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Fused-core silica column ultra performance liquid chromatography – ion trap tandem mass spectrometry for determination of global DNA methylation status¹

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Abstract

Epigenetic modifications, such as DNA methylation, play key roles in transcriptional regulation of gene expression. More recently, global DNA methylation levels have been documented to be altered in several diseases, including cancer, and as the result of exposure to environmental toxicants. Based on the potential use of global DNA methylation status as a biomarker of disease status and exposure to environmental toxicants, we sought to develop a rapid, sensitive, and precise analytical method for the quantitative measurement of global DNA methylation status using ultra performance liquid chromatography with detection by ion trap tandem mass spectrometry. Using a fused-core silica column, 2'-deoxyguanosine (2dG) and 5-methyl-2'-deoxycytidine (5mdC) were resolved in less than 1 minute, with detection limits of 0.54 and 1.47 fmol for 5mdC and 2dG respectively. The accuracy of detection was 95% or above and the day-to-day coefficient of variations was found to be 3.8%. The method was validated by quantification of global DNA methylation status following treatment of cells with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine, which reduced DNA methylation from 3.1% in control cells to 1.1% in treated cells. The sensitivity and high throughput of this method render it suitable for large scale analysis of epidemiological or clinical DNA samples.

Keywords

Ultra performance liquid chromatography (UPLC); electrospray ionization ion trap mass spectrometry (ESI ITMS); global DNA methylation

Introduction

DNA methylation is an epigenetic modification that consists in the addition of a methyl group to position 5 on the cytosine ring of CpG dinucleotides. In mammals, DNA

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methylation is involved in several processes such as genomic imprinting and stability, X chromosome inactivation and gene silencing. DNA methylation is ubiquitous in the sense that all categories of DNA sequences (genes, transposons and inter-genic DNA) are methylated [1], however, the pattern of methylation varies from one cell type to another. Aside from gene-specific DNA methylation status, global DNA hypomethylation has been strongly associated with a variety of cancers [2] and it has been proposed that global DNA demethylation may lead to genomic instability therefore enhancing susceptibility of cells to genetic changes [3]. Alterations of DNA methylation have also been identified in other disease states, such as systemic lupus erythematosus [4] and numerous neurological diseases [5]. There is also growing evidence that aberrant DNA methylation during development could increase susceptibility to adult diseases [6–7], and that exposure to environmental contaminants may interfere with the proper maintenance of the DNA methylation patterns and levels [8–9]. Given the potential use of global DNA methylation status as a biomarker of environmental exposures and disease status, there is a need for accurate and efficient assessment of the global DNA methylation status of human cells and tissues.

Following enzymatic or acid DNA hydrolysis, it is possible to determine the global state of DNA methylation by analytical means. Chromatographic methods are faster and more sensitive than restriction landmark genomic scanning techniques [10]. Previous studies have used liquid chromatography coupled with uv detection to assess global DNA methylation status. However, these methods typically require a large amount of starting material [11–12], which render them less suitable for quantification of human samples. High performance liquid chromatography-mass spectrometry (HPLC/MS) approaches provide better sensitivity in general, but analytical runs are frequently long, which limits sample throughput [13–15]. Most recently, improvements in analytical times were attained by using ultra performance liquid chromatography (UPLC) coupled with quadrupole MS for detection [16]. With the advent of UPLC, newer fused-core silica particles columns, with shorter diffusional mass transfer paths [17–19] have become available. These sub-3 μm fused-core silica particles are made by coating a non-porous solid silica core with a superficially porous silica shell. This architecture enables faster separation, better peak resolution, higher sample throughput, and less back pressure when compared to conventional fully porous sub-2 μm silica particle UPLC columns typically used in the analysis of pharmaceuticals [20–21]. Sub-2 μm (1.7 μm) fused-core silica particles UPLC columns were recently commercialized and demonstrated greater chromatographic performance than conventional fully porous sub-2 μm silica particle UPLC [22]. However, these fused-core silica particles columns have yet to be applied to the separation of nucleosides. Finally, there was also need for a simple, effective generic mobile phase and gradient program for nucleoside separation regardless of reverse phase (RP) particle types or column dimensions.

