Preface

Epigenetics can be defined as the study of heritable changes in gene expression without alteration of the DNA sequence itself. This means that epigenetic variants are stable alterations that are heritable during somatic cell divisions (and possibly transmitted through germ line transmissions in some occasions) but do not involve mutations of the DNA itself. Epigenetic phenomena are mediated by various molecular mechanisms, including histone modifications and core histone variants; ATP-dependent chromatin-remodeling complexes; polycomb/trithorax protein complexes; small RNAs, including siRNA and miRNAs as well as other noncoding RNAs; and last but not least DNA methylation. This volume in the *Methods in Molecular Biology*™ series focuses entirely on protocols for the analysis of DNA methylation, which is the only genetically programmed DNA modification in mammals occurring almost exclusively at the carbon 5 position of cytosines followed by a guanine.

Realizing the importance of epigenetic changes in development and disease, a variety of techniques for the study of DNA methylation have been developed over the last few years. **Figure 1** gives an overview of many of the commonly used technologies, but many more methods and variants of the named assays do exist. No single method has emerged as the "gold" standard technique unifying quantitative accuracy and high sensitivity or possibilities for whole genome analysis and precise investigations of individual CpG positions. The choice of the method mainly depends on the desired application. Although by no means complete, this second edition of "DNA methylation" gives a comprehensive overview of available technologies together with detailed step-by-step protocols for all experimental procedures required to successfully perform DNA methylation analysis.

This is the second edition of the DNA methylation protocols; however, the field has dramatically changed within the 6 years that have passed since the first edition edited by K.I. Mills and B.H. Ramsahoye was published. As DNA methylation technologies and our knowledge of DNA methylation patterns have been advancing at a breathtaking pace over the past few years and most of the techniques described in the first edition have been further optimized and/or replaced by novel, easier, refined, and/or more quantitative technologies, I have entirely remodeled the contents of this book. The increase in available methods is also reflected in the great expansion of the number of chapters within this book. While the first edition contained 14 chapters, this second edition consists now of 27 chapters. Only three chapters have been retained from the first edition and these have been completely rewritten by the authors to accommodate the changes and improvements made in the last years. The analysis of gene-specific DNA methylation patterns has been complemented or superseded by genome-wide approaches and epigenomics has taken a central place in many laboratories.

The selection of different technologies enables the analysis of the global DNA methylation content as well as precise quantitative data on single CpG positions. Methods for the high-resolution analysis of CpG positions within a target region identified by one of the multiple available genome-wide technologies are presented, and emphasis has been placed on array-based approaches that permit a hypothesis-free-driven research to identify

