SPMSP: A Solid-phase PCR with Colorimetric Read-out for Quantitative Methylation Analysis

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Abstract. *Background: As DNA methylation has been determined as an important diagnostic biomarker in cancer management, methods for the detection of quantitative DNA methylation which are high-throughput and low-cost are needed. Materials and Methods: In this work, we introduce the solid-phase methylation-specific PCR (SPMSP) as a method for quantitative methylation analysis. SPMSP combines methylation-specific DNA amplification on a solid phase with subsequent colorimetric detection in an ELISA-based format. Results: In contrast to existing methods for quantitative methylation analysis, SPMSP can be carried out with standard laboratory equipment, i.e. a thermocycler and an ELISA reader. With this method, DNA methylation in the promoter of the APC tumour suppressor gene was quantified in a set of colorectal carcinoma samples. Conclusion: As SPMSP can be modified to the analysis of other promoter sequences and is also adaptable to a high-throughput system, SPMSP offers a platform for methylation profiling with widespread applications in cancer research and management.*

DNA methylation has been recognized as an important mode of epigenetic gene regulation in normal development and disease (1). The hypermethylation of CpG islands in the promoters of many genes, in concert with other epigenetic modifications, renders the local chromatin inaccessible to transcription factors and leads to the silencing of the respective genes. In tumorigenesis, this epigenetic silencing involves tumour suppressor genes and thus represents an important functional hit (2). For example, the inactivation of the *APC* gene in colorectal cancer is likewise brought about by sequence mutations, loss of heterozygosity and promoter hypermethylation (3). Moreover, the concerted promoter hypermethylation of several genes, described as the methylation profile, has been shown to be a valuable diagnostic and prognostic biomarker (4) in cancer management. Thus, developing methods for the detection of DNA methylation which are accurate, cost-effective and suitable for high-throughput is crucial.

The most common way of detecting DNA methylation is *via* chemical modification with bisulfite (5). Upon treatment with sodium bisulfite, all unmethylated cytosines are converted to uracils, whereas methylated cytosines are not converted. The resulting sequence differences between the methylated and the unmethylated strands can then be analyzed by polymerase chain reaction-based methods, such as methylation-specific PCR (MSP) (6). However, as standard MSP is a very sensitive but not a quantitative method, the extent of promoter hypermethylation is readily overestimated which makes it difficult to reveal correlations between methylation and clinical features (7). It was shown, for example, that neoplastic cells can bear low level methylation which may not be associated with transcriptional silencing (8). Thus, quantitative methylation analysis seems desirable in evaluating the role of promoter hypermethylation in cancer formation.

A cost-effective approach to the quantitative analysis of PCR products is their detection by ELISA-like techniques (9). In PCR-ELISAs, the PCR product is captured onto a microtiter plate and detected *via* an anti-tag antibody or by hybridization with a specific internal oligonucleotide probe. An improvement of this technique is the generation of the PCR product directly on the surface of the microtiter plate ("solid-phase PCR") as this approach minimizes the risk of cross-contamination and reduces hands-on time.

We sought to combine the need for quantitative methylation analysis with the advantages of solid-phase PCR assays. Therefore we developed SPMSP, a novel solid-phase methylation-specific PCR which offers the prospect of low-cost methylation profiling.

Abbreviations: APC, adenomatous polyposis coli; ELISA, enzyme-linked immunosorbent assay; MSP, methylation-specific PCR; SPMSP, solid-phase methylation-specific PCR; qPCR, quantitative real-time PCR.

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Materials and Methods

DNA isolation and bisulfite modification. DNA from fresh frozen tumour tissue samples from 14 patients (median age = 74.5 years, range 30-80 years) with colorectal carcinoma (Table I) and DNA from human embryonic kidney 293 (HEK293) cells was isolated with the QiaAmp DNA Mini Kit (Qiagen, Germany). Methylated control DNA was prepared by treatment of HEK293 cell DNA with M.SssI methylase (NEB, USA).

Bisulfite modification of 1 μg DNA was carried out with the EZ DNA Methylation-Gold Kit (Zymo Research, USA). Exemplary PCR products were cloned into the pDrive cloning vector (Qiagen) and individual clones were sequenced.

