Published online 20 August 2008

Nucleic Acids Research, 2008, Vol. 36, No. 18 ***e119***
doi:10.1093/nar/gkn534

DNA methylation determination by liquid chromatography–tandem mass spectrometry using novel biosynthetic [U-^{15}N]deoxycytidine and [U-^{15}N]methyldeoxycytidine internal standards

Eoin P. Quinlivan[1,*] and Jesse F. Gregory III[2]

[1]Biomedical Mass Spectrometry Laboratory, General Clinical Research Center and [2]Food Science and Human Nutrition Department, University of Florida, Gainesville, FL, USA

Received April 24, 2008; Revised July 24, 2008; Accepted August 4, 2008

ABSTRACT

Methylation of the promoter CpG regions regulates gene transcription by inhibiting transcription factor binding. Deoxycytidine methylation may regulate cell differentiation, while aberrations in the process may be involved in cancer etiology and the development of birth defects (e.g. neural tube defects). Similarly, nutritional deficiency and certain nutra-genomic interactions are associated with DNA hypomethylation. While LC-MS has been used previously to measure percentage genomic deoxycytidine methylation, a lack of a secure source of internal standards and the need for laborious and time-consuming DNA digestion protocols constitute distinct limitations. Here we report a simple and inexpensive protocol for the biosynthesis of internal standards from readily available precursors. Using these biosynthetic stable-isotopic [U-^{15}N]-labeled internal standards, coupled with an improved DNA digestion protocol developed in our lab, we have developed a low-cost, high-throughput (>500 samples in 4 days) assay for measuring deoxycytidine methylation in genomic DNA. Inter- and intraassay variation for the assay (%RSD, $n = 6$) was <2.5%.

INTRODUCTION

CpG islands are 5′-deoxycytidine-deoxyguanosine-3′-rich regions of DNA. In mammalian cells these regions are initiation sites for DNA transcription (1). Deoxycytidine methylation (dCyt→MdCyt) of the CpG island plays an important role in regulating gene transcription by blocking transcription factor binding. The inhibition of transcription factor binding increases exponentially with methylation density (2) but can be overcome with increased transcription factor concentrations. Alternatively, the methylated CpG promoter region can act as an initiation site for heterochromatin formation (3), which irreversibly inhibits transcription. By promoting heterochromatin formation, DNA methylation is thought to be a major component of epigenetic gene regulation, i.e. inheritance of information on the basis of gene expression as opposed to gene sequence (genetics). Examples of epigenetic gene regulation include autosomal gene inheritance, where one parental copy is silenced (4) and X-chromosome inactivation in mammals (5). DNA methylation also is thought to play a role in cell differentiation, as methylation levels are tissue specific (6,7) and change during tissue maturation (6).

CpG hypomethylation has been associated with a number of carcinoma types including Wilms tumors (8) (typified by frequent chromosome 1 and chromosome 16 pericentromeric rearrangements), ovarian epithelial carcinoma (8) and breast cancer (9). DNA hypomethylation also may cause chromosomal instability by promoting stand breakage (10) while the pericentromeric rearrangements observed in the rare genetic disease, immunodeficiency, centromeric heterochromatin instability and facial anomalies (ICF), may be due to the associated hypomethylation of chromosome 1 (11). DNA methyltransferase inhibitors cause similar a hypomethylation and pericentromeric rearrangement of chromosome 1. Subjects homozygous for the methylenetetrahydrofolate reductase (MTHFR) 677 C→T polymorphism have been shown to exhibit (12) global hypomethylation of DNA, especially when coupled with low plasma folate (<12 nmol/l) or red cell folate (<1.13 nmol/g hemoglobin) concentrations. It has been suggested that the aberrations in DNA methylation associated with the MTHFR 677 TT genotype may explain why this gene polymorphism is a risk factor for breast (13), cervical (14) and some colon cancers (15), and why mothers homozygous for the genotype are at increased risk (16) of having a child with a neural tube defect (NTD).

*To whom correspondence should be addressed. Tel: +1 352 381 6925; Fax: +1 352 846 0990; Email: epq@ufl.edu

© 2008 The Author(s)
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.0/uk/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Despite the potential importance of assessing global DNA methylation no 'gold standard' exists for its measurement. Cytosine extension assays (17) are motif specific, are unable to distinguish between hemi- and fully methylated motifs, and exhibit large inter- (~15%) and intra (~4%) assay variability. Methyl-acceptance assays (18) similarly exhibit large inter- (~9%) and intra (~5%) assay variability. For both assays intersample variability is probably higher, as both assays are dependent on the quality of the DNA sample (e.g. strand breaks, denatured DNA) which may vary among samples, and the reproducibility with which the DNA concentration can be measured (a single sample of homogenous quality and concentration is typically used to determine inter- and intraassay variability). Furthermore, both of these assays are only semiquantitative, as they provide only a relative measure of dCyt methylation (or rather a measure of unmethylated dCyt in the CpG motif), not an absolute measure. This makes comparisons of samples measured in different labs, or measured on different days, difficult.

