Pyrosequencing® assays to study promoter CpG site methylation of the *O⁶-MGMT*, *hMLH1*, *p14ARF*, *p16INK4a*, *RASSF1A*, and *APC1A* genes

ZARAH M. LÖF-ÖHLIN and TORBJÖRN K. NILSSON

Department of Clinical Chemistry, Örebro University Hospital, S-70185 Örebro, Sweden

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Abstract. DNA methylation of CpG sites in promoter regions of several cancer related genes, such as *O⁶-MGMT, hMLH1, p14ARF, p16INK4a, RASSF1A* and *APC1A*, has been actively explored recently. Much of the data has been obtained using a variation of an allele-specific PCR assay known as methylation specific PCR. This technique is objectionable for a number of methodological limitations and drawbacks. We wanted to study the promoter regions of the above mentioned genes using bisulfite-treated genomic DNA amplified by PCR, using primers designed to bind only to CpG-free sequences. The methylated fraction (%) of each CpG site was measured by Pyrosequencing® technology. For three of the genes, *O⁶-MGMT*, *hMLH1* and *p14ARF*, two amplicons were designed to cover all relevant CpG sites. Several of the amplicons were analyzed by two different Pyrosequencing assays. In all, we designed nine optimized PCR protocols and 13 Pyrosequencing assays covering the promoters of all six studied genes. In all cases, standard PCR generated sufficient quantities of pure amplicons to be further analyzed by Pyrosequencing technology. Thus, a total of 119 CpG sites in six genes could be quantified. We conclude that standard PCR followed by Pyrosequencing is a workable, more specific and quantitative alternative to 'methylation specific' PCR. This approach provides a more comprehensive picture of the distribution of DNA methylation throughout the promoter regions of the studied set of six genes, which will be of benefit in oncological research.

Introduction

Epigenetic regulation, through methylation or demethylation of CpG sites in promoter regions of cancer-related genes by methyltransferases (2), plays an important role in the pathogenesis of cancer (1). This mode of regulation is considered activating or silencing depending upon action.

DNA methylation is particularly common in tumor-suppressor (1) and DNA-repair genes. Methylation of the CpG sites in gene promoter regions may cause the genes to lose their activity. This loss of activity usually involves cell-cycle regulation, cell adhesion or DNA reparation. In this study, we focused on developing new, quantitative methylation assays for six genes frequently implicated in cancer research. *O⁶-MGMT* is a DNA repair protein involved in tumor-cell resistance to the cytostatic activity of chemotherapeutic alkylating agents, but also protects against genotoxic and carcinogenic effects of DNA alkylation (3). *hMLH1* forms a heterodimer that is responsible for the recruitment of proteins needed for mismatch repair (4). An alternate reading frame product of the CDKN2A locus, *p14ARF*, can interact with the MDM2 protein within the nucleus, thus modulating the activity of the p53 protein (5). Another alternate reading frame product of the CDKN2A locus, *p16INK4a*, inhibits the cyclin D-dependent kinases (CDK) that control the phosphorylation of the Rb protein and cell proliferation (5,6). *RASSF1A* regulates the stability of mitotic cyclins and the timing of mitotic progression (7). *APC1A* is an important regulator of cell adhesion as it binds to and inactivates ß-catenin (8,9).

The methylation status of the promoter regions of *O⁶-MGMT*, *hMLH1*, *p14ARF*, *p16INK4a*, *RASSF1A* and *APC1A*, has previously been studied using methylation specific PCR (10,11). Methylation-specific PCR (MS-PCR) is limited in that the methylation status of only a few CpG sites (i.e., those interfering with the PCR primer binding) can be interrogated at once. The technique also has the drawback of providing only a qualitative indication of the methylation status of the sites. Like all 'allele-specific' PCR methods, the judgment of the presence or absence of a band on an agarose gel is largely a matter of the combination of the number of PCR optimization experiments made and the subjective analysis of the bands detected during gel electrophoresis. We feel that these limitations and drawbacks are a real cause of concern that may have stalled progress in the understanding of the role of DNA methylation in clinical cancer research.

Our goal was to develop methods to quantitatively measure the methylated fraction of the CpG sites located in the promoter regions within the above-mentioned genes that

Correspondence to: Dr Torbjörn Nilsson, Department of Clinical Chemistry, Örebro University Hospital, S-70185 Örebro, Sweden
E-mail: torbjorn.nilsson@orebroll.se

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Figure 1. Our strategy for designing PCR primers exemplified by the *O⁶-MGMT* gene. The highlighted CpG sites (large font against a gray background) show where the primers for the 'methylation specific' PCR were placed in previous studies (10,11). Our PCR primers were designed to only cover unique sequences lacking CpG sites and are underlined in the figure above.

were previously implicated in cancer transformation. The PCR primers should be uniquely designed and devoid of CpG sites in primer-binding areas such that amplification will always take place independent of methylation status. Furthermore, we wanted to study the PCR amplicons using Pyrosequencing® technology; a technique that generates a quantitative measure of methylation and automatically calculates and reports percent methylation for each CpG site in the studied sequence, thus allowing the detection of partially methylated CpG sites. We succeeded in developing methods to quantify a large number of consecutive CpG sites, including previously studied CpG sites, in addition to adjacent sites considered novel up until now. This study provides a more comprehensive picture of the distribution of DNA methylation throughout the promoter regions of the studied set of six genes.

