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Genotypic analysis of multiple loci in somatic cells by whole genome amplification

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ABSTRACT

To screen multiple loci in small purified samples of diploid and aneuploid cells a PCR-based technique of whole genome amplification was adapted to the study of somatic lesions. DNA samples from different numbers of flow-sorted diploid and aneuploid cells from biopsies were amplified with a degenerate 15mer primer. Aliquots of these reactions were then used in locus-specific reactions using a single round of PCR cycles with individual sets of primers representing polymorphic markers for different regions. As a result, polymorphic markers for different chromosomal regions, including VNTRs and dinucleotide repeats, can be used to perform up to 30 locus-specific PCR assays with a single sample obtained from fewer than 1000 cells.

INTRODUCTION

It is now widely accepted that cancer develops as a result of a genetic instability that predisposes to the evolution of abnormal clones of cells with accumulated genetic abnormalities (1–3). The development of these abnormal clones can be studied with molecular markers that assess genetic abnormalities, such as loss of heterozygosity (LOH) and microsatellite instability. However, the detection of these abnormalities in human biopsies usually requires relatively large amounts of purified tissue per assay. As a result, it is often not feasible to screen individual samples efficiently for abnormalities at the different loci involved in the pathogenesis and progression of cancer.

We have been investigating the premalignant stages of esophageal adenocarcinoma for several years using a combination of flow cytometric cell sorting and polymerase chain reaction (PCR) assays. Esophageal adenocarcinoma occurs predominantly in patients with a condition known as Barrett's esophagus. Barrett's esophagus develops as a complication of chronic gastroesophageal reflux disease and is characterized by replacement of the normal esophageal squamous epithelium with a metaplastic columnar epithelium. There is substantial evidence that the progression to malignancy in Barrett's esophagus also occurs through a process of genetic instability and clonal evolution (4–6). Evolution from a normal cell to a malignancy in Barrett's esophagus is associated with the development of aneuploid cell populations and allelic losses involving known tumor suppressor genes (7,8).

As part of our ongoing studies into the molecular basis of esophageal adenocarcinoma we have been investigating the order in which genetic abnormalities develop during neoplastic progression using polymorphic markers in PCR-based assays. However, our studies have been limited by small biopsies of interest, the time and the expense of flow sorting multiple samples from each patient and the amount of DNA required for PCR assays. Recently a PCR-based method, termed primer extension pre-amplification (PEP), has been developed that can amplify the genome of a single cell to an estimated minimum of 30 copies (9). PEP uses a degenerate pentadecanucleotide primer, consisting of 4^{15} different combinations of the four possible bases, that anneals randomly throughout the genome to prime amplification. PEP has been used in combination with nested PCR analysis to detect germline abnormalities at different loci in single cell analysis (9,10). However, the presence of low levels of normal tissue in biopsy samples could obscure the detection of somatic lesions in neoplastic tissue, especially when using multiple steps of locus-specific PCR amplification. The availability of flow-sorted material in our studies stimulated us to determine whether PEP could be adapted to the detection of polymorphic markers in somatic tissue with a single step of PCR.

MATERIALS AND METHODS

Isolation of diploid and aneuploid cells from esophageal adenocarcinomas

Tissue samples were obtained from surgical specimens using our previously published mapping techniques that permit the localization of abnormalities within the esophageal mucosa (11). DNA content cell sorting was used to isolate diploid populations and aneuploid populations from patients whose constitutive and tumor samples had been evaluated previously with polymorphic markers using standard PCR techniques (1,7,8,12). DNA was extracted from each sample by our standard protocol (11) and resuspended to a concentration corresponding to 1000 sorted cells/μl.

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PEP and locus-specific PCR analyses

Stock DNA samples from the diploid and the aneuploid samples were serially diluted in ddH_20 for the concentrations used in the PEP reactions. All PEP reactions were done in 60 µl volumes according to previously published procedures (9). We assayed 2 µl aliquots from each PEP reaction for locus-specific markers using standard PCR conditions. The PCR primer sequences and the thermocycling conditions used in this study have been described previously for 17p (1), 13q (7), 5q (13) and 8p (14). Radioactively labeled primers and denaturing gels were used in order to visualize the small repeats associated with the polymorphisms and to increase the sensitivity of detection with only a single step of PCR. For each PCR reaction the forward primer was labeled with [γ-^{32}P]ATP (ICN, Irvine, CA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). All locus-specific PCR reactions were done in 25 µl volumes and subsequently mixed with an equal volume of stop mix (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.02% xylene cyanol). Aliquots (5 µl) from each reaction were loaded onto a 6% polyacrylamide denaturing gel with 1× TBE, electrophoresed at 80 W constant power and then transferred to Gel Blot paper (Schleicher and Schuell, Keane, NH), vacuumed dried and exposed to X-ray film (Kodak, Rochester, NY).

