# Chapter 7

# Detection of DNA Methylation in Genomic DNA by UHPLC-MS/MS

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## Abstract

DNA methylation serves to mark DNA as either a directed epigenetic signaling modification or in response to DNA lesions. Methods for detecting DNA methylation have become increasingly more specific and sensitive over time. Conventional methods for detecting DNA methylation, ranging from paper chromatography to differential restriction enzyme digestion preference to dot blots, have more recently been supplemented by ultrahigh performance liquid chromatography coupled with mass spectrometry (UHPLC-MS/MS) to accurately quantify specific DNA methylation. Methylated DNA can also be sequenced by either methylated DNA immunoprecipitation followed by sequencing (MeDIP-seq) or single-molecule real-time sequencing (SMRTseq) for identifying genomic locations of DNA methylation. Here we describe a protocol for the detection and quantification of epigenetic signaling DNA methylation modifications including, N6-methyladenine (6mA), N4-methylcytosine (4mC) and C5-methylcytosine (5mC) in genomic DNA by triple quadrupole liquid chromatography coupled with tandem mass spectrometry (QQQ-LC-MS/MS). The high sensitivity of the UHPLC-MS/MS methodology and the use of calibration standards of pure nucleosides allow for the accurate quantification of DNA methylation.

**Key words** N6-methyladenine, 6mA, C5-methylcytosine, 5mC, N4-methylcytosine, 4mC, UHPLC-MS/MS, methylated DNA

## 1 Introduction

DNA methylation is induced as either a nonenzymatic DNA damaging lesion; such as 1mA, 3mA, 7mA, 3mC, 2mG, 6mG, 7mG, 3mT, or 4mT, or as a directed signaling modifications; such as 4mC, 5mC, or 6mA [1–5]. The directed methylation marks have been demonstrated to regulate gene expression, DNA replication, DNA stability and the restriction modification system in prokaryotes and differentiation, stem cell pluripotency, genomic imprinting, and epigenetic memory [6–11] in more recently evolved organisms. Therefore, accurate detection and quantification techniques are necessary to delineate the functional consequences of DNA methylation.

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DNA methylation detection techniques have evolved rapidly since the initial identification of 5mC by crystallization in 1925 [12]. Paper chromatography and ultraviolet absorption spectra were used to compare synthetically generated modified nucleotides to DNA extracted from various prokaryotes and eukaryotes [1, 13–15]. This was subsequently complemented by differential restriction enzymedigestion techniques [16, 17], which are limited to detection of methylated sites in specific recognition motifs. Dot blots can also be used to detect DNA methylation marks [18] but are dependent on the methyl-specific antibody exclusively recognizing the intended modification and not detecting RNA contamination. More recently capillary electrophoresis and laser-induced fluorescence (CE-LIF) have been used to quantify 6mA with a lower limit of detection of 0.01% 6mA [19]. The most sensitive technique for quantifying DNA methylation, however, is high-performance liquid chromatography coupled with mass spectrometry [20] which can detect 6mA as low as 0.00001% [21]. While each of these techniques is powerful on its own, it is essential to have a thorough understanding of the caveats involved in each technique. This includes extraction of pure genomic DNA (gDNA) material devoid of RNA or microbiota contamination, and correction of any contaminations introduced during the detection technique [22]. Additionally, complementation with multiple independent techniques as well as manipulation of methyltransferases or demethylases and observing a concomitant increase or decrease of DNA methylation can help to definitively quantify DNA methylation concentrations.