Quantitative assessment of APC promoter methylation by SPMSP. Coating of the solid-phase. The wells of an 8-well NucleoLink™ strip (Nunc, Germany) were coated with the primer 836: 5'-P-TTTTTTTTTTGGGTTGTATTAA TATAGTTATAT-3'. The coating primer carries a stretch of 10 thymidines at the 5'-end to allow for the proper spacing of the primer with respect to the surface (10) and a 5'-phosphate which enables the covalent attachment to the activated surface of the NucleoLink™ strip (Figure 1). NucleoLink™ (Nunc) is a thermostable chemically derivatized polystyrene surface (11). The coating reaction was carried out overnight at 50°C in 10 mM methylimidazole, 10 mM EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) and 5 μM primer 836. The wells were then washed three times with wash buffer (100 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20), soaked for 5 min and washed again three times. These washing steps were carried out once again with water.

First indiscriminate PCR. A fragment of the *APC* promoter (nt 628-829 of U02509.1) was amplified with primers 832: 5'-GGG TTGTATTAATATAGTTATAT-3' and 833: 5'-TTCCTTACTTACT AAAAACTAAAA-3' for 25 cycles at the following temperatures: 15 min at 95°C, 30 s each at 95°C, 50°C and 72°C, 10 min at 72°C. The reactions were performed using 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.6 μM of each primer, 0.5 mM tetramethylammonium chloride (TMAC) and 0.5 U HotStarTaq DNA polymerase (Qiagen).

Control MSP reactions for the first PCR. The semi-nested control PCR was carried out with the same indiscriminate forward primer 832 and methylation-specific reverse primers which were 892: 5'-CGAAATACGAATCGAAAAACG-3' for the methylated product and 893: 5'-CAAAATACAAATCAAAAAACA-3' for the unmethylated product. Amplification was carried out for 35 cycles at an annealing temperature of 58°C for the methylated product and 56°C for the unmethylated product.

Solid-phase PCR. The solid-phase PCR was carried out as a semi-nested methylation-specific PCR as described for the control reactions. Thereby the primer for the methylated product was biotinylated and the primer for the unmethylated product was unlabelled. The solid-phase PCR consisted of an initial denaturation at 95°C for 15 min, followed by 28 cycles of each 30 s at 95°C, 54°C and 72°C, and a final extension at 72°C for 10 min. The PCR was performed in 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 μM of each reverse primer, 0.06 μM forward primer, 0.5 mg/ml BSA and 1.25 U HotStarTaq DNA polymerase (Qiagen) in a total volume of 50 μl.

Table I. *Analysis of the APC promoter methylation in colorectal carcinoma by SPMSP and quantitative methylation-specific PCR: percentages of methylation in the 14 tumours analyzed, grading of the tumours and age of the corresponding patients.*

Tumour #	Grade	Age (years)	% Methylation as determined by SPMSP	% Methylation as determined by qPCR
53	G2	61	0%	0%
54	G2	30	0%	0%
55	G3	79	15%	39%
58	-	61	8%	8%
60	G2	79	23%	38%
61	G2-G3	79	0%	2%
62	G1-G2	80	0%	1%
63	G2	69	0%	0%
172	G2	75	0%	0%
173	G2	73	0%	0%
174	G2	79	0%	0%
175	G3	74	0%	0%
176	G2	63	0%	0%
177	G2-G3	76	76%	67%

After the PCR, the wells were washed as described. The wells were then incubated with streptavidin/biotin-horseradish-peroxidase (HRP) complex [0.1 μg/ml streptavidin (Perbio, Belgium), 25 μg/l biotin-HRP (Perbio) in wash buffer] for 1 h at 37°C. The wells were washed again and the detection solution was added (1.2 mM tetramethylbenzidine, 3 mM hydrogen peroxide-urea adduct in phosphate buffer). After 15 min, the reaction was stopped with 0.1 M H_2SO_4, and the wells were read-out at 450 nm in a Dynatech MR5000 ELISA plate reader (Dynex Technologies, USA). An uncoated well and a water-only well were included as controls on each strip. The absorption values of the samples were normalized by subtracting the absorption values of the uncoated well. All quantification reactions were carried out in triplicates, unless otherwise stated.