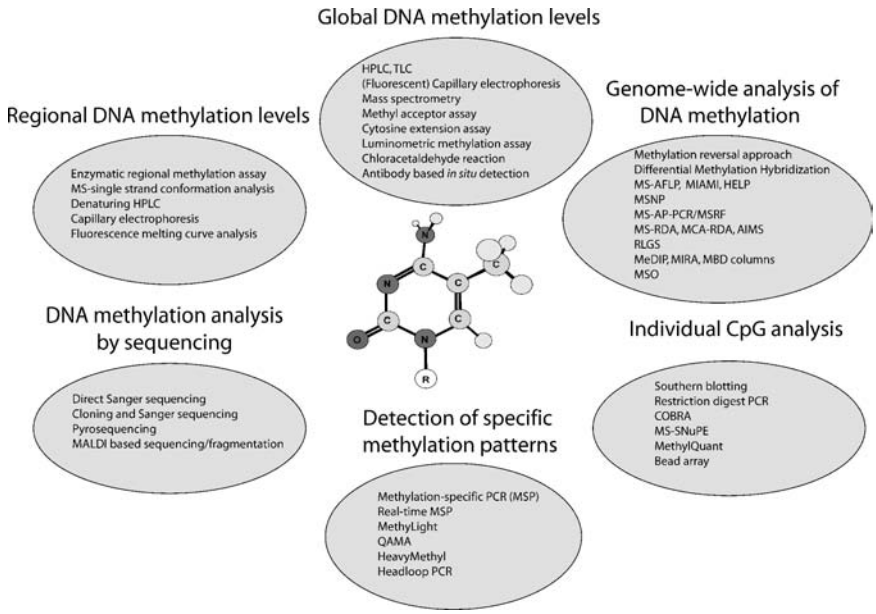

Fig. 1. An overview of the different technologies used for the analysis of DNA methylation. MS: Methylation sensitive; HPLC: High-performance Liquid Chromatography; TLC: Thin-layer Chromatography; MS-AFLP: Methylation-sensitive Amplified Fragment Length Polymorphism; MIAMI: Microarray-based Integrated Analysis of Methylation by Isochizomers; HELP: *Hpa*II tiny fragment Enrichment by Ligation-mediated PCR; MSNP: Methylation Single Nucleotide Polymorphism; MS-AP-PCR: Methylation-sensitive Arbitrarily-primed PCR; MSRF: Methylation-sensitive Restriction Fingerprinting; MS-RDA: Methylation-sensitive Representational Difference Analysis; MCA-RDA: Methylated CpG island Amplification—Representational Difference Analysis; AIMS: Amplification of intermethylated Sites; RLGS: Restriction Landmark Genomic Scanning; MeDIP: Methylated DNA ImmunoPrecipitation; MIRA: Methylated CpG Island Recovery Assay; MSO: Methylation-specific Oligonucleotide array; MALDI: Matrix-assisted Laser Desorption/Ionization mass spectrometry; COBRA: Combined Bisulfite Restriction Analysis, MS-SNuPE: Methylation-sensitive Single Nucleotide Primer extension; QAMA: Quantitative Analysis of Methylated Alleles. Reproduced with permission from Tost, J. (2008) Methods for the genome-wide and gene-specific analysis of DNA methylation levels and patterns. In: *Epigenetics* (Tost, J., ed.), Horizon Scientific Press, Norwich, UK, pp 63–103.

DNA methylation patterns of interest. In the final chapters of this book, more specialized applications like the sensitive detection of aberrant methylation patterns in body fluids, prevention of contamination, and whole genome amplification of bisulfite-treated DNA are described. Methods requiring special instruments are presented along technologies that can be performed with a simple thermocycler. This volume of the *Methods in Molecular Biology*™ series contains widely used methods, such as cloning and sequencing and methylation-specific PCR as well as novel and promising techniques such as the immunodetection array that have only very recently passed the proof-of-principle stage.

This book is addressed to postdoctoral investigators and research scientists that are implicated in the different aspects of genetics and cellular and molecular biology as well as to clinicians involved in diagnostics or choice of treatment of diseases that have an epigenetic component. The presentation in this volume is equally suited for laboratories that already have a great deal of expertise in a certain technology to analyze DNA methylation, but might want to obtain other or complementary data using a second technique, and for genetics/genomics/biology groups that want to initiate research in this exciting area and want to identify the method best suited to answer their question. Notes and tips from

the experts and/or pioneers of the different methods will enable a rapid implementation of the different protocols in the laboratory and avoid time-consuming and cost-intensive mistakes. With the tools and protocols available, our knowledge and understanding of DNA methylation will increase rapidly, and this book will contribute to spreading of the "savoir faire" to analyze DNA methylation.

I am indebted to all the authors for their hard work and outstanding contributions to this second edition of "DNA methylation". It was a pleasure to work with them on this project. I hope that the protocols described in detail in this volume will help to accelerate the analysis and description of the "methylome" of different species and will enhance our understanding of the molecular processes that determine the genomic DNA methylation landscape.

Evry, March 2008 ***Jörg Tost***

Chapter 2

Quantification of Global DNA Methylation by Capillary Electrophoresis and Mass Spectrometry

María Berdasco, Mario F. Fraga, and Manel Esteller

Abstract

Two approaches for the evaluation of the relative degree of global DNA methylation through the quantification of 2′ deoxynucleosides are described. Detection and quantification of 5-methyl 2′-deoxycytidine in genomic DNA is performed using both high-performance capillary electrophoresis (HPCE) with UV–Vis detection or liquid chromatography with electrospray ionization mass spectrometric detection (LC-ESI/MS). Treatment of genomic DNA with a ribonuclease and generation of nucleosides through enzymatic hydrolysis notably increases the specificity of both techniques. Both approaches have been demonstrated to be highly specific and sensitive, being useful for the rapid quantification of the degree of global DNA methylation and its exploitation for the analysis of poorly purified and/or concentrated DNA samples, such as tumor biopsies.

Key words: Capillary electrophoresis, mass spectrometry, global DNA methylation, 2′-deoxynucleosides, 5-methyl 2′ deoxycytidine.

1. Introduction

DNA methylation research can be approached from several standpoints since there are a wide range of techniques available for the study of the occurrence and localization of methylcytosine in the genome *(1)*. Each technique has its own peculiarities implying that there is a best-suited technique for each specific problem. The available methods for studying the degree of DNA methylation can be classified with respect to the type of information they produce: the degree of global genomic DNA methylation, the DNA methylation status of specific sequences, and the discovery of new methylation hot spots. With respect to the quantification of global levels of methylcytosine in the genomic

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DNA, measurements can be performed by high-performance separation techniques or by enzymatic/chemical means. The latter are never as sensitive as the former and sometimes their resolution is restricted to endonuclease cleavage sites *(2)*. Despite the drawbacks, enzymatic/chemical approaches are still commonly used since, unlike separation techniques, they do not require expensive and complex equipment, which is not always available. Although almost all actual efforts are focused on the characterization of the gene-specific methylation patterns or the construction of DNA methylation maps of the entire genome (*methylome*), global measurements of DNA methylation remain a valuable tool for understanding the molecular pathology of human cancer, for measuring the potential effect of tumor-preventive or -promoting compounds, and for monitoring therapeutic responses to hypomethylating agents undergoing evaluation in human clinical trials *(3)*.

