Hypersensitive quantification of global 5-hydroxymethylcytosine by chemoenzymatic tagging

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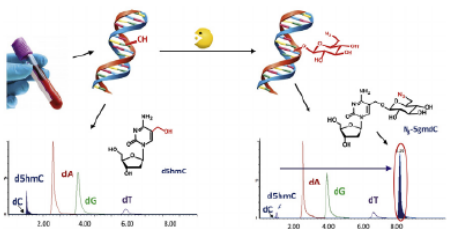
HIGHLIGHTS

- Specific chemoenzymatic tagging of 5hmC results in eight folds signal enhancement in LC-MS/MS measurements.
- A detection limit of 0.001% 5hmC from only 300 ng (9 fmol) DNA achieved with a basic LC-MS/MS device.
- A reduction in %5hmC in leukemia patients was detected relative to healthy individuals, from only 200 ng DNA.
- Proven reproducibility and accuracy of %5hmC levels in blood, solid tissues and synthetic DNA samples.
- A potential for routine 5hmC% detection in clinical laboratories, using cost-effective LC/MS-MS devices.

GRAPHICAL ABSTRACT



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ABSTRACT

5-hydroxymethylcytosine (5hmC) is an epigenetic DNA modification. Tissue-specific reduction in global 5hmC levels has been associated with various types of cancer. One of the challenges associated with detecting 5hmC levels is its extremely low content, especially in blood. The gold-standard for reliable global 5hmC quantitation is liquid chromatography-tandem mass spectroscopy (LC-MS/MS) operating in a multiple reaction monitoring (MRM) mode. Difficulties associated with 5hmC detection by LC-MS/MS include its low abundance, low ionization efficiency and possible ion suppression from co-eluted compounds. Hence, detecting 5hmC levels in blood samples for diagnosis of leukemia and other blood malignancies presents a unique challenge. To overcome these difficulties we introduce a simple chemoenzymatic method for specifically tagging 5hmC, resulting in an eight-fold increase in detection sensitivity. We demonstrate that we could quantitatively detect 5hmC levels in various human tissues, including blood samples from healthy individuals and leukemia patients, using the most basic quadrupole mass-analyzer instrument and only 200 ng of DNA. The limit of detection (LOD) of our technique is 0.001% 5hmC from 300 ng DNA, sufficient for future mass-spectroscopy based diagnostics of diseases associated with low 5hmC levels such as leukemia.

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1. Introduction

5-hydroxymethylcytosine (5hmC) is an epigenetic modification formed by the oxidation of 5-methylcytosine (5 mC) by the ten-eleven translocation (TET) family of enzymes, an iron- and α-ketoglutarate (α-KG)-dependent dioxygenases [1–3] (Scheme 1A). Levels of 5hmC are dynamic in non-differentiated cells from, e.g., pre-implantation embryos and in primordial germ cells, during development [4]. However, in fully differentiated cells, 5hmC was shown to be a stable DNA modification, with levels that are tissue-type specific [5–10]. An important observation concerning 5hmC levels is its association with various diseases. Studies have shown that 5hmC levels decrease significantly in several types of cancers [11] such as colon [10,12], melanoma [13], breast, prostate [14] and Leukemia [15–18], indicating the potential of 5hmC as a biomarker for early cancer diagnosis.

Various methods are used for detecting global 5hmC levels. The most common include dot blot [9,19], immunohistochemistry [14] and enzyme-linked immunosorbent assays (ELISA) [20,21]. These methods are based on anti- 5hmC and a secondary reporter antibody. They are simple to use, however, their limit of detection (0.02% 5hmC) is higher than the 5hmC percentage in various important tissues such as blood. Non-specific binding of α-5hmC and the requirement for several micrograms of starting DNA are additional drawbacks of these techniques [9]. Sequencing of captured 5hmC- containing fragments may provide information about the locus of the 5hmC modification. However, this technique is inaccurate for monitoring exact 5hmC levels: Fragments containing one to several 5hmC sites would contribute equally to 5hmC quantification [22–24]. Tet-assisted bisulfite whole genome or loci specific sequencing (TAB-seq) is a useful method for detecting both 5hmC levels and 5hmC location within the genome [25,26]. However, 5hmC requires extremely expensive high sequencing depth due to its low abundance and takes several weeks. In addition, the preparation of TAB- treated samples itself, is costly and elaborative. Recently, two high throughput colorimetric assays were developed for assessing 5hmC levels [6,27]. Both methods rely on the attachment of a glucose or a glucose azide molecule to the hydroxyl group of 5hmC, utilizing the highly specific T4 phage enzyme, β-glucosyltransferase (β-GT) (Scheme 1B) [6,22,27–31]. This high throughput assay is accurate and easy to use, however, it requires large amounts of starting DNA material (6 μg per sample).

Since the rediscovery of 5hmC in Purkinje neurons, in 2009 [1,32] liquid chromatography-mass spectroscopy (LC-MS/MS) has been considered as the "gold standard" technique for global 5hmC analysis in cell culture [33], different types of tissues [8,34] and even human urine, using ammonium bicarbonate (NH_4HCO_3) as an additive to the mobile phase [35]. This concept is related to the techniques' unique specificity and sensitivity. In addition, the sample preparation procedure is simple, requires low amount of DNA, the measurement is cost effective and takes only several minutes.