In contrast, the LC-MS (or LC-MS/MS) methods provide a quantitative measure of dCyt methylation independently of DNA quality. However, most LC-MS methods lack a readily available and secure source of MdCyt internal standard. For example, while Friso *et al.* (19) used a labeled MdCyt internal standard when developing their LC-MS method, they needed a custom synthesis. To compensate for a lack of an internal standard, some developers of LC-MS methods have used external standards [intraassay %RSD = 4% (20)] or pseudo-internal standards, e.g. deoxyguanosine [%RSD = 1.7–9.2% (21,22)]. In the absence of an internal standard, no correction can be made for sample recovery during preparation and for changes in ionization efficiency between runs (e.g. due to ion suppression, detector contamination, changes in gas pressures, detector saturation or changes in running buffer).

Consequently, we devised a simple and inexpensive protocol for synthesizing [U-$^{15}N_3$]-labeled deoxynucleosides for use as LC-MS/MS internal standards. The starting materials and biological reagents used in the synthesis are readily available commercially (ensuring a secure source of internal standards for future studies), and the biosynthesis can be conducted in a laboratory equipped with standard biochemical or biological equipment. We report the use of these stable-isotopic [U-^{15}N]-labeled deoxynucleosides, specifically [$^{15}N_3$]dCyt and [$^{15}N_3$]MdCyt, as internal standards in a LC-MS/MS assay for the determination of percentage DNA methylation (%MdCyt).

EXPERIMENTAL SECTION

Biosynthesis and purification of [U-^{15}N]-labeled DNA

Cryopreserved *Escherichia coli* K12 was inoculated into 10-ml growth medium (23) containing 50 mM NaCl, 25 mM KH_2PO_5, 70 μM CaCl, 2.1 mM $MgSO_4$, 10 mM glucose and 50 mM [^{15}N]NH_4Cl (Cambridge Isotopes, Andover, MA), pH 7.2. The sample was incubated at 37°C in a shaking incubator. While in log-phase growth (~12-h growth) 10 μl of culture was used to inoculate 50 ml of fresh [^{15}N]-labeled growth medium. This second culture was grown to lag-phase (~24-h growth), at which point the bacteria were harvested by centrifuging at 300 *g* for 10 min. Bacterial DNA was extracted using a Bactozol Kit (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. The Bactazol Kit includes an RNA hydrolysis step allowing for the selective precipitation of DNA from cell lysates. The DNA pellet was washed twice with 75% v/v ethanol and reconstituted (~50 μg/ml) in 10 mM Tris–HCl, 50 mM NaCl, 1 mM dithiothreitol, 10 mM $MgCl_2$ (pH 7.9).

[U-^{15}N]DNA methylation

To increase [$^{15}N_3$]MdCyt abundance in the standard the [U-^{15}N]DNA was hypermethylated by adding (per 100 μg of DNA) 100 U SssI (CpG) methyltransferase (New England Biochemicals, Ipswich, MA) and 20 μl SAM (32 mM), and incubating at 37°C for 4 h.

Preparation of [U-^{15}N]internal standard

The hypermethylated [U-^{15}N]DNA was hydrolyzed to nucleosides (24) by adding 250 U Benzonase (Merck, Darmstadt, Germany), 300 mU phosphdiesterase (Sigma, St Louis, MO) and 200 U alkaline phosphatase (Sigma), and incubating at 37°C for 12 h. The percentage unlabeled deoxynucleoside was determined by analyzing a portion of the digested internal standard (equivalent to ~1 μg [^{15}N]DNA) by LC-MS/MS. The peak corresponding to the unlabeled (M + 0) and labeled (M + 3) deoxynucleosides was integrated. [In the case of the unlabeled (M + 0) peak this was mainly background noise]. The area of each unlabeled deoxynucleoside peak was expressed as a percentage of the corresponding M + 3 labeled deoxynucleoside peak.

DNA digestion

DNA was quantified using a PicoGreen kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Digest Mix (enough for 100 samples) was prepared using 250 U Benzonase, 300 mU phosphdiesterase, 200 U alkaline phosphatase, 200 μl of digested [U-^{15}N]DNA (equivalent to ~10 μg DNA) and 0.5 ml 10× Buffer [100 mM Tris–HCl, 500 mM NaCl, 100 mM $MgCl_2$ (pH 7.9)], and made up to 5 ml with water. DNA samples (1 μg in 50-μl water) were digested by adding 50 μl Digest Mix and incubating at 37°C for 6 h.