Among high-performance separation techniques, capillary electrophoresis (HPCE) and liquid chromatography (HPLC) are used most frequently. The development of capillary electrophoretic (CE) techniques, based on the separation of molecules by the use of a narrow-bore fused-silica capillary, has given rise to a methodological approach that has several advantages over other current methodologies used for the separation of various DNA components, including a number of base adducts *(4)*. Molecules are separated on the basis of differences in size, charge, structure, and hydrophobicity under application of specific and strong voltages. CE has been shown to be extremely useful for the quantification of the extent of DNA methylation. Due to the sensitivity, specificity, and economy of these methods, HPCE had taken an advantage with regard to HPLC-based methods during the last years. However, the application of HPLC methods for the study of global DNA methylation has recently been enforced with the development of mass spectrometry (MS). LC/MS refers to the combination of liquid chromatographic (LC) separation with MS detection. The combination of these two powerful techniques enables the analysis of a great number of molecules, due to the resolution of each technique. In this way, it has been estimated that LC provides a consistent mechanism for the separation of molecules in over 80% of known organic species *(5)*. In addition, MS is a useful tool to provide information about structure, molecular weight, or the empirical formula about a specific analyte. The development of electrospray ionization enables LC/MS to be utilized for the quantitative determination and structural characterization of a great number of polar/ionic molecules, such as nucleic acids, in biological samples *(6)*.

2. Materials

All enzymes and reagents are available from Sigma–Aldrich if not otherwise stated.

2.1. Enzymes

1. Ribonuclease A (RNase A),
2. Nuclease P1: 200 U/mL in 30 mM sodium acetate, and
3. Alkaline phosphatase: 50 U/mL in 2.5 M ammonium sulphate.

2.2. Buffers and Other Reagents

1. 10 mM zinc sulphate,
2. 0.5 M Tris–HCl, pH 8.3,
3. Ethanol, and
4. 2-Isopropanol.

2.3. High-Performance Capillary Electrophoresis (HPCE)

1. 14 mM sodium bicarbonate (pH 9.6, equilibrated with 0.1 M sodium hydroxide) containing 20 mM sodium dodecyl sulphate (SDS),
2. 0.1 M sodium hydroxide,
3. 0.45-μm filters (Sartorius, Göttingen, Germany), and
4. Uncoated fused-silica capillary of 60.2 cm × 100 cm, with an effective length of 50 cm (Waters Chromatography S.A., Madrid, Spain).

2.4. High-Pressure Liquid Chromatography (HPLC)

1. 0.1% formic acid (HPLC grade) in water and
2. 0.1% formic acid in 50% water:50% methanol (HPLC grade).

2.5. Nucleotide Standards

All nucleosides standards are dissolved at 5 mM in Milli-Q grade water.

1. 2′-deoxyadenosine 5′monophosphate (dA),
2. 2′-deoxythymidine 5′monophosphate (dT),
3. 2′-deoxyguanosine 5′monophosphate (dG),
4. 2′-deoxycytidine 5′monophosphate (dC), and
5. 5-methyl 2′-deoxycytidine 5′monophosphate (5mdC).

2.6. Equipment

1. A HPCE P/ACE MDQ system (Beckman-Coulter, Fullerton, CA, USA) connected to a data-processing station (32 Karat™ Software);
2. An Agilent Serie 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with an online vacuum-degassing system, a quaternary pumping system, an autosampler with internal refrigeration and ultraviolet and visible lamps for variable wavelength detection;
3. Reverse-phase column Atlantis dC18 column (2.1 × 150 mm; 5 μm particle size);

4. Guard column (2.1 × 20 mm; 5 μm particle size, Agilent); and
5. An Agilent LC/MSD VL MS equipped with an electrospray ionization source (Agilent) coupled to the HPLC system.

3. Methods

In this chapter, we describe two different approaches for the separation of nucleosides: a HPCE-based method and a HPLC-based method. As shown in **Fig. 2.1**, the first steps and the relative quantification of global DNA methylation signals are shared between both techniques.