UHPLC-MS/MS is highly sensitive and provides accurate quantitative analysis of the amounts of modified nucleosides, such as 6mA and 5mC and unmodified nucleosides such as deoxyadenosine (dA) and dC (deoxycytidine). In this method, the gDNA or RNA sample is first enzymatically digested to nucleotides by a nuclease followed by dephosphorylation by an alkaline phosphatase to generate nucleosides. Subsequently, a sample of the nucleoside mix is injected in the UHPLC-MS/MS instrument so that different nucleosides are first separated by reverse-phase chromatography in a gradient of the mobile phase (methanol–water) using a C18 column (packed with an octadecyl carbon chain (C18)-bonded silica) followed by ionization and detection by the Triple Quadrupole Mass Spectrometer (Fig. 1). More specifically, each ionized nucleoside (Precursor Ion) is selectively detected in the quadrupole mass filter Q1 based on the *m/z* (mass-to-charge ratio). Next the precursor ion enters the quadrupole collision cell Q2, where it is fragmented to the product ion by nitrogen gas and finally the product ion is detected in the quadrupole mass filter Q3 based on the *m/z*. By this method, each nucleoside is depicted as a peak that is retained in the column for a specific amount of time (retention time) and its area (peak area) reflects the abundance of

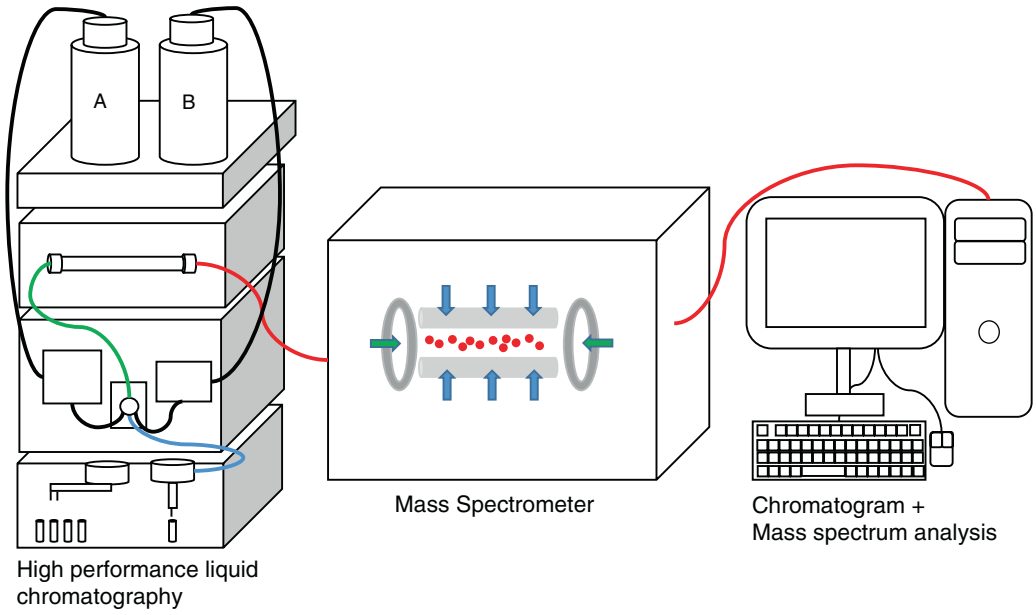


**Fig. 1** Schematic representation of the UHPLC-MS/MS. Vials are loaded into the HPLC machine autosampler and injected into the column before being read by the mass spectrometry machine and analyzed

the nucleoside in the sample (Fig. 2). In the initial optimization phase, pure standards of individual modified or unmodified nucleosides are run in the UHPLC-MS/MS to identify the retention time of the peak that corresponds to each nucleoside under a specific gradient of methanol and water. When properly optimized, UHPLC-MS/MS can accurately detect in a quantitative manner each nucleoside resolved from the liquid chromatography column, such as 6mA and unmodified dA based on the unique *m/z* values during the transition from precursor ion to product ion.

Here we describe a detailed method for the detection and quantification of 6mA/dA, 4mC/dC, and 5mC/dC ratios in gDNA samples using the 6470A QQQ LC-MS/MS system from Agilent using the MassHunter software for data acquisition and qualitative and quantitative analysis. While this protocol describes a method for detecting directed DNA methylation events, a similar approach can be utilized to detect nonenzymatically methylated DNA or methylations on RNA as well.

