1396–1400 *Nucleic Acids Research, 1998, Vol. 26, No. 6* *© 1998 Oxford University Press*

Denaturing high performance liquid chromatography (DHPLC) used in the detection of germline and somatic mutations

Wanguo Liu, David I. Smith, Keri J. Rechtzigel, Stephen N. Thibodeau and C. David James*

Department of Laboratory Medicine and Pathology, Division of Experimental Pathology, Mayo Clinic and Foundation, Rochester, MN 55905, USA

Received December 17, 1997; Revised and Accepted January 27, 1998

ABSTRACT

Denaturing high performance liquid chromatography (DHPLC) has been described recently as a method for screening DNA samples for single nucleotide polymorphisms and inherited mutations. Thirty-eight DNAs, 22 of which were heterozygous for previously characterized rearranged transforming gene (*RET*) or cystic fibrosis transmembrane conductance regulator gene (*CFTR*) mutations or polymorphisms, were examined using DHPLC analysis to assess the accuracy of this scanning method. Ninety-one per cent (20/22) of the PCR amplicons from specimens with heterozygous *RET* or *CFTR* sequence showed elution profiles distinct from corresponding homozygous normal patterns; whether the profiles for two amplicons containing heterozygous *RET* sequence were distinct from homozygous cases was equivocal. To investigate the usefulness of this method for detecting mutations in tumor DNAs, each of the phosphatase and tensin homologue deleted on chromosome ten gene (*PTEN*) exons were examined for mutations in 63 malignant gliomas. Seventeen *PTEN* PCR products from this series of brain tumors showed elution profiles indicating sample heterozygosity and in each instance conventional sequencing confirmed the presence of a mutation. *PTEN* amplicons containing exons 1, 3 and 5 were sequenced for each of the 63 tumor DNAs to determine whether any mutations may have escaped DHPLC detection, and this analysis identified one such alteration in addition to the eight mutations that DHPLC had revealed. In total, DHPLC identified 37 of 40 (92.5%) PCR products containing defined sequence variation and no alterations were indicated among 196 amplicons containing homozygous normal sequence.

INTRODUCTION

It is of fundamental importance to both basic and clinical research to efficiently and accurately detect gene sequence variation within DNA samples. Several methods have been developed to scan DNAs for polymorphisms and mutations to accommodate this need, and these techniques have been reviewed on multiple occasions (1–4).

A relatively new addition to DNA scanning methods uses denaturing high performance liquid chromatography (DHPLC; 5–9). In its early stage of application to the analysis of nucleic acids, HPLC was shown to provide an effective means for separating oligonucleotides (10), PCR fragments (11) and for analyzing the products formed in competitive RT–PCR reactions to determine relative levels of gene expression (12).

Mutation/polymorphism scanning by DHPLC involves subjecting PCR products to ion-pair reverse-phase liquid chromatography in a column containing alkylated non-porous particles. Under conditions of partial heat denaturation within a linear acetonitrile gradient, heteroduplexes that form in PCR samples having internal sequence variation display reduced column retention time relative to their homoduplex counterparts. In the majority of cases the elution profiles for such samples are distinct from those having homozygous sequence, making the identification of samples harboring polymorphisms or mutations a straightforward procedure. The major advantages of this method include the use of automated instrumentation, speed of analysis (~5 min per sample) and the size of the DNA fragment that can be analyzed (up to 1.5 kb).

No previous report has addressed the accuracy of mutation/polymorphism detection by DHPLC analysis. One of the objectives of the investigation reported here was to determine the reliability of DHPLC for detecting inherited gene sequence variation. To accomplish this we used DHPLC to examine PCR fragments produced from several DNAs, having previously identified germline mutations or polymorphisms in the rearranged transforming proto-oncogene (*RET*) or the cystic fibrosis transmembrane conductance regulator gene (*CFTR*). Our other major interest was to assess the usefulness of DHPLC for screening tumor DNAs for mutations of tumor suppressor genes (TSGs), a potentially powerful application of this technology that had not previously been examined. However, as the method requires heteroduplex DNA for detection of intra-sample sequence variation, it is reasonable to question whether mutations would escape detection in instances where loss of a wild-type TSG occurs in combination with mutation of the remaining allele since the predominant double-stranded DNA formed would be mutant homoduplex. To address this question, a large panel of malignant glioma DNAs were examined for phosphatase and tensin homologue deleted on chromosome ten gene (*PTEN*) mutations.

