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SIRPH Analysis

SNuPE With IP-RP-HPLC for Quantitative Measurements of DNA Methylation at Specific CpG Sites

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Summary

This chapter describes a detailed protocol using single-nucleotide primer extension (SNuPE) for quantitative analysis of DNA methylation on specific CpG sites. The first step DNA sample to be studied is treated with sodium bisulfite, which converts selectively unmethylated cytosines to uracil, while methylated cytosines remain unconverted. Subsequently, a SNuPE reaction is performed, with an oligo just flanking a CpG site, using a purified polymerase chain reaction product derived from bisulfite-treated DNA as a template. The oligo is extended by either ddCTP or ddTTP depending on whether the site is methylated or unmethylated, respectively. The reaction is quantitative and linear, and two to three sites can be studied simultaneously in a multiple reaction. The SNuPE product, without further purification, is separated by ion-pair reverse-phase (IP RP) high-performance liquid chromatography (using an alkylated nonporous polysterene-divinylbenzene cartridge) that allows an easy, semiautomated method for separation of the extended and unextended products and an accurate quantification of the extended products. The ratio of the ddCTP to the ddTTP gives the fraction of the methylated cytosines at that specific CpG site.

Key Words: Bisulfite; CpG methylation; SNuPE; DHPLC; ion pair reverse-phase HPLC; quantitative DNA methylation analysis.

1. Introduction

In the postgenome era, and after deciphering the human genome code, functional genomics has attracted a great deal of interest. This includes the functional analysis of proteins, and their interactions, and gene expression patterns. Another layer of information is the epigenetic modifications of DNA that influence the patterns of gene expression, including factors that affect chromatin structure, such as histone modifications and cytosine methylation. In mam-

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mals, cytosine methylation occurs mainly in a CpG context. DNA methylation is involved in silencing genes in a tissue-specific manner and during specific developmental stages ***(1,2)***. Moreover, alterations in normal patterns of methylation are associated with many human diseases such as imprinting diseases and tumors formation ***(3,4)***. Hence there is great interest in the analysis and accurate quantification of methylation levels.

Many methods exist for analyzing DNA methylation patterns; these can be divided into two approaches (reviewed in **ref. *5***). The first is the total genome approach which gives the overall content of methylated cytosines in a given DNA, such as the enzymatic cleavage of DNA to individual nucleosides followed by separations on high-performance liquid chromatography (HPLC). The second is the sequence-specific approach which helps quantify methylation with a high degree of accuracy. This includes restriction enzyme analysis with methylation-sensitive enzymes and the widely used bisulfite analysis.

Bisulfite analysis was introduced by Frommer et al. in 1992 *(6)* and became the method most used to provide sequence-independent information (vs the use of methylation-sensitive restriction enzymes, which is restricted by the enzyme recognition sequence). Bisulfite analysis gives detailed information on methylation levels and patterns in a given region. It is based on the ability of sodium bisulfite to interact selectively with cytosines at their carbon 6 position to form sulfonated cytosine intermediates. These intermediates are then converted to uracil by pH-dependent deamination and desulphonation steps. 5-Methyl cytosine remains non-reactive under such conditions. The uracil in the bisulfite-converted DNA is replaced by thymine in the subsequent polymerase chain reaction (PCR). Several protocols and modifications of the original method have been published that allow analysis of only a few cells ***(7–9)***.

After successful amplification of a region of interest (using bisulfite-treated DNA as a template), the ultimate aim is to quantify the methylated (CpG) and the unmethylated (TpG) portions of the PCR product. The literature is very rich in a wide variety of methods that vary in the accuracy of the methylation levels they provide and with the machines required for analysis. The traditional method of cloning and sequencing bisulfite PCR products provides the most detailed information. This approach, however, is very time-consuming and laborious, and large numbers of clones must be analyzed to provide statistically significant results. Therefore, several groups have developed alternative methods. One such method is combined bisulfite retriction analysis (COBRA), which is based on restriction enzyme digestion of bisulfite PCR products ***(10)***. The use of this method is limited, however, since it only allows analysis of CpG methylation within (newly generated) restriction sites of the bisulfite PCR products. Another method is methylation-specific PCR (MSP), which is based on using two pairs of specific primers to amplify methylated or

unmethylated alleles specifically, taking advantage of the sequence differences between methylated and unmethylated CpG sites that occur after bisulfite conversion ***(11)***. Although MSP is very sensitive for low amounts of methylated or unmethylated product, it does not give a detailed picture of the methylation patterns and the exact quantity of methylated/unmethylated product. Recently a more flexible method, based on differential hybridization of bisulfite PCR fragments using oligonucleotide-containing chips, was introduced ***(12)***. Although this method allows high-throughput screening, it requires a high technological laboratory standard and a sophisticated and laborious chip design and analysis tools.