Materials and methods

DNA isolation and bisulfite treatment of whole blood leukocytes. Genomic DNA (gDNA) was extracted from 200 μl whole EDTA blood using a BioRobot EZ1 (Qiagen Inc., Valencia, CA, USA) with the QIAamp EZ1 DNA blood 200 μl kit according to the manufacturer's instructions (Qiagen Inc.) utilizing a BioRobot EZ1 (Qiagen Inc.). Freshly extracted DNA (~1000 ng) was used for the bisulfite treatment. The bisulfite treatment was performed with the EZ DNA Methylation kit according to the manufacturer's instructions (Zymo Research, Orange, CA, USA). In summary, DNA was diluted with M-Dilution buffer and incubated for 15 min at 37˚C. CT conversion reagent was added to the DNA samples from the previous step and incubated again at 50˚C for 16 h. Finally, the samples were incubated on ice for 10 min and then M-binding buffer was added. The samples were centrifuged and then washed with M-Wash buffer. The dsDNA was eluted in 10 μl M-Elution buffer and then diluted 5 times with TE buffer (10 mmol/l Tris-HCl, 0.05 mmol/l EDTA, pH 7.5).

PCR. PCR primers for Pyrosequencing® were designed using Methprimer (http://www.urogene.org/methprimer/index1.html). The purpose of the designs of PCR primers for *O⁶-MGMT, hMLH1, p14^{ARF}, p16^{INK4a}, RASSF1A* and *APC1A* were to amplify the previously identified promoter regions of the six genes (10,11). Our designs should also interrogate and quantify the methylated fraction % at the CpG sites located in the primer-binding regions of previously published studies that have utilized methylation-specific PCR; therefore, longer PCR amplicons were required compared to previously published data. Primer sequences and PCR conditions can be seen in Table I.

PCR was performed using an Eppendorf Mastercycler (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) and the HotStarTaq DNA polymerase kit (Qiagen Inc.); we used 30 or 60 μl volume reactions depending on the number of amplicons required to analyze in the Pyrosequencer (PSQ 96MSA sys tem, Biotage AB). Each reaction contained 0.4 μmol/l of each primer, 1.25 units of Taq polymerase, 1.5 mmol/l $MgCl_2$ and 0.2 mmol/l each of dGTP, dATP, dTTP and dCTP. The diluted bisulfate-treated gDNA (5 μl) served as the PCR template. The PCR program consisted of an initial polymerase activation step at 95˚C for 15 min followed by 53 cycles of denaturation at 94˚C for 30 sec, primer annealing at 48-57˚C for 30 sec and extension at 72˚C for 1 min. A final extension step at 72˚C for 7 min finalized the program. The PCR primers, annealing temperatures and amplicon sizes for Pyrosequencing are shown in Table I.

Pyrosequencing. PCR products from bisulfite-treated genomic DNA samples were analyzed with Pyrosequencing technology, in order to quantify site-specific methylation; sequence primers are summarized in Table I. The post-PCR (see above) samples were prepared with the Vacuum Prep workstation (Biotage AB, Uppsala, Sweden) according to the following protocol summary: 30 μl of the amplicon, 3 μl streptavidin Sepharose HP beads (Amersham Biosciences, Uppsala, Sweden), 37 μl binding buffer (10 mmol/l Tris-HCl, 2 M NaCl, 1 mmol/l EDTA, 0.1% Tween-20, Milli-Q (18.2 MΩ x cm) water, pH 7.6) and 15 μl Milli-Q water were mixed and used in the Vacuum Prep workstation. The biotinylated amplicons were immobilized onto the streptavidin Sepharose beads and then passed through one denaturation step and two washing steps using the Vacuum Prep workstation. The first step was 70% ethanol, the second 0.2 mol/l NaOH (denaturation step) and the third was washing buffer (10 mmol/l Tris-Acetate, Milli-Q water). The amplicons were transferred to a plate containing sequencing primer (0.4 μmol/l) in 40 μl annealing buffer (20 mmol/l Tris-acetate, 2 mmol/l magnesium acetate, pH 7.6). The sequencing primer was annealed to the template at 80˚C for 2 min. Pyrosequencing was performed using the PSQ96

Table I. PCR primer sequences, annealing temperatures, amplicon sizes and sequencing primer sequences for the 6 cancer related genes amplified in 9 different amplicons.

Gene	PCR primer sequence 5'-3'	Annealing temp (°C)	$MgCl_2$ conc (mmol/l)	Size (bp)	Sequencing primer 5'-3'
O⁶-MGMT Fw	F: GGATATGTTGGGATAGTT R: Biotin-ACAACACCTAAAAAACACTTAAAAC	50	1.5	259	Py1: GATTTGGTGAGTGTTTGGGT
O⁶-MGMT Re	F: Biotin-GGATATGTTGGGATAGTT R: ACAACACCTAAAAAACACTTAAAAC	50	1.5	259	Py1: AAATAAATAAAAATCAAAAC Py 2: ACCCAAACACTCACCAAATC
hMLH1 Fw	F: TTTTTTTAGGAGTGAAGGAGGTTA R: Biotin-CCCAAAAAAAAACAAAATAAAAATC	55	2	274	Py 1: TTTTTTTAGGAGTGAAGGAGGTTA
hMLH1 Re	F: Biotin-TTTTTTTAGGAGTGAAGGAGGTTA R: CCCAAAAAAAAACAAAATAAAAATC	55	2	274	Py 1: ATAAAACCCTATACCTAATCTATC
p14ARF Start	F: TTTATTTGGTTTTTTAGGAAG R: Biotin-CAAATTCTTAATAACCCTCC	47	1.5	190	Py1: GTTGGTTTTTTAGTAGTATTAGTA Py 2: GGGATGTGAATTA
p14ARF End	F: Biotin-GGAGGGTTATTAAGAATTTG R: TCTACAATTAAAAAAACAAAAATAA	55	2	122	Py 1: TCACCTCTAATACCAAAAAA
P16INK4a	F: Biotin-TAGATTTTTTATTTATTTGGAT R: CTAACTAATCACCAAAAAATAAAAC	52	2	254	Py 1: ACTAACTAACTAACCAC Py 2: CTAACTAATCACCAAAAAATAAAAC
RASSF1A	F: Biotin-AGTTTAATGAGTTTAGGTTTTTT R: CTACACCCAAATTTCCATTAC	52	2	186	Py 1: ATCTAAATCCTAAAAAAAAC
APC1A	F: Biotin-TTTTTTTGTTTGTTGGGGATT R: ACTACACCAATACAACCACATATC	50	1.5	247	Py 1: ACACAACTACTTCTCTCTCC Py 2: CCCACACCCAACCAA