Liquid hromatography–tandem mass spectroscopy

Sample injection was 10 μl (equivalent to 100 ng DNA). DNA digests were chromatographed using a 3 μm Luna $C_{18}(2)$ column (50 × 3 mm, Phenomenex, Torrance, CA) eluted with 5 mmol/l ammonium acetate/0.1% (v/v) acetic acid using a methanol gradient at a flow rate of 0.5 ml/min. The methanol gradient increased linearly from 4% to 18% over 3.5 min. where it was held for 1.5 min, after which it returned to baseline (4%). There was a 1-min delay before the next injection—not allowing the column to fully equilibrate improved the

peak shape. Total run time (injection to injection) was 6 min.

Samples were analyzed in ESI mode using a triple quadrupole mass spectrometer. For the determination of isotopic nucleosides enrichment scanning was performed in positive ionization mode using selected reaction monitoring (SRM) mode, monitoring the mass to charge (m/z) transitions of dCyt: 228.1→112.0; [$^{15}N_3$]dCyt: 231.1→115.0; MdCyt: 242.1→126.0; [$^{15}N_3$]MdCyt 245.1→129.0. Chromatograms were analyzed using the Xcalibur software package (Thermo; Version 2.0).

Preparation of standard curves

Stock solutions of dCyt (Sigma) and MdCyt (ChemGenes Corporation, Wilmington, MA) were prepared in water. The concentration of the stock solutions was determined by diluting 1 in 10 with 0.1 M HCl and measuring their absorbance. The molar absorptivity coefficients (Є) used were: dCyt Є = 13 400 $M^{-1}cm^{-1}$ at 280 nm and MdCyt Є = 12 200 $M^{-1}cm^{-1}$ at 286 nm (25).

A stock mixture of the two standards was carefully prepared to give an exact known concentration ratio of dCyt to MdCyt (typically on the order of 12:1). The stock solution was further diluted to make a working solution. A standard curve was prepared from the working solution by serially dilution with water (Figure 1). To 50 µl of each standard dilution was added 50 µl of the Digest Mix (containing the [U-^{15}N] internal standards). Standard curves were prepared by plotting the analyte/internal standard ratio (M + 0/M + 3) against nucleoside concentration.

Determining percentage deoxycytidine methylation

The concentration of dCyt and MdCyt in each sample was calculated from the standard curves using the analyte/internal standard ratio (M + 0/M + 3) by reverse prediction. Percentage dCyt methylation (%MdCyt) was calculated by dividing the MdCyt concentration by the total dCyt concentration ([dCyt] + [MdCyt]).

Inter- and intraassay variability

Intraassay variability was determined by running six replicate DNA samples within the same run. Interassay variability was determined by running six replicate DNA samples on 6 consecutive days.

Effect of [U-^{15}N]DNA concentration on measured deoxycytidine methylation

To determine the effect of variations in the volume (e.g. due to pipetting error) of added [U-^{15}N]DNA internal standard had on %MdCyt, 50 µl of a stock dCyt and MdCyt mixture were spiked (in triplicate) with 25, 50 or 75 µl of the Digest Mix. Concentrations of dCyt and MdCyt were determined as described above, as was %MdCyt.

Preparation of stock DNA mixtures of varying percentage DNA methylation

Using a REPLI-g Midi kit (Qiagen, Valencia, CA) 100 ng of pooled lymphocyte DNA was amplified to ~40 µg according to the manufacturer's instructions. Amplification produces an unmethylated end product that, according to the manufacturer's literature, is essentially fully double stranded. The amplified DNA was divided, and half was methylated using SssI (CpG) methyltransferase (New England Biochemicals). The DNA was purified using a Qiaex II Gel Extraction Kit (Qiagen). The methylated and unmethylated DNA was mixed to give a series of samples with known ratios of methylated to unmethylated DNA. Percentage DNA methylation for each sample was determined by LC-MS/MS as described above.

Methyl-acceptance assay

DNA samples showing wide variability in methylation (as determined by LC-MS/MS) were identified from a folate intervention study (26). The relative abundance of unmethylated cytosines in the CpG motif in these samples was determined using a modification (27) of the Balaghi and Wagner [^{3}H]methyl-acceptance assay (28). In brief, DNA (0.5 µg) was incubated with *Sss*I methyltransferase (New England Biolabs, Beverly, MA) and [^{3}H]methyl *S*-adenosylmethionione (New England Nuclear, Boston) at 30°C for 4 h. The DNA was collected on a DE81 ion-exchange filter. After washing, to remove unincorporated