*To whom correspondence should be addressed at: Mayo Clinic and Foundation, 200 First Street S.W., Hilton Building Room 820-D, Rochester, MN 55905, USA. Tel: +1 507 284 8989; Fax: +1 507 266 5193; Email: james.charles@mayo.edu

Table 1. Comparison of mutation detection by DHPLC and by sequencing

Amplicon	Length	G-C%	MPT[1]	Sequence Alteration[2]	#Cases	DHPLC[3]
CFTR exon 7	606 bp	38	58°C	None	9	HM
				C->G (1059)	1	HT
				G->A (1096)	1	HT
				C->G (1104)	1	HT
				C->T (1129)	1	HT
				C->T (1132)	2	HT
				T->A (1139)	1	HT
				G->C (1172)	1	HT
				T->A (1175)	1	HT
				G->A (1187)	2	HT
RET exon 10	198 bp	64	61°C	None	7	HM
				G->A (1826)	1	HT
				T->C (1831)	1	HT
				G->A (1832)	1	HT
				T->A (1852)	1	HT
				T->C (1852)	1	*HM*
				T->G (1852)	1	HT
				G->C (1853)	1	HT
				G->T (1853)	1	HT
				C->G (1854)	1	HT
				T->G (1858)	1	HT
				T->C (1858)	1	HT
				G->A (1859)	1	*HM*
				C->G (1860)	1	HT
PTEN exon 1	482 bp	55	59°C	None	61	HM
				A->C (69)	1	HT
				+A (70)	1	HT
PTEN exon 3	433 bp	27	58°C	None	62	HM
				+T (splice acceptor)	1	*HM*
PTEN exon 5	732 bp	34	57°C	None	57	HM
				T->G (279)	1	HT
				T->C (302)	1	HT
				-AATCA (364-8)	1	HT
				-T (370)	1	HT
				G->A (376)	1	HT
				T->A (404)	1	HT

[1]Mobile phase temperature.
[2]cDNA sequence location of alteration in parentheses.
[3]HT, heterozygous; HM, homozygous.
Italicized type indicates cases in which the results of DHPLC and conventional sequencing were discrepant. Altering the mobile phase temperature in these instances, however, resolved sample homoduplex and heteroduplex fractions (see Results and Discussion).

The results of these analyses indicate that DHPLC offers a reliable approach for the detection of germline and somatic mutations.

MATERIALS AND METHODS

Amplicon synthesis

DNAs from peripheral blood leukocytes and tumor tissue snap frozen by immersion in liquid nitrogen were isolated and purified as described (13). Samples used for mutation screening and sequencing were generated in 50 µl reaction volumes containing 10–100 ng of genomic DNA, 20 pmol of forward and reverse primers for either *PTEN* exons 1–9 (14), *CFTR* exon 7 (15) or *RET* exon 10 (16), 200 µM dNTPs (Perkin-Elmer, Foster City, CA), 1.25 U of *Taq* polymerase (AmpliTaq Gold: Perkin-Elmer) and 1× buffer supplied by the manufacturer. PCR amplifications were for 35 cycles: 95°C for 30 s, 60°C for 30 s and 72°C for 1 min (final extension at 72°C for 10 min) following sample denaturation at 95°C for 9 min. Synthesis of appropriately sized PCR reaction products was confirmed by agarose gel electrophoresis.