More flexible methods are based on single-nucleotide primer extension (SNuPE) ***(13)*** techniques, whereby an oligo, just flanking the 5' end of a CpG site, is extended by either ddCTP or ddTTP for methylated and unmethylated templates, respectively. The SNuPE product is then detected and measured by various methods. Gonzalgo and Jones ***(14)*** were the first to apply such an approach for DNA methylation analysis by developing methylation-sensitive SNuPE (MS-SNuPE), which uses incorporation of radioactive nucleotides; the extended products are separated on acrylamide gels and quantified by autoradiography. A second method is MethyLight, a real-time PCR-based SNuPE technique, which is quantitative and highly sensitive but requires special fluorescently labeled oligonucleotides ***(15)***. A third method is matrix-assisted laser desorption ionization (MALDI) mass spectrometry-based separation technique of the SNuPE reaction that is accurate but requires special modified primers ***(16)***. Recently we and others ***(17,18)*** have developed an inexpensive, nonradioactive variation of such a SNuPE protocol using ion-pair reverse-phase HPLC ***(19)*** as a separation and detection method (SNuPE–IP RP HPLC or SIRPH). This protocol gives an accurate quantification of methylation at selected CpG sites.

The PCR product to be analyzed is purified to remove residual PCR oligos and dNTPs. Subsequently, unmodified primers immediately 5' to a CpG site are hybridized to the denatured single-stranded PCR product. The primers used are identical in sequence to the bisulfite-treated DNA strand that contains CpGs and/or TpGs (not GCs and/or ACs on the opposite strand), and thus it will hybridize to the opposite strand that contains GCs and/or ACs; this allows addition of ddCTP and/or ddTTP nucleotides at the 3' position of the primer (**Fig. 1**). Temperature cycling using Thermo Sequenase™ in the presence of both ddCTP and ddTTP extends the annealed primers. The ddTTP (for unmethylated CpG) or ddCTP (for methylated CpG; *see* **Fig. 1**) extended products are then directly loaded on an HPLC column (Wave DNA Fragment Analysis System, Transgenomics). Because of incorporation of the more hydrophobic ddTTP, the retention time of such an extended product is longer compared with that of