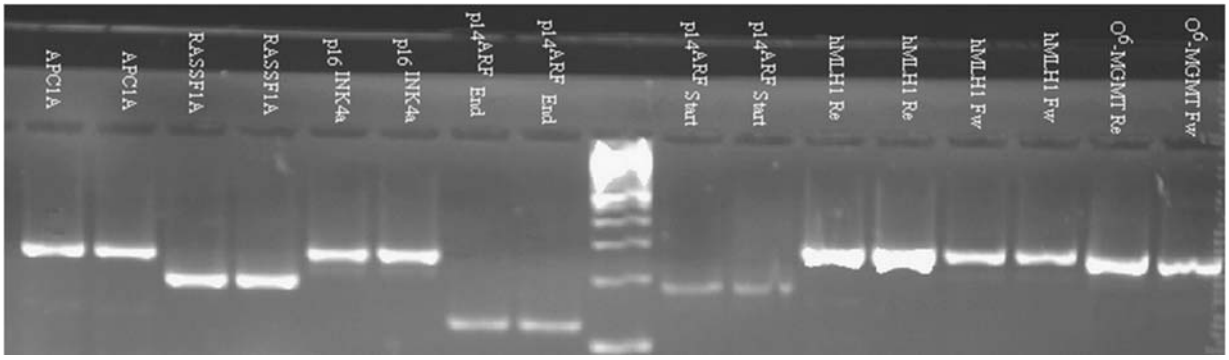

Figure 2. Agarose gel electrophoresis showing the optimization of the PCR amplifications. The gel shows a 100-bp ladder in the middle, and (right to left) amplicons of *O⁶-MGMT* Fw + Re (259 bp), *hMLH1* Fw + Re (274 bp), *p14ARF* Start (190 bp), *p14ARF* End (122 bp), *p16INK4a* (254 bp), *RASSF1A* (186 bp) and *APC1A* (274 bp).

SNP reagent kit and the PSQ 96MSA system (Biotage AB). Pyrograms showing the nucleotide addition order can be seen in Figs. 3-8. Pyro Q-CpG™ software v. 1.0.9 was used to determine the optimal order of nucleotide addition, when designing the assays. The software also automatically analyzed the methylation results. The % methylated fraction (C/T ratio) is displayed in a small colored box just above each CpG site in the analyzed sequence (see Figs. 3-8). Each site is analyzed as a C/T-polymorphism where a 100% C-reading denotes a fully methylated C in the original gDNA sample and a 100% T-reading denotes that this C was unmethylated in the gDNA. Intermediate C/T percentages denote partial methylation at the level of the sample.

Results and Discussion

The aim of the project was to develop methods to quantitatively measure the methylated fraction of the CpG sites in the promoter regions of the *O⁶-MGMT*, *hMLH1*, *p14ARF*, *p16INK4a*, *RASSF1A* and the *APC1A* genes. What we wanted to do was to move the primer binding sites to unique sequences outside of the CpG-containing sequences previously studied by MS-PCR by van den Donk and van Engeland (10,11), to be able to always generate one and the same amplicon, irrespective of the methylation status of the CpG sites in the promoter region that we wanted to analyze. In Fig. 1, comparing the old and new methylation assay designs of the *O⁶-MGMT* gene, the