Denaturing HPLC analysis

DHPLC analysis was carried out using automated instrumentation identical to that described by Underhill *et al.* (9). Four to seven µl of each PCR product, containing ~50–100 ng DNA, was denatured for 3 min at 95°C and then gradually reannealed by decreasing sample temperature from 95 to 65°C over a period of 30 min. PCR products were then separated (flow rate of 0.9 ml/min) over a period of time and through a linear acetonitrile gradient, the values for which were determined by the size and G–C content of the amplicon (Table 1).

The column mobile phase consisted of a mixture of 0.1 M triethylamine acetate (pH 7.0) with (buffer A) or without (buffer B) 25% acetonitrile. The mobile phase temperatures required for optimal resolution of homoduplex and heteroduplex DNAs were determined empirically by injecting one PCR product for each exon at increasing temperatures until a significant decrease in sample retention time was observed. Specific values for the gradient ranges (buffer A component indicated), separation times and mobile phase temperatures used to analyze the amplicons described above are as follows: 57.0–64.2%, 4 min and 58°C for

CFTR exon 7; 53.0–59.3%, 3.5 min and 61°C or 53.0–59.3%, 3.5 min and 59°C for *RET* exon 10; 55.2–56.2%, 5 min and 59°C for *PTEN* exon 1; 52.0–57.4%, 3 min and 58°C for *PTEN* exon 3 and 54.5–60.8%, 3.5 min and 57°C for *PTEN* exon 5. Between sample analyses the column was regenerated with a 19:1 mixture of buffers A and B (40 s) and a solution whose buffer A content was 5% less than the low end of the desired gradient range (40 s).

Sequence analysis

Solutions (10 µl) were prepared with 10–20 ng of product from previous PCR reactions, 0.05 U of *Taq* polymerase, 1× buffer, 10% DMSO, 400 µM ddATP, 600 µM ddTTP, 60 µM ddGTP, 200 µM ddCTP, 10 µM each of dATP, dTTP and dCTP, 20 µM 7-deaza-dGTP (Boehringer Mannheim) and 0.05 µM 5′-^{32}P-labeled sequencing primer. Sequencing reactions were carried out for 30 cycles at 95°C for 20 s, 58°C for 30 s and 72°C for 1 min, using a 1 min ramp time between annealing and elongation phases. Following sample denaturation, reaction products were loaded onto a 6% sequencing gel. Electrophoresis was at 75 W and room temperature for 1–3 h, after which the gels were dried and exposed to Kodak XAR film.

Microsatellite analysis

PCR reactions for determination of tumor loss of heterozygosity contained ~10 ng of genomic DNA, 8–10 pM forward and reverse primers for either the D10S541 or D10S1765 locus (Research Genetics, Huntsville, AL), 0.8 µCi [α-^{32}P]dCTP and 0.2–0.35 U of *Taq* polymerase in 10–15 µl of 1× buffer containing 200 µM dGTP, dATP and dTTP, and 25–34 µM dCTP. Samples were placed in 96-well plates and amplified at 95°C denaturation (30 s), 55°C annealing (30 s) and 72°C extension (1 min) for 43 cycles. At completion of PCR, an equal volume of denaturing buffer was added to each reaction. Samples were then heated to 95°C and quenched on ice. Two µl of each sample were applied to 4 or 6% acrylamide sequencing gels and electrophoresed for 1.5–3 h at 75 W. Gels were dried and exposed to X-ray film for 4–48 h.

RESULTS AND DISCUSSION

PCR fragments were synthesized from 22 peripheral blood leukocyte specimens heterozygous for previously identified exon 10 *RET* or exon 7 *CFTR* mutations or polymorphisms (Table 1). Each PCR reaction product was subjected to DHPLC analysis and their corresponding elution profiles were compared with patterns associated with homozygous normal sequence controls, nine of which were included for the analysis of *CFTR* sequence alterations and seven for the analysis of *RET* alterations.