The goal of this study is to develop a fast, sensitive and reliable analytical method to assess the percentage of DNA methylation in genomic DNA by quantifying the ratio of 5-methyl-2'-deoxycytidine (5mdC) to 2'-deoxyguanosine (2dG). Previously reported HPLC triple quadrupole MS/MS methods for the measurement of global DNA methylation [23–24] were modified and improved using a sub-2 μm fused-core silica particles column UPLC separation and an ion trap tandem mass spectrometry (ITMS/MS) detection platform.

Methods

Cell culture, treatment and DNA isolation

Human neuroblastoma SK-N-AS cells were obtained from ATCC (Cat # CRL-2137) and cultured in minimum essential medium (MEM, Cellgro, 10-010-CV) containing 10% fetal calf serum (Cellgro, 35015-CV), 5 mM sodium pyruvate (Cellgro, 25-000-CI), 5 x nonessential amino acids (Cellgro, 25-025-CI) and supplemented with 100 units/ml

penicillin and 100 µg/ml streptomycin (Cellgro, 30-001-CI) at 37 °C in 5% CO₂. Cells were seeded at a concentration 3×10^5 cells/ml in 100 mm tissue culture dish and grown overnight. They were then cultured with or without the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5azadC) (Sigma, A3656) [25] at concentrations of 0 (vehicle only), 1.25 or 2.5 µM for a total of 48 h. The media was then replaced and cells were grown for another 24 h without inhibitor prior to being harvested and lysed in AL buffer (Qiagen, 19075). DNA was extracted using Qiagen DNAeasy Kit (Qiagen, 69504) according to the manufacturer's protocol and DNA purity was assessed with a NanoDrop 2000 at 230, 260 and 280 nm. As an additional control, nuclear protein extracts were prepared using the Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit according to the manufacturer's protocol (Thermo, 78833) and DNMTs activity was measured in the samples with an EpiQuik DNA Methyltransferase Activity/Inhibition Assay Kit from (Epigentek, P-3001) following the manufacturer's protocol.

Enzymatic hydrolysis

The genomic DNA (gDNA) from the 5azadC treated and non-treated SK-N-AS cells and commercial gDNA from human embryonic kidney cells HEK293 (GenScript, M00094) were utilized for method validation. The DNA digestion was performed according to the protocol published by Crain [26] with minimal modifications. For each sample, 1 µg of DNA was brought to a volume of 70 µl in RNase and DNase free water and denatured at 100 °C for 3 min and chilled on ice. Then 7 µl of 0.1 M ammonium acetate (pH 5.3) and 2 units of nuclease P1 are added and the tubes are incubated at 45 °C for 2 h. 7.9 µl of 1 M ammonium carbonate and 0.002 unit of venom phosphodiesterase 1 are subsequently added and tubes were incubated at 37 °C for 2 h. Finally 0.5 units of alkaline bovine phosphatase are added to each tube and the samples are incubated for 1 h at 37 °C. The nuclease P1, venom phosphodiesterase I, and alkaline bovine phosphatase were purchased from Sigma (Sigma, N8630, P3242 and P6774, respectively). Samples were then kept at -80 °C until analysis.

UPLC Separation

Pesticide residue analysis grade water, methanol, formic acid, HPLC grade ammonium bicarbonate, and molecular biology grade ammonium acetate were purchased from VWR international, Inc. (West Chester, PA). 2dG was purchased from Sigma Chemical Co. (St. Louis, MO) and 5mdC was obtained from MP Biochemicals, LLC (Solon, OH). The 5mdC and 2dG in digested DNA samples were separated using a Thermo Accela autosampler coupled to a Thermo Accela pump system (Thermo Scientific, San Jose, CA) and one of the following UPLC capable RP columns: Hypersil GOLD 200 × 2.1 mm (1.9 µm particle size, Thermo Scientific, San Jose, CA), Hypersil GOLD phenyl 100 × 2.1 mm (1.9 µm particle size, Thermo Scientific, San Jose, CA) and Kinetex C18 100 × 2.1 mm (1.7 µm particle size, Phenomenex, Torrance, CA).