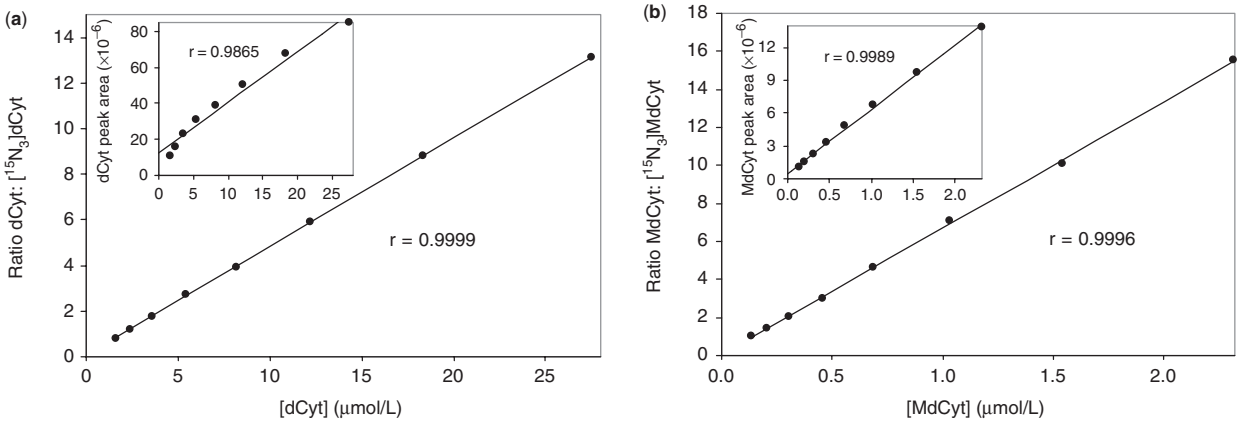

Figure 1. Standard curves for (**a**) deoxycytidine and (**b**) 5-methyldeoxycytidine, corrected for recovery of their respective [$^{15}N_3$]-labeled internal standard. Insets, uncorrected standard curves.

label, the dried filter paper was counted in a liquid scintillation counter. Each DNA sample was analyzed in triplicate. Typical inter- and intraassay variability determined using a pooled lymphocyte DNA sample were 10 and 7%, respectively.

RESULTS AND DISCUSSION

[U-^{15}N]internal standard

Using only a portion of the [U-^{15}N]-labeled DNA extracted from each 50 ml *E. coli* culture, we were able to produce several milligrams of hypermethylated DNA. After digesting to deoxynucleosides this produced enough [U-^{15}N]-labeled internal standard for several tens-of-thousands of assays. All of the components used are inexpensive and readily available commercially. This biosynthetic method produced highly enriched [$^{15}N_3$]dCyt and [$^{15}N_3$]MdCyt internal standards containing <0.5% of the unlabeled (M + 0) isotopomer. Similarly, the contribution of unlabeled dCyt and MdCyt standards to the M + 3 signal (e.g. due to natural enrichment) was minor (<0.1%).

Liquid chromatography–tandem mass spectroscopy

The chromatographic conditions gave good separation of dCyt and MdCyt (Figure 2). The relatively high specificity of the mass transitions employed yielded good signal-to-noise ratios with negligible appearance of contaminating peaks. [$^{15}N_2$]Deoxyuridine and [$^{15}N_2$]thymidine (derived from the [U-^{15}N]-labeled DNA) have, respectively, similar mass transition to [$^{15}N_3$]dCyt (231.1→115.0) and [$^{15}N_3$]MdCyt (245.1→129.0). However, their retention time (deoxyuridine = 1.59 min; thymidine = 3.13 min) were sufficiently dissimilar from dCyt (retention time = 1.20) and MdCyt (retention time = 1.94) so as not to interfere with the analysis. Furthermore, under normal experimental conditions [$^{15}N_2$]deoxyuridine was not present at detectable concentrations.

Standard curves

After adjusting for internal standard recovery (M + 0/M + 3), standard curves were linear over the range of interest (dCyt: 1–100 μmol/l; MdCyt: 0.1–10 μmol/l; $P \geq 0.999$) and over a range at least an order of magnitude higher and lower.

Inter- and intraassay reproducibility and variability

The assay exhibited good reproducibility and little inter- (4.36%; %RSD = 2.36%; $n = 6$) and intraassay (4.33%; %RSD = 2.49%; $n = 6$) variability. The importance of using an internal standard was evident from the large variability observed for the unadjusted dCyt and MdCyt peak areas (Table 1). This was particularly evident for MdCyt determination, where a large decrease in peak area (~25%) was observed between the third and subsequent samples, resulting in a large variation in peak area (%RSD ≈ 20%). However, as the response of the [$^{15}N_3$]MdCyt internal standard was similarly affected (as would be expected), the %RSD for the corrected MdCyt peak area (M + 0/M + 3) was only 2.54%.