RESULTS

PEP was originally developed for amplifying the genome of single sperm cells for haplotype analyses (9) and more recently has been used for germline analysis in single diploid cells (10). The use of PEP samples in haplotyping requires that a single allele be scored in locus-specific PCR assays. However, in screening for somatic abnormalities with polymorphic markers one allele of a heterozygous diploid cell may be preferentially amplified during initial amplification cycles with low DNA template concentrations. The use of polymorphic markers under these conditions would generate an LOH artifact. In addition, both previous studies required nested PCR to detect the alleles of interest in the PEP samples. Multiple steps of locus-specific PCR could make the detection of LOH sensitive to the presence of a normal diploid cell in a biopsy sample. Therefore, to adapt PEP to the study of genetic lesions in somatic neoplastic cells we determined: (i) the minimum number of cells required for the accurate detection of LOH using only a single step of PCR; (ii) whether low numbers of diploid cells containing two alleles obscured detection of LOH; (iii) whether multiple polymorphic markers from different loci could be detected in a single PEP sample; (iv) whether a locus-specific assay can be used to screen multiple patients. As a source of material to address the first three criteria we selected a patient with an adenocarcinoma that was known to have LOH on 17p, 13q and 8p by standard PCR analysis. In all assays DNA from flow-sorted diploid and aneuploid cells were serially diluted and then amplified in separate PEP reactions.

17p VNTR

One of the most frequent lesions reported in human cancers, including esophageal adenocarcinoma, is LOH at the *p53* locus on 17p (1,15). Therefore, aliquots from each PEP reaction were screened with a 17p VNTR located within the *p53* gene (Fig. 1). The single 17p allele in the aneuploid cells (even numbered lanes) was detected at every dilution from 500 cells (lane 2) down to the single cell level (lane 12). However, in the diploid cells (odd numbered lanes) both alleles were detected only to the 100 cell level (lane 5). Further decreases in cell numbers for the PEP reaction resulted in the apparent 'loss' of either the upper (lane 7) or the lower allele (lane 9) in the subsequent locus-specific PCR assay. This pattern in the diploid cells is consistent with a random preferential amplification of one allele in the initial cycles of the PEP–PCR assay due to limiting amounts of DNA template.

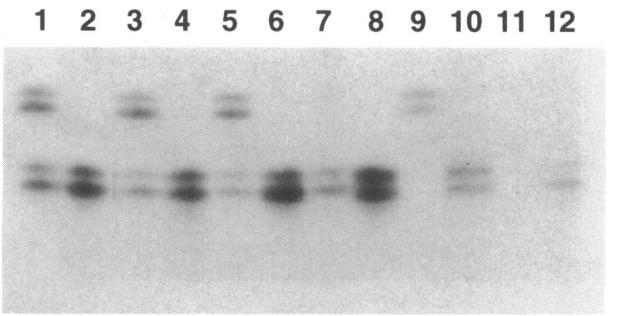

Figure 1. PCR amplification of 17p VNTR from PEP samples. Genomic DNA isolated from flow-sorted diploid (odd numbered lanes) and aneuploid (even numbered lanes) cells were serially diluted. Concentrations corresponding to DNA from 500 (lanes 1 and 2), 250 (lanes 3 and 4), 100 (lanes 5 and 6), 50 (lanes 7 and 8), 10 (lanes 9 and 10) and 1 cells (lanes 11 and 12) were used in separate PEP reactions. Aliquots of 2 µl from each reaction were subsequently used in locus-specific PCR amplification containing 1 µl ^{32}P-labeled forward primer.

13q VNTR

Another frequent target of LOH in human cancers is the retinoblastoma (*Rb*) locus on 13q (7,16). Therefore, we evaluated a VNTR associated with this locus. Aliquots from the same PEP reactions used for the 17p analysis were screened with a marker for this region in separate PCR reactions. The diploid cells (odd numbered lanes) were informative for the 13q polymorphism and the aneuploid cells (even numbered lanes) had LOH on 13q (Fig. 2). Heterozygosity was detected using as few as 50 cells in the PEP reaction (Fig. 2, lane 7). Further dilution of template resulted in loss of detection of both alleles in the heterozygous samples (Fig. 2, lane 9) and the single allele in the aneuploid population (Fig. 2, lane 12).