Four different UPLC methods (A, B, C, and D) were developed using three different UPLC columns and two different mobile phase gradient programs (I and II). The same mobile phases, (A) composed of water with 0.15% of formic acid and (B) composed of acetonitrile with 0.15% of formic acid, were used for all of experimental UPLC methods presented in this study. To test and compare chromatographic performance of the three different UPLC columns listed in Table 1, a generic mobile phase gradient (Gradient Program I) was developed for the separation of 5mdC and 2dG. Gradient Program I was as follows: 0.0 min (5% B), 3.5 min (22.5% B), 5.0 min (50% B), 6.0 min (50% B), and returned to baseline at 6.5 min (5% B). The mobile phase flow rate was constant at 0.35 ml/min. Gradient Program I was used for UPLC methods A, B and C as listed in Table 1. After identifying the fused-core silica particles column as the most promising, the mobile phase gradient was optimized

for this particular column (Gradient Program II). Gradient Program II was as follow: 0.0 min (10% B), 0.8 min (12% B), 1.0 min (50% B), 1.1 min (50% B), 1.15 min (10% B) and returned to baseline at 1.5 min (10% B). In this gradient, the mobile-phase flow rate was initially 0.35 ml/min but increased to 0.4 ml/min at 1.0 min.

ESI-ITMS/MS

The UPLC elute was directly introduced into an electrospray ionization (ESI) source attached to a Thermo LTQ XL mass spectrometer (Thermo Scientific, San Jose, CA) and the analytes were ionized in positive-ion mode. ESI full scan mass spectra were obtained for UPLC injections of the mixture standard of 5mdC (25 µg/ml) and 2dG (125 µg/ml) in 75% mobile phase A and acquired in the m/z range 100–300. Optimized ESI conditions were achieved for the MS/MS experiment as follows: ESI temperature, ESI source voltage, needle current, capillary temperature, and capillary voltage for ITMS/MS scans were 270 °C, 5.0 kV, 100 µA, and 39 V, respectively. Optimized ITMS/MS conditions were as follows: collision induced dissociation, Q value, maximum ionization time, isolation window for precursor ions, and isolation window for selective reaction monitoring (SRM) scans were 31.0%, 0.21, 10 ms, m/z 3.0 and m/z 5.0 for 2dG, and 38.0%, 0.25, 10 ms, m/z 3.0 and m/z 5.0 for 5mdC respectively. The 5mdC and 2dG were identified by observing precursor/product ion pair of m/z 242.1/126.1 and m/z 268.1/152.2 respectively. Data acquisition was performed using the Xcalibur 2.0.7 and the data were analyzed by Qual Browser and Quan Browser 2.0.7 (Thermo Fisher Scientific Inc, San Jose, CA). DNA Methylation rates have previously been measured by taking a molar concentration ratio of 5mdC and 2dG [12–23] and it was determined here using integrated peak areas from the total ion chromatographs (TIC).

To achieve the highest sensitivity for these ESI-ITMS/MS measurements, each step of the ESI-ITMS/MS method was optimized. First, the ESI/ITMS was tuned to maximize the intensity of the precursor ions of m/z 268.1 for 2dG and m/z 242.1 for 5mdC in ITMS to 100% in full scan mode. In the second step, the MS system was set to maximize the product ions of m/z 152.2 for 2dG and m/z 126.1 for 5mdC in the MS/MS experiment. SRM was performed by accumulating two product ions in the ion cavity; increasing ion injection time (ionization time) and then selectively scanning the two ions.

Method characterization and data analysis

The DNA methylation levels were calculated using the ratio of the molar amounts of 5mdC to 2dG measured in each sample. For the optimized experimental method D, dilutions of a working solution of 5mdC:2dG at a molar ratio of 22.2% (corresponding to 5.2 to 103 fmol/µl of 5mdC) were used to calculate the standard deviations for each concentrations ($n = 3-5$). This allowed the determination of the method's detection limit (MDL) by interpolation of the standard deviation at a "0" concentration to obtain s_0 ; the method's detection limit is defined as $3s_0$ [27]. The accuracy across a range of sample concentrations was also evaluated for experimental method D with a working solution of 5mdC:2dG at a molar ratio of 8.9%. The working solution was diluted serially to obtain final on column amounts ranging from 5.2 pmol to 47 fmol of 5mdC with a 10 µl injection volume and standard concentrations of 518 to 4.7 fmol/µl. The percentage of DNA methylation was also measured in HEK293 cells gDNA on two different days ($n = 3/\text{day}$) with an approximate initial gDNA concentration 1 ng/µl in the sample (total volume ≈ 90 µl; 20 µl injected). The intra and inter assay variability was measured for standard solutions corresponding to a 5mdC:2dG molar ratio of 8.9% and on column amounts of 1.25 and 6.25 µg for 5mdC and 2dG respectively. Means, standard deviations, % of coefficient of variation, accuracy, and detections limit were calculated in Excel (2003) from Microsoft™ Professional Suite.