## 2 Materials

For the preparation of buffers used in digestion of gDNA to nucleosides, use ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C). Use only LC-MS/MS grade reagents in all buffers used for UHPLC-MS/MS analysis.

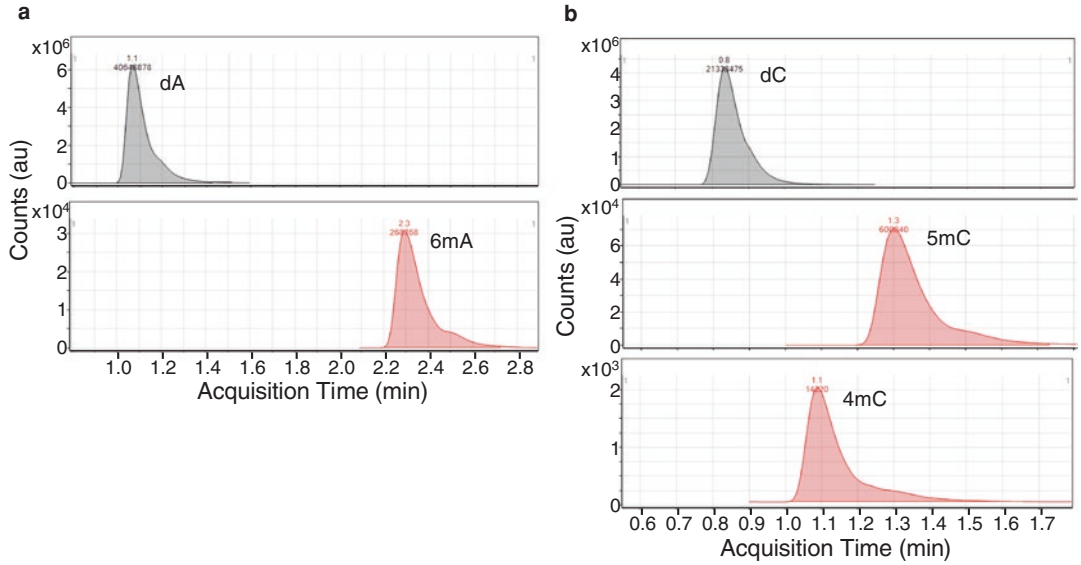


**Fig. 2** Representative chromatographs of (**a**) dA, 6mA, and (**b**) dC, 4mC, and 5mC that were extracted using the MassHunter Qualitative Analysis software

Diligently follow all waste disposal regulations when disposing of waste materials.

### *2.1 Digestion of gDNA to Nucleosides*

#### *2.1.1 Reagents*

1. DNA Degradase Plus (Zymo Research).

   (Alternatively) Nuclease P1 or Nuclease S1 and Fast AP (*see* **Note 1**)

2. 1.5 ml Eppendorf tubes
3. Insulin syringes and needles.
4. 0.22 μm Millex Syringe Filters
5. UHPLC-MS/MS vials and caps.
6. 2′-Deoxyadenosine. Prepare 1 mM stock in ultrapure water. Store at −20 °C.
7. N6-Methyl-2′-deoxyadenosine. Prepare 1 mM stock in ultrapure water. Store at −20 °C.
8. 2′-Deoxycytidine. Prepare 1 mM stock in ultrapure water. Store at −20 °C.
9. C5- Methyl-2′-deoxycytidine. Prepare 1 mM stock in ultrapure water. Store at −20 °C.
10. N4-Methyl-2′-deoxycytidine-5′-Triphosphate (4mdCTP (Trilink). From 100 mM stock prepare 1 mM 4mdCTP in ultra-pure water (store at −20 °C) and treat with DNA Degradase Plus enzyme mix as described in the methods section to remove the triphosphate and generate 4mC.