5q and 8p dinucleotide repeats

Recently the expansion and contraction of small repetitive 'microsatellite' sequences have been associated with the development of cancer (17,18). Markers for these repeats are highly polymorphic and can be used to score microsatellite instability and LOH. We used a 5q dinucleotide repeat marker, D5S299, to determine the level of detection for these repeats in flow-sorted samples (Fig. 3). This region is a frequent target of LOH in many tumor cells, including esophageal cancers (12,19). Although the 5q locus was not polymorphic in our patient, the repeats can be clearly seen down to the 100 cell level in both diploid (lane 5) and aneuploid samples (lane 6). Further decreases in template concentration inhibited accurate detection of the repeats (lanes 7–10). To determine whether LOH could be accurately scored with this class of markers we selected an 8p dinucleotide repeat, D8S87, for analysis. A polymorphism with LOH in the aneuploid

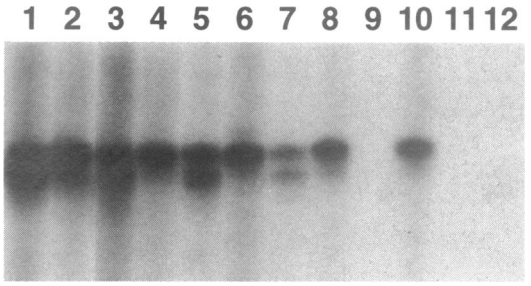

Figure 2. PCR amplification of 13q VNTR with PEP samples. Genomic DNA isolated from flow-sorted diploid (odd numbered lanes) and aneuploid (even numbered lanes) cells were serially diluted. Concentrations corresponding to DNA from 500 (lanes 1 and 2), 250 (lanes 3 and 4), 100 (lanes 5 and 6), 50 (lanes 7 and 8), 10 (lanes 9 and 10) and 1 cells (lanes 11 and 12) were used in separate PEP reactions. Aliquots of 2 µl from each PEP reaction were subsequently used in locus-specific PCR amplification containing 1 µl ^{32}P-labeled forward primer.

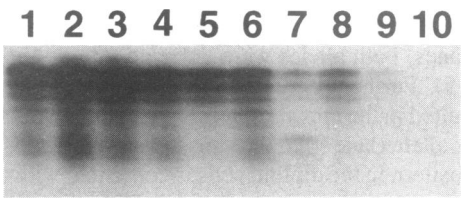

Figure 3. PCR amplification of the 5q dinucleotide repeat with PEP samples. Genomic DNA isolated from flow-sorted diploid (odd numbered lanes) and aneuploid (even numbered lanes) cells were serially diluted. Concentrations corresponding to DNA from 1000 (lanes 1 and 2), 500 (lanes 3 and 4), 100 (lanes 5 and 6), 50 (lanes 7 and 8) and 10 cells (lanes 9 and 10) were used in separate PEP reactions. Aliquots of 2 µl from each PEP reaction were used for locus-specific PCR amplification with 1 µl ^{32}P-labeled forward primer.

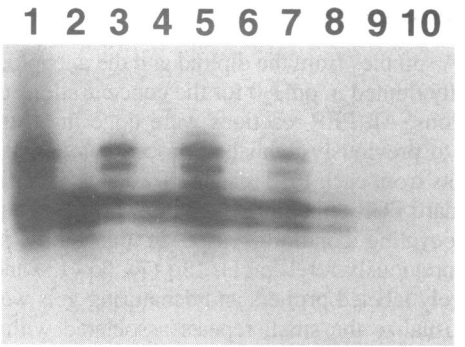

Figure 4. PCR amplification of 8p dinucleotide repeats with PEP samples. Genomic DNA isolated from flow-sorted diploid (odd numbered lanes) and aneuploid (even numbered lanes) cells were serially diluted. Concentrations corresponding to DNA from 500 (lanes 1 and 2), 250 (lanes 3 and 4), 100 (lanes 5 and 6), 50 (lanes 7 and 8) and 10 cells (lanes 9 and 10) were used in separate PEP reactions. Aliquots of 2 µl from each PEP reaction were used for allele-specific amplification with 1 µl ^{32}P-labeled forward primer.