The elution profiles for the control *CFTR* PCR products were all highly similar and showed a single peak of homoduplex DNA. In contrast, each of the nine PCR products with internal *CFTR* sequence variation produced a distinct profile with multiple peaks due to the reduced column retention time of heteroduplex DNA (examples shown in Fig. 1A). All samples with heterozygous *CFTR* sequence were identified using the same separation conditions (Materials and Methods). G–C content of the 60 bases surrounding each *CFTR* alteration varied between 35 and 54%, suggesting that the detection of sample heteroduplex within a specific amplicon is not greatly influenced by differences in the melting point of sequences flanking the site of base mismatch.

To determine whether DHPLC detection of sample heterozygosity is influenced by nucleotide identity at a specific site of sequence variation, several patient DNAs with heterozygous mutations effecting *RET* cysteine codons 609, 611, 618 and 620 were examined. For nucleotide substitutions at position 1852 of the coding sequence, split-peak elution profiles distinct from the profiles associated with normal homoduplex DNAs were evident for T→A and T→G alterations (Fig. 1B). However, a T→C substitution at this position failed to produce a profile with multiple peaks; this was also the case for an amplicon containing a G→A alteration at base 1859 (Table 1). The peaks for these two cases, however, were noticeably wider than control peaks, and thereby suggested the presence of homoduplexes and heteroduplexes in the corresponding eluates. An alternative DHPLC protocol (mobile phase temperature of 59°C) resulted in a slight resolution of homoduplex and heteroduplex fractions in each sample (see inset for the T→C substitution at base 1852, Fig. 1B). Nine additional heterozygous samples with mutations effecting the cysteine codons showed unique profiles using the initial separation protocol, including two associated with different nucleotide substitutions at position 1853 (Fig. 1B). Significantly, there were no false positives associated with the analysis of either *CF* or *RET* sequence alterations.

Mutation screening by DHPLC has not been applied to the analysis of DNA samples extracted from neoplastic tissue, and to assess the potential of this technology for analyzing tumor specimens, we examined DNAs from 63 malignant gliomas for mutation of the *PTEN* gene. Elution profiles indicated sequence variation within 17 of the 567 PCR products examined, and in each of these cases the presence of a mutation was determined by conventional sequencing (elution profiles for DNAs with exon 5 mutations are shown in Fig. 2A). As opposed to the *RET* and *CFTR* germline mutations which involved single nucleotide substitutions in all instances, the *PTEN* mutations included deletions and insertions, as well as several nucleotide substitutions (Table 1). The overall incidence of DHPLC-detected *PTEN* mutations among this series of tumors (17 of 63; 27%) compares favorably with those reported previously (14,17) and, as was the case for the analysis of DNAs for germline *CFTR* or *RET* mutations, there were no false positives associated with the *PTEN* analysis.

To determine whether some alterations had escaped DHPLC detection, *PTEN* PCR fragments for exons 1, 3 and 5 were conventionally sequenced for all 63 cases. This analysis revealed a single exon 3 mutation that DHPLC had failed to identify. Interestingly, the exon 3 PCR product had the lowest G–C composition (27%) of any of the amplicons examined in this study. Comparison of mobile phase temperatures (MPTs) against corresponding amplicon G–C contents in Table 1 suggests that the temperature used to analyze exon 3 amplicons may have been too high and prevented mutation detection by ‘melting open’ sample duplexes. Consequently, we compared our empirically derived MPTs (Materials and Methods) against MPTs recommended by a recently installed internet program (http://lotka.stanford.edu/dhplc/melt.html). This analysis revealed a close correspondence between experimental and recommended MPTs for all amplicons other than exon 3, for which the internet program recommended a temperature of 53°C. DHPLC analysis at this temperature clearly revealed homoduplex and heteroduplex fractions in the tumor specimen containing the exon 3 mutation (data not shown).