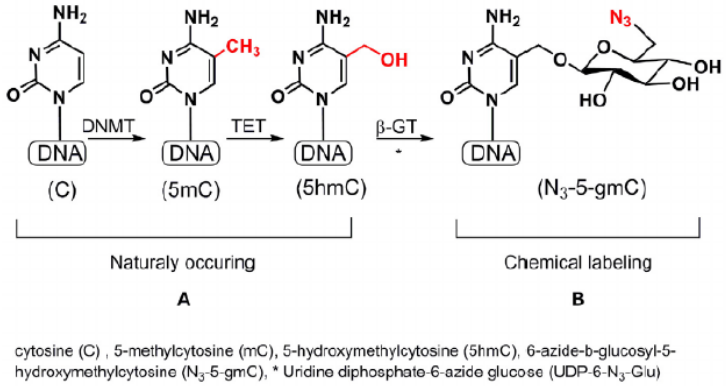
Although LC-MS/MS is most adequate for global 5hmC assessment, detecting 5hmC levels in tissues containing low 5hmC levels is challenging. The nucleoside, 5-hydroxymethyl-2′-deoxycytidine (5hmdC) suffers from relatively low ionization efficiency and possible ion suppression from co-eluted cytosine, which hinders 5hmC quantification. Hence, detecting 5hmC levels in Leukemia samples in which the already low 5hmC levels (of healthy blood) are even further reduced, is extremely challenging. Tang et al. used chemical derivatization for increasing LC-MS/MS sensitivity [36]. However, such derivatization is not specific to 5hmC and results in longer retention times for all nucleosides. The significantly increased sample run time is incompatible with high throughput analysis. Using top-end LC-MS/MS equipment for 5hmC quantification may also improve the LC-MS/MS sensitivity to 5hmC. However, many research and diagnostic laboratories do not have access to such costly top-end instruments.

Here we introduce a robust chemoenzymatic approach for specific labeling of 5hmC with a molecule of high ionization efficiency, to enhance the LC-MS/MS response to 5hmC. We utilize β-GT enzyme to selectively convert 5hmC to 6-azide-β-glucosyl-5-hydroxymethylcytosine (N_3-5gmC) (Scheme 1B). This labeling induced a significant increase in the LC-MS/MS sensitivity to 5hmC. With this method, we can detect reduced 5hmC levels in chronic lymphocytic leukemia (CLL) patients although utilizing a basic LC-MS/MS triple quadrupole instrument. This illuminates the technique's potential for routine high-throughput diagnosis of leukemia.

2. Experimental section

2.1. Reagents

Deoxynucleosides: 2′-Deoxycytidine (dC), 2′-Deoxyguanosine monohydrate (dG), 2′-Deoxyadenosine monohydrate (dA) and



cytosine (C) , 5-methylcytosine (mC), 5-hydroxymethylcytosine (5hmC), 6-azide-b-glucosyl-5-hydroxymethylcytosine (N_3-5-gmC), * Uridine diphosphate-6-azide glucose (UDP-6-N_3-Glu)

Scheme 1. Epigenetic modifications of the cytosine base (A) and chemoenzymatic labeling of 5hmC (B).
Scheme 1. A) Structure of cytosine (C) and its epigenetic transformations: Cytosine methylation is catalyzed by DNA methyltransferase (DNMT) resulting in the formation of 5-methylcytosine (mC), mC oxidation to 5-hydroxymethylcytosine (5hmC) is catalyzed by TET family of enzymes. The process described above occurs naturally in genomic DNA. B) 5hmC labeling with a glucose azide is catalyzed by β-glucosyltransferase (β–GT) utilizing uridine diphosphate-6-azide glucose (UDP-6-N_3-Glu).

Thymidine (dT), were from Sigma-Aldrich. Modified cytosine nucleosides; 5-Methyl-2′-deoxycytidine (5mdC) and 5-hydroxymethyl-2′-deoxycytidine (5hmdC), were from Caymanchem (Michigan, USA) and from Carbosynth (Compton- Berkshire, UK), respectively. HPLC grade water, LC-MS grade acetonitrile, and formic acid were from Sigma. Proteinase K used for DNA extraction was from Sigma—Aldrich; absolute ethanol and 2-propanol were from Merck (Whitehouse Station, NJ, USA). The enzymatic glucosylation reaction was performed using T4 Phage β-GT, 10 × NEB buffer 4 and uridine diphosphate glucose (UDP-Glu), all from New England Biolabs (NEB, Ipswich, MA, USA) or with Uridine diphosphate-6-azide-glucose (UDP-6-N_3-Glu), synthesized in-house according to Nifker et al. [30] (can also be purchased from Jena Bioscience). PCR fragments for the standard samples were prepared from: My Taq Red Mix (Bioline, London, UK), Lambda DNA template (NEB) and primers from Integrated DNA Technologies (IDTs, Coralville, IA, USA). PCR fragments for calculating the labeling efficiency were prepared from 5-hydroxymethyl-dCTP (Bioline), dATP, dGTP, dTTP, and dCTP (Sigma—Aldrich), Vent (exo-) DNA polymerase in ThermoPol Reaction Buffer Reagents from NEB and primers from IDTs. PCR fragments were purified on a Qiaquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany). The length of the DNA fragments was determined by electrophoresis on 2% agarose gels (Ameresco, Framingham, MA) pre-stained with SYBR Safe DNA gel stain (Life Technologies). DNA digestion was performed by Nuclease S1 and phosphodiesterase from Sigma-Aldrich and Antarctic Phosphatase, from NEB.

2.2. Patients and study design

Commercially available DNA, from human peripheral blood cells, was purchased from (BioServe, Reprocell, Beltsville, MD, USA). We measured DNA from five healthy individuals and four chronic lymphocytic leukemia (CLL) patients, men, and women 49—82 years old. A peripheral blood sample from a healthy human donor was collected with informed consent for research use and approved by the Tel-Aviv University and Meir medical center ethical Review Boards, in accordance with the declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll Paque Plus (GE Healthcare) according to the manufacturer's instructions.