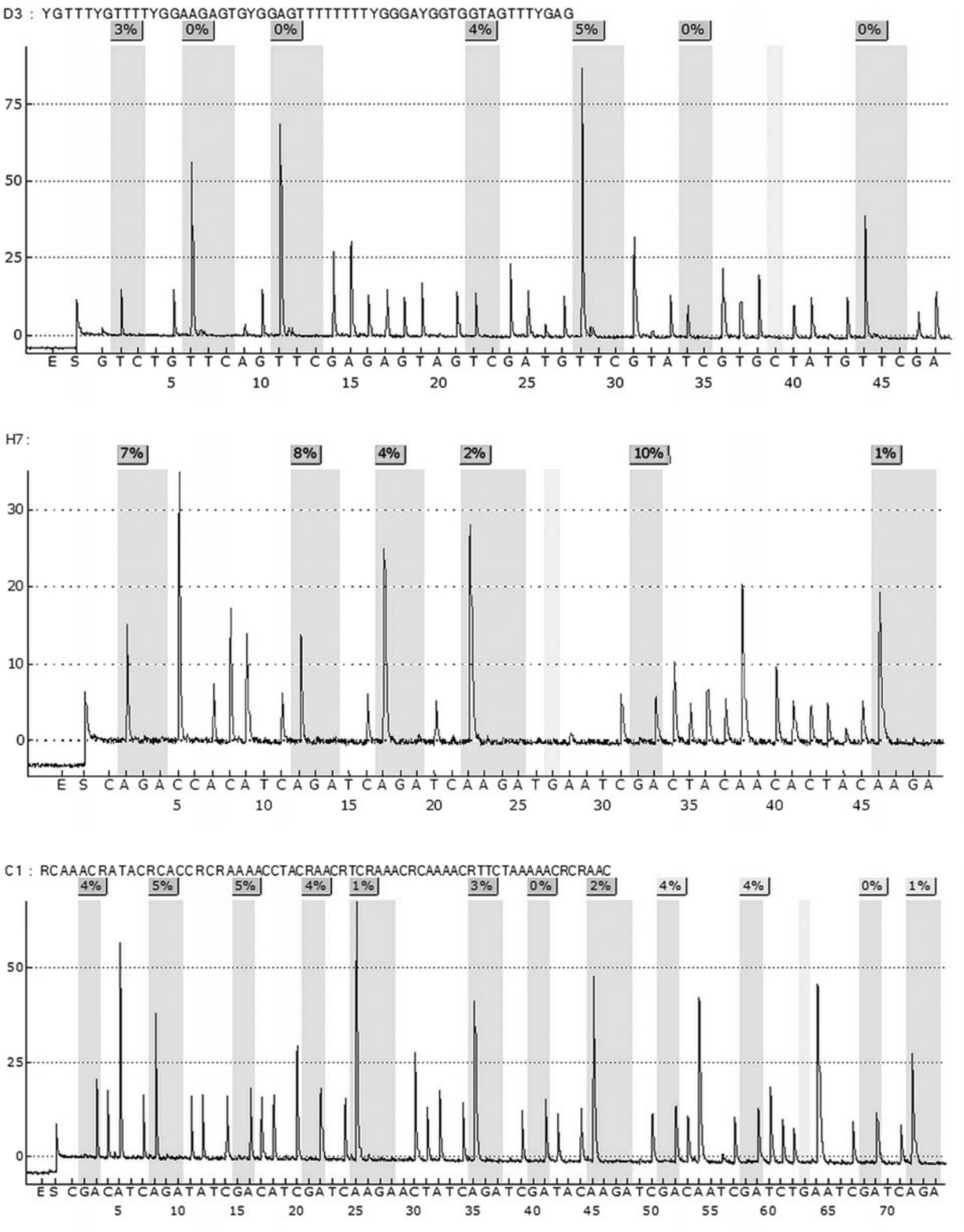

Figure 3. Typical pyrograms showing the sequences analyzed in the *O⁶-MGMT* gene by the three different Pyrosequencing® assays, O6-MGMT Fw - Py1 (upper panel) *O⁶-MGMT* Re - Py1 (middle panel) and *O⁶-MGMT* Re - Py2 (lower panel). Dispensation order of nucleotides is shown underneath each program.

CpG sites where the MS-PCR primers used in previous studies (10,11) bind are highlighted. Our primer binding sites (underlined) are moved outside of the sequence analyzed before (enlarged sequence in the middle of Fig. 1), and now only cover sequences where no CpG sites are present, meaning that even the CpG sites that were located in the binding regions of the MS PCR primers, are themselves assayed in our designs. The requirement of longer amplicons than in the previous studies, turned out to be a real challenge due to the extensive fragmentation of the bisulfite treated DNA, but as can be seen