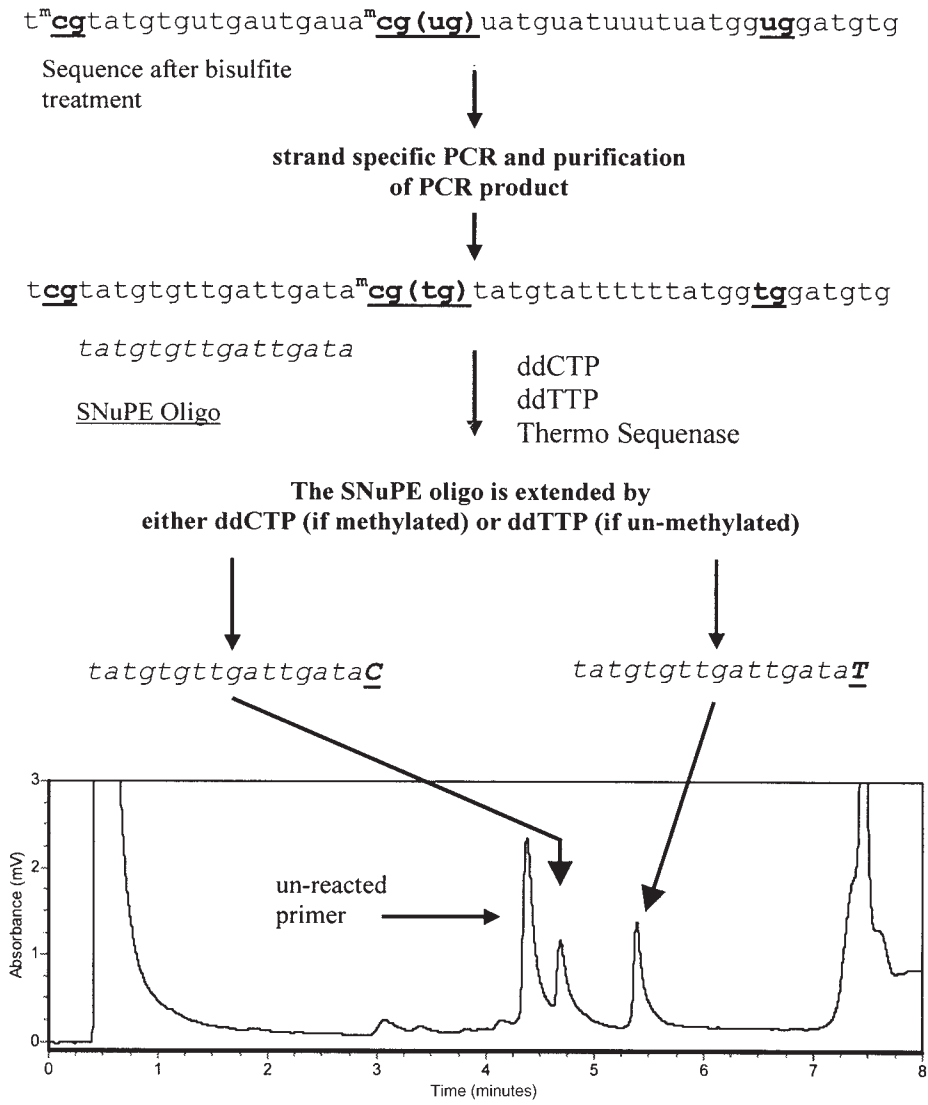

Fig. 1. General scheme of the SIRPH analysis.

products containing ddCTP (**Fig. 1**). The amount of the ddTTP and ddCTP extended products can then be quantified by measuring the height of the peaks and calculating their percentage ratios. The reaction produces highly reproducible results while maintaining linearity (**Fig. 2**).