2.3. Extraction of genomic DNA from peripheral mononuclear blood cells (PBMCs)

DNA was extracted from solid tissue samples and from PBMCs using the archive pure DNA tissue kit (5 Prime, Hilden, Germany) according to the manufacturers' protocol with minor variations: Digestion with proteinase K was carried out overnight at 55 °C. The protein precipitation step was repeated at least three times, each followed by incubation on ice for 30 min. These repeated long incubation steps on the ice were essential for obtaining protein-free DNA samples. Extracted DNA was hydrated in autoclaved, nuclease-free water and its concentration was determined using a spectrophotometer (Pearl UV/vis NanoPhotometer; Implen, Munich, Germany); the absence of protein and salt impurities was verified by values of A_{260}/A_{280} 1.8 and A $_{260}/A_{230}$ 2.0.

2.4. Preparation of 5hmC standards

The percentage of 5hmC in genomic DNA samples was interpolated from a calibration curve formed by a linear regression plot, fitted to a set of DNA standards with known 5hmC percentages (Fig. S4). The standards were prepared by a mixture of two types of synthetic 1 kb DNA PCR fragments; "cold" and "hot". The "cold" fragments were 1 kb long containing no 5hmC, and the "hot" fragments were prepared from forward and reverse primers, each containing internal 5hmC. Hence, 100% "hot" PCR fragment is a 0.1% 5hmC/total nucleobases (in 1 kb "hot" PCR fragment, two 5hmC for every 2000 bases). A typical PCR-amplification reaction was performed in a 50-μL volume containing 200 ng of Lambda DNA as a template, 1 × My Taq Ready Mix, 0.4 μM of forward primer, 5′-CTCATGCTGAAAACGTGGTG-3′ and 0.4 μM of reverse primer 5′-GGACAGGACCAGCATACG-3'. In a PCR reaction of a "hot" 1 kb fragment, the forward and reverse primers were: 5′-CTCATGCTGAAAA/i5HydMe-dC/GTGGTG and 5′-GGACAGGAC/i5HydMe-dC/AGCATACG, respectively. The PCR reaction was carried out on Eppendorf MC Pro gradient S, using the following cycling protocol: incubation at 95 °C for 1 min as an initial step, followed by 35 cycles of 30 s at 95 °C, 60 s at 53 °C, and 60 s at 72 °C, and finally 5 min at 72 °C. Free nucleotides and primers were removed from PCR products using a QIAGEN QIAquick PCR purification kit. The length of the DNA fragments was confirmed by gel electrophoresis at 50 V for 60 min on 1% agarose gel pre-stained with SYBR Safe (Fig. S1). Standards with physiologically relevant 5hmC percentages (0.002—0.1%) were obtained by appropriate mixing between the "cold" and "hot" PCR fragments. To each mixed standard, 5 mC nucleosides were added at physiological percentages of 1—4%.

2.5. Tagging of 5hmC

The labeling procedure of 5hmC with glucose or glucose azide proceeded as follows: DNA was mixed with 3 μL 2 mM UDP-Glu or 2 μL of 3 mM UDP-6-N_3-Glu (200 μM final concentration), 1 μL β-GT (10 units), 1 × NEB buffer 4, and Milli-Q water to a final volume of 30 μL, and incubated overnight at 37 °C. The labeled 5hmC nucleobases are converted to β-glucosyl-5-hydroxymethylcytosine (5gmC) and N_3-5gmC, respectively. The negative control was a 5hmC-free 1 kb DNA fragment that was treated according to the same labeling protocol.

2.6. DNA hydrolysis for LC-MS/MS measurements

Genomic and PCR-amplified DNA samples were hydrolyzed by two steps of enzymatic reaction. In the first step, 1 × Nuclease S1 buffer and 2.4 μL 1 M HCl (for naturalizing the NEB buffer 4) were added. These DNA samples were heat denatured for 5 min at 98 °C and then directly put on ice for 2 min followed by the immediate addition of 100 U Nuclease S1 and incubation at 37 °C for 3 h. In the second step, 5 U antarthicphosphetase and 0.1 U phosphodiesterase were added in the presence of 18 mM NaOH and 1 mM MgCl and incubated overnight at 37 °C to complete the DNA hydrolysis. Enzymes inactivation was performed by incubation for 10 min at 80 °C in the presence of 33 mM EDTA. The resulting nucleoside mixtures were filtered by amicon ultra 0.5 ml 10 K preconditioned columns (Merck), the eluted solution was applied to glass vials with inserts and 10 μL was injected into the LC-MS/MS system. Throughout the text, the non-hydrolyzed nucleobases are referred to as 5hmC and N_3-5gmC or 5gmC, in the labeled form and the hydrolyzed nucleosides are referred to as 5-hydroxymethyl-2′-deoxycytidine (5hmdC), 6-azide-β-glucosyl-5-hydroxymethyl-2′-deoxycytidine (N_3-5gmdC) or β-glucosyl-5-hydroxymethyl-2′-deoxycytidine (5gmdC).

2.7. LC-MS/MS system, conditions, and analysis

The chromatographic separations were performed on an Acquity UPLC system (Waters) using Xselect HSS T3 column (2.5 μm, 2.1 × 75 mm, Waters), which was maintained at 40 °C. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1%

formic acid in acetonitrile (solvent B). Nucleosides were eluted at a flow rate of 0.3 ml/min with a gradient of 100% solvent A 0 1 min, then linear increase 1.0 2 min to 4.0% solvent B, 2.0 4 min to 9.0% solvent B and 4.0 5 min to 20.0% solvent B. mobile phase was maintained at 20% solvent B up to 5.5 min. The composition was then returned to the initial conditions at 5.51 min and held under these elution conditions up to 8.0 min. The effluent from the column was directed into an electrospray ion source and then streamed into a Xevo TQD triple quadrupole mass spectrometer (Waters). The measurements of targeted nucleosides were conducted in a positive ion mode by multiple reaction monitoring (MRM) with the following transitions: dC m/z 228 112, 250 134, dT m/z 243 117, 127, dA m/z 252 136, 119, dG m/z 268 135, 152, 5hmdC m/z 258 124, 142, 5mdC m/z 242 126, 264 148, 5gmdC m/z 421 269, N $_3$-5gmdC m/z 445 124, 329. The MRM parameters (e.g., cone and collision cell voltage) of each nucleoside were automatically optimized to achieve maximal detection sensitivity.