3.1. Genomic DNA Extraction and RNase Treatment

DNA from animal tissues is extracted by standard methods *(7)*. It is important to obtain high-purity DNA to assure an effective

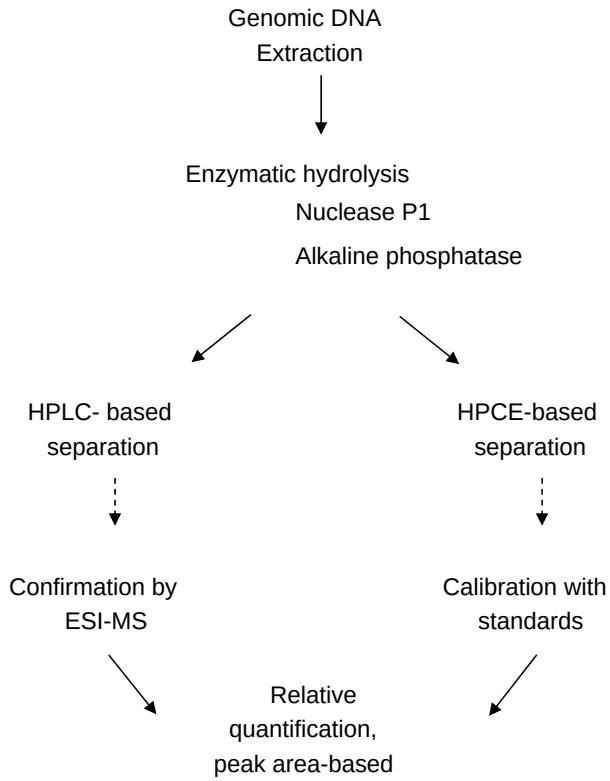

Fig. 2.1. Simplified representation of the two alternative procedures described in this chapter, which are used for separation of DNA nucleosides and quantification of global DNA methylation levels. After enzymatic hydrolysis of genomic DNA, nucleosides could be separated by high-performance capillary electrophoresis (HPCE) or liquid chromatography coupled to an electrospray ionization mass spectrometry (LC-ESI/MS). In both cases, relative quantification of 5-methyl-2′-deoxycytidine (5mdC) levels are extrapolated from HPCE or HPLC chromatograms.

action of the next steps of the protocol. A potential problem in the measurement of genomic DNA methylation is interference from RNA contamination (*see* **Note 1**); therefore, treatment with a ribonuclease is recommended before DNA hydrolysis.

1. Add RNase A to a final concentration of 20 μg/μL. Mix gently and incubate the mixture at 37°C for 30 min.
2. Following the incubation, add an equal volume of cold 2-isopropanol and mix thoroughly in order to enhance genomic DNA precipitation.
3. Centrifuge for 10 min at 11,000*g* and carefully decant the supernatant.
4. Wash the DNA pellet by adding cold 70% ethanol. Centrifuge for 5 min at 11,000*g* and resuspend the resulting pellet in Milli-Q grade water. Genomic DNA can be stored at 4°C till used.

3.2. DNA Hydrolysis

1. Prepare DNA samples (2–7 μg) in 10 μL of total volume. If necessary, dilute the samples in distilled water.
2. Denature the samples by heating for 2 min in a boiling water bath and cool rapidly in ice for 5 min.
3. Add nuclease P1 to a final concentration of 1.5 μg/μL and zinc sulphate to a final concentration of 1 mM (*see* **Note 2**). Incubate overnight at 37°C.
4. Add 0.75 μL of alkaline phosphatase and 1.25 μL of 0.5 M Tris–HCl, pH 8.3 (*see* **Note 2**). Incubate the mixtures for 2 h at 37°C.
5. In order to eliminate any solid residue, centrifuge samples at 10,000*g* for 3 min. Supernatant must be stored at 4°C till used.

3.3. Nucleoside Separation by High-Performance Capillary Electrophoresis (HPCE)

We have previously described the quantification of the relative methylcytosine content of the genomic DNA using a HPCE system to analyze hydrolyzed genomic DNA *(8, 9)*. In this context, separation and quantification of cytosine and methylcytosine is only possible by the use of a sodium dodecylsulphate (SDS) micelle system. This method is faster than HPLC (taking less than 10 min per sample) and is also reasonably inexpensive since it does not require continuous running buffers and displays a great potential for fractionation (up to 10^6 theoretical plates). Nevertheless, no or almost no preparative analyses are possible with HPCE systems because of the low injection volumes.

For the separation of nucleosides after genomic DNA hydrolysis, the following procedure must be applied:

1. Before each run, prepare all buffers and washing solutions with Milli-Q water and filter them through 0.45-μm filters (*see* **Note 3**).
2. Condition the capillary system just before each run by washing with 0.1 M NaOH for 3 min.