Establishment of a standard curve. For the setup of a standard curve, different percentages of *in vitro* methylated and untreated HEK293 cell DNA were mixed, bisulfite modified and applied in the SPMSP assay. The observed methylation values were fitted to a hyperbolic curve of the formula $y=y_0+ax/(b+x)$ and were correlated to the applied methylation percentages. The standard curve was performed three times. The standard curve formula was used to calculate the methylation percentages x from the absorption values y of the tumor samples, whereby $x=[by/a-(y_0/a)/(1-y/a)]$.

Quantitative methylation-specific PCR (qMSP) of the APC promoter. The qMSP assay consisted of two separate PCRs: one that is indiscriminate and therefore quantifies the total product (qPCR), and one that amplifies the methylated product only. The qMSPs were carried out using the ABI 5700 Sequence Detection System (Applied Biosystems, Germany). QPCR reactions were composed of 1 × ImmoMix [0.15 U/μl Immolase DNA polymerase, 16 mM $(NH_4)_2SO_4$, 62.5 mM Tris-HCl (pH 8.3), 0.01% Tween, 1 mM dNTPs, 1.5 mM $MgCl_2$ (Bioline, Germany)], 0.3 μM of each primer, 1 × Sybr Green Solution and 0.5 mM tetramethylammonium

Figure 1. *Bisulfite-modified sequence of the methylated 252 bp PCR product of the APC promoter. 832-833 are the primers of the indiscriminate PCR. For immobilization on the solid phase, primer 832 is modified by a (dT)10 spacer and a 5'-phosphate. The reverse primer 892, containing four CpG sites, is specific for the methylated product and is biotinylated. Primer sequences are underlined. CpG sites are printed in bold. The biotin tag is indicated by the polygon.*

chloride. After an initial denaturation step for 7 min at 95°C, 40 cycles of amplification were performed as follows: 20 s at 95°C, 30 s at 56°C and 30 s at 72°C. The indiscriminate qPCR primers were identical to those of the first PCR. The qMSP was carried out with primers 832 and 892. PCR efficiencies for the indiscriminate and the MSP were determined *via* calibration dilution curve and slope calculation using the REST program (12). The percentage of methylation was calculated from the ratios of the C_t values of the two PCR reactions by the $2^{-\Delta\Delta Ct}$ method, whereby $\Delta\Delta C_t = C_t(\text{msPCR–indPCR})_{x\%} - C_t(\text{msPCR–indPCR})_{100\%}$.

Results

Our work demonstrates for the first time, the application of a solid-phase PCR assay for the quantitative detection of DNA methylation.

The basic principle of SPMSP is that an MSP is carried out on a solid phase and is coupled to a colour reaction which is then quantified in a standard ELISA reader. For the SPMSP, both PCR primers require crucial modifications. The forward primer carries a 5' phosphate group which enables the covalent attachment to the surface of the NucleoLink™ wells. The reverse primer is labelled with biotin which can be directly detected *via* a streptavidin/biotin-horseradish-peroxidase complex.

SPMSP is designed as a semi-nested PCR (positions of primers are shown in Figure 1). In the first PCR, a region of the *APC* promoter is amplified with primers which do not contain CpG sites and therefore amplify in an indiscriminate manner for DNA methylation. The first PCR product is then applied in the solid-phase reaction and is amplified using the same indiscriminate forward primer and a mixture of reverse primers for the methylated and the unmethylated products. However, only the primer for the methylated product is biotin-tagged, whereas the primer for the unmethylated product is untagged. Consequently, only the methylated product is detected by the horseradish-peroxidase complex which converts the colourless substrate tetramethylbenzidine into a blue end-product.

For the setup of the SPMSP assay, we first assessed the quality of the untreated and *in vitro*-methylated HEK cell

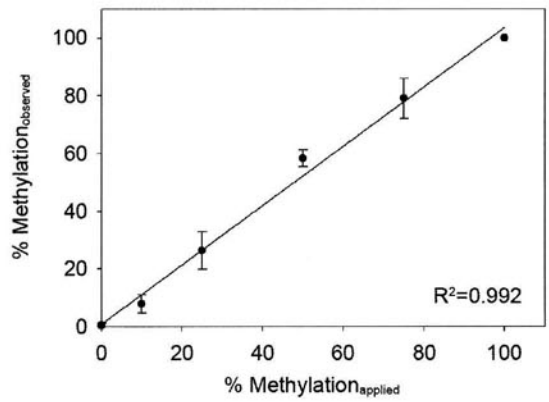

Figure 2. *Quantitative methylation-specific PCR (N=3). The same mixtures of methylated and unmethylated DNA as in the solid-phase PCR were applied in the qMSP. The observed methylation values were correlated to the applied methylation values. Values are means ± standard deviations.*

DNA as standards by cloning and bisulfite sequencing of exemplary PCR products of the first indiscriminate PCR. Sequencing of eight clones each from unmethylated and methylated DNA (corresponding to 320 CpG sites in total) revealed an error rate of 0.3% (1/320).