Results

Optimization of UPLC conditions

Sample chromatograms using three different columns and two gradient programs are depicted in Figure 1 (A–C) and Figure 2. The UPLC conditions used for this latter separation (method D) reduced the total chromatographic analysis time reported in other studies from 10–60 min [12; 23; 28] to just under one min with retention times of 0.75 and 0.91 min for 5mdC and 2dG, respectively. A comparison of our results to other published methods is presented in Table 2.

Method detection limit, accuracy and variability

The inter assay variability as well as the intra assay variability were better or approximately the same as those of other published methods. In addition, the current method's LOD and LOQ demonstrated no loss of sensitivity despite an LTQ XL platform and a faster UPLC separation method. The MDL for 5mdC and 2dG were estimated to be 0.54 fmol and 1.47 fmol on column respectively. The accuracy of the analytical approach was obtained for sample dilutions ranging two orders of magnitude with a constant molar ratio of 5mdC to 2dG of 8.9%. The measured ratios ranged from 8.7 to 9.3% (mean $\pm$ SD of 8.9 ± 0.20 %) and the accuracy ranged from 95.2 to 99.9% (mean $\pm$ SD of 98.4 ± 1.5 %). The accuracy in the measurement of the 5mdC/2dG ratio was 98.0% at the lowest concentrations tested with on column amounts of 4.0 and 45.6 fmol of 5mdC and 2dG, respectively.

Validation of the method to measure the percentage of global DNA methylation in gDNA

As a first step to validate our method, we determined the global methylation status of genomic DNA isolated from HEK293 cells. Measurements were taken on two consecutive days to determine the reproducibility of the method. Total methylation status in percentage (mean $\pm$ SD) was: 5.9 ± 0.4 % and 5.6 ± 0.3 % on day 1 and 2, respectively, with an inter-day coefficient of variation (%CV) of 3.8%. Next, we assessed the ability of our method to determine changes in methylation status following treatment of cells with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza2dC). Treatment of SK-N-AS neuroblastoma cells with 2.5 μ M 5-aza2dC for 48 h reduced DNA methyltransferase activity by 80% (data not shown). This inhibition resulted in the total methylation status being reduced from 3.1% in control cells to 1.1% in treated cells.

Discussion

The goal of every new analytical method is high throughput with the rapid and accurate measurement of the target analytes on an inexpensive platform. This goal was achieved for the determination of the % of methylation of genomic DNA by the innovative combination of a sub-2 μ m fused-core silica particles UPLC separation scheme to at a linear ion trap (LTQ XL) mass spectrometer. The detailed methods comparison presented in Table 2 highlights the improvements in both sensitivity and throughput. Gains in sensitivity, as shown by the reduced amounts of gDNA required for analysis and lower detection limits, were obtained using the affordable ion trap mass spectrometer despite the common belief that ITMS are less sensitive than triple quadrupole instruments due to the loss of ions with each successive MS experiment. The resolution of the analytes using sub-2 μ m fused-core silica particles UPLC was another reason for the reduction in LOD, possibly because of the improved transfer efficiency into the MS. Overall this method is faster and requires less DNA than comparable methods.

Chromatography

The UPLC method was optimized for throughput making it roughly five times faster than the next fastest HPLC or UPLC based method. Taking into account that faster elution times often co-occur with a loss in resolution; multiple solid and mobile phases were tested (Table 1) to minimize the retention times and increase sensitivity of both analytes without any significant loss of precision. Method A is a versatile set of conditions that can be adapted to either UPLC or HPLC separation. Method D provided the best compromise conditions considering the aforementioned factors. Other studies have employed reversed phase HPLC or UPLC separation of these analytes using conventional fully porous silica particles (summarized in Table 2). However, it is the use of the sub-2 μm fused-core silica particles along with this particular gradient profile and flow rate in a UPLC chromatograph that allowed for better MDL and the faster elution of these analytes.