3. After washing, equilibrate the capillary system with the running buffer for 3 min. The optimal running buffer is 14 mM sodium bicarbonate, pH 9.6 containing 20 mM SDS, which allows for the micelle formation of the nucleosides.
4. Filtered hydrolyzed samples (*see* **Section 3.2**) through 0.45-μm pore filters.
5. Inject samples under pressure (0.3 psi) for 3 s. Running conditions, optimized in *(9)* consist of a temperature of 25°C and an operating voltage of 17 kV (*see* **Note 3**). Absorbance is monitored at 254 nm. **Figure 2.2** shows a representative electropherogram obtained for standard nucleosides and the DNA extracted from a human tumor cell line.

3.4. Nucleoside Separation by HPLC and Detection of Nucleosides Peaks by ESI/MS

The basic principles of both techniques are represented in **Fig. 2.3.** The separation mechanism in reverse phase (RP)-HPLC depends on the hydrophobic-binding interaction between the solute molecule of the sample in the mobile phase and the

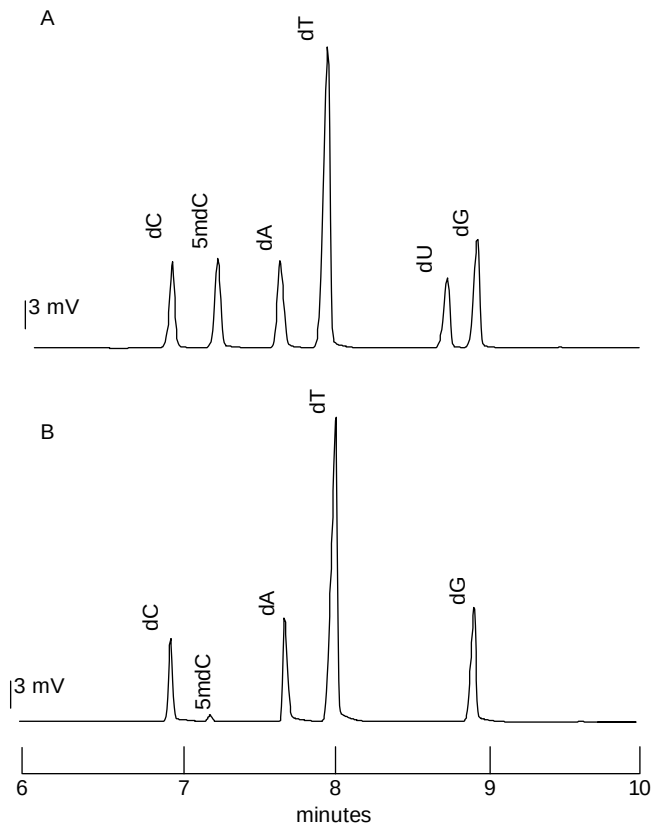

Fig. 2.2. Separation of nucleosides by HPCE. (**A**) Electropherogram for standard nucleosides (dC, 5mdC, dA, dT, and dG) dissolved in Mili-Q grade water at 5 mM. (**B**) Resolution of nucleosides obtained from enzymatic hydrolysis of genomic DNA from a human tumor cell line. Analytical conditions are described in **Section 3.3**.

