations present in the clinical samples, the PNA-mediated FLAG-KRAS reactions were performed as described in Figure 2. Representative PNA-mediated FLAG-KRAS data are depicted in Figure 6. A fluorescent signal is produced in each reaction performed, except for the one containing the PNA probe fully complementary to the KRAS mutated sequence present in the sample (Figure 6). Samples showing a Ct < 38 in the FLAG assay, in absence of PNA were considered to be mutants since amplification of wild-type alleles is prevented by PspGI cleavage. The exact nucleotide change was identified by PNA-mediated FLAG-KRAS. In this case, Ct > 38 indicates suppression of the amplification by the PNA probe fully complementary to the KRAS mutated sequence and we therefore assigned to the sample the genotype complementary to that specific PNA. The results were compared with those obtained by ASP-S. The two techniques show 81.5% concordance (Supplementary Table 1). Out of 27 samples tested, we identified 22 concordant positive calls, 4 positives for FLAG-KRAS (Ct < 38) that were negative for ASP-S and a negative sample (TL140, Ct = 38.1) for FLAG-KRAS that was positive for ASP-S. The excess positives identified via FLAG-KRAS may potentially be a result of the high selectivity of the FLAG

Figure 6. Representative PNA-mediated FLAG-KRAS obtained from analysis of clinical samples. Four FLAG-KRAS reactions were performed on clinical sample TL132 in presence of different PNA probes, specific for GTT, GAT, TGT or GCT mutations. The assays produced fluorescent amplicons in presence of all PNA, except for the one specific for GCT mutation. The data indicate that sample T132 contains the mutation GCT.

method that allows the detection of four mutated samples missed by ASP-S due to the very low-abundance KRAS mutations. Overall, there is a satisfactory agreement between the two independent methods.

DISCUSSION

FLAG provides a novel, simple method for real-time signal generation during PCR that is based on the use of the exceptionally thermophilic enzyme PspGI acting on the formed double-stranded PCR amplicons. FLAG does not require fluorogenic probes as the Taqman or Molecular Beacons approaches (4,5,25) since the fluorophores are on the PCR primers, thus providing an alternative platform for multiplexing real-time PCR reactions. As with other non-probe-based real-time PCR approaches (33–36), the basic requirement for a specific signal generation by FLAG is avoiding primer–dimer formation, which would result in false positive calls. We addressed this problem by designing our primers taking advantage from accurate simulations performed by the VisualOMP4 Software to exclude any problematic primer pairs. Because PspGI remains active during the entire PCR course, fluorescence is generated continuously during the reaction resulting to robust signals. A potential limitation of FLAG is that the amplified region must not contain PspGI sites other than those incorporated at the 5′-ends of the primers. This requirement restricts the application to amplicons that are devoid of 5′-CCWGG-3′ sequence strings. Given that the amplicons are short, we found that this is not a severe limitation.

We adapted FLAG to the single-step detection and genotyping of somatic KRAS mutations within a large excess of wild-type sequences, starting directly from genomic DNA. The KRAS mutation selection is enabled by the primer-mediated formation of restriction sites that are recognized by the same enzyme used for signal generation, the exceptionally thermophilic PspGI. The KRAS mutation genotyping is enabled by the novel use of PNA probes designed to interrogate specific nucleotide changes in a set of real-time FLAG reactions. While PNA

is frequently employed for suppression of wild-type KRAS alleles (37,38), the present adaptation extends the use of PNA to the genotyping of specific nucleotide changes, thus circumventing the need for di-deoxy-sequencing. This application can either be performed in two steps (single FLAG-KRAS reaction to determine presence of mutation, followed by a set of four parallel reactions for genotyping, if mutation-positive) or in a single step of five parallel real-time PCR reactions. The first approach reduces the total number of reactions performed, as the wild-type samples are not processed for a second step. The second approach provides the advantage of speed and a closed-tube approach since no PCR tube needs to be opened to perform a second reaction. The closed-tube approach used for PNA-mediated FLAG-KRAS decreases the risk of contamination that is more elevated for alternative technologies based on multi-step reactions (16,39–41). The selectivity of the assay was demonstrated to be 0.1% (detection of 1 mutant sequence in presence of 999 wild-type alleles). Current protocols based on thermophilic enzymes other than PspGI (42) require multi-step approaches to reach similar sensitivity (43). The high selectivity of the assay can potentially be useful for homogenous detection and genotyping of mutations present at low prevalence in plasma samples. Nanogram quantities of circulating DNA are present in the plasma of normal individuals (44), and increased quantities circulate in patients with cancer (45–47). Detection of mutated DNA in plasma DNA may precede conventional cancer diagnosis (48,49) resulting to a non-invasive tool for initial detection of malignancy as well as an indicator of recurrence during follow-up. Detection of specific KRAS nucleotide changes in plasma using PNA-mediated FLAG-KRAS could contribute to a more accurate prognosis and therapy choice in certain cancers (24,25).