### 2.2 LC-MS/MS Analysis

#### 2.2.1 Reagents

1. Acetonitrile LC-MS/MS grade.
2. Ultrapure water LC-MS/MS grade .
3. Methanol LC-MS/MS grade.
4. Formic acid LC-MS/MS grade.
5. C18 reversed-phase column Eclipse XDB-C18, 2.1 × 50 mm, 1.8 μm (Agilent).

All LC-MS/MS buffers described below should be prepared in a sterile hood and stored at room temperature. Before each run it is important to ensure that a sufficient quantity of buffers is prepared for the entire run.

1. Mobile Phase A: water, 0.1% (v/v) formic acid. Mix 1 ml ampule formic acid LC-MS/MS grade in 1 l water LC-MS/MS grade.
2. Mobile Phase B (methanol, 0.1% (v/v) formic acid. Mix 1 ml ampule formic acid LC-MS/MS grade in 1 l methanol LC-MS/MS grade.
3. Wash buffer for injection needle (50% methanol, 50% water).

#### 2.2.2 Software

1. MassHunter Data Acquisition (Agilent).
2. MassHunter QQQ Qualitative analysis (Agilent).
3. MassHunter QQQ Quantitative analysis (Agilent).

## 3 Methods

### 3.1 Digestion of gDNA to Nucleosides

1. Digest 1–2 μg (*see* **Note 2**) of DNA to free nucleosides by adding 10 U of DNA Degradase Plus enzyme mix in 1× DNA Degradase plus buffer in 25–30 μl reactions incubated in a 1.5 ml eppendorf tube for 2–3 h at 37 °C. Also include a mock reaction consisting of DNA Degradase Plus enzyme and digestion buffer in water, without any added DNA. This control establishes the background level of methylated and unmethylated bases present in the enzyme mix that must be subtracted from the measured values for accurate quantification (*see* **Note 1**).
2. After digestion of samples, add 70–75 μl (to a total volume of 100 μl) of ultra-pure water and mix well by pipetting up and down.
3. Filter the samples using 0.22 μm Millex Syringe Filters directly into the HPLC vials. An insulin syringe can be used to help transfer the entire sample from the eppendorf tube to the vial through the filter. Close each vial with a cap and gently flick the vial to make sure there is no air trapped at the bottom of the vial. The UHPLC-MS/MS needle will reach close to the bot-

tom of the vial, so it is important to avoid injecting air instead of the sample. It is important to avoid touching the vial cap where the needle will pierce the vial cap to avoid introducing contaminants from gloves into the HPLC machine. Filters, vials, and caps should be selected individually without touching other empty filters, vials, and caps to avoid contamination.

4. Prepare calibration standards by performing serial dilutions of a mix of 6mA and dA or 5mC (or 4mC) and dC that is prepared from the pure stock concentrations. A typical range of calibration standards is usually between 10 μM to 1 pM for each nucleoside but this will also depend on how abundant or rare each modification is in the gDNA samples. For example, if the expected ratio of 6 mA/dA is around 1%, then prepare 10–14 calibration standards by performing twofold dilutions of a mix containing 10 μM dA and 100 nM 6 mA. The range of the concentration of the calibration standards will depend on the concentration of the nucleosides in the actual samples, as accurate quantification requires the peak area values of the samples to fall within the range of peak area values of the calibration standards used to generate the standard curve (for more details *see* Subheading 3.2.7).