The following features of the SPMSP assay were analyzed in parallel by qMSP. As the two methylation-specific primers contain four CpG and TpG sites, respectively, we expected the SPMSP assay to achieve a high specificity. In standard MSP reactions (35 cycles), the primers for the methylated product were found to only give an amplification product with methylated DNA and the primers for the unmethylated product only with untreated DNA. In the qMSP, the primers for the methylated product amplified the unmethylated product with a delay of 8 cycles. In the SPMSP, however, a false detection of methylation is prevented because the untagged primer for the unmethylated product is included in the assay. Thus, the amplification of unmethylated DNA with the primer for the methylated product is suppressed (data not shown).

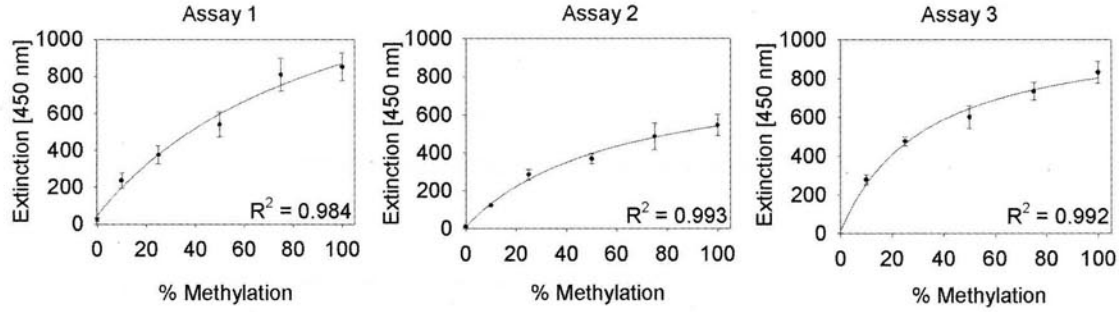

Figure 3. *SPMSP calibration curves. Mixtures of different percentages of methylated and unmethylated DNA were used to establish the calibration curves: the observed signals were plotted against the percentage of methylation. Values are means ± standard deviations.*

Table II. *Intra-assay and interassay variations: absorption values of the methylation mixtures of the standard curves obtained with SPMSP.*

	Assay 1			Assay 2			Assay 3			Interassay variation		
% Methylation	Mean	SD	C_v (%)	Mean	SD	C_v (%)	Mean	SD	C_v (%)	Mean	SD	C_v (%)
100%	850.33	74.77	8.79	545.33	55.61	10.20	831.00	56.31	6.78	742.22	170.78	23.01
75%	808.00	89.1	11.02	486.00	70.06	14.42	733.67	45.50	6.20	675.89	168.60	24.94
50%	540.00	67.91	12.58	368.33	26.50	7.19	600.33	59.01	9.83	502.89	120.37	23.94
25%	374.67	49.22	13.14	283.67	27.30	9.62	475.33	22.05	4.64	377.89	95.87	25.37
10%	235.67	40.10	17.02	122.00	3.46	2.84	278.33	25.01	8.98	212.00	80.81	38.12
0%	25.67	10.50	-	8.33	4.73	-	–0.33	2.89	-	11.22	13.24	-

SD: standard deviation.

As most of the DNA is degraded during bisulfite modification (13), the SPMSP procedure starts with a pre-amplification step with primers indiscriminate of methylation. To test if the methylation status of a sample was preserved after the preamplification step, mixtures of different percentages of methylated and unmethylated DNA were used as templates in the first PCR. We then analyzed the products of the first PCR by qMSP and found that the applied methylation values showed a linear correlation with the observed methylation values (R^2=0.99) (Figure 2). We thus conclude that at the end of the preamplification step the first PCR is still in its linear phase.

