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Liquid Chromatography Tandem Mass Spectrometry for the Measurement of Global DNA Methylation and Hydroxymethylation

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Abstract

DNA methylation is an important epigenetic phenomenon. Global DNA methylation levels in the genome are tissue-specific and are reduced in the context of specific developmental and pathological states. Hence accurate assays to quantify global DNA methylation are required. In this review we describe different methods for global DNA methylation measurement and explain the methodology, strengths and limitations of the gold standard assay liquid chromatography tandem mass spectrometry (LC-MS/MS) for 5-methylcytosine and 5-hydroxymethylcytosine quantitation. Measurement of global DNA methylation using LC-MS/MS has helped to elucidate the effects of environmental exposures and demethylating drugs that can alter DNA methylation levels and further studies using this technique may provide insights on the prevention and/or reversal of DNA hypomethylation.

Keywords: Liquid chromatography mass spectrometry; LC-MS/MS; Global DNA methylation; 5-methylcytosine; 5-hydroxymethylcytosine

Abbreviations: DNMT: DNA Methyltransferase; 5mC: 5-Methycytosine; 5hmC: 5-Hydroxymethylcytosine; LUMA: Luminometric Methylation Assay; HPLC: High Performance Liquid Chromatography; SAM: S-Adenosylmethionine; LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry

Introduction

DNA methylation is essential for normal development, cellular differentiation and tissue-specific gene expression [1]. In differentiated mammalian cells, DNA methylation occurs almost exclusively at cytosine bases located 5' to a guanine, in a CpG dinucleotide. The distribution of DNA methylation across the genome is non-random. Most of the CpGs located in the genome are dispersed throughout the intergenic and intronic DNA regions such as repetitive DNA sequences where they are normally methylated to ensure DNA stability and transcriptional silencing of transposable elements [2]. In contrast, dense stretches of CpG dinucleotides found within the promoter regions of 70% of all genes (CpG islands) [3] are normally unmethylated.

Mammalian DNA methylation is established and maintained by a family of DNA methyltransferase (DNMT) enzymes, the mechanisms of which have been reviewed comprehensively elsewhere [4]. In normal tissue DNA, 3.5-5% of cytosines are methylated [5], representing methylation at up to 80% of all CpG dinucleotides [6]. The factors that determine the 5mC content of different tissues remain unclear. Early studies found no obvious associations between 5mC content and average cell turnover rates in different tissues or the percentage of the genome that is transcribed [7]. A reduction in the percentage of 5-methycytosines (5mC) in the genome (global DNA hypomethylation) is an important and frequent phenomenon occurring in many disease states including cancer, autoimmune diseases (e.g. lupus) and metabolic diseases (e.g. cardiovascular disease) [8]. Hence, accurate assays to measure global DNA methylation are particularly relevant when examining dietary and environmental exposures known to alter methylation patterns. Hypomethylation may have important biological consequences including DNA instability and the activation of retroelements which is relevant given the use of medications that act via inhibition of DNMTs (e.g. 5-aza-2'-deoxycytidine) for the treatment of myelodysplastic syndrome and other diseases [9].

While DNA methylation is well accepted as a stable and somatically heritable epigenetic modification, recent studies in embryonic stem cells and murine brain have demonstrated that methylation can be dynamic in some circumstances. Acute changes in methylation can occur due to the activity of specific enzymes that are able to remove 5mC through a base excision repair mechanism, leading to active DNA demethylation [10]. This involves the oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) achieved via the coordinated activity of multiple proteins including the Ten-eleven translocation (Tet) family of enzymes [11]. Numerous recent reports have highlighted the importance of hydroxymethylation in embryonic development and pluripotency [12]. Current evidence suggests the function of hydroxymethylation is distinct from CpG methylation and thus it has been increasingly described as a separate epigenetic entity [13].

This article will review the different methods of measuring global DNA methylation and hydroxymethylation and in particular describe the role of LC-MS/MS in global DNA methylation and hydroxymethylation analysis. Potential research and clinical applications of measuring global DNA methylation using LC-MS/MS will also be discussed.

Global DNA Methylation Assays

Several methods have been used for global DNA methylation measurement. These include restriction enzyme digestion based methods (e.g. luminometric methylation assay (LUMA) and end-specific PCR), M.SssI methyl acceptance assay, high performance liquid chromatography (HPLC), LC-MS/MS, cytosine extension assays and enzyme immunoassays (Table 1). Both restriction enzyme based methods [14] and cytosine extension assays [15] are sequence specific and hence may not be entirely representative of global DNA