### 3.2 LC-MS/MS Analysis

1. Place the vials in the Infinity 1290 autosampler of UHPLC-MS/MS 6470 system (*see* **Note 3**).
2. Wash the C18 reversed-phase column to clear any contaminated nucleosides from previous runs off of the column by flushing with 100% acetonitrile for 5–10 min followed by a wash with 99.9% methanol and 0.1% formic acid for 5–10 min.
3. After washing the column with acetonitrile and methanol, equilibrate the column by running 2–4 blanks of ultrapure water before starting with the injection of mock reactions, samples reactions and calibration standards. Before proceeding with running the samples it is necessary to confirm that no peaks that correspond to the nucleosides are being detected in the blank reactions (for more details *see* Subheading3.2.6).
4. Run the samples in the UHPLC-MS/MS system in positive electrospray ionization mode using the MassHunter Data Acquisition software and following a specific method for detection of 6mA and dA or 4mC, 5mC, and dC. The detection and quantification of the nucleosides is performed in dynamic multiple reaction monitoring (dMRM) mode, by monitoring the mass transitions from precursor to product ion for dA, 6mA, 4mC, 5mC and dC. The mass transitions used in our methods and the retention times on the C18 column for each nucleoside is shown in Table 1. Note that 4mC and 5mC have the same

mass transitions, but can still be separated because of their different retention time in the chromatography column (Fig. 2b).

5. We typically inject 5 μl of the filtered sample solution using the following parameters for the, liquid chromatography and the detection of 6mA and dA (Tables 1, 2, and 4) and 5mC or 4mC and dC (Tables 1, 3 and 4). Between detecting different nucleosides, it is necessary to run 2–4 blank reactions and ensure that no peaks are detected.

### 3.3 LC-MS/MS Qualitative Analysis

After running the samples, confirm that dA and 6mA or dC, 4mC, and 5mC, were successfully detected by running the MassHunter Qualitative analysis software to extract the chromatographs for each nucleoside (Fig. 2).

1. Open all data files in MassHunter Qualitative analysis and then extract the chromatographs for each compound by reaction monitoring (MRM) analysis (this is done by selecting "find compound by MRM").
2. Review the extracted chromatographs and confirm that all blanks have background levels of dA, 6mA, dC, 4mC, and 5mC, (no peaks are detected at the expected retention times) and that nucleosides in the samples can confidently be detected by the presence of clear peaks for dA, dC, 4mC, 5mC, and 6mA at the expected retention times based on pure standards (*see* **Note 4**).

### 3.4 LC-MS/MS Quantitative Analysis

Quantify the ratio of 6mA/dA, or 5mC/dC or 4mC/dC, in gDNA samples using the MassHunter Quantitative Analysis software using the calibration standards from serial dilutions of pure dA and 6mA, and 4mC or 5mC and dC.

1. Create a new batch of quantification analysis and select all data to be analyzed including the samples and calibration standards so that all the data are imported to the quantification analysis batch.
2. Create the quantification method by selecting "new method from the acquired MRM data". This is the easiest way of creating a new method as information about the compounds, precursor and product ion mass, retention times, will be extracted from the data files.
3. Select the calibration standards concentration tab and set up the concentrations used of each nucleoside (e.g., dA and 6mA) for each calibration standard level used in the analysis (*see* **Note 5**).
4. Apply the final method to the data by selecting "exit" and then select "use the method to analyze the data." The data will be analyzed according to the method and retention times and peak area values will be calculated for all samples.

**Table 1**
**Data acquisition parameters for 6mA, dA, 4mC, 5mC, and dC detection**

| Compound | Precursor ion | Product ion | Retention time | Fragmentor | Collision energy | Cell accelerator voltage |
|---|---|---|---|---|---|---|
| dA | 252.1 | 136.0 | 1.1 | 90 | 14 | 5 |
| 6mA | 266.1 | 150.0 | 2.3 | 90 | 16 | 5 |
| dC | 228.2 | 112.2 | 0.8 | 70 | 7 | 6 |
| 5mC | 242.2 | 126.1 | 1.15 | 70 | 7 | 6 |
| 4mC | 242.2 | 126.1 | 1.35 | 70 | 7 | 6 |

**Table 2**
**Liquid chromatography parameters for 6 mA and dA detection**

| Run time (min) | A: Water, 0.1% FA | B: Methanol, 0.1% FA |
|---|---|---|
| 0.00 | 98% | 2% |
| 3.00 | 92% | 8% |
| 3.01 | 2% | 98% |
| 5.00 | 2% | 98% |
| Post-run time (min) | | |
| 2.5 | 98% | 2% |
| Flow rate: 0.5 ml/min, column temperature: 35 °C | | |
| Run cycle time: 5 min | | |
| Post run time: 2.5 min | | |