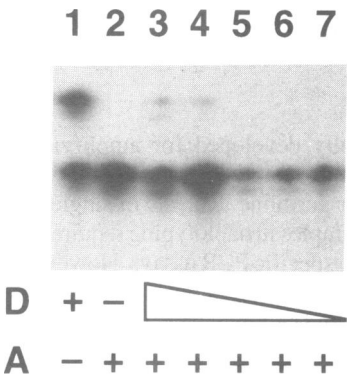

Figure 5. The effect of diploid cells retaining two alleles on the detection of LOH in aneuploid cells. Genomic DNA corresponding to 1000 diploid (lane 1) and 1000 aneuploid cells (lane 2) were used in different PEP–PCR reactions. In addition, DNA from 1000 aneuploid cells was mixed with decreasing concentrations of DNA from diploid cells, corresponding to 500 (lane 3), 100 (lane 4), 50 (lane 5), 10 (lane 6) and 1 cells (lane 7), and used in separate PEP–PCR assays. Aliquots of 2 µl from each PEP reaction were used with the 17p VNTR primers for the locus-specific PCR reaction.

cells (even numbered lanes) was observed with the 8p marker (Fig. 4). Both diploid alleles and the single allele present in the aneuploid cells were detected down to the 50 cell level (lanes 7 and 8). Further dilution of template in the PEP reactions resulted in loss of detection of alleles (lanes 9 and 10).

Detection of LOH in the presence of normal diploid cells in PEP samples

A frequent problem in the detection of LOH is the presence of contaminating normal diploid cells in a tumor sample. The use of flow-sorted samples has been critical to our studies by allowing us to separate diploid and aneuploid populations from the same biopsy. However, the sensitivity of PCR assays raises the possibility that even low levels of normal cells may interfere with the detection of allelic losses in PEP samples. To investigate the level of 'contamination' that can be tolerated in our assays prior to the PEP reaction we diluted the DNA from a fixed number (1000) of aneuploid cells that had an allelic loss with DNA from decreasing numbers of diploid cells that retained both alleles. The different mixes were then used for PEP reactions followed by the 17p locus-specific PCR assay (Fig. 5). Pure populations of 1000 diploid (lane 1) and 1000 aneuploid (lane 2) cells were used as a control for detection of the 17p LOH. The results in Figure 5 show that even with a relatively high level of diploid contamination the difference between aneuploid (lanes 3–7) and diploid cells (lane 1) can be determined.

Detection of alleles in different patients

To determine the order in which genetic abnormalities develop during neoplastic progression multiple patients need to be studied. Therefore, to test the accuracy of using PEP samples for allelotyping multiple patients a fixed number (1000) of diploid and corresponding aneuploid cells were pre-amplified and then assayed for 17p alleles (Fig. 6). In all cases the 17p alleles of the diploid cells (odd numbered lanes) and the aneuploid cells (even numbered lanes) were consistent with our previous results (1,7,8,12). This included cases who were informative with (A, B,

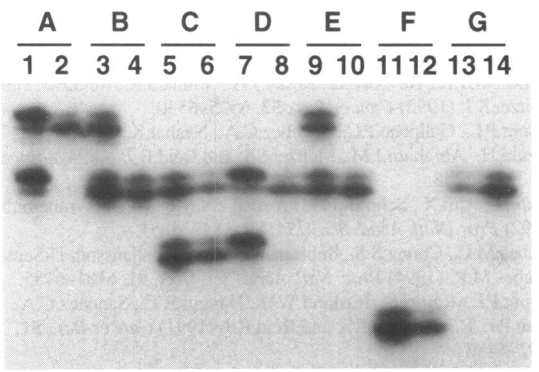

Figure 6. PCR amplification of 17p VNTR in multiple patients. Genomic DNA isolated from 1000 flow-sorted diploid (odd-numbered lanes) and 1000 aneuploid (even numbered lanes) cells from different patients (**A–G**) were used in separate PEP reactions. Aliquots of 2 µl from each PEP reaction were subsequently used in locus-specific PCR amplification containing 1 µl ^{32}P-labeled forward primer.

D and E) or without (C) LOH in the aneuploid cells and cases that were non-informative with different sizes of alleles (F and G).