The relative content of modified cytosine in each sample was calculated from the MRM peak area of 5mdC or 5hmdC, (mostly monitored as labeled 5hmC: 5gmdC or N_3-5gmdC) divided by the sum of peak areas of all other nucleosides or by the peak area of dG. This procedure allowed the normalization of concentration discrepancies in injected DNA samples. The relative 5hmC or 5 mC contents were converted to actual %5 mC or %5hmC by extrapolation from calibration graphs prepared from standard samples of known 5hmC and 5 mC percentages, as described in 2.4 and in Fig. S4. The total amount of injected DNA in each sample was calculated by extrapolation from a second calibration graph of standard samples containing different known concentrations of total nucleosides. Each set of samples was accompanied by a 5hmC and 5 mC% calibration graphs and a negative control sample containing neither 5hmC nor 5 mC, to account for slight variations in the labeling efficiency and the instrument performance. The amount of DNA in each standard is in accordance with the amount of DNA in the analyzed sample.

2.8. Calculating tagging ef ciency

Synthetic DNA fragments in which all cytosines were replaced by 5hmC were used to measure the efficiency of 5hmC tagging and are referred to as all-5hmC-1kb, (supporting information, table S2, Figs. S2 and S3). Calculation of the labeling efficiency was conducted by measuring the percentage of the remaining unlabeled 5hmC content, following a labeling reaction, and the subtraction of that value from one hundred percent labeling efficiency as illustrated in Fig. S3 and Table S2. Unlabeled, remaining 5hmC percentages were calculated from the ratios of normalized 5hmdC signals obtained from labeled and from non-labeled all-5hmC-1kb fragments, respectively. The 5hmdC signals were normalized to the signals obtained for all nucleosides in the digested sample.

The synthetic DNA fragments were prepared by a PCR reaction in a total volume of 50 µL which contained: 0.5 µM of forward primer: 5′- CTCATGCTGAAAACGTGGTG-3′ and 0.5 µM of reverse primer: 5′- GGACAGGACCAGCATACG -3′, 1 unit of Vent (exo-), 200 µM of dATP, dGTP, dTTP, and hydroxymethyl-dCTP nucleotides and 200 ng lambda DNA (as a template) in 1 × NEB ThermoPol buffer. PCR (Eppendorf MC Pro gradient S) was carried out using the following cycling protocol: incubation at 95 °C for 300s as an initial step, followed by 35 cycles of 30 s at 95 °C, 30 s at 53 °C, and 90 s at 72 °C, and finally 600 s at 72 °C. Free nucleotides and primers were removed from PCR products using a QIAGEN QIAquick PCR purification kit. The length of DNA fragments was confirmed by gel electrophoresis at 50 V for 60 min on 1% agarose gel pre-stained with SYBR Safe.

2.9. Method validation

2.9.1. Accuracy, precision, and linearity of the calibration graphs

The accuracy of our method was determined by comparing the nominal %5hmC values of four different standards to the observed values calculated from the linear regression curve of the calibration graph. The equation used for accuracy calculations is:

$$100 - \left(\frac{(nominal\ \ alue - obser\ ed\ \ alue)^2}{nominal\ \ alue} \times 100 \right.$$

The precision of the standards is of samples from different preparations and presented as %coefficient of variance (%CV). The data represents an average of two to three different samples for each standard having the same amount of 5hmC content and total DNA (within CV < 8).

The linearity of the calibration curves was evaluated using the coefficient of determination (R-square) from nine different calibration graphs.

Intraday and interday precision are calculated from sequential measurements of 200 ng DNA sample, extracted from blood and presented as %CV.

2.9.2. Limit of quanti cation

The limit of quantification (LOQ) was obtained by the sequential dilution of 0.001% standard sample. The LOQ of a 0.001% 5hmC sample, is determined as the DNA concentration at which the peak to peak, signal to noise ratio (S/N) is higher than 10 and accuracy is between 80 and 120% of nominal value (Table S3, and Fig. S5). The limit of detection (LOD) of a 0.001% 5hmC sample is determined as the DNA concentration at which the peak to peak, signal to noise ratio (S/N) is higher than 5.

3. Results and discussion

3.1. Effect of dC nucleoside on the LC-MS/MS response to 5hmdC

The assessment of 5hmC levels in tissues containing low 5hmC content is challenging due to two main factors that reduce the LC-MS/MS sensitivity: The first is related to the low ionization efficiency of 5hmdC [35–37], a process required for its efficient transfer into the detector. The second factor is related to the chromatographic separation between the rare 5hmdC and the abundant dC nucleosides. Good chromatographic separation of these analytes is essential for removing interference from abundant dC nucleoside leading to ion suppression of 5hmdC. However, the chromatographic separations of 5hmdC and dC are, in many cases, unsatisfactory as they are closely eluted or co-eluted off the LC column [38,39].