5. Although the software automatically integrates the peaks for each nucleoside in the samples and standards, it is important to inspect and review each peak separately to confirm that the retention times are accurate and the peak areas were precisely selected (Fig. 3a and b). If the automatic integration is not accurate (Fig. 3b), perform corrections by using the manual integration tool. This allows for manual editing of the peak area that will be analyzed.
6. For each nucleoside, generate a standard curve by altering the line of best fit (for example linear or quadratic function) in order to achieve $R^2$ values as close to 1 as possible (Fig. 3c and d). Different nucleosides will increase in a linear or nonlinear fashion depending on the abundance of the specific nucleoside being measured. Therefore, it is important that the standard curves for each nucleoside are optimized so that the accuracy of

**Table 3**
**Liquid chromatography parameters for 4mC, 5mC, and dC detection**

| Run time (min) | A: Water, 0.1% FA | B: Methanol, 0.1% FA |
|---|---|---|
| 0.00 | 99.5% | 0.5% |
| 2.50 | 99% | 1% |
| 2.51 | 2% | 98% |
| 4.00 | 2% | 98% |
| Post-run time (min) | | |
| 2.5 | 99.5% | 0.5% |
| Flow rate: 0.3 ml/min, column temperature: 35 °C | | |
| Run cycle time: 4 min | | |
| Post run time: 2.5 min | | |

**Table 4**
**Source parameters for 6 mA, dA, 4mC, 5mC and dC detection**

| | 6 mA and dA method | | 4mC, 5mC and dC method | |
|---|---|---|---|---|
| Gas temp | 250 °C | | 250 °C | |
| Gas flow | 10 l/min | | 10 l/min | |
| Nebulizer | 25 psi | | 45 psi | |
| Sheath gas temp | 375 °C | | 400 °C | |
| Sheath gas flow | 12 l/min | | 12 l/min | |
| Capillary voltage | 2500 positive | 2500 negative | 2500 positive | 2500 negative |
| Nozzle voltage | 0 positive | 0 negative | 500 positive | 500 negative |

quantification is as close to 100% as possible. This is especially critical for the set of standards with peak area values that flank the peak area values of the samples. If the peak area values of the samples fall outside of the calibration standard curve than the quantitative values will be inaccurate (Fig. 3e and f). If this is the case, it is necessary to regenerate a calibration curve including a larger range of detection so that quantification of experimental samples is accurate.

7. Analyze the experimental samples based on the calibration standard curve for each nucleoside by selecting the analyze function. In this way the final concentration of each nucleoside in the samples is calculated based on the standard curve.