DISCUSSION

In our previous studies of neoplastic progression in Barrett's esophagus we relied on standard PCR techniques to screen for somatic genetic lesions in flow-sorted samples. Our ability to screen multiple loci in flow-sorted material has been limited by the number of cells, often less than 10^4, available for study. To determine whether PEP could be used to extend our ability to investigate somatic genetic lesions in these small samples we have used constitutive and tumor samples that had been evaluated previously with polymorphic markers using standard PCR techniques (1,7,8,12) to address five main criteria: (i) the appearance of LOH artifacts at low template concentration; (ii) the detection of multiple loci in a single sample; (iii) the effect of normal cell 'contamination' on the detection of LOH; (iv) the use of different classes of polymorphic markers; (v) the detection of alleles in samples from multiple patients.

LOH artifact

LOH artifact in diploid cells was observed with the 17p VNTR at or below the 50 cell level in PEP reactions (Fig. 1, lane 7). The appearance of either the lower (lane 7) or the upper (lane 9) allele is consistent with a random preferential amplification of one allele during the intial rounds of PCR cycles. In addition, further decreases in cell number often resulted in loss of detection in both diploid and aneuploid cells with all the markers used in this study. These observations suggest that PEP samples from 50–100 cells are equivalent to the theoretical limit of 50 DNA fragments required for the accurate detection of heterozygosity in single locus-specific PCR assays (20). In our ongoing studies of neoplastic progression in Barrett's esophagus we routinely use 1000 cells in our PEP assays for primary screens of biopsies, which provides a 10- to 20-fold margin of safety over the minimum requirements. Assaying 2 µl aliquots from each 60 µl PEP reaction allows us to rapidly and accurately perform up to 30 locus-specific PCR assays/1000 cells from each biopsy and to extend our work with previously limiting amounts of flow-sorted material. In addition, the use of 1000 cells in PEP is sufficient to visualize single step PCR products on ethidium bromide stained gels (data not shown).

In our previous studies we routinely used 5000–20 000 cells/locus-specific asssay with a single round of PCR cycles (1,7,8). Therefore, to allelotype all 40 arms of the non-acrocentric somatic chromosomes would require 200 000–800 000 cells/biopsy. This is often in excess of the material available from each biopsy for all analyses. The adaptation of PEP to our work has allowed us to complete allelotyping of individual patients using fewer cells than the minimum previously required for a single PCR assay.

Multiple loci in a single PEP sample

PEP has been shown to be effective in single cell analysis for germline mutations in both haploid and diploid genomes (9,10). To determine whether we could accurately screen multiple loci in somatic diploid and aneuploid cells from the same PEP samples we selected markers for separate chromosomal regions, 17p, 5q, 13q and 8p, that are often associated with tumor development. In addition, we have also used an 18q-specific VNTR on these same samples, with similar results (data not shown). In each case we were able to detect alleles in both diploid and aneuploid samples with a single round of PCR cycles using DNA from a minimum of 50–100 cells in the PEP reaction.

A possible limitation to the use of PEP is the under-representation of large PCR products in the sample.The alleles detected in this study range from 80 (17p VNTR) to 350 bp (13q VNTR). Previous studies have reported up to 800 bp PCR products using PEP samples as template (9,10). We encountered problems with the detection of a second 17p-associated marker, YNZ22, whose alleles can exceed 1.0 kb (1). Thus our data and those of others suggest that larger PCR products may be under-represented in the PEP reaction (9).

Effect of normal cell 'contamination'

A problem for the detection of LOH in primary somatic tissue is the presence of normal cells in tumor biopsies. Our use of flow sorting to obtain highly purified cell fractions from each biopsy sample has been critical for genetic analysis in our studies. However, the number of cycles in the PEP reaction and the sensitivity of PCR assays raises the possibility that detection of LOH in PEP samples could be obscured by even very low levels of normal cells. Our mixing experiments (Fig. 5) have shown that LOH can still be detected in the presence of relatively high levels, up to 33% (lane 5), of diploid cells with two alleles.

Different classes of polymorphic markers

In this initial report we have shown that alleles of loci that often contain abnormalities in tumor cells could be detected in a single step of PCR with PEP samples. The markers used in this study include VNTRs and dinucleotide repeats. These represent the most widely used classes of polymorphisms in somatic studies. We have also used an 8p-specific RFLP on these same samples, with similar results (data not shown). The patient sample selected in this study did not have microsatellite instability at 5q (Fig. 3) or 8p (Fig. 4). However, we have detected this form of instability in other patients during our ongoing studies with PEP samples. In

all cases the results with PEP samples were in agreement with those obtained by standard PCR assays (1,7,8,12)

Detection of alleles in multiple samples

Finally, we have also validated the use of PEP samples in genotypic studies by screening a series of patients with the 17p VNTR (Fig. 6). To avoid potential LOH artifacts we used 1000 cells in the PEP reactions. In each case the 17p allelic status of both diploid and aneuploid cells determined in PEP samples was the same as determined by standard PCR techniques.