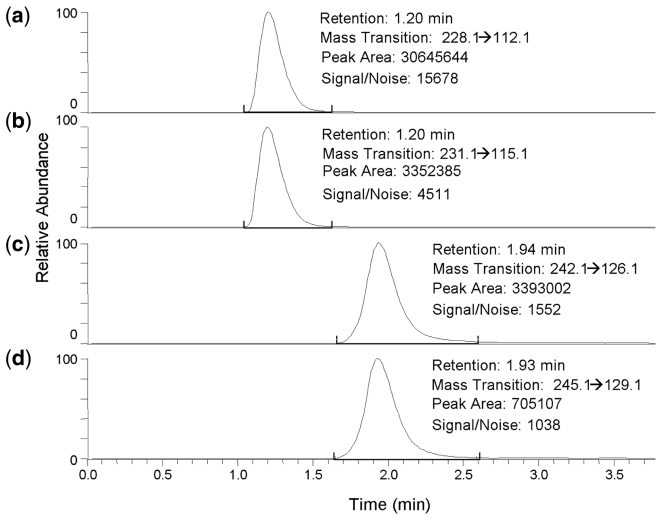

Figure 2. LC-MS/MS chromatogram of a typical sample, showing (**a**) deoxycytidine, (**c**) 5-methyldeoxycytidine peak, (**b**) and (**d**) their respective [$^{15}N_3$]-labeled internal standard peaks.

Table 1. An example of interassay peak area variability observed in the assay, and its correction using recovery of the [$^{15}N_3$]-labeled internal standard

Sample[a]	dCyt peak area			MdCyt peak area		
	Analyte[b]	[$^{15}N_3$][c]	Ratio[d]	Analyte[b]	[$^{15}N_3$][c]	Ratio[d]
1	99 681 858	12 915 824	7.72	20 661 241	4 261 932	4.85
2	96 111 965	12 214 937	7.87	20 358 454	4 175 934	4.88
3	100 721 386	13 179 525	7.64	19 816 002	4 174 776	4.75
4	116 390 992	14 718 118	7.91	15 388 665	3 056 475	5.03
5	109 791 299	15 053 509	7.29	13 830 853	2 859 798	4.84
6	99 047 865	13 201 706	7.50	13 496 698	2 888 077	4.67
Mean	103 624 228	13 547 270	7.66	17 258 652	3 569 499	4.84
%RSD	7.5	8.1	3.0	19.5	19.5	2.54

[a]Six replicates of a pooled sample were independently digested and analyzed consecutively by LC-MS/MS, as described in the text.
[b]Peak area of the analyst (M + 0).
[c]Peak area of the [$^{15}N_3$]-labeled internal standard (M + 3).
[d]Ratio of analyte (M + 0) to [$^{15}N_3$]internal standard (M + 3).

Table 2. The effect of varying the volume of [U-^{15}N]-internal standard added on the apparent concentrations of deoxycytidine (dCyt) and 5-methyldeoxycytidine (MdCyt), and on apparent percentage deoxycytidine methylation (%MdCyt)

	dCyt (μmol/l)		MdCyt (μmol/l)		%MdCyt[a]	
Stock	10.93[b]		0.93[c]		7.81	
Digest Mix[d]	Mean ± SD[e]	Accuracy (%)	Mean ± SD[f]	Accuracy (%)	Mean ± SD	Accuracy (%)
25 μl	20.83 ± 0.06	190.6	1.79 ± 0.08	192.5	7.91 ± 0.31	101.3
50 μl	10.58 ± 0.10	96.8	0.92 ± 0.02	98.9	8.00 ± 0.25	102.4
75 μl	6.98 ± 0.04	63.9	0.60 ± 0.01	64.5	7.95 ± 0.17	101.8

[a]Precentage MdCyt to total dCyt (MdCyt + dCyt).
[b]Final concentration of dCyt if 50 μl of Digest mix was used.
[c]Final concentration of MdCyt in a final volume of 100 μl.
[d]Volume of Digest Mix (containing digested [U-^{15}N]DNA as internal standard) added to sample (normal amount added to the sample is 50 μl).
[e]Measured concentration of dCyt, estimated from a standard curve using the M + 0/M + 3 ratio.
[f]Measured concentration of MdCyt, estimated from a standard curve using the M + 0/M + 3 ratio.

Effect of varying internal standard or sample DNA concentrations

As expected, the volume of internal standard added to the sample greatly affected (Table 2) apparent dCyt and MdCyt concentration. In contrast, %MdCyt values were independent of the internal standard concentration. Similarly, when changing the sample DNA concentration (using 0.5, 1.0 or 1.5 μg) the measured concentrations of dCyt and MdCyt increased with DNA concentration, while the %MdCyt was consistent (%RSD = 2.3%; data not shown). Consequently, the amount of internal standard or sample DNA used in the assay has little effect on the measured %MdCyt.