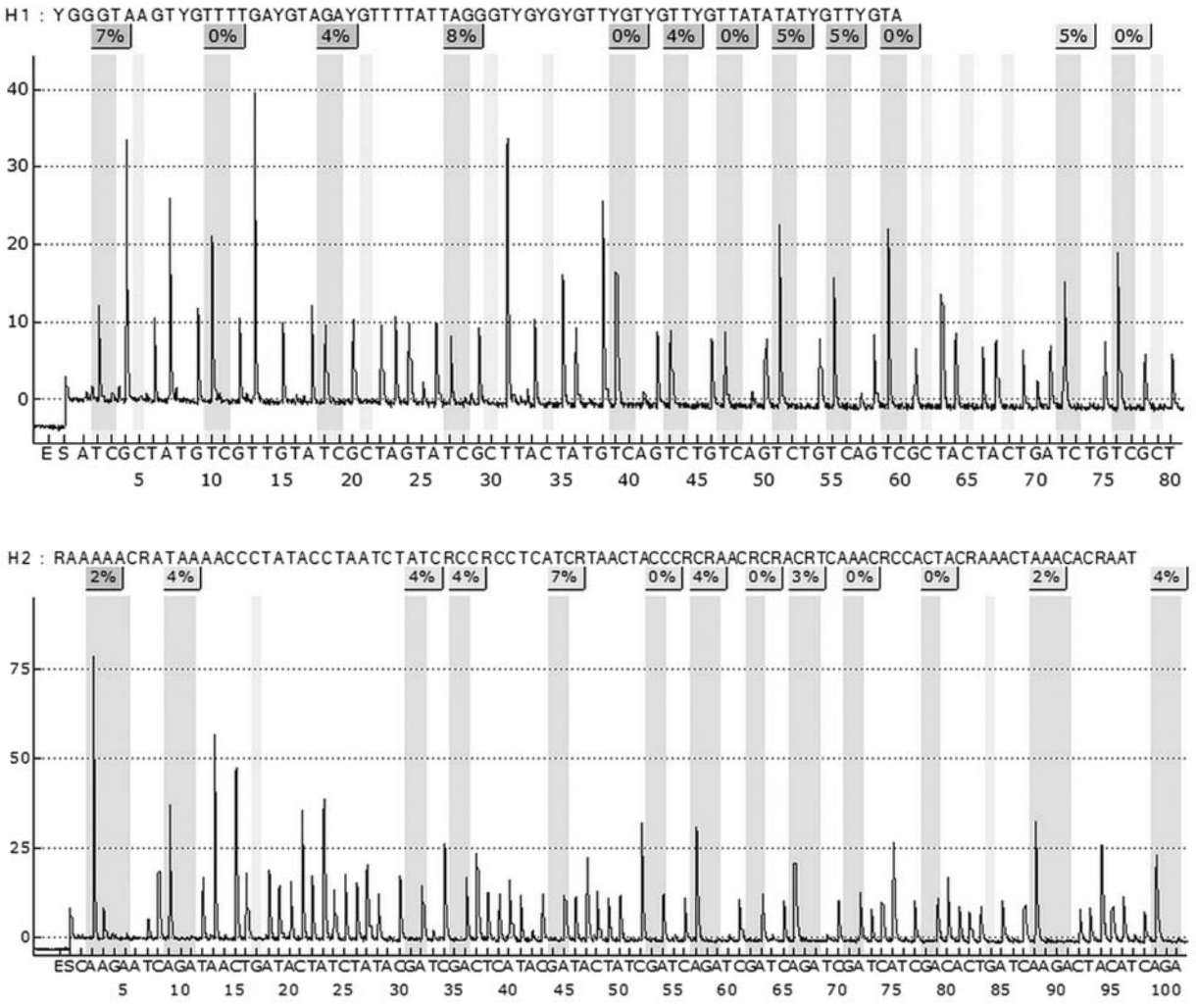

Figure 4. Typical pyrograms showing the sequences analyzed in the *hMLH1* gene by the two different Pyrosequencing assays, *hMLH1* Fw - Py1 (upper panel) and *hMLH1* Re - Py1 (lower panel).

on the agarose gel electrophoresis in Fig. 2, PCR products could be yielded from all of the genes. The conditions for each PCR are given in Table I.

Pyrosequencing assays were applied to the previously mentioned PCR amplicons. Pyrosequencing technology allows each CpG site to be specifically quantified and analyzed as a C/T-polymorphism. In principle, a 100% C-reading denotes a fully methylated-C in the original gDNA sample and a 100% T-reading denotes that this C is unmethylated in the gDNA. Intermediate C/T percentages denotes partial methylation at the level of the sample. Representative Pyrograms are displayed in Figs. 3-8 and shall be commented gene for gene in the following sections.

O^6-MGMT. The *O^6-MGMT* PCR assay generated a 259-bp amplicon that was analyzed by three different Pyrosequencing assays consisting of one assay in the forward direction and two assays in the reverse direction, in order to interrogate the non-coding strand. The forward assay contained 7 CpG sites, including sites one to four shown in Fig. 3 (upper panel, *O^6-MGMT* Fw - Py1) have previously been covered by the 'methylation specific' reverse primer used by van den Donk and van Engeland *et al* (see Table II in refs. 10,11). The two reverse Pyrosequencing-assays covered 18 CpG sites including six previously un-analyzed CpG sites within the first assay (Fig. 3 middle panel, *O^6-MGMT* Re - Pyl). The second reverse assay (*O^6-MGMT* Re-Py2), covering 12 CpG sites, included CpG sites 6-8 (Fig. 3, lower panel) that had been previously analyzed by the forward primer used in van den Donk and van Engeland *et al* (see Table II in refs. 10,11). A total of 25 CpG sites were analyzed in the promoter region of the *O^6-MGMT* gene, 18 of these have not been previously reported for methylation status.

hMLH1. The *hMLH1* PCR assay generated a 274-bp amplicon that was analyzed by two different Pyrosequencing assays consisting of a single assay in the forward direction and a single assay in the reverse orientation. The forward assay included 12 CpG sites, of which sites 3-7 (Fig. 4, upper pane, *hMLH1* Fw - Py1) have been previously covered by the forward primer assay reported in van den Donk and van Engeland *et al* (see Table II in refs. 10,11). The reverse assay also included 12 CpG sites, of which sites 3-5 (Fig. 4, lower panel, *hMLH1* Re - Py1) were covered by the reverse