In summary, PNA-mediated FLAG-KRAS is a novel and practical method for simultaneous selection and genotyping of KRAS sequence alterations in clinical samples. The technology has the specificity and the selectivity required for analysis of surgical/biopsy tumor samples and can be potentially used for identification of KRAS codon 12 variations in fluid specimens (needle aspiration, plasma-circulating DNA). In principle it should also be applicable for additional somatic mutations. However, using a mutagenic primer may also reduce the priming efficiency of the PCR reaction. Therefore adaptation of the FLAG assay to additional mutations may need substantial optimization and remains to be proven in practice.

Finally, besides KRAS mutation detection, FLAG provides a generally applicable new method for fluorescent signal generation in real-time PCR and could be used for multiple additional applications (e.g. in genetic testing, infectious diseases or virology).

SUPPLEMENTARY DATA

Supplementary data are available at NAR Online.

REFERENCES

1. Eberhard,D.A., Johnson,B.E., Amler,L.C., Goddard,A.D., Heldens,S.L., Herbst,R.S., Ince,W.L., Jänne,P.A., Januario,T. *et al.* (2005) Mutations in the epidermal growth factor receptor and in KRAS are predictive and prognostic indicators in patients with non-small-cell lung cancer treated with chemotherapy alone and in combination with erlotinib. *J. Clin. Oncol.*, **23**, 5900–5909.
2. Di Fiore,F., Blanchard,F., Charbonnier,F., Le Pessot,F., Lamy,A., Galais,M.P., Bastit,L., Killian,A., Sesboüé,R. *et al.* (2007) Clinical relevance of KRAS mutation detection in metastatic colorectal cancer treated by Cetuximab plus chemotherapy. *Br. J. Cancer*, **96**, 1166–1169.
3. Kumar,R. and Dunn,L.L. (1989) Designed diagnostic restriction fragment length polymorphisms for the detection of point mutations in ras oncogenes. *Oncogene Res.*, **4**, 235–241.
4. Tyagi,S., Bratu,D.P. and Kramer,F.R. (1998) Multicolor molecular beacons for allele discrimination. *Nat. Biotechnol.*, **16**, 49–53.
5. Marras,S.A., Kramer,F.R. and Tyagi,S. (1999) Multiplex detection of single nucleotide variations using molecular beacons. *Genet. Anal.*, **14**, 151–156.
6. Newton,C.R., Graham,A., Heptinstall,L.E., Powell,S.J., Summers,C., Kalsheker,N., Smith,J.C. and Markham,A.F. (1989) Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acid Res.*, **17**, 2503–2516.
7. Okayama,H., Curiel,D.T., Brantly,M.L. and Holmes,M.D. (1989) Crystal RG.Rapid, nonradioactive detection of mutations in the human genome by allele-specific amplification. *J. Lab. Clin. Med.*, **114**, 105–113.
8. Chen,X. and Kwok,P.Y. (1997) Template-directed dye-terminator incorporation (TDI) assay: a homogeneous DNA diagnostic method based on fluorescence resonance energy transfer. *Nucleic Acid Res.*, **25**, 347–353.
9. Chen,X., Zehnbauer,B., Gnirke,A. and Kwok,P.Y. (1997) Fluorescence energy transfer detection as homogeneous DNA diagnostic method. *Proc. Natl Acad. Sci.*, **94**, 10756–10761.
10. Chen,X., Levine,L. and Kwok,P.Y. (1999) Fluorescence polarization in homogeneous nucleic acid analysis. *Genome Res.*, **9**, 492–498.
11. Pastinen,T., Partanen,J. and Syvanen,A.C. (1996) Multiplex, fluorescent, solid-phase minisequencing for efficient screening of DNA sequence variation. *Clin. Chem.*, **42**, 1391–1397.
12. Syvanen,A.C., Aalto-Setälä,K., Harju,L., Kontula,K. and Söderlund,H. (1990) A primer-guided nucleotide incorporation assay in the genotyping of apolipoprotein E. *Genomics*, **8**, 684–692.
13. Baron,H., Fung,S., Aydin,A., Bähring,S., Luft,F.C. and Schuster,H. (1996) Oligonucleotide ligation assay (OLA) for the diagnosis of familiar hypercholesterolemia. *Nat. Biotechnol.*, **14**, 1279–1282.
14. Lyamichev,V., Mast,A.L., Hall,J.G., Prudent,J.R., Kaiser,M.W., Takova,T., Kwiatkowski,R.W., Sander,T.J., de Arruda,M. *et al.* (1999) Polymorphism identification and quantitative detection of genomic DNA by invasive cleavage of oligonucleotide probes. *Nat. Biotechnol.*, **17**, 292–296.
15. Amicarelli,G., Adlerstein,D., Shehi,E., Wang,F. and Makrigiorgos,M.G. (2006) Genotype-specific signal generation based on digestion of 3-Way DNA junctions: application to KRAS variation detection. *Clin. Chem.*, **52**, 1855–1863.
16. Parsons,B.L. and Heflich,R.H. (1997) Genotypic selection methods for the direct analysis of point mutations. *Mutat. Res.*, **387**, 97–121.