Linearity of DNA admix

The REPLI-g amplified product exhibited little (<0.1%) DNA methylation while the post methylated (with SssI) DNA had 3.94% DNA methylation. Admixes of the methylated and unmethylated DNA, containing different fixed ratios of the methylated and unmethylated DNA, exhibited (Figure 3) good linearity ($r = 0.9998$) when assayed by LC-MS/MS.

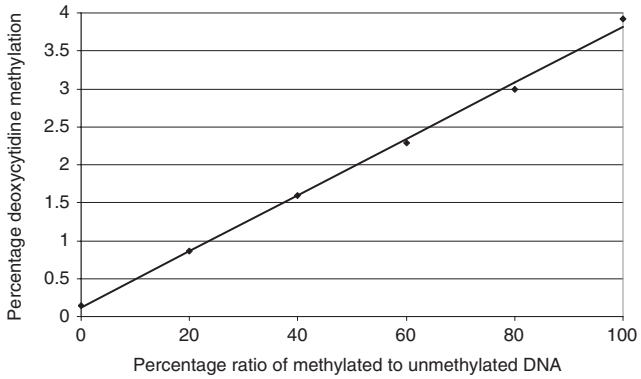

Figure 3. Percentage methylation of DNA admixes, each containing a fixed ratio of methylated to unmethylated DNA. The plot shows the percentage DNA methylation for each admix versus the percentage fully methylated DNA in each sample. DNA admixes were prepared by mixing known ratios of methylated and unmethylated DNA. The resulting mixtures were analyzed by LC-MS/MS to determine their percentage DNA methylation. Unmethylated DNA was prepared by amplifying genomic DNA as described in the text. Methylated DNA was prepared by methylating the amplified DNA with SssI (CpG) methyltransferase.

Comparison of LC-MS/MS and [^{3}H]methyl-acceptance assay results

Results from the LC-MS/MS assay and the [^{3}H]methyl-acceptance assay are not directly comparable as the LC-MS/MS assay quantitatively measures the percentage of methylated dCyt in the sample, while the [^{3}H]methyl-acceptance assay semiquantitatively measures the number of unmethylated dCyt in the CpG motif (e.g. the results from both assays are, at best, inversely proportional). However, within the confines of the assay (particularly the [^{3}H]methyl-acceptance assay) the two assays showed good agreement with each other (Table 3), with samples with a relatively low %MdCyt exhibiting a relatively high methyl-acceptance capacity.

Sample throughput and clinical applications

Using a Repeater Pipetter (Eppendorf) it was possible to add Digest Mix to each well of a 96-well plate in <2 min. In this manner >500 pre-plated samples (26,29) could be digested in a single day. These >500 samples could then be analyzed LC-MS/MS in <3 days, due to the short chromatography time for each sample (6 min. from injection to injection).

CONCLUSION

We have devised a simple and inexpensive protocol for synthesizing [U-$^{15}N_3$]-labeled deoxynucleoside. Using these [U-$^{15}N_3$]internal standards we have devised a LC-MS/MS protocol for the high-throughput determination of percentage DNA methylation. The one-step sample digestion procedure, and ability to prepare and analyze large numbers of samples (>500 samples) in a single assay, greatly reduce the risk of sample error and potentially limits intersample variability. Furthermore, as this method gives an absolute measure of DNA methylation (rather than the relative values determined by the cytosine extension or [^{3}H]methyl-acceptance

Table 3. Comparison methylation in human DNA determined by LC-MS/MS, or by [^{3}H]methyl-acceptance

	Methyldeoxycytidine[a] (%)	[^{3}H]methyl-acceptance (d.p.m./0.5 µg DNA)[b]
Normally methylated DNA		
Subject 1	4.46	10 044 ± 707
Subject 2	4.28	9 972 ± 699
Subject 3	4.42	13 598 ± 1292
Subject 4	4.26	10 230 ± 4165
Hypomethylated DNA		
Subject 5	0.46	33 300 ± 6138
Subject 6	0.54	87 794 ± 3071
Subject 7	0.65	66 555 ± 1905
Subject 8	0.93	45 800 ± 677
Controls		
Blank (no DNA added)	NA	139 ± 82
SssI methylated DNA[c]	5.71	364 ± 30
Human placental DNA[d]	4.13	13 022 ± 656
Human lymphocyte DNA[e]	4.33	6 794 ± 407
DNMT1$^{-/-}$, DMNT3b$^{-/-}$ DNA[f]	1.50	41,000 ± 964