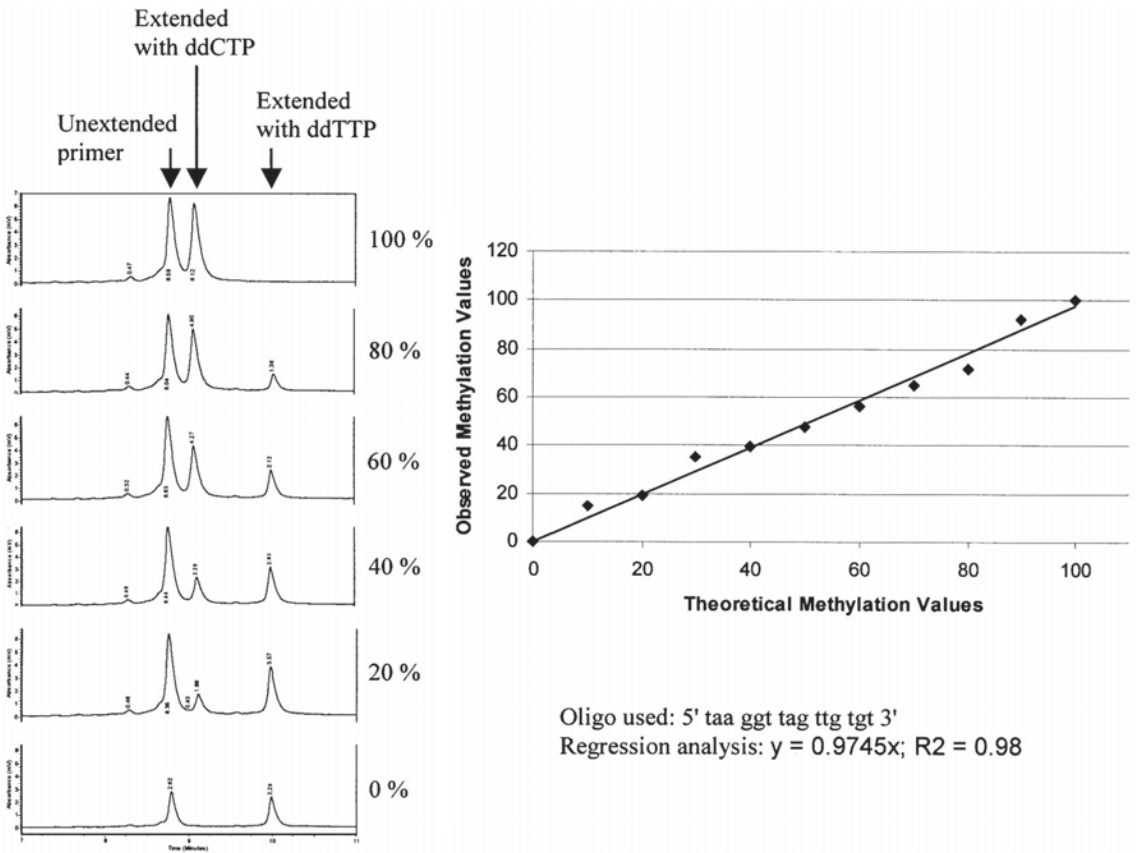

Fig. 2. Linearity of the reaction. A PCR product and a primer in the *SNRPN* gene was used with a serial mixes of methylated and umnethylated alleles in 10% increments.

2. Materials

2.1. Removal of Excess Primers and dNTPs

1. For gel extraction or direct PCR purification: QIAquick Gel Extraction kit, Qiagen, cat. no. 28704 or QIAquick PCR purification kit, cat. no. 28106.
2. A mixture of exonuclease I and shrimp alkaline phosphatase which will degrade the unreacted primers and inactivate dNTPs: ExoSap-IT, Amersham, cat. no. US78201.

2.2. SNuPE

1. ddTTP and ddCTP (Amersham, cat. no. 27-2081-01 and 27-2061-01).
2. Thermosequenase enzyme (Amersham, cat. no. E79000Y).
3. Standard unmodified oligos from *n*-1 secondary products. (Oligos by polyarcylamide gel electrophoresis [PAGE] are of sufficient quality.)

2.3. IP RP HPLC

1. For all HPLC analyses, we recommend the Wave system from Transgenomic together with the IP RP HPLC column, the DNASep (cat. no. DNA-99-3510, Transgenomic). The stationary phase in the column is made of alkylated nonporous polystyrene-divinylbenzene 2-µm beads particles (cat. no. PS/DVD-C18).
2. TEAA buffer (Transgenomic, cat. no. 553303).
3. Acetonitril (ROTH Art 8825.2).
4. HPLC-grade water (Merck, cat. no. 1.15333.2500).

3. Method

The SIRPH protocol can be divided into three parts: (1) generating the PCR product, (2) performing the SNuPE reaction, and (3) separating the products on HPLC and quantification of the peaks. In this chapter I describe the last two steps; protocols for bisulfite treatment can be found in Hajkova et al. ***(8)*** or El-Maarri et al. ***(9)***.

3.1. Purification of PCR Product

The PCR product can be purified by one of two methods (*see* **Note 1**):

1. Run the product on 1% agarose gel until separation is optimal, excise the specific band, and recover the product by using a standard PCR-gel extraction kit (QIAquick Gel Extraction Kit, Qiagen), which yields very pure products with high rates of recovery. Alternatively, when there is no nonspecific PCR product(s), a PCR purification kit can be used directly without the need for separation on agarose (QIAquick PCR purification kit).
2. Add 2 µL of exonuclease I and shrimp alkaline phosphatase (ExoSap-IT, Amersham) to 5 µL of PCR product, and heat at 37°C for 15 min followed by 15 min at 80°C to deactivate the enzyme mixture.

3.2. SNuPE Reaction

1. Set up the SNuPE reaction in a total volume of 20 µL with the following components (*see* **Note 2**):
   a. 2 µL Reaction buffer (10 X buffer).
   b. 1 µL SNuPE Oligos (n) (12.5 pmole solution/for each oligo).
   c. SNuPE template:
      i. 1–5 µL PCR product (50–100 ng of 200–400 bp PCR product).
      ii. 1 µL ddCTP (1 m*M* solution).
      iii. 1 µL ddTTP (1 m*M* solution).
      iv. n µL Thermo Sequenase (diluted to 1 U/µL).
      v. Up tp 20 µL H_2O.
      vi. *n* = the number of oligos used for multiplex in the reaction.
   d. For SNuPE primers used in the reaction, *see* **Notes 3–8**.
2. Subject the above mix to the following thermocycles (*see* **Note 9**): cycle 1, 94°C for 2 min; cycle 2, 92°C for 10 s; cycle 3, 30°C for 1 min; cycle 4, 60°C for 1 min. Repeat **steps 2–4** 50 times.