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REFERENCES

1 Blount,P.L., Meltzer,S.J., Yin,J., Huang,Y., Krasna,M.J. and Reid,B.J. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 3221–3225.
2 Sidransky,D., Mikkelson,T., Schwechheimer,K., Cavanee,W., Rosenblum,M.L. and Vogelstein,B. (1992) *Nature (Lond.)*, **355**, 846–847.
3 Vogelstein,B., Fearon,E.R., Hamilton,S.R., Kern,S.E., Preisinger,A.C., Leppert,M., Nakamura,Y., White,R., Mits,A.M. and Bos,T.L. (1988) *New Engl. J. Med.*, **319**, 525–532.
4 Reid,B.J. (1991) *Gastroenterol. Clin. North. Am.*, **20**, 817–834.
5 Rabinovitch,P.S., Reid,B.J., Haggitt,R.C., Norwood,T.H. and Rubin,C.E. (1988) *Lab. Invest.*, **60**, 65–71.
6 Reid.B.J., Blount,P.L., Rubin,C.E., Levine,D.S., Haggitt,R.C. and Rabinovitch,P.S. (1992) *Gastroenterology*, **102**, 1212–1219.
7 Huang,Y., Boynton,R.F., Blount,P.L., Silverstein,R.J., Yin,J., Tong,Y., McDaniel,T.K., Newkirk,C., Resau,J.H., Sridhara,R., Reid,B.J. and Meltzer,S.J. (1992) *Cancer Res.*, **52**, 6525–6530.
8 Blount,P.L., Galipeau,P.C., Sanchez,C.A., Neshat,K., Levine,D.S., Yin,J., Suzuki,H., Abraham,J.M., Meltzer,S.J. and Reid,B.J. (1994) *Cancer Res.*, **54**, 2292–2295.
9 Zhang,L., Cui,X., Schmitt,K., Hubert,R., Navidi,W. and Arnheim,N. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 5847–5851.
10 Snabes,M.C., Chong,S.S., Subramanian,S.B., Kristjansson, DiSepio,D. and Hughes,M.R. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 6181–6185.
11 Blount,P.L., Ramel,S., Raskind,W.H., Haggitt,R.C., Sanchez,C.A., Dean,P.J., Rabinovitch,P.S. and Reid,B.J. (1991) *Cancer Res.*, **51**, 5482–5486.
12 Boynton,R.F., Blount,P.L., Yin,J., Huang, Y., Tong, Y., Brown, V.L., McDaniel,T., Newkirk, C., Resau, J.H. and Meltzer,S.J. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 3385–3388.
13 van Leewen,C. (1991) *Nucleic Acids Res.*, **19**, 5805.
14 Weber,J.L. (1990) *Nucleic Acids Res.*, **18**, 4038.
15 Hollstein,M., Sidransky,D., Vogelstein,B. and Harris,C.C. (1991) *Science*, **253**, 49–53.
16 Horowitz,J.M., Park,S.H., Bogenmann,E., Cheng,J.C., Yandell, D.W., Kaye,F.J., Minna,J.D., Dryja,T.P. and Weinberg,R.A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 2775–2779.
17 Bronner,C.E., Baker,S.M., Morrison,P.T., Warren,G., Smith,L.G., Lescoe,M.K., Kane,M., Earabino,C., Lipford,J., Lindbloom,A., Tannergard,P., Bollag,R.J., Godwin,A.R., Ward,D.C., Nordenskjold,M., Fishel,R., Kolodner,R. and Liskay,M. (1994) *Nature*, **368**, 258–261.
18 Fishel,R., Lescoe,M.K., Rao,M.R.S., Copeland,N.G., Jemkins,N.A., Garber,J., Kane,M. and Kolodner,R. (1993) *Cell*, **75**, 1027–1038.
19 Fearon,E.R., Cho,K.R., Nigro,J.M., Kern,S.E., Simons,J.W., Ruppert,J.M., Hamilton,S.R., Preisinger,A.C., Thomas,G., Kinzler,K.W. and Vogelstein,B. (1990) *Science*, **247**, 49–56.
20 Navidi,W., Arnheim,N. and Waterman,M.S. (1992) *Am. J. Hum. Genet.*, **50**, 347–359.