The reduction in retention times displayed in Figures 1A–C and Figure 2 demonstrates the ability of the gradient program to reduce retention time with the newer silica particles and higher pressures generated with modern UPLC systems. The fused-core silica particles column preserves analytes resolution by minimizing the time spent by the analytes with each individual particle. Column differences: length, particle size and fused core; allowed for a decrease in retention time from approximately four minutes to just over one minute. The final reduction in retention time from 1.1 min to 0.9 min for 2dG (method C to D) was attributed to a more rapid gradient step to 12% A at 0.8 min coupled with an increase in flow rate from 350 to 400 $\mu\text{L}/\text{min}$. This significant reduction in analysis time from other literature methods makes the present method cost-efficient and suitable for high-throughput analysis of DNA methylation status.

Mass spectrometry

Comparing sensitivity across methods is at best difficult and worst unrealistic based on the different techniques used to estimate noise or signal to noise ratios. Given these constraints, an attempt was made to compare relative sensitivities (LODs and LOQs) of other published methods for the measurement of the percentage of methylation in gDNA. With detection limits of 0.54 fmol and 1.47 fmol for 5mdC and 2dG respectively, our method was comparable or better than those currently found in the literature demonstrating that the retention time reductions did not come at the expense of sensitivity.

With multiple ionization sources and mass spectrometric methods to choose from, optimization of the MS method becomes a focal point for method improvement. Often a single ion monitoring experiment is more sensitive but the MS/MS method with ESI provided the greatest signal and no measurable background. Atmospheric pressure chemical ionization methods were also examined, but were not as sensitive for these compounds. Maximizing the precursor ion in the first stage and the product ion in the second stage created a single reaction monitoring experiment that improved the LOD by roughly 2 to 5 times. The MS/MS experiment essentially eliminated the noise and allowed for both improved LODs and decreased the amount of sample required to make the measurement. Creating a more sensitive method with an ion trap mass spectrometer suggests that adaptation of this method to a triple quadrupole platform might achieve and even lower limit of quantitation.

Conclusion

A rapid sub-2 μm fused-core silica particles UPLC method for the separation of 5mdC and 2dG coupled to an ion trap mass spectrometer was developed for the determination of the global percentage of methylation in genomic DNA. The optimized method was as sensitive

as or better than other published methods. Its applicability was demonstrated by measuring the percentage of DNA methylation in cells cultured in the presence or absence of 5azadC, a known inhibitor of DNA methylation. The decrease in retention time to just over one minute and the corresponding high throughput coupled with the LOD in the low fmol range and the small amounts of starting material required rend this method suitable for large scale processing of epidemiological or clinical sample sets.

Abbreviations used

2dG	2'-deoxyguanosine
5azadC	5-aza-2'-deoxycytidine
5mdC	5-methyl-2'-deoxycytidine
%CV	coefficient of variation (%)
DNMT	DNA methyltransferase
ESI	electrospray ionization
gDNA	genomic DNA
ITMS	ion trap mass spectrometer
LOD	limit of detection
LOQ	limit of quantification
MDL	methods detection limit
MRM	multiple reaction monitoring
RP	reverse phase
SD	standard deviation
SRM	selective reaction monitoring
TIC	total ion chromatograph
UPLC	ultra performance liquid chromatography

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Fig 1.

Total ion chromatographs (TIC) of 5mdC and 2dG standard injections using three different columns. (A) Hypersil GOLD C18 200 × 2.1 mm (1.9 μm particle size); (B) Hypersil GOLD phenyl 100 × 2.1 mm (1.9 μm particle size) and (C) Kinetex C18 100 × 2.1 mm (1.7 μm particle size). For all three experiments, the gradient program I was used and the ITMS/MS conditions were identical. The fused-core silica particle column (C) allowed the shortest retention times and was chosen for optimization.

Fig 2.

TIC of a 0.55 ng injection of HEK293 cells genomic DNA digest using the optimized UPLC conditions (experimental method D: Kinetex C18 100 × 2.1 mm (1.7 μm particle size) fused-core silica particle UPLC column and gradient program II).