3.3. Run the Products on HPLC

1. Load 10–15 µL of the PCR product directly on the HPLC machine (Wave, Transgenomics). Set the oven temperature to 50°C (*see* **Note 10**) and the elution gradient (mixture of buffers A and B) at 0.9 mL/min for 10 min:

Step	Time (min)	%A (0.1 *M* TEAA)	%B (0.1 *M* TEAA, 25% Acetonitril)
Loading	0.0	100-b1	b1
Start gradient	0.1	100-b1	b1
Stop gradient	10.0	100-b2	b
Start clean	10.01	0	100
Stop clean	11.1	0	100
Start equilibrate	11.2	100-b1	b1
Stop equilibrate	12.2	100-b1	b1

Where b1 is the start percentage of buffer B in the elution buffer that will steadily increase over a 10-min period to reach b2. The values of b1 and b2 are defined empirically for each set of SNuPE oligos (*see* **Note 11**).

2. Calculation of percent methylation: the percent of the methylated portion of the DNA can be calculated according to the formula: $M = [HC/(HC + HT)] \times 100$, where HC and HT are the peak heights of the ddCTP and ddTTP extended oligos, respectively (*see* **Notes 12–14**). The WaveMaker software automatically calculates the AC and AT.

4. Notes

1. Enzymatic treatment is more expensive but has the advantage that it is rapid and easier to perform, especially when analyzing large numbers of samples. Gel

extraction, on the other hand, is more laborious, but it has the advantage of concentrating a faint PCR product in a smaller volume. It also offers the possibility of isolating the specific product when nonspecific products are present.

2. The amount of template to be used is flexible; up to 1 µg could be used without affecting the quantification results, However, with less than 50 ng, the yield of the SNuPE reaction may not be high enough to give reproducible quantitative results. The oligos used in the SNuPE reaction should always be in excess; their corresponding band (on HPLC separation) can be used as a reference for the extended product(s) that should come shortly after.
3. The 3' end of the SNuPE oligo has to be just 5' (flanking) of the specific CpG site to be studied.
4. Avoid placing the oligo on a T-rich region, as this could increase mispriming and lead to inaccuracy in the methylation measurements; however, if possible, it is preferable to have the 3' end (region) of the oligo on a C to T (but not on an initial CpG) converted region so that the specificity to the bisulfite converted product is higher.
5. Oligos should not include a CpG site, as this will bias the linearity of measurements.
6. Oligos that are too short have a higher chance of mispriming. Oligos as short as 10 bases can still produce accurate data. However, for routine use we prefer oligos 15–18 bases long when possible.
7. For multiplex SNuPE reactions that are run simultaneously on the HPLC, the retention time of the individual oligos and their elongation products should be different. If, for practical reasons, two oligos have to be designed that give similar retention times on HPLC, we recommend extending one of the oligos by adding thymidine to its 5' end. In our experience this addition has no effect on the annealing to the template or on the SNuPE reaction. The number of Ts to be added has to be determined empirically; however, each additional T has a stepwise additional retardation effect in a linear fashion.
8. All oligos have to be tested for self-annealing and self-extension in the absence of a template.
9. The annealing temperature used is 30°C; there is no detectable change in either the yield of the reaction or the quantification results when using a range of 20°C (from 30 to 50°C) for the annealing. Therefore, as a standard procedure for all oligos used in the SNuPE reaction, a 30°C annealing is used. An extension time of 1 min should give a good reaction yield for most oligos; however, increasing the extension as well as the annealing times could give higher yields for some oligos. This has to be tested individually.
10. An oven temperature of 50°C (compared with 60, 70, and 80°C) was found to give the highest difference in retention time between the ddCTP and the ddTTP extended oligos.
11. When setting up a new assay, run with a wide gradient of 10% (b1) to 60% (b2) buffer B. Most short oligos of 10–20 bp should be eluted by this gradient. At a later stage, and depending on the retention time of the oligos, the gradient can be