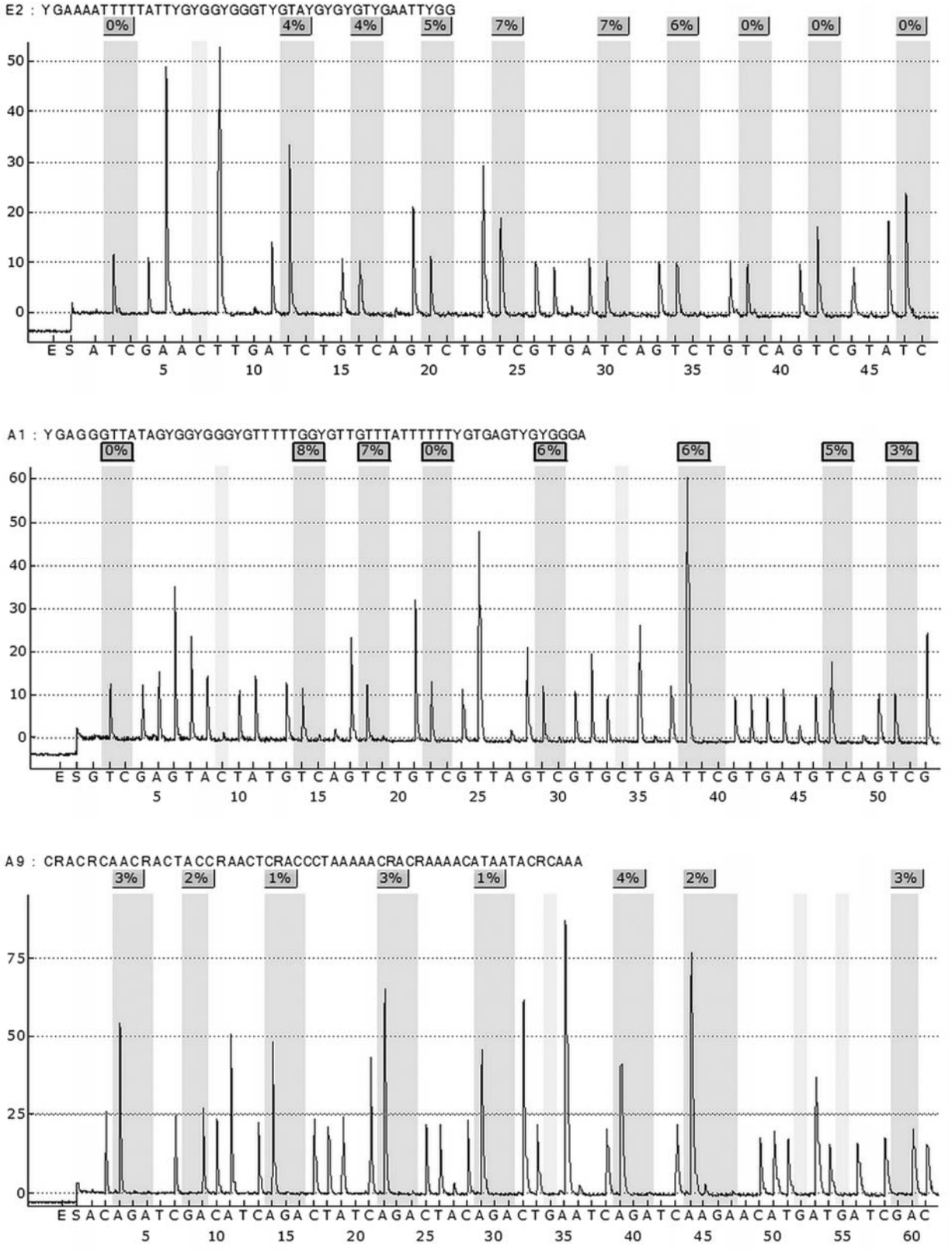

Figure 5. Typical pyrograms showing the sequences analyzed in the *p14*^{ARF} gene by the three different Pyrosequencing assays, *p14*^{ARF} Start - Py1 (upper panel), *p14*^{ARF} Start - Py2 (middle panel) and *p14*^{ARF} End - Py1 (lower panel).

primer used by van den Donk and van Engeland *et al* (see Table II in refs. 10,11). A total of 24 CpG sites were analyzed in the promoter region of the *hMLH1* gene, 16 of these have not been previously reported for methylation status.

p14^{ARF}. The *p14*^{ARF} PCR assays generated two separate amplicons (*p14*^{ARF} Start; 190 bp and *p14*^{ARF} End; 122 bp) to enable the Pyrosequencing assays to analyze as many CpG sites as possible in the promoter region of the gene. The