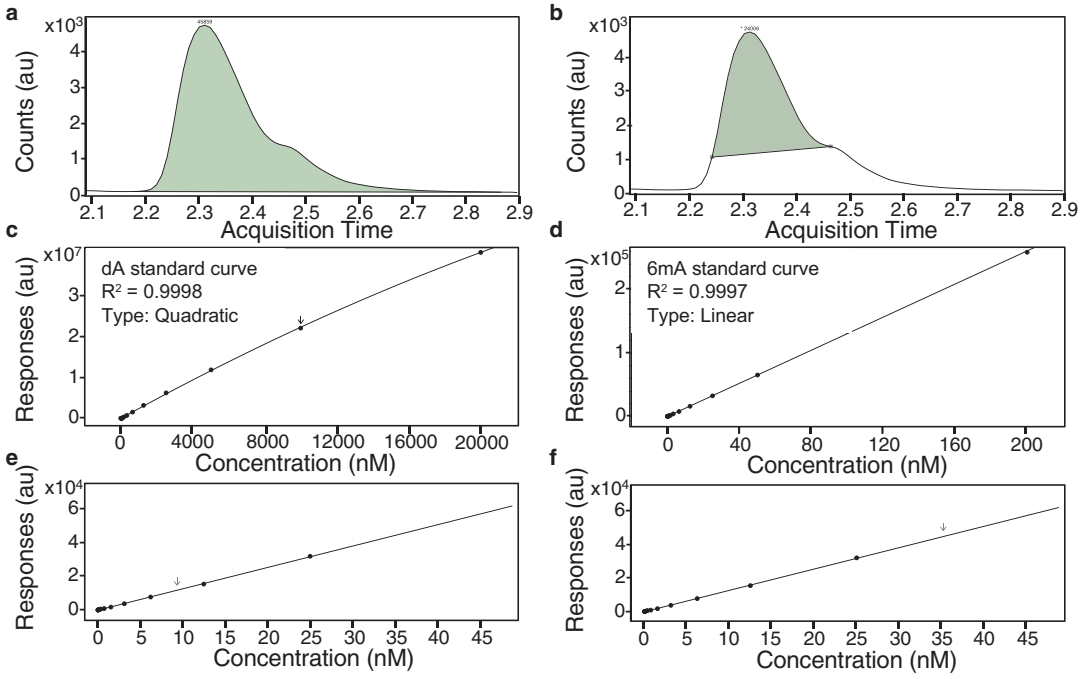


**Fig. 3** Quantitative analysis of UHPLC-MS/MS samples requires accurate peak integration, standard curve calibration curves, and sample measurements within the range of the standards. (**a**–**b**) Representative chromatographs of 6mA in which the peak area was (**a**) successfully integrated by the MassHunter Quantitative analysis software or (**b**) required manual integration. (**c**–**d**) Calibration standard curves need to be fitted differently for different nucleosides. The standard curve should be fitted so that the $R^2$ value is as close to 1 as possible. Examples of a (**c**) quadratic standard curve for dA or a (**d**) linear standard curve for 6mA are shown. (**e**–**f**) It is important that the samples being measured fall within the range of the calibration standards to ensure measurement accuracy. Examples of 6mA samples measured (**e**) within or (**f**) outside of the range of the calibration standards are shown

8. Export the data (final concentration, peak area, and retention time for each sample) as an Excel file by selecting the export table function.
9. In Excel, calculate the ratios of methylated to unmethylated bases. If any detectable 6mA or dA is present in the mock reaction, these values must be subtracted from the experimental sample values of dA or 6mA in each gDNA sample and calculate the normalized 6mA/dA ratio afterwards.

## 4 Notes

1. We routinely use DNA Degradase Plus for the digestion of gDNA samples to nucleosides as this enzyme mix conveniently contains both a nuclease and an alkaline phosphatase and has been optimized for DNA digestion to nucleosides for UHPLC-MS/MS analysis. In addition, we have found that the

DNA Degradase Plus mix has minimal levels of contaminating DNA [22]. Alternatively, gDNA can be digested to nucleosides in two steps: Nuclease P1 or Nuclease S1 digestion followed by alkaline phosphatase treatment [22]. As above, mock reactions without DNA template should be included to test for potential contaminating DNA in the enzyme preparations.

2. The amount of gDNA required for quantifying methylated DNA bases will depend on the species that is being analyzed and the abundance of the methylated nucleoside. If unsure of the concentration 1 μg is a good starting point.
3. If an autosampler is not being used, individual vials should be placed below the injection needle before each injection or samples should be injected directly into the machine.
4. In the initial technique optimization phase it is necessary to spike digested gDNA samples with pure standards to confirm that the detected peak for each nucleoside is migrating at the expected retention time.
5. It is important to ensure that when entering the values for the serial dilution of the standards, they are entered with the same prefix (pM, nM, or μM) so that they can be directly compared to modified nucleosides, which are much less prevalent in gDNA compared to the unmodified nucleosides.

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