PTEN mutations in malignant gliomas are often accompanied by loss of the remaining wild-type allele (14,17,18). To assess whether this had occurred in any of the tumors examined here, microsatellite

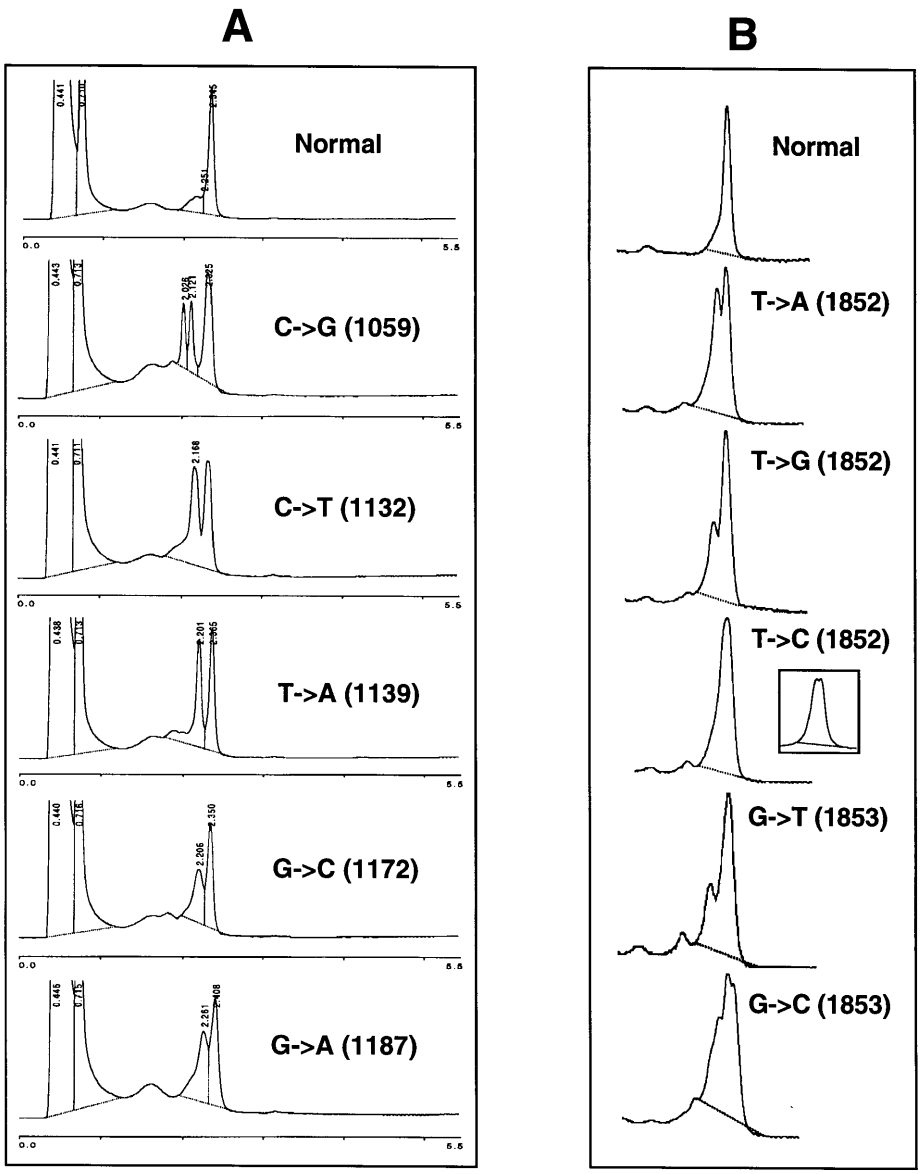

Figure 1. DHPLC detection of *CFTR* and *RET* germline mutations. Elution profiles associated with the DHPLC analysis of PCR amplicons containing either *CFTR* exon 7 (**A**) or *RET* exon 10 (**B**). Retention times for homoduplex and heteroduplex fractions are indicated above the *CFTR* elution peaks. For the *RET* exon 10 results, only the portion of the profile containing the homoduplex and heteroduplex peaks are shown. cDNA sequence location of mutations identified previously in each sample are indicated. The inset profile shown for the T→C substitution at base 1852 was obtained using a mobile phase temperature of 59°C.