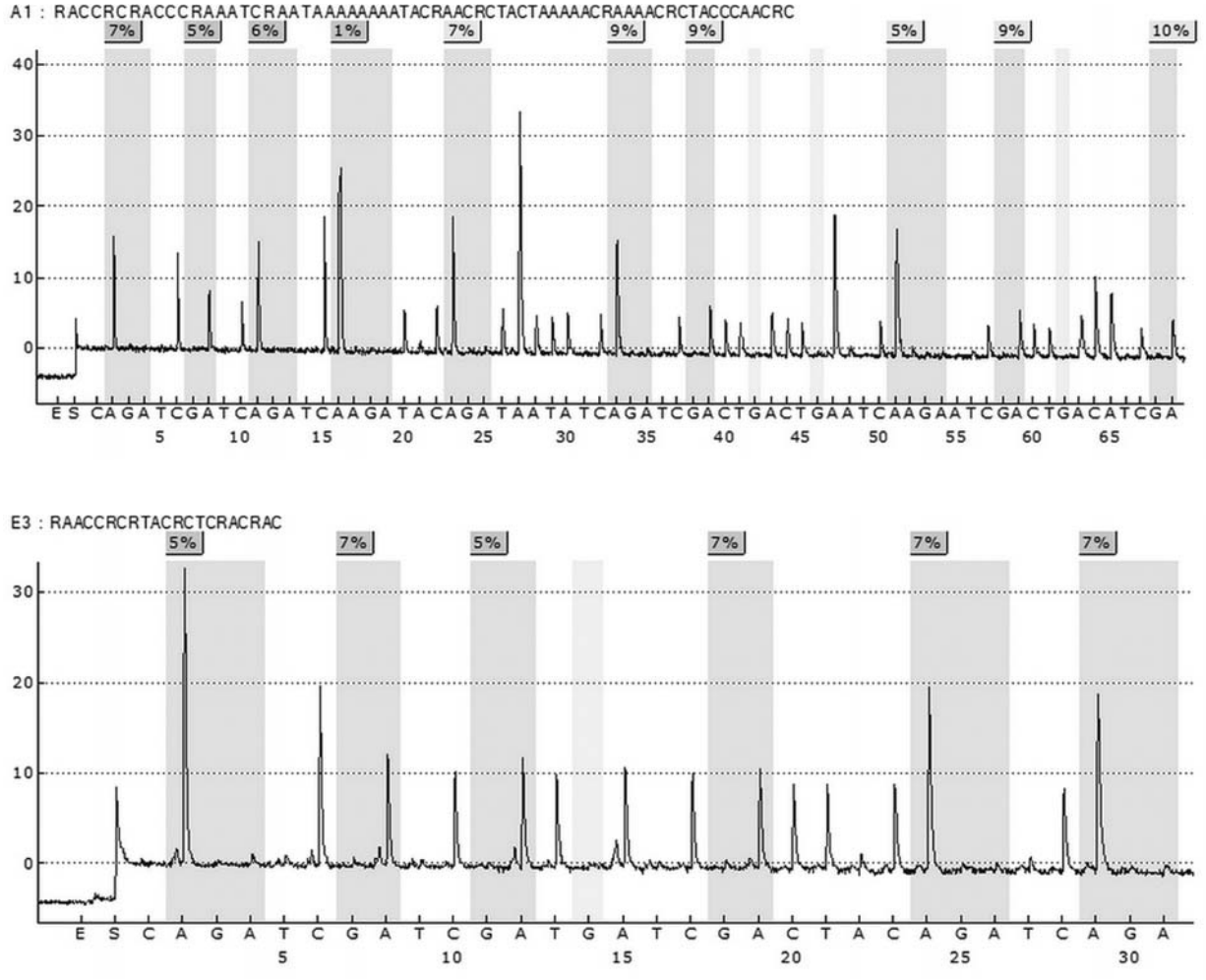

Figure 6. Typical pyrograms showing the sequences analyzed in the *p16*^{INK4a} gene by the two different Pyrosequencing assays, *p16*^{INK4a} - Py1 (upper panel) and *p16*^{INK4a} - Py2 (lower panel).

sequence of the promoter region made the design of a single large amplicon impossible due to the lack of CpG free potential primer-binding sites. The Start amplicon included 18 CpG sites covered by two Pyrosequencing assays run in the reverse direction. The first Pyrosequencing assay, *p14*^{ARF} Start - Py1, covered 10 CpG sites, of which sites 1-4 (Fig. 5, upper panel) were previously analyzed by van den Donk and van Engeland *et al* (see Table II in refs. 10,11). The second assay, *p14*^{ARF} Start - Py2, covers 8 CpG sites, of which none has been analyzed before (Fig. 5, middle panel). The End amplicon, including 8 CpG sites, was analyzed with a single Pyrosequencing assay run in the forward direction. Sites 1-3 (Fig. 5, lower panel - *p14*^{ARF} End - Py1) were previously covered by the reverse primer reported in van den Donk and van Engeland *et al* (see Table II in refs. 10,11). A total of 26 CpG sites were analyzed in the promoter region of the *p14*^{ARF} gene, 19 of these have not been previously been reported for methylation status.

p16^{INK4a}. The *p16*^{INK4a} PCR assay generated a 254-bp amplicon analyzed by two Pyrosequencing assays, both in the reverse orientation. The first assay, *p16*^{INK4a} - Py1, covered 10 CpG sites, of which sites1-5 (Fig. 6, upper panel) were previously analyzed by the forward primer used by van den Donk and van Engeland *et al* (see Table II in refs. 10,11). The second assay, *p16*^{INK4a} - Py2, covered 6 CpG sites, of which sites 1-3 (Fig. 6, lower panel) were previously analyzed by the reverse primer used by van den Donk and van Engeland *et al* (see Table II in refs. 10,11). A total of 16 CpG sites were analyzed in the promoter region of the *p16*^{INK4a} gene, 8 of these have not been previously reported for methylation status.

RASSF1A. The *RASSF1A* PCR assay generated a 186-bp amplicon that we analyzed with a single Pyrosequencing assay in the reverse direction. The assay, *RASSF1A* - Py1, covered 11 CpG sites, of which sites 1-4 (Fig. 7) were previously analyzed by the reverse primer reported in van den Donk and van Engeland *et al* (10,11). Sites 8 to 10 (Fig. 7) were previously covered by the forward primer used by van den Donk and van Engeland *et al* (see Table II in refs. 10,11). A total of 11 CpG sites were analyzed in the promoter region of the *RASSF1A* gene, 4 of these have not been previously reported for methylation status.

APC1A. The *APC1A* PCR assay generated a 247-bp amplicon, which we analyzed by two different Pyrosequencing assays,

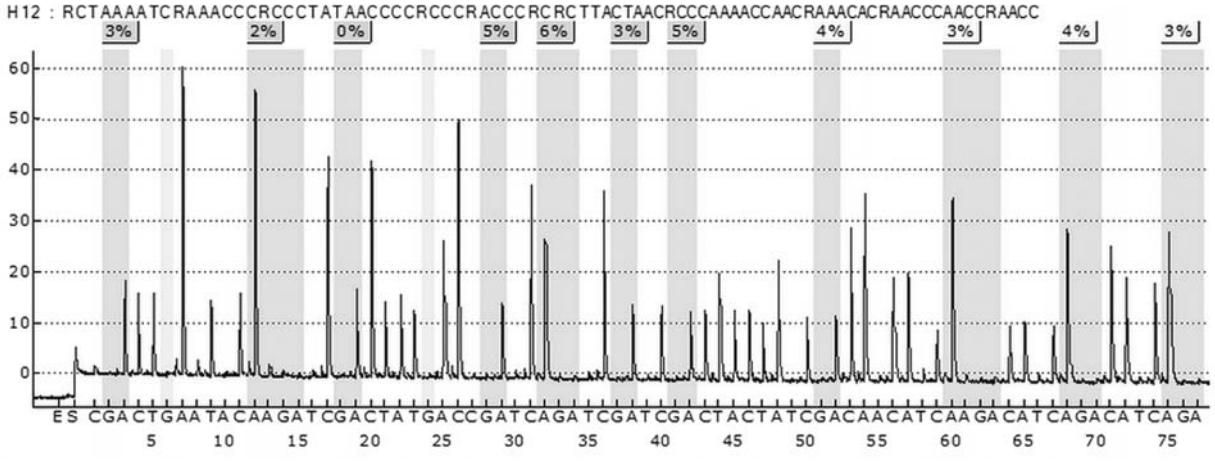

Figure 7. Typical pyrogram showing the sequence analyzed in the *RASSF1A* gene by the Pyrosequencing® assay, *RASSF1A* - Py1.