Fig. 1A shows superimposed chromatograms of the MRM transitions of dC and 5hmdC, nucleosides, from a mixture containing 0.02% 5hmdC/total nucleosides (780 nM, 2 ng per injection) and 1 mM of dC, dG, dA and dT (2.3, 2.7, 2.5, 2.4 µg per injection of dC, dG, dA and dT, respectively). The response to 5hmdC was enlarged by 36 times for visualization and the co-elution of dC and 5-hydroxymethyl-2′-deoxycytidine (5hmdC) is clearly observed. In Fig. 1B we have examined the effect of dC on the response of LC-MS/MS to 5hmdC. We prepared samples which contained standards at constant concentrations of 2 µM 5hmdC (5.1 ng in each injection) and 1 mM of dG, dT, dA nucleosides (2.7, 2.5, 2.4 µg dG, dA, and dT, respectively, per injection), with increasing concentration of dC from 50 µM to 1 mM (from 115 ng to 2.3 µg, per injection). Our results show an attenuation of the LC-MS/MS response to 5hmdC with increasing dC concentration, indicating that the presence of

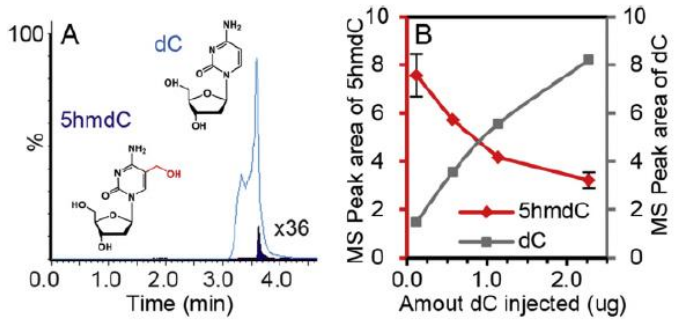


Fig. 1. A) superimposed chromatograms of 1 mM, (2.3 μg dC per injection) and 0.02% (780 nM, 2 ng per injection) 5hmdC standard, monitored by LC-MS/MS in MRM mode. The 5hmdC signal shown is a 36 times enlargement for visual clarity. B) The MS/MS response (presented as the peak area of dC or 5hmdC) to a constant concentration of 2 μM 5hmdC (5.1 ng per each injection) as the amount of dC injected to the LC increases.

dC leads to suppression of 5hmdC ionization as they co-elute from the LC- column. This phenomenon is strongest when working with large amounts of DNA, with low 5hmC content. One of the problems arising from this observation, in quantitative studies, is that the 5hmdC signal may not only correlate with 5hmdC concentrations but also with the amount of dC (and hence DNA) injected to the LC-MS/MS. The LC-MS/MS responses to all other nucleosides (dT/dA/dG), which are well separated from dC in the chromatogram, were not affected by dC concentration in the sample (data not shown). Many studies use spiked in stable isotope internal standard to calibrate the signal for co-eluted abundant nucleotides and matrix-interferences. However, the attenuation of the 5hmdC signal due to ion suppression and the low ionization efficiency of 5hmdC, introduce major sensitivity limitations which require a different approach when assessing 5hmC levels in tissues containing low amounts of 5hmC.

3.2. Enhancing LC-MS/MS sensitivity by chemoenzymatic labeling of 5hmC

In order to increase the sensitivity of the LC-MS/MS to 5hmC detection, we utilize a robust chemoenzymatic approach for specifically tagging 5hmC with a molecule of high ionization efficiency. In this approach, 5hmC is linked to a sugar molecule in a single step, specific enzymatic reaction, using β-GT and UDP-Glu or UDP-6-N_3-Glu (synthesized in-house [30]), resulting in the formation of 5gmC and N_3-5gmC (Scheme 1), respectively. Fig. 2A shows superimposed chromatograms of MRM signals to five nucleosides (dC, 5hmdC, dA, dG, dT) prepared by enzymatic digestion of 1 kb PCR fragments in which all Cs were replaced by 5hmC apart from the Cs on the primers (referred to as all-5hmC-1kb PCR fragment). The LC-MS/MS response obtained for 5hmdC is much lower than the response for the other nucleosides, although very little dC is in the sample, indicating that 5hmC has low ionization efficiency regardless of interference from abundant dC. Fig. 2B shows superimposed chromatograms of nucleosides digested from all-5hmC-1kb DNA fragments, which were selectively pre-labeled with a glucose moiety by β-GT (yielding all-5gmC-1kb). This labeling procedure has resulted in a shift of the retention time from 0.92 min (for 5hmdC) to 1.46 min (for 5gmdC). However, the signal response obtained for 5gmdC was five times lower than that of 5hmdC indicating that the 5gmdC ionization efficiency is even lower than that of 5hmdC. Specific labeling of all-5hmC-1kb with glucose azide (yielding all-N_3-5gmC-1kb) resulted in a significant shift in retention time (from 0.92 min to 8.26 min) and a four-fold

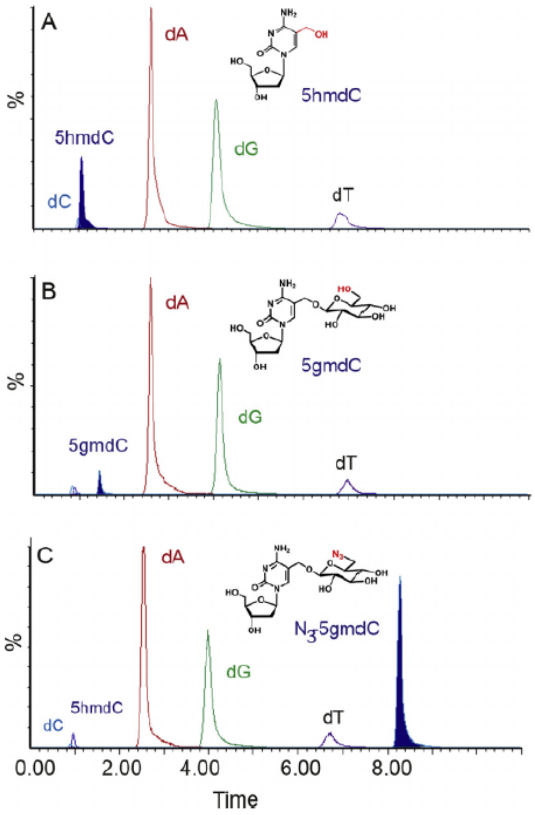


Fig. 2. Superimposed MRM chromatograms of dC, 5hmdC, dA, dG, dT nucleosides, digested from synthetic 1 kb DNA fragment in which all cytosine nucleotides were replaced by 5hmC, (all-5hmC-1kb) (A) no pre-treatment, (B) all-5hmC-1kb was enzymatically labeled with glucose before digestion, yielding 5gmC-1kb and (C) 5hmC-1kb was chemoenzymatically labeled with glucose azide before digestion, yielding N_3-5gmC-1kb. The blue filled peaks are for 5hmdC and its derivatives. The light blue outlined peaks are for the cytosine nucleotides in the primers, and the dark blue outlined peaks in B and C are for 5hmC that were not labeled by glucose and glucose azide, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