analysis was performed on 14 samples with *PTEN* mutations for which there was corresponding normal DNA available. This analysis revealed loss of heterozygosity in 13 instances and, consequently, these results suggest that DHPLC can detect the formation of heteroduplex even when the ratio of normal:mutant DNA sequence in a tumor DNA is quite low. To formally test this hypothesis, we mixed varying amounts of DNA from normal tissue and cell line U251, homozygous for a dinucleotide insertion mutation in *PTEN* exon 7 (14), and analyzed resulting exon 7 PCR amplicons. Elution profiles from the DHPLC analysis of these samples indicate that substantial heteroduplex is formed even in instances where the tumor DNA is 4-fold more abundant than normal (Fig. 2B); similar results were obtained for the reverse situation where normal DNA represented the majority component of the mixture. Taken together, these results indicate that DHPLC requires between 10 and 20% of the minority DNA species for detecting heteroduplex DNA, and extend the use of this method to tumor mutational analysis. In addition, this experiment shows that DHPLC can be used to detect alterations in a homogenous mutant DNA (e.g. cell line) sample by adding an approximately equal amount of normal DNA to the clonal, mutant specimen.

In summary, this survey of different exon sequences indicates that DHPLC offers a reliable and sensitive means for the detection of germline and somatic mutations. The few exceptions encountered may relate to the extreme G–C content of the associated amplicons (64 and 27% for *RET* exon 10 and *PTEN* exon 3, respectively). The differential sensitivity for detecting the transversion and transition mismatches at *RET* positions 1852 and

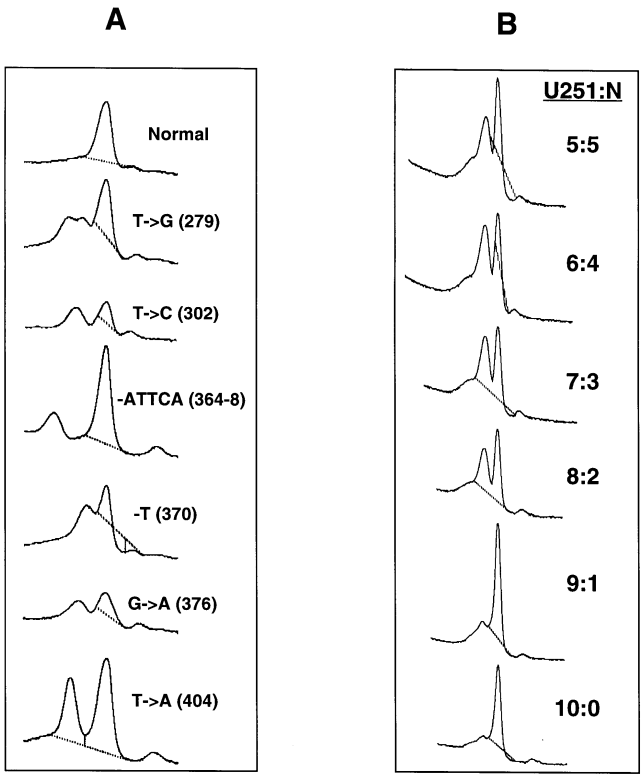

Figure 2. DHPLC detection of *PTEN* mutations in tumor DNAs. Portions of elution profiles are shown for homoduplex and heteroduplex peaks resulting from the analysis of normal or tumor *PTEN* exon 5 PCR products (**A**) and for *PTEN* exon 7 PCR products synthesized from a mixed sample containing homozygous normal DNA and DNA from a glioblastoma cell line with a *PTEN* exon 7 mutation (**B**). Locations of corresponding exon 5 mutations are shown with the profiles to the left (A) and corresponding proportions of cell line:normal DNA are indicated for the profiles shown to the right (B).