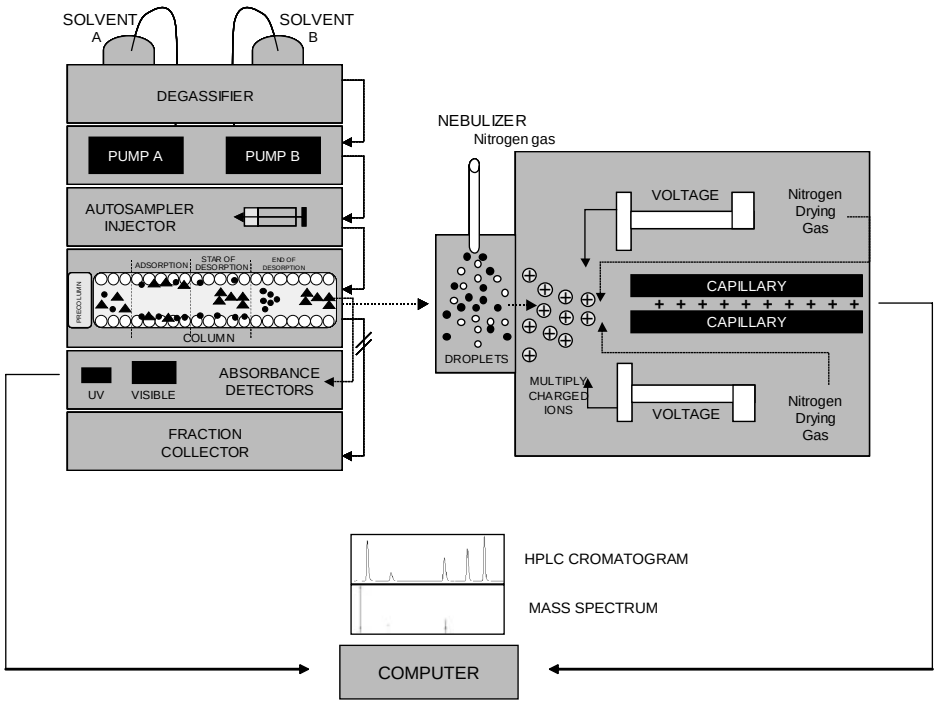

Fig. 2.3. Representative diagram of a LC-ESI/MS apparatus. First, samples are introduced into a HPLC system and analytes are separated in function of their individual hydrophobicity under specific conditions in a reverse-phase column. Then, the resulting mobile phase with the eluted molecules is introduced into the ESI/MS apparatus and subjected to fragmentation, ionization, and desorption processes under a constant nitrogen flow. HPLC and ESI/MS modules are connected to a computer, allowing the combined representation of HPLC chromatograms and mass spectra.

immobilized hydrophobic ligand (stationary phase) that constitutes the column. The capacity of solute molecule binding to the stationary phase can be controlled by manipulation of the hydrophobic properties of the mobile phase. The initial mobile phase-binding conditions used in RP-HPLC are primarily aqueous allowing the formation of a structured layer of water around both the matrix and the analyte. The sample must be applied to the column in such a flow rate that allows the optimal adsorption of the sample components. Transport and elution of analytes is achieved by increasing the concentration of the organic component in the mobile phase. Once the molecules are eluted from the column they get introduced into the electrospray system of the mass spectrometer. At this point, it is important to note that buffers must be free of salts, which could potentially damage the mass spectrometer. The electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) methods are the major techniques based on the atmospheric pressure ionization (API). In the ESI, both solvent and sample are nebulized with the help of a gas stream and broken into small droplets. The mobile phase solvent evaporates from the droplets

(desolvation). Droplets undergo Coulomb explosions when the charge density increases until the Raleigh limit (10^8 V/cm^3) and new smaller droplets are formed. Ions in solution are desorbed under the influence of high potential of the electrospray fields in the spray chamber. The ESI technique can be applied to a wide range of molecule sizes, except for small (<1000 mw) and extremely nonpolar molecules. However, one of the disadvantages of ESI is that the solution chemistry could influence the ionization process and some adducts could be generated, such as $[M + H]^+$, $[M + Na]^+$, and $[2M + H]^+$.

A LC-ESI/MS approach for analyzing enzymatic hydrolysates of DNA was previously described *(6)*. Although this method provided a good quantitative analysis of DNA methylation in less than 15 min, conditions for the LC included buffers with ammonium salts which are inconvenient for the maintenance of the LC-ESI/MS system and also favor the production of single ammonium adducts in the ESI/MS. Here we describe a protocol in which adequate separation of the DNA and RNA components is achieved within 25 min. Buffers without salts are employed, making the direct flow of solvents from LC to ESI/MS system feasible.

LC-ESI/MS conditions required for the analysis of the 2′-deoxyribonucleotide-5′-monophosphate levels are as follows:

1. Before each run, equilibrate the HPLC column with the running buffer. The mobile phase consists of two buffers: 0.1% formic acid in water (Solvent A) and 0.1% formic acid in 50% water:50% methanol (Solvent B) (*see* **Note 4**). Equilibration must be done by maintaining the initial conditions, 95% Solvent A–5% Solvent B in an isocratic mode during 5 min at constant flow of 0.220 mL/min. The employed Atlantis dC18 column permits to minimize the loss in retention in a 100%-aqueous mobile phase *(3)*. It is strongly recommended to protect the column by the use of a guard column (*see* **Note 3**).
2. Dilute the hydrolyzed DNA (*see* **Section 3.2**) in water to a final volume of 50 μL and filter it through a 0.45-μm pore filter just before injection (*see* **Note 3**).
3. HPLC separation must be performed with an initial gradient of 5% solvent B, then an increase of solvent B to 50% within 9 min and an isocratic gradient (50% of solvent B) during 25 min. The acquisition of HPLC signals is obtained by UV detection at 254 nm and 280 nm. It is important to point out that the HPLC separation under the previously described conditions is achieved in solvents without salt compounds. As a consequence, no desalting before the entry of the solvents into the ESI/MS is needed.
4. Source conditions for ESI/MS are as described in *(6)*, with minor modifications. A drying gas flow of 10.0 L/min was

employed, with auxiliary 35 psis gas to assist nebulization and a drying temperature of 350°C. The mass spectrophotometer was operated at a capillary voltage of 4,000 V, and spectra were collected in positive ion mode.