We next questioned if the SPMSP assay is suitable for quantitative analysis. To this end, mixtures of different percentages of *in vitro*-methylated and unmethylated DNA were used as templates in the SPMSP assay. We then compared the applied to the observed methylation values. The obtained standard curves showed correlation coefficients between R^2=0.993 and R^2=0.984 (Figure 3). The coefficients of variation (C_vs) for each triplicate assay were between 2.8% and 17.0% (mean=9.55%), indicating a good reproducibility of SPMSP. The inter-assay C_vs were between 24% and 38% (Table II). Few wells showed outlier values which broadened the C_v range. We speculate that these outliers may be due to manufacturing errors. On the other hand, we experienced similar variations in standard ELISA measurements using the same horseradish-peroxidase/tetramethylbenzidine detection system. Therefore, we suppose that these variations are not due to the solid-phase amplification and may be overcome by the use of another detection system.

We then calculated the sensitivity of SPMSP based on the absorption values of the 0% methylated samples. Presuming a normal distribution, the mean value of the 0% methylated samples±3 SDs was defined as the detection limit of SPMSP. The detection limit corresponds to 2.7% methylation under the currently applied 28 PCR cycles.

Finally, we applied SPMSP to the analysis of tumour samples by determining the methylation status of the *APC* promoter in 14 samples from colorectal carcinoma patients. The measurements were performed in duplicate. Samples 55, 58, 60 and 177 showed elevated methylation levels ranging from 8% to 76% (Table I). All of the tumour samples were also analyzed by qMSP and the same samples showed methylation values ranging from 8% to 67% (Table II) while samples 61 and 62 showed low level methylation of 1% and 2%, respectively. These low methylation values might be an artifact caused through the use of the $2^{-\Delta\Delta Ct}$

method for the calculation of the methylation values. The $2^{-\Delta\Delta Ct}$ method is based on the assumption that the PCR efficiencies of the indiscriminate and the MSP are identical. Indeed, we found a slight difference in the efficiencies of the indiscriminate PCR (E_{ind}=2.38) and the MSP (E_{ms}=2.29). In summary, we were able to verify the elevated methylation levels of samples 55, 58, 60 and 177 whereas the low methylation levels of samples 61 and 62, which are below the detection limit of SPMSP, likely are an artefact of the MSP method.

The fact that the methylation values obtained by SPMSP and MSP are not identical can be ascribed to the different principles of measurement of the two methods. In the PCR, the methylation percentages are calculated based on a comparison of the products of the methylated and the indiscriminate PCR whereas in the SPMSP, the methylated product is directly detected and the calculation is based on a standard curve. Therefore the exact methylation values of the two methods cannot be directly compared.

Discussion

In the last few years, an abundance of new methods for the quantitative detection of DNA methylation has been published. Some of them are based on fluorescent real-time PCR, such as MethyLight, QAMA, HeavyMethyl, or MethylQuant (14-17). While MethyLight and QAMA rely on TaqMan probes and TaqMan probes with minor groove binder technology, respectively, HeavyMethyl uses methylation-specific oligonucleotide blockers and MethylQuant is a Sybr Green assay with a locked nucleic acid at the 3' end of the discriminative primer. These methods provide accurate and sensitive methylation quantification but are very expensive in their use of reagents and need special equipment. Other methods also heavily rely on specific instrumental platforms such as: Bio-Cobra or ms-SNuPE (18-20). In contrast, SPMSP promises to be low-cost in reagents as well as in equipment as it only needs a standard thermocycler and an ELISA reader, which are available in most laboratories.

Recently, multigene methylation analysis revealed so-called DNA methylation profiles which are specific for the respective tumour entities. Thereby, methylation profiles have not only been correlated with diagnostic subgroups (21), but also with patient survival (22, 23). Provided that a convenient method is available, methylation profiling will experience widespread clinical application in the future. SPMSP offers the ideal platform for the development of diagnostic methylation arrays as it can easily be modified to the analysis of multiple promoters and is also adaptable to high-throughput. Hence, SPMSP has the potential to be developed into a valuable diagnostic and prognostic tool for cancer management.

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Competing Interests Statement

The described method was filed as a patent (EP 05020789.3).

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