increase in the LC-MS/MS sensitivity to 5hmC, as seen in Fig. 2C. We attribute the increase in sensitivity to the addition of an easily chargeable $-N_3$ group. Also, due to the longer retention time of N_3-5gmdC on the LC column, its extraction occurs with a more volatile, higher ratio organic mobile phase. Thus, allowing more efficient ionization of N_3-5gmdC in ESI due to better desolvation. A significant 5hmC signal enhancement was also observed by Tang et al. following the addition of an easily chargeable tertiary amine group and a hydrophobic phenyl group to 5hmC, by chemical derivatization with 2-bromo-1-(4-dimethylamino-phenyl)-ethanone (BDAPE) [36]. However, the BDAPE labeling reaction is not specific to 5hmC; it increases the hydrophobicity of dC, dA and all cytosine oxidation products. Hence, the chromatographic separation between abundant dC and 5hmdC is not optimal and the retention times are longer, resulting in longer run time per sample, which is incompatible with high throughput analysis. Our simple, specific tagging procedure, changes the retention time of only the required analyte, hence preventing interference from similarly structured co-eluted nucleosides, while preserving short measurement duration.

3.3. Comparison of LC-MS/MS response to non-labeled and labeled 5hmC signals in physiologically relevant concentrations

The 5hmC content in fully differentiated healthy cells is constant and tissue-type specific ranging from 0.2%, in the brain to 0.004% 5hmC/total nucleotides, in blood. The sensitivity and quantitative capabilities of LC-MS/MS to 5hmC at this range (60 pg–6 ng 5hmC from 3 μg DNA per injection) were examined. Calibration graphs were prepared from standard samples, containing known ratios of 5hmC. According to the slope of the calibration graphs, the response of LC-MS/MS to N_3-5gmdC was eight times higher than that of 5hmCs (Fig. 3A).

The insert at the top of Fig. 3A illustrates the capability of the LC-MS/MS to detect extremely low glucose azide tagged 5hmC content (N_3-5gmdC) allowing the distinction between control, 0.002% and 0.004% 5hmC samples, a range of values associated with leukemia blood samples. The LC-MS/MS response to non-tagged or glucose labeled 5hmC did not reach this sensitivity and hence cannot be used for blood samples analysis.

Fig. 3B shows the difference between the signal to noise ratios (S/N) of the peaks obtained for 0.002% 5hmdC (left), 5gmdC (middle) and N_3-5gmdC (right) samples, digested from 3 to 3.5 μg DNA. The signal to noise ratio of N_3-5gmdC is approximately 2.5 times higher than that of 5hmdC.

The accessibility of DNA samples from human patients is often limited. Hence, our goal is to be able to use as low as 200 ng DNA per LC-MS/MS measurement. Considering that the signals in Fig. 3 were obtained from 3 to 3.5 μg DNA, the S/N needed to reach the required limit of quantification (LOQ) could not be achieved with non-modified 5hmC samples. However, labeling 5hmC with glucose azide may allow such measurements.

3.4. Ef ciency of the chemoenzymatic labeling reaction

The efficiency of the enzymatic labeling reaction was determined as 87.6 ± 3.4% for glucose azide, (N_3-5gmC) and 91.2 ± 5.5% for glucose, (5gmC) (Fig. S3 and Table S2). The high reaction efficiency supports the method's suitability for detecting 5hmC in samples containing low 5hmC levels.

3.5. Method validation

3.5.1. Accuracy, precision, and linearity of the calibration graphs

The high accuracy and precision of our method are represented in Table 1, 0.002% 5hmC from 267 ng DNA (16.2 fmol) samples were reliably quantified with an accuracy of 90 ± 1%. This value is below the required limit of quantification (LOQ) for blood cancer studies. Our method is also suitable for detecting 0.001% 5hmC in 282 ng