1853 are surprising, but imply that DHPLC profiles may serve as a type of 'fingerprint' revealing the precise sequence alteration associated with sample heterogeneity. At a minimum, the accuracy of the method suggests a potential for increased use.

ACKNOWLEDGEMENTS

This work was supported by NCI grants CA-55728 (C.D.J.) and CA-48031 (D.I.S.).

REFERENCES

1 Grompe, M. (1993) *Nature Genet*., **5**, 111–117.
2 Forrest, S., Cotton, R., Landegren, U. and Southern, E. (1995) *Nature Genet*., **10**, 375–376.
3 Mashal, R.D. and Sklar, J. (1996) *Curr. Opin. Genet. Dev*., **6**, 275–280.
4 Cotton, R.G.H. (1997) *Trends Genet*., **13**, 43–66.
5 Oefner, P.J. and Underhill, P.A. (1995) *Am. J. Hum. Genet*., **57** (Suppl.), A266.
6 Underhill, P.A., Jin, L., Zemans, R., Oefner, P.J. and Cavalli-Sforza, L.L. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 196–200.
7 Ophoff, R.A., Terwindt, G.M., Vergouwe, M.N., van Eijk, R., Oefner, P.J., Hoffman, S.M., Lamerdin, J.E., Mohrenweiser, H.W., Bulman, D.E., Ferrari, M., Haan, J., Lindhout, D., van Ommen, G.J., Hofker, M.H., Ferrari, M.D. and Frants, R.R. (1996) *Cell*, **87**, 543–552.
8 Hayward-Lester, A., Chilton, B.S., Underhill, P.A., Oefner, P.J. and Doris, P.S. (1996) In Ferre, F. (ed.), *Gene Quantification*. Birkhauser Verlag, Basel Switzerland, pp. 44–77.
9 Underhill, P.A., Jin, L., Lin, A.A., Mehdi, S.Q., Jenkins, T., Vollrath, D., Davis, R.W., Cavalli-Sforza, L.L. and Oefner, P.J. (1997) *Genome Res*., **7**, 996–1005.
10 Huber, C.G., Oefner, P.J. and Bonn, G.K. (1993) *Anal. Biochem*., **212**, 351–358.
11 Huber, C.G., Oefner, P.J., Preuss, E. and Bonn, G.K. (1993) *Nucleic Acids Res*., **21**, 1061–1066.
12 Hayward-Lester, A., Oefner, P.J., Sabatini, S. and Doris, P.A. (1995) *Genome Res*., **5**, 494–499.
13 James, C.D., Carlbom, E., Dumanski, J., Hansen, M., Nordenskjold, M., Collins, V.P. and Cavenee, W.K. (1988) *Cancer Res*., **48**, 5546–5551.
14 Steck, P.A., Pershouse, M.A., Jasser, S.A., Yung, W.K.A, Lin, H., Lignon, A.H., Langford, L.A., Baumgard, M.L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D.H.F. and Tavtigian, S.V. (1997) *Nature Genet*., **15**, 356–362.
15 Zielenski, J., Rozmahel, R., Bozon, D., Kerem, B., Grzelczak, Z., Riordan, J.R., Rommens, J. and Tsui, L.C. (1991) *Genomics*, **10**, 214–228.
16 Tsai, M.S., Ledger, G.A., Khosla, S., Gharib, H. and Thibodeau, S.N. (1994) *J. Clin. Endocrinol. Metab*., **78**, 1261–1264.
17 Rasheed, B.K.A., Stenzel, T.T., Mclendon, R.E., Parsons, R., Friedman, A.H., Friedman, H.S., Bigner, D.D. and Bigner, S.H. (1997) *Cancer Res*., **57**, 4187–4190.
18 Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Janusz, P., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S.H., Giovanella, B.C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M.H. and Parsons, R. (1997) *Science*, **275**, 1943–1947.