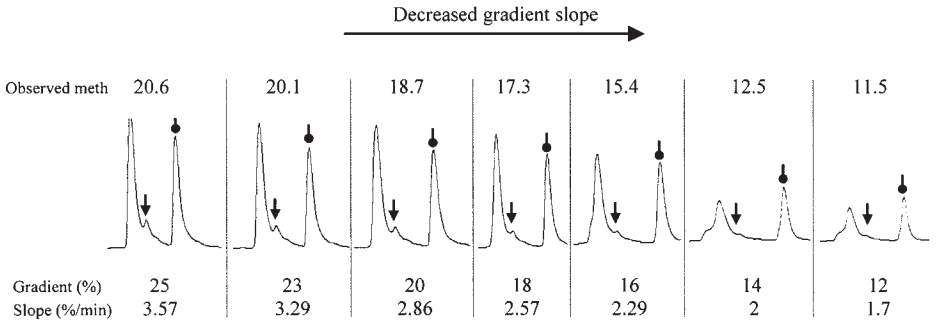

Fig. 3. Effect of the slope of the acetonitrile buffer on the separation of the minor allele (the methylated allele, in this case 12% methylated and 88% unmethylated) when it is too close to the unextended primer. The (↓) and (⇃) represent the ddCTP and ddTTP extended primers, respectively.

narrowed down from either the left side, the right side, or both sides simultaneously to give the best spatial resolution between the oligos.

12. If the unreacted primer, after HPLC separation, is close to the ddCTP extended primer, the integration of the area under the ddCTP extended primer curve may not be accurate. Therefore it is more accurate to use the peak height for measurements. The gradient used has great influence on both the separation efficiency and the accuracy of quantification, a slope of at least 2% increase of buffer B over 1 min gives a good separation (*see* **Fig. 3**). It is recommended to use the lowest slope that will still give a distinguishable peak for the minor allele (the ddCTP extended allele).
13. Reproducibility of the HPLC measurements: it is recommended that one test the accuracy of the measurement reproducibility on the HPLC machine. This can be done by injecting the same SNuPE product several times and calculating the standard deviation for each sample. Such measurements are shown in **Fig. 4**; for most oligos and at different ratios of ddCTP to ddTTP, the standard deviation ranges between 1 and 3%.
14. Limit of detection for the minor allele: this differs from one primer to another. Mainly two factors can have an influence: first, the yield of the SNuPE reaction, as some primers can be easily extended, giving a high yield that produces high peaks, whereas others are less efficient; and second, the separation between the unextended oligo and the ddCTP extended oligo, when the two peaks are partially overlapping, could lead to ambiguity in distinguishing the small peak of the ddCTP extended primer. However, for most oligos the limit of detection of the minor allele is between 5 and 10%.

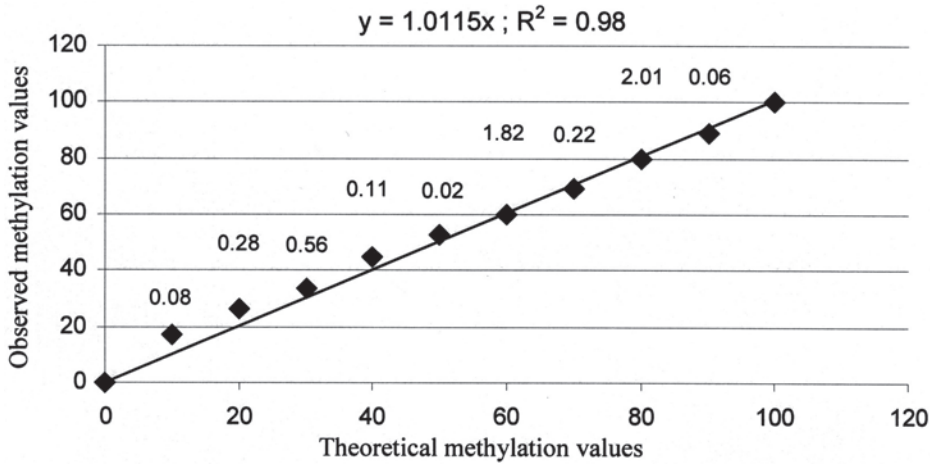

Fig. 4. Sensitivity and accuracy of HPLC measurements at different methylated/unmethylated ratios. The same samples were reinjected three times; the standard deviation values are shown above each group of measurements.

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