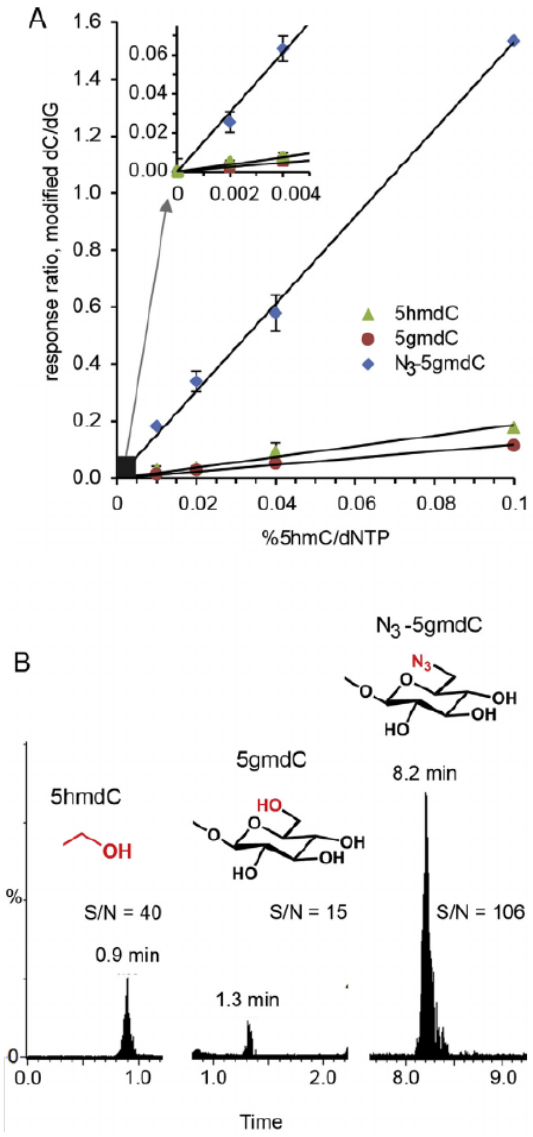


Fig. 3. (A) Calibration graphs prepared from standards containing 0.002%–0.2% 5hmC. The green marks are for non-modified 5hmC; the red and blue marks are for 5hmC labeled with glucose (5gmdC) or with glucose-azide (N3-5gmdC), respectively. The top insert is an enlargement of the lower scale of the 5hmC percentage. (B) Signal to noise ratios of the LC-MS/MS response obtained for 0.002% 5hmdC (left), 0.002% 5gmdC (middle) and 0.002% N3-5gmdC (right), calculated by the peak to peak method. 5hmdC peaks were normalized to the peak areas of dG in each sample. An amount of 3–3.5 μg total DNA was used for each measurement. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Accuracy and precision of standards quantification.

Nominal %5hmC	Observed %5hmC	Total DNA (ng)	Nominal 5hmC (fmol)	% Accuracy[a]	% Precision[b]
0.001	0.0006 ± 0.0001	282	8.6	62 ± 12	16.7
0.002	0.0020 ± 0.0003	267	16.2	90 ± 1	15.0
0.005	0.0043 ± 0.0003	251	38.0	86 ± 7	7.0
0.01	0.0106 ± 0.0001	233	69.8	94 ± 5	0.9

[a] Accuracy is $100 - \left(\frac{(nominal\ \ alue - obser\ ed\ \ alue)^2}{nominal\ \ alue}\right) \times 100$.

[b] Precision is the coefficient of variance (%CV).

DNA (8.6 fmol); however, due to its relatively low accuracy, quantifying samples with this amount of 5hmC may not be reliable.

Each set of samples is accompanied by a calibration graph and a negative control which is a 5hmC and 5 mC- free DNA sample. None of the negative controls had a signal at the retention time of N_3-5gmdC. An average of 0.9905 R-square with a CV of 0.68% was determined for linear regression curves fitted to calibration standard sets (Fig. S4). The good linear fit and the high accuracy of our method at extremely low 5hmC contents emphasis its suitability for measuring 5hmC content in tissues containing low 5hmC levels.

3.5.2. Limit of quanti cation

The limit of quantification was assessed for 0.001% 5hmC, the lowest point in our calibration graphs. The quantification accuracy of 0.001% 5hmC from 282 ng DNA was relatively low; hence, we checked what would be the minimal amount of total DNA, required for reliable 0.001% quantification. Our results show that 0.001% 5hmC levels can be reliably quantified from 474 ng DNA samples (14 fmol) with a peak to peak (PtP) signal to noise ratio (S/N) of 21 ± 7 and an accuracy of 80 ± 13% (Table S3 and Fig. S5). We determine this value as our limit of quantification (LOQ). Our limit of detection (LOD) is 0.001% 5hmC from 297 ng DNA (9 fmol) with PtP S/N of 7 ± 2 (Table S3).

A recent review by Chowdhury et al. summarizes the advances in the global 5hmC analysis [40]. Key techniques and their sensitivities are presented in supporting information (table S4). The most sensitive commercial ELISA kit has a LOD of 0.02% 5hmC in 100 ng DNA [41]. This sensitivity is insufficient for detecting blood malignancies. Our method was found to be at least seven times more sensitive than the commercial ELISA kit. In addition, a single molecule study conducted by Gilat at el. showed inconsistencies between the 5hmC levels measured using the ELISA kit and the 5hmC levels measured by the single molecule method and several other LC-MS studies [12]. Recently Chowdhury et al. [42] have developed an enhanced ELISA method based on biotin-avidin mediated enzyme-based immunoassay (EIA), with a 5hmC LOD of 1.6 fmol. However, the 5hmC% calculated by this method, for blood gDNA, was 0.023 ± 0.006%, about five times the reported 5hmC percentage in blood. LC-MS/MS yet remains the most accurate, reproducible and sensitive technique for detecting global 5hmC levels. Tsuji et al. have detected 0.001% 5hmC in 200 ng DNA with a LOD of 6 fmol [33] using an MS device which is 10 times more sensitive and 30% more expensive than the MS device used in this study. Using our signal enhancement method we have managed to reach the same sensitivity as reported by Tsuji et al.

Since our LOQ is below the required sensitivity for quantifying 5hmC content in cancer and healthy blood samples, ranging from 0.0035 to 0.006% 5hmC/total nucleosides we assumed that we would be able to use as low as 200 ng DNA per measurement, as discussed in section 3.5.3.

3.5.3. Intraday and interday precision of %5hmC in healthy PBMC

The quantitative reliability of our chemoenzymatic 5hmC modification technique has been further examined in blood samples. The intraday and interday precision of 5hmC in peripheral blood mononuclear cell (PBMC) sample was 11% and 8%, respectively (Table 2). The low variations are attributed to the relatively high signal to noise ratio of 70 ± 4 obtained following 5hmC detection from only 200 ng digested DNA. We have measured 0.0043 ± 0.0004% 5hmC content in PBMC, in good agreement with other studies [12,17,43]. A typical chromatogram of DNA from blood is presented in Fig. 4.