[a]Percentage of 5-methyldeoxycytidine to total deoxycytidine, measured by LC-MS/MS.
[b]A relative measure of unmethylated CpG. Measured using Sss I methyltransferase and [methyl-^{3}H]SAM. Mean ± SD (n = 3)
[c]DNA was fully methylated using unlabeled SAM and SssI methyltransferase prior to the [^{3}H]methyl-acceptance assay.
[d]Human placental DNA (Sigma)
[e]Human lymphocyte DNA pool.
[f]DNA from a cell line containing a DNMT 1 and DNMT 3b double knock-out (30), cells exhibit ~5% normal dCyt methylation.

assays) results from different studies or different labs are directly comparable.

ACKNOWLEDGEMENT

The authors wish to thank David Maneval for performing the REPLI-g DNA amplification and [^{3}H]methyl-acceptance assays.

FUNDING

National Institutes of Health (DK72398, partial); General Clinical Research Center (MO1-RR00082); Florida Agricultural Experiment Station. Funding for open access charge: NIH [grant number DK 72398].

Conflict of interest statement. None declared

REFERENCES

1. Delgado,S., Gomez,M. and Bird,A. (1998) Antrquera, F. Initiation of DNA replication at CpG islnds in mammalian chromosomes. *EMBO J.*, **17**, 2426–2435.
2. Hsieh,C.-L. (1994) Dependence of transcriptional repression on CpG methylation density. *Mol. Cell Biol.*, **14**, 5487–5494.
3. Razin,A. (1998) CpG methylation, chromatin structure and gene silencing – a three-way connection. *EMBO J.*, **17**, 4905–4908.
4. Bartolomei,M.S. and Tilghman,S.M. (1997) Genomic imprinting in mammals. *Annu. Rev. Genet.*, **31**, 493–525.
5. Heard,E and Clerc,P. (1997) Avner, P. X-chromosome inactivation in mammals. *Annu. Rev. Genet.*, **31**, 571–610.
6. Gama-Sosa,M.A., Midgett,R.M., Slagel,V.A., Githens,S., Kuo,K.C., Gehrke,C.W. and Ehrlich,M. (1983) Tissue-specific differences in DNA methylation in various mammals. *Biochim. Biophys. Acta*, **740**, 212–219.
7. Ehrlich,M., Gama-Sosa,M.A., Huang,L.-H., Midgett,R.M., Kuo,K.C., McCune,R.A. and Gehrke,C. (1982) Amount and distribution of 5-methylcytosine in human DNA from different types of tissues or cells. *Nucleic Acids Res.*, **10**, 2709–2721.
8. Qu,G., Grundy,P.E., Narayan,A. and Ehrlich,M. (1999) Frequent hypomethylation in Wilms tumors of pericentromeric DNA in chromasomes 1 and 16. *Cancer Genet. Cytogenet.*, **109**, 34–39.
9. Bernardino,J., Roux,C., Almeida,A., Vogt,N., Gibaud,A., Gerbault-Seureau,M., Magdelenat,H., Bourgeois,C.A., Malfoy,B. and Dutrillaux,B. (1997) DNA hypomethylation in breast cancer: an independent parameter of tumor progression? *Cancer Genet. Cytogenet.*, **97**, 83–89.
10. Chen,R.Z., Pettersson,U., Beard,C., Jackson-Grusby,L. and Jaenisch,R. (1998) DNA hypomethylation leads to elevated mutation rates. *Nature*, **395**, 89–93.
11. Ji,W., Hernandez,R., Zhang,X.Y., Qu,G.Z., Frady,A., Varela,M. and Ehrlich,M. (1997) DNA demethylation and pericentromeric rearrangements of chromosome 1. *Mutat. Res.*, **379**, 33–41.
12. Friso,S., Choi,S.-W., Girelli,D., Mason,J.B., Dolnikowski,G.G., Bagley,P.J., Olivieri,O., Jacques,P.F., Rosenberg,I.H., Corrocher,R. *et al.* (2002) A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. *Proc. Natl Acad. Sci. USA*, **99**, 5606–5611.
13. Shrubsole,M.J., Gao,Y.-T., Cai,Q., Shu,X.O., Dai,Q., Hebert,J.R., Jin,F. and Zheng,W. (2004) MTHFR polymorphisms, dietary folate intake, and breast cancer risk: results from the Shanghai breast cancer study. *Cancer Epidemiol. Biomark. Prev.*, **13**, 190–196.
14. Gerhard,D.S., Nguyen,L.T., Zhang,Z.Y., Borecki,I.B., Coleman,B.I. and Rader,J.S. (2003) A relationship between methylenetetrahydrofolate reductase variants and the development of invasive cervical cancer. *Gynecol. Oncol.*, **90**, 560–565.
15. Oyama,K., Kawakami,K., Maeda,K., Ishiguro,K. and Watanabe,G. (2004) The association between methylenetetrahydrofolate reductase polymorphism and promoter methylation in proximal colon cancer. *Anticancer Res.*, **24**, 649–654.
16. Whitehead,A.S., Gallagher,P., Mills,J.L., Kirke,P.N., Burke,H., Molloy,A.M., Weir,D.G., Shields,D.C. and Scott,J.M. (1995) A genetic defect in 5,10-methylenetetrahydrofolate reductase in neural tube defects. *Q. J. Med.*, **88**, 763–766.