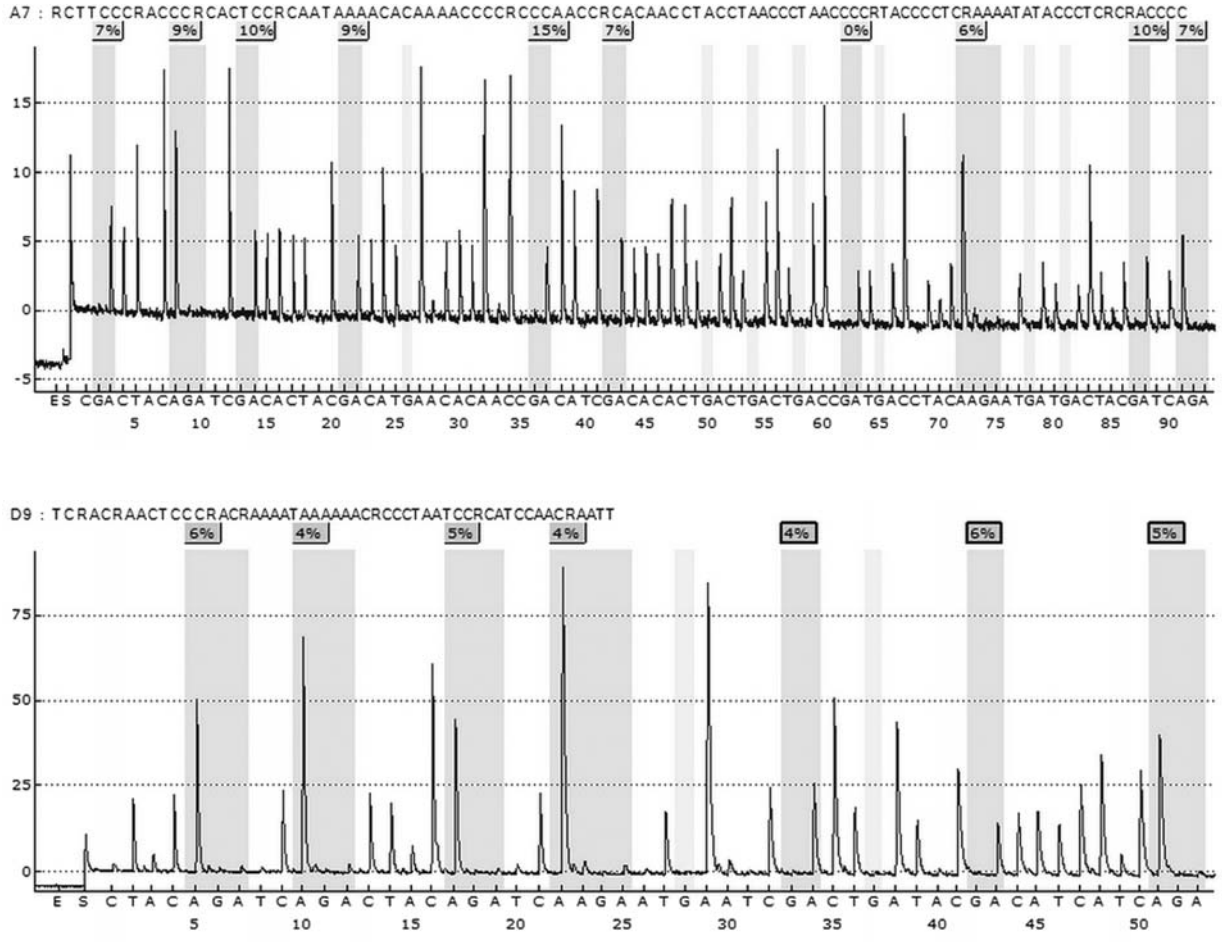

Figure 8. Typical pyrograms showing the sequences analyzed in the *APC1A* gene by the two different Pyrosequencing assays, *APC1A* - Py1 (upper panel) and *APC1A* - Py2 (lower panel).

both run in the reverse direction. The first assay, *APC1A* - Py1, covers 10 CpG sites, of which sites 2-4 (Fig. 8, upper panel) are the sites previously covered by the forward primer used by van den Donk and van Engeland *et al* (see Table II in refs. 10,11). The second assay, *APC1A* - Py2, covers 7 CpG sites, of which sites 1-4 (Fig. 8, Lower panel) are the ones previously covered by the reverse primer used by van den Donk and van Engeland *et al* (see Table II in refs. 10,11). A total of 17 CpG sites were analyzed in the promoter region of the *APC1A* gene, 10 of these have not been previously reported for methylation status.

In conclusion, we successfully developed 13 Pyrosequencing assays enabling us to analyze 119 CpG sites in the promoter regions of the O^6-*MGMT*, *hMLH1*, $p14^{ARF}$,

p16^{INK4a}, *RASSF1A* and *APC1A* genes. DNA from human non-neoplastic blood leukocytes appeared to be unmethylated, as expected, in all CpG sites of the genes studied (Figs. 3-8). Due to the low damping of the Pyro Q-CpG Software version 1.0.9, genes that visually contain no C peaks in the Pyrograms, and hence are unmethylated, were nonetheless often assigned a single-digit-methylated-fraction (<5-10%). By visual inspection of the Pyrograms, one can easily confirm their unmethylated status.

We conclude that standard PCR followed by Pyro-sequencing technology is a workable, sequence-specific and quantitative alternative to 'methylation-specific' PCR in oncology research, which provides a more comprehensive picture of the distribution of DNA methylation throughout the promoter regions of the studied set of six genes.

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