17. Lee,L.G., Connell,C.R. and Block,W. (1993) Allelic discrimination by nick-translation PCR with fluorogenic probes. *Nucleic Acid Res.*, **21**, 3761–3766.
18. Kostrikis,L.G., Tyagi,S., Mhlanga,M.M., Ho,D.D. and Kramer,F.R. (1998) Spectral genotyping of human alleles. *Science*, **279**, 1228–1229.
19. Blomeke,B., Sieben,S., Spotter,D., Landt,O. and Merk,H.F. (1999) Identification of *N*-acetyltransferase 2 genotypes by continuos monitoring of fluorogenic hybridization probes. *Anal. Biochem.*, **275**, 93–97.
20. Germer,S. and Higuchi,R. (1999) Single-tube genotyping without oligonucleotide probes. *Genome Res.*, **9**, 72–78.
21. Massanger-Stephan,K., Tag,C., Reiser,A. and Gressner,A.M. (1999) Rapid genotyping of hemochromatosis gene mutations on the LightLycler with fluorescent hybridization probes. *Clin. Chem.*, **45**, 1875–1878.
22. Nauk,M., Wieland,H. and Marz,W. (1999) Rapid, homogeneous genotyping of the 4G/5G polymorphism in the promoter region of the *PAII* gene by fluorescence resonance energy transfer and probe melting curves. *Clin. Chem.*, **45**, 1141–1147.
23. Matsubara,Y., Fujii,K., Rinaldo,P. and Narisawa,K (1999) A fluorogenic allele-specific amplification method for DNA-based screening for inherited metabolic disorders. *Acta Paediatr.*, **88**(Suppl.), 65–68.
24. Kwiatkowski,R.W., Lyamichev,V., de Arruda,M. and Neri,B. (1999) Clinical, genetic, and pharmacogenetic applications of the invader assay. *Mol. Diagn.*, **4**, 353–364.
25. Whitcombe,D., Brownie,J., Gillard,H.L., McKechnie,D., Theaker,J., Newton,C.R. and Little,S.A (1998) homogeneous fluorescence assay for PCR amplicons: its application to real-time, single-tube genotyping. *Clin. Chem.*, **44**, 918–923.
26. Dabritz,J., Hanfler,J., Preston,R., Stieler,J. and Oettle,H. (2005) Detection of Ki-ras mutations in tissue and plasma samples of patients with pancreatic cancer using PNA-mediated PCR clamping and hybridisation probes. *Br. J. Cancer*, **92**, 405–412.
27. Ward,R., Hawkins,N., O'Grady,R., Sheehan,C., O'Connor,T., Impey,H., Roberts,N., Fuery,C. and Todd,A. (1998) Restriction endonuclease-mediated selective polymerase chain reaction: a novel assay for the detection of KRAS mutations in clinical samples. *Am. J. Pathol.*, **153**, 373–379.
28. Morgan,R., Xiao,J. and Xu,S. (1998) Characterization of an extremely thermostable restriction enzyme, PspGI, from a Pyrococcus strain and cloning of the PspGI restriction-modification system in Escherichia coli. *Appl. Environ. Microbiol.*, **64**, 3669–3673.
29. Pingoud,V., Conzelmann,C., Kinzebach,S., Sudina,A., Metelev,V., Kubareva,E., Bujnicki,J.M., Lurz,R., Luder,G. *et al.* (2003) PspGI, a type II restriction endonuclease from the extreme thermophile Pyrococcus sp.: structural and functional studies to investigate an evolutionary relationship with several mesophilic restriction enzymes. *J. Mol. Biol.*, **20**, 913–929.
30. Le Calvez,F., Mukeria,A., Hunt,J.D., Kelm,O., Hung,R.J., Tanière,P., Brennan,P., Boffetta,P., Zaridze,D.G. *et al.* (2005) TP53 and KRAS mutation load and types in lung cancers in relation to tobacco smoke: distinct patterns in never, former, and current smokers. *Cancer Res.*, **65**, 5076–5083.
31. Hilbe,W., Dlaska,M., Duba,H.C., Dirnhofer,S., Eisterer,W., Oberwasserlechner,F., Mildner,A., Schmid,T., Kühr,T. *et al.* (2003) Automated real-time PCR to determine KRAS codon 12 mutations in non-small cell lung cancer: comparison with immuno-histochemistry and clinico-pathological features. *Int. J. Oncol.*, **23**, 1121–1126.