17. Axume,J., Smith,S.S., Pogribny,I.P., Moriarty,D.J. and Caudill,M.A. (2007) The methylenetetrahydrofolate reductase 677TT genotype and folate intake interact to lower global leukocyte DNA methylation in young Mexican American women. *Nutr. Res.*, **27**, 13–17.
18. Pufulete,M., Al-Ghnaniem,R., Leather,A.J., Appleby,P., Gout,S., Terry,C., Emery,P.W. and Sanders,T.A. (2003) Folate status, genomic DNA hypomethylation, and risk of colorectal adenoma and cancer: a case control study. *Gastroenterology*, **124**, 1240–1248.
19. Friso,S., Choi,S.-W., Dolnikowski,G.G. and Selhub,J. (2002) A method to assess genomic DNA methylation using high-performance liquid chromatography/electrospray ionization mass spectrometry. *Anal. Chem.*, **74**, 4526–4531.
20. Shelnutt,K.P., Kauwell,G.P., Gregory,J.F. III, Maneval,D.R., Quinlivan,E.P., Theriaque,D.W., Henderson,G.N. and Bailey,L.B. (2004) Methylenetetrahydrofolate reductase 677C–>T polymorphism affects DNA methylation in response to controlled folate intake in young women. *J. Nutr. Biochem.*, **15**, 554–560.
21. Liu,Z., Liu,S., Xie,Z., Blum,W., Perrotti,D., Paschka,P., Klisovic,R., Byrd,J., Chan,K.K. and Marcucci,G. (2007) Characterization of in vitro and in vivo hypomethylating effects of decitabine in acute myeloid leukemia by a rapid, specific and sensitive LC-MS/MS method. *Nucleic Acids Res.*, **35**, e31.
22. Song,L., James,S.R., Kazim,L. and Karpf,A.R. (2005) Specific method for the determination of genomic DNA methylation by liquid chromatography-electrospray ionization tandem mass spectrometry. *Anal. Chem.*, **77**, 504–510.
23. Quinlivan,E.P., McPartlin,J., Weir,D.G. and Scott,J.M. (2000) Mechanism of the antimicrobial drug trimethoprim revisited. *FASEB J.*, **14**, 2519–2524.
24. Quinlivan,E.P. and Gregory,J.F. (2008) DNA digestion to deoxyribonucleosides: a simplified one-step procedure. *Anal. Biochem.*, **373**, 383–385.
25. Dawson,R.M.C., Elliott,D.C., Elliott,W.H. and Jones,J.M. (1969) *Data for Biomedical Research*, 2nd edn. Oxford University Press, New York, pp. 169–179.
26. Quinlivan,E., Crider,K., Berry,R.J., Hao,L., Li,Z., Zhu,J.-H., Maneval,D., Young,T.P. and Bailey,L.B. (2008) Global DNA methylation changes in response to chronic consumption and withdrawal of low, moderate, and high folic acid doses. *FASEB J.*, **22**, 689.7.
27. Rampersaud,G.C., Kauwell,G.P., Hutson,A.D., Cerda,J.J. and Bailey,L.B. (2000) Genomic DNA methylation decreases in response to moderate folate depletion in elderly women. *Am. J. Clin. Nutr.*, **72**, 998–1003.
28. Balaghi,M. and Wagner,C. (1993) DNA methylation in folate deficiency: use of cpg methylase. *Biochem. Biophys. Res. Commun.*, **193**, 1184–1190.
29. Zhu,J.-H., Ling,L., Li,Z., Quinlivan,E., Maneval,D., Yang,T.P., Berry,R.J., Crider,K., Rasmussen,S. and Bailey,L.B. (2008) Low vitamin B12 status is negatively associated with global DNA methylation in young Chinese women. *FASEB J.*, **22**, 689.8.
30. Rhee,I., Bachman,K.E., Park,B.H., Jair,K.W., Yen,R.W., Schuebel,K.E., Cui,H., Feinberg,A.P., Lengauer,C., Kinzler,K.W. *et al.* (2002) DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature*, **416**, 552–556.