After 14 min, all the DNA and RNA compounds are completely separated as shown in the LC chromatogram (**Fig. 2.4**). The ESI/MS spectra are used to verify the identity of each HPLC peak used for the estimation of the DNA methylation levels. As expected, the ESI source with the mass spectrometer in positive ion detection mode shows protonated molecules as well as fragments ions and other known adducts derived from nucleosides. **Figure 2.4** shows the LC chromatogram and the product ion spectra of the five deoxyribonucleosides (5mdC, dC, dG, dA, and dT) and the five ribonucleosides (5mC, C, G, A, and U) after hydrolysis of a 4 μg of a tumor sample without RNase treatment during nucleic acid extraction. The transitions pairs of *m/z* 242.1/126.1, 228.1/112.1, 268.1/152.1, 252.1/136.0, and 243.1/127.0 corresponded to 5mC, 5mdC, dC, dG, dA, and T, respectively, while 258.1/126.0, 244.1/112.1, 284.1/152.2, 268.1/136.1, and 245.1/113.0 were acquired for 5mC, C, G, A, and U, respectively. The presence of T and U in the LC chromatogram is less prominent than the other nucleosides,

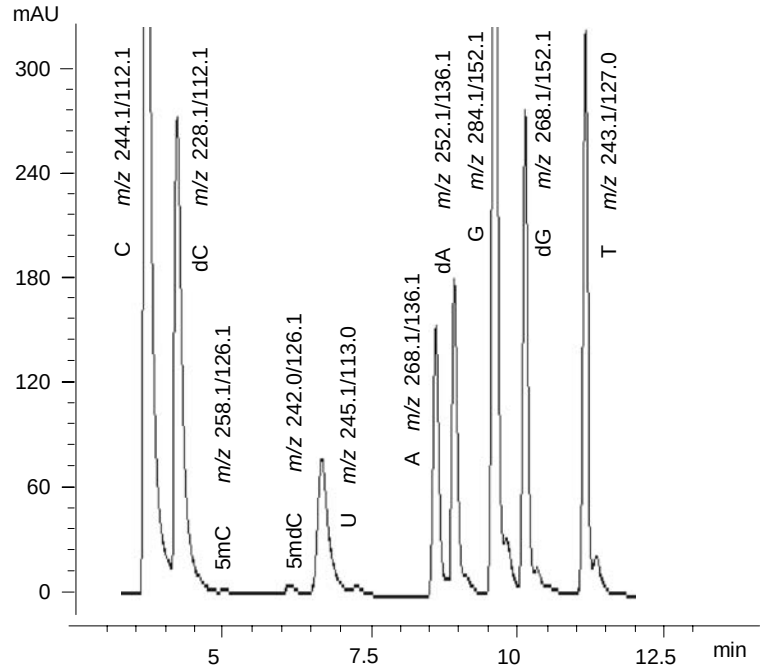

Fig. 2.4. LC-ESI/MS chromatogram and specific product ions of 10 nucleosides corresponding to the DNA and RNA compounds. DNA hydrolysis was carried out from 4 μg of DNA from a tumor cell line without RNase treatment. LC and ESI/MS conditions are described in **Section 3.4.**

which may be attributed to the weaker proton affinity of these nucleosides.

In the case of RNase-treated samples, the chromatogram shows only peaks corresponding to the five deoxyribonucleotides (**Fig. 2.5**). The HPLC peak eluting after 4.0±0.5 min corresponds to 2′-deoxycytidine (dC), and the HPLC peak eluting after 5.5±0.5 min correspond to 5-methyl-2′-deoxycytidine (5mdC). **Figure 2.5B** and **C** report the full-scan spectra (ESI/MS spectra) of dC and 5mdC, respectively. The $[M + H]^+$ adduct appears at *m/z* 228.1 and 242.1 for dC and 5mdC, respectively. Also present are the [2M] and $[2M + H]^+$ adducts at *m/z* 455.1 and 456.0 for dC and *m/z* 483.1 and 484.0 for 5mdC, respectively. In some samples, the $[M + 23]^+$ and the $[2M + 23]^+$adducts can also be found, which correspond to sodium adducts. It is important to point out that sodium adducts are frequently detected in ESI mass spectra of organic compounds, because they are normal compounds of glass vials used for HPLC