32. Orum,H., Nielsen,P.E., Egholm,M., Berg,R.H., Buchardt,O. and Stanley,C. (1993) Single base pair mutation analysis by PNA directed PCR clamping. *Nucleic Acids Res.*, **21**, 5332–5336.
33. Fuery,C.J., Impey,H.L., Roberts,N.J., Applegate,T.L., Ward,R.L., Hawkins,N.J., Sheehan,C.A., O'Grady,R. and Todd,A.V. (2000) Detection of rare mutant alleles by restriction endonuclease-mediated selective-PCR: assay design and optimization. *Clin. Chem.*, **46**, 620–624.
34. Thelwell,N., Millington,S., Solinas,A., Booth,J. and Brown,T. (2000) Mode of action and application of Scorpion primers to mutation detection. *Nucleic Acids Res.*, **28**, 3752–3761.
35. Nazarencho,I. (2006) Homogeneous detection of nucleic acids using self-quenched polymerase chain reaction primers labeled with a single fluorophore (LUX primers). *Methods Mol. Biol.*, **335**, 95–114.
36. Peirson,S.N. and Butler,J.N. (2007) Quantitative polymerase chain reaction. *Methods Mol. Biol.*, **362**, 349–362.
37. Thiede,C., Bayerdorffer,E., Blasczyk,R., Wittig,B. and Neubauer,A. (1996) Simple and sensitive detection of mutations in the ras proto-oncogenes using PNA-mediated PCR clamping. *Nucleic Acids Res.*, **24**, 983–984.
38. Luo,J.D., Chan,E.C., Shih,C.L., Chen,T.L., Liang,Y., Hwang,T.L. and Chiou,C.C. (2006) Detection of rare mutant K-ras DNA in a single-tube reaction using peptide nucleic acid as both PCR clamp and sensor probe. *Nucleic Acids Res.*, **34**, e12.
39. Li,J., Harris,L., Mamon,H., Kulke,M.H., Liu,W.H., Zhu,P. and Mike Makrigiorgos,G. (2006) Whole genome amplification of plasma-circulating DNA enables expanded screening for allelic imbalance in plasma. *J. Mol. Diagn.*, **8**, 22–30.
40. Li,J., Wang,F., Mamon,H., Kulke,M.H., Harris,L., Maher,E., Wang,L. and Makrigiorgos,G.M. (2006) Antiprimer quenching-based real-time PCR and its application to the analysis of clinical cancer samples. *Clin. Chem.*, **52**, 624–633.
41. El Housni,H., Heimann,P., Parma,J. and Vassart,G. (2003) Single-nucleotide polymorphism genotyping by melting analysis of dual-labeled probes:examples using Factor V Leiden and phrotrombin 20210A mutations. *Clin. Chem.*, **49**, 1669–1672.
42. Ehrich,M, Bocker,S. and Van den boom,D. (2005) Multiplexed discovery of sequence polymorphisms using base-specific cleavage and MALDI-TOF MS. *Nucleic Acids Res.*, **33**, e38.
43. Baris,I., Koksal,V. and Etlic,O. (2004) Multiplex PCR-RFLP assay for detection of factor V Leiden and prothrombin G20210A. *Gen. Test.*, **8**, 381–383.
44. Simundic,A.M., Topic,E. and Stefanovic,M. (2003) Detection of factor V Leiden by PCR-SSCP using GMA precast Elchrom scientific gels. *Clin. Appl. Thromb. Hemost.*, **9**, 227–231.
45. Uemura,T., Hibi,K., Kaneko,T., Takeda,S., Inoue,S., Okochi,O., Nagasaka,T. and Nakao,A. (2004) Detection of KRAS mutations in the plasma DNA of pancreatic cancer patients. *J. Gastroenterol.*, **39**, 56–60.
46. Steinman,C.R. (1975) Free DNA in serum and plasma from normal adults. *J. Clin. Invest.*, **56**, 512–515.
47. Leon,S.A., Shapiro,B., Sklaroff,D.M. and Yaros,M.J. (1977) Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res.*, **37**, 646–650.
48. Kopreski,M.S., Benko,F.A., Borys,D.J., Khan,A., Mc Garrity,T.J. and Gocke,C.D. (2000) Somatic mutation screenings: identification of individuals harbouring KRAS mutations with the use of plasma DNA. *J. Natl Cancer Inst.*, **92**, 918–923.
49. Maebo,A. (1990) Plasma DNA level as a tumour marker in primary lung cancer. *Jpn. J. Thorac. Dis.*, **28**, 1085–1091.