Table 1

UPLC methods developed for the separation of 5dmC and 2dG

Experimental UPLC method	UPLC column (particle size (μm), d × l (mm))	Retention time			Peak area/height		Coefficient of variation ^c (%)	Accuracy ^d (%)	
		Gradient program ^a	5dmC (min)	2dG (min)	Peak resolution (min)	5dmC ^b			2dG ^b
A	Conventional Thermo C18 (1.9, 2.1 × 200)	I	2.72	3.95	1.20	100/100	100/100	0.85	99.3
B	Conventional Thermo phenyl (1.9, 2.1 × 100)	I	1.31	1.77	0.46	104/129	89/105	1.74	98.7
C	Fused-core particle Kinetex C18 (1.7, 2.1 × 100)	I	0.84	1.16	0.32	108/275	74/123	1.83	98.6
D	Fused-core particle Kinetex C18 (1.7, 2.1 × 100)	II	0.73	0.91	0.18	157/334	110/214	1.44	98.9

^a Described in the methods section

^b Based on 25 pg of 5dmC and 125 pg of 2dG injections; expressed as a relative % of method A

^c N= 3, 4 or 5

^d Calculated as: 100% - (100*|Expected Ratio-Observed Ratio|/Expected Ratio)

Table 2
Comparison of chromatographic methods to determine the percentage of global DNA methylation

Separation	Yang et al. (2010)	Song et al. (2005)	Stogia et al. (2010)	Vandeghechichte et al. (2009)	Kok et al. (2007)	Ma et al. (2009)	Liu et al. (2009)
	Thermo Accela UPLC	Agilent 1100 HPLC	Waters Acquity UPLC	Waters Acquity UPLC	Perkin-Elmer Series 200 HPLC	Agilent 1100 HPLC	Shimadzu HPLC
Column (d × l (mm))	Kinetex (2.1 × 100)	Atlantis dC18 (2.1 × 150)	BEH HILIC (2.1 × 100)	HSS T3 (2.1 × 100)	Xterra MS C18 RP (4.6 × 50)	Atlantis dC18 (2.1 × 150)	Hypersil Aquasil C18 (2.1 × 150)
Particle size (µm)	1.7	5	1.7	1.8	3.5	5	5
Ionization mode	ESI	ESI	---	ESI	ESI	ESI	ESI
Detection apparatus	Thermo LTQXL ion trap MS	AB API300 triple quadrupole MS MRM ^a	Waters PDA ea (UV-visible)	Waters Tandem Quadrupole MRM ^a	API3000 triple quadrupole MS MRM ^a	AB API4000 triple quadrupole MS MRM ^a	PerkinElmer Sciex API 300 triple quadrupole MRM ^a
Minimal amount of DNA required for determination	0.3 ng	5 ng	10 µg	2 µg ^b	0.1 µg ^b	---	1 to 5 ng
DNA Hydrolysis	Enzymatic	Enzymatic	Acid	Enzymatic	Acid	Enzymatic	Enzymatic
Ratio	5mdC/2dG	5mdC/2dG	5mdC/(5mdC+2dC)	5mdC/2dG	5mdC/(5mdC+2dC)	5mdC/(5mdC+2dC)	5mdC/2dG
LOQ 5mdC (S/N) (fmol, on column)	1.7(10) ^c	1 (10) ^b	1.7 (10) ^c	---	>27 (10) ^b	---	40
LOD 5mdC (S/N) (fmol, on column)	0.5(3) ^c	0.2 (2)	0.5 (3) ^c	28	8.2 (3) ^b	4.1 (5.6) ^b	---
Retention time 2dC (min)		---	3.8	---	3	5	4.7
Retention time 5mdC (min)	0.7	8.8	4.4	3.4	4.3	9	5.1
Retention time 2dG (min)	0.9	13.2	---	3.8	---	16	---
Intra-assay variation (%)	≤1.5	<2.0	<4	---	1.7	4	<3.3
Inter-assay variation (%)	1.1	<1.4	<4	≤4.2	3.5	5	<6.0
Comments	8.9 and 22% 5mdC/2dG ratio standard solution	5 and 10% 5mdC/2dG ratio standard solution	Calf thymus DNA; up to 100µg digested	QC origin not specified	Calf thymus DNA	Calf thymus DNA	2 and 10% 5mdC/2dG ratio standard solutions

^aMRM: multiple reaction monitoring

^bCalculated based on reported data

^cMeasured with serial dilutions according to Taylor (1987) [26].