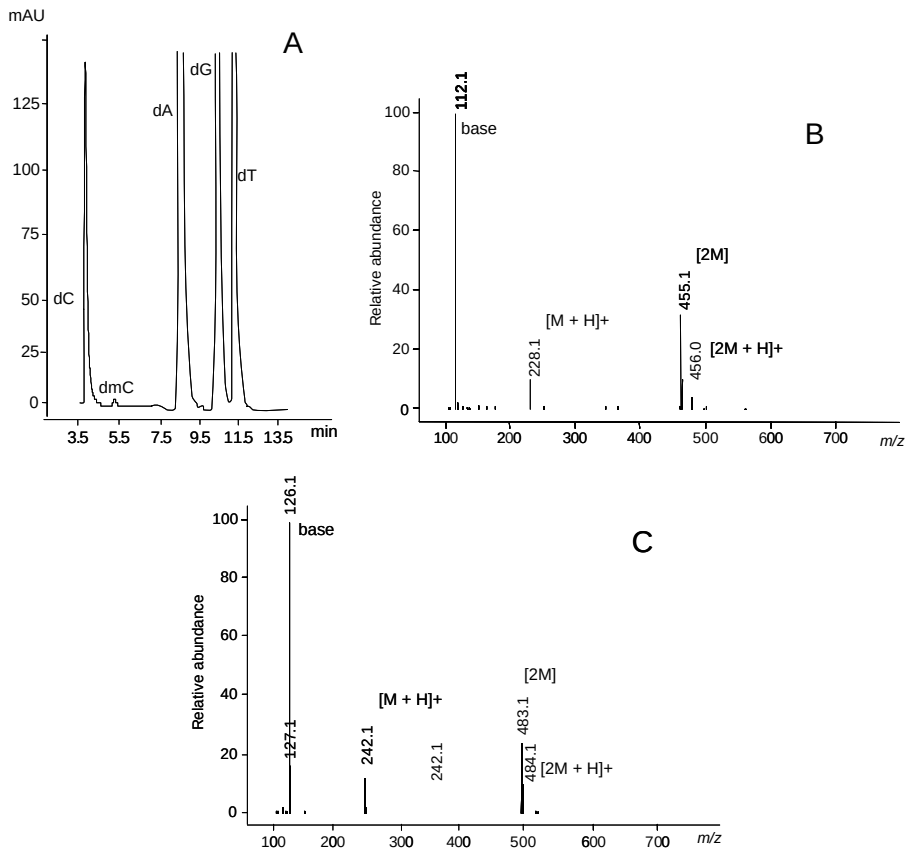

Fig. 2.5. LC-ESI/MS chromatogram of a human lymphocyte sample containing 3 μg of RNA-free genomic DNA. (**A**) Separation of the five deoxynucleosides in the HPLC chromatogram obtained by UV detection at an absorbance of 254 nm. (**B** and **C**) Full spectra obtained in ESI/MS for 2′-deoxycytidine (dC) and 5-methyl-2′-deoxycytidine (5mdC), respectively. LC and ESI/MS conditions are described in **Section 3.4**.

separation. After the separation of the DNA bases the fragmentation conditions established in the ESI/MS cause the separation of the pentose moiety from the pyrimidine ring of both dC and 5mdC resulting in the production of cytosine (*m/z* 112.1) and 5-methylcytosine (*m/z* 126.1). In this way, conditions for ESI/MS can be optimized to change the intensity of $[M + H]^+$adducts with respect to the formation of dimers, sodium adducts, and nitrogen bases *(3)*.

3.5. Quantification

To determine the 5mdC abundance, the percentages of global genomic DNA methylation are calculated by integration of the peak areas of 5mdC relative to global cytidine (methylated or not). Area peaks are obtained directly from HPCE or HPLC chromatograms, depending of the selected approach. The following equation was used in both cases: 5mdC peak area × 100/(dC peak area + mdC peak area).

4. Notes

The most common considerations for preventing failures in the separation of nucleosides by HPCE and HPLC techniques which could influence results are

1. One of the major problems of this technique is the incomplete digestion of RNA compounds. As the estimation of global DNA methylation is based on a relative index between methylated and unmethylated cytidines, this index could be underestimated in the presence of RNA compounds. Treatment with a ribonuclease assures the fidelity of the results as shown in **Fig. 2.5**.
2. Adjustment of the pH and molarity of the Tris and sulphate buffers is important to assure the complete and specific DNA hydrolysis. Unspecific hydrolysis could influence results, especially for the HPCE technique.
3. Temperature and voltage are the two main variables that determine the best separation of the nucleosides. Small particles can permanently block the capillaries. It is important to use filtered solvents always both for the HPCE and the HPLC method. Furthermore, in HPLC the employment of precolumns is strongly recommended. If not, the pressure of the system might not be constant and the resolution of the method might noticeably decrease. The temperature must be lower than 30°C for HPCE and column temperature should be controlled in the HPLC.
4. Solutions of organic acids, such as formic acid, in organic solvents act as corrosive factors of all steel components. Although the HPLC method uses a low concentration, a 0.1% solution of formic acid in methanol, the acid can attack steel.

Consequently, it is important to remove the running buffers by washing the system with methanol: water solutions before switching off the apparatus. However, a low concentration of acid is necessary for the positive ion mode detection in the mass spectrometry.

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