3.6. Quanti cation of 5hmC in mouse tissues

The 5hmC content in various mouse tissues was measured following labeling of 5hmC with glucose azide. The values of 5hmC % in each tissue were calculated by extrapolating the peak area ratios of N_3-5gmdC to dG, from a calibration graph (Fig. S6). These results are in agreement with 5hmC values we have obtained using a colorimetric assay as published by Shahal et al. [6,27] and with other tissue-dependent 5hmC studies [7,9,44]. The colorimetric assay is a simple approach to 5hmC quantification, which does not require costly, specialized instrumentation nor expertise; however, it requires large quantities of DNA per sample (6 μg). The LC-MS/MS technique required only 40 ng for the brain tissue and few hundreds of ng for the low-level 5hmC tissues.

3.7. Detecting 5hmC levels in DNA from healthy and CLL blood samples

Following validating the qualitative reliability of our method in blood, we examined whether we can differentiate between healthy and leukemia blood samples, based on 5hmC levels. Healthy and chronic lymphocytic leukemia (CLL) DNA samples, extracted from whole peripheral blood, were tested for 5hmC levels. The results show a significant decrease in the average 5hmC levels of CLL compared to healthy blood samples (0.0055 ± 0.0007% and 0.0036 ± 0.0001% 5hmC, respectively), in agreement with Gilat et al. [12] (Fig. 5A). This significant reduction in 5hmC levels could

Table 2
Precision and signal to noise ratios of 5hmC in PBMC.

%5hmC	5hmC (fmol)	DNA (ng)	Intraday variation (%)	Interday variation (%)	S/N PtP
0.0043 ± 0.0004	26 ± 2	200	≤11	8	70 ± 4

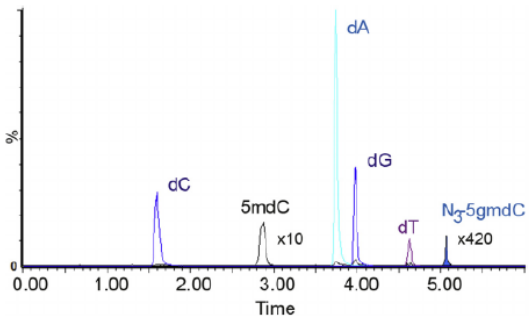


Fig. 4. A typical chromatogram obtained from 200 ng blood sample pre-labeled with glucose azide.

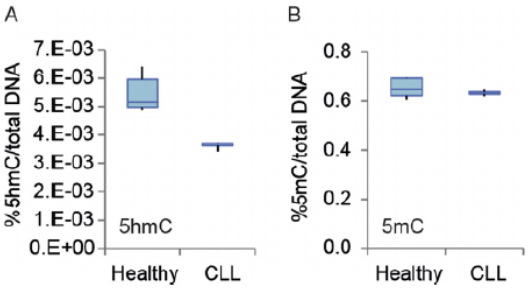


Fig. 5. 5hmC and 5 mC levels in healthy and CLL samples. A) 5hmC levels B) 5 mC levels, both calculated by extrapolating the ratio of 5hmdC peak area and total nucleotides peak area from a calibration graph.

not have been detected for untagged 5hmC, utilizing our basic quadrupole mass-analyzer instrument (according to Fig. 3A). The reduction in 5hmC levels was reported for several types of hematological malignancies [12,16–18] and various solid tissue cancers [12–15,45]. As appose to 5hmC, the average levels of 5 mC, in healthy and CLL samples, were similar (Fig. 5B), as previously reported for various solid tissues [10,46]. This indicates 1) a critical role for 5hmC in malignancy transformation and 2) the potential of global 5hmC levels but not of 5 mC levels as a biomarker for cancer diagnosis. The mechanisms involved in 5hmC reduction, in leukemia have been the topic of many recently published papers. Several independent reports have associated low levels of 5hmC, in leukemia, with mutations in the TET2 gene (responsible for the oxidation of 5 mC to 5hmC) [15,17,47–49] or in other genes, encoding enzymes related to 5hmC production [17,47]. Other studies have reported downregulation in the expression of such enzymes [13,17,50]. However, in some cases 5hmC reduction was neither accompanied by genetic mutations nor by downregulation of gene expression, suggesting alternative pathways for 5hmC reduction. One of the alternative pathways suggested is a passive dilution of 5hmC due to enhanced cell proliferation rate which, in some cases, may be the cause of 5hmC reduction in cancer [8,46].

4. Conclusions

Diverse pathways may lead to malignant tumor formation, indicating that specific pathway-associated factors, such as a mutation in a particular gene, may not always serve as a good marker for cancer diagnostics. The global reduction in 5hmC levels has been, however, comprehensively and independently associated with many types of cancers. The difficulty in using 5hmC as a diagnostic marker is its extremely low frequency, especially in blood. Cost-effective, fast and straightforward LC-MS/MS analysis offers the sensitivity required for global 5hmC detection in most solid tissues, however, detecting 5hmC in blood is challenging even for LC-MS/MS, especially when top-end MS instruments are not available. We reported here a technique for eight folds 5hmC signal enhancement which involves specific chemoenzymatic labeling of 5hmC with glucose azide, a molecule with relatively high ionization efficiency. This procedure allowed the differentiation between healthy and CLL samples, based on 5hmC levels using the most basic quadrupole mass-analyzer instrument. While most derivatization techniques for GC/MS or LC/MS analysis, are based on non-specific chemical labeling, this simple, specific tagging procedure, changes the retention time of only the required analyte, hence preventing interference from similarly structured co-eluted nucleosides, while preserving short measurement duration. The high sensitivity, accuracy, and precision achieved make this method a potential protocol for routine detection of 5hmC levels in clinical laboratories, using cost-effective quadrupole mass-analyzer instruments.

Notes

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2018.08.035.

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