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Assay/References	DNA preparation/treatment	Strengths	Limitations	Other comments
High performance liquid chromatography (HPLC) [29]	Total genomic DNA hydrolysed to deoxyribonucleotides and treated with ribonuclease to remove contaminating RNA. Deoxyribonucleotides converted to deoxyribonucleosides (treat with alkaline phosphatase). Products separated by reverse phase HPLC. Quantification with external standards or UV absorbance at 254nm.	Highly quantitative and reproducible	Requires large amounts of DNA (several micrograms). Not suitable for high throughput. Contaminants may co-elute with DNA and impact on accuracy.	Has been the preferred quantitative technique for determining global DNA methylation prior to the development of LC-MS/MS assays
M.SssI acceptance assay [16,30]	Genomic DNA is incubated with M.SssI DNA methyltransferase in the presence of tritiated S-adenosylmethionine and immobilised on nitrocellulose paper. Radiation is measured in scintillation counter.	Simple and rapid procedure.	High inter-assay variability. Semi-quantitative	Difficult to compare results with those generated by other laboratories. Requires radiation.
Enzyme immunoassay Epigenetek (P-1034); Cell Biolabs (STA-380 and STA-381)	Genomic DNA is denatured, immobilized and incubated with monoclonal antibodies against 5mC. Levels of 5mC determined by intensity of staining determined by incubation with fluorescein-conjugated secondary antibody.	Can be used to map the location of DNA methylation on chromosomes in individual cells e.g. juxtacentromeric regions.	Qualitative assay. High inter-assay variability.	Commercially available kits
Luminometric methylation assay (LUMA) [14]	Based on combined DNA cleavage by methylation-sensitive restriction enzymes and polymerase extension assay by pyrosequencing	Sensitive (requires 200-500 ng DNA), semi-quantitative	Assay is sequence-specific and may not truly represent global DNA methylation levels	Difficult to compare results with those generated by other methods
Bisulfite sequencing of repetitive elements [21, 31, 32]	Bisulfite treatment of DNA and real-time PCR or standard PCR amplification of repetitive DNA elements (using combined bisulfite restriction analysis [COBRA] or pyrosequencing)	Very sensitive (<100 ng), high-throughput, relatively quantitative for each repeat element	May not truly represent global DNA methylation levels. High inter-assay variability, bisulfite conversion of DNA introduces errors due to spontaneous deamination of methylated cytosines into thymine which occur frequently at repetitive elements	Very popular assay for applications where only small quantities of DNA are available.
End-specific PCR [33]	Quantitative assessment of the level of unmethylated LINE-1, Alu and LTR elements using methylation-sensitive restriction enzyme digestion and real-time PCR with a fluorescence-based readout	Ultra sensitive (only 1-5ng needed), high-throughput.	Surrogate for global DNA methylation. Only able to analyze specific subsets of LINE and Alu repeats. Assay is only suitable for human DNA samples. Semi-quantitative.	Useful when only extremely small quantities of DNA are available.
LC-MS/MS [34]	Identical to HPLC except quantitation is performed using spiked internal standards and measured using mass spectrometry	Sensitive (100-500 ng), quantitative, reproducible, absolute quantitation	High instrument setup costs, expensive to synthesize or purchase internal standards, requires mass spectrometry expertise	Considered the gold standard method.

Table 1: Summary and evaluation of key methods of global DNA methylation measurement.

methylation. Furthermore, the cytosine extension assay has high variability and is unable to distinguish between single stranded methylation and fully methylated sequences. The methyl acceptance assay is a semi-quantitative method that relies on the ability of the DNA methyltransferase M.SssI to methylate all unmethylated CpG sites within a sample. However this technique can be confounded by suboptimal enzyme activity and stability of the methyl donor S-adenosylmethionine (SAM), thus resulting in a large intra- and inter-assay variability [16]. Enzyme immunoassays, developed in the form of commercialised kits have also emerged in recent years (e.g. Methylflash Methylated DNA Quantification Kit (Epigentek) [17] and Global DNA Methylation or Hydroxymethylation ELISA kit (Cell Biolabs) [18]) and several studies using these assays have also been published [19,20]. While these commercialised kits are convenient, the methylation content of a sample is extrapolated from the degree of binding of monoclonal antibodies against 5-methylcytosine, which is quantified using a fluorescein-conjugated secondary antibody. Factors that affect the efficacy of antibody binding or inadequate washing of excess antibody limit the reproducibility and reliability of this technique. Since the manufacturers do not disclose the individual components of these kits, these assays can be extremely difficult to troubleshoot.

Assays of repeat element methylation have been increasingly used in the literature as a surrogate for global DNA methylation since a correlation between global DNA methylation and repeat element methylation have been reported in different cell lines [21,22] and tumor samples [23]. These assays interrogate regions that are highly heterogenous, thus yielding a high background 'noise'. For example, a commonly used bisulfite PCR assay that measures hypomethylation at repeat elements [22] has been reported to have background of ~10 times higher than the changes typically observed in cancers. It is not well established whether inter-individual differences in global DNA methylation levels in humans is reflective of methylation at LINE-1 and Alu repeat elements. Several studies have found little correlation between repeat element methylation and global DNA methylation in human blood and bowel samples [24,25], suggesting that in particular contexts methylation levels at repeat elements can be a poor surrogate for global methylation levels. Ultimately, the greatest limitation of all the above studies is the fact that these assays provide an indirect relative measure of 5mC content [26].

In contrast, HPLC and LC-MS/MS assays provide absolute measures of 5mC content in DNA samples that are independent of DNA quality. The limitation of HPLC assays however, is the requirement of large quantities of DNA (several micrograms) and the difficulty of incorporating internal standards [27]. In contrast, LC-MS/MS is highly accurate and sensitive and is regarded as the 'gold standard' for global DNA methylation measurement [28].

LC-MS/MS for Global Methylation Analysis

The proportion of 5mC as a percentage of all cytosines in genomic DNA can be accurately measured using stable isotope-dilution LC-MS/MS. The LC-MS/MS assay produces three modes of selectivity. Firstly, HPLC separates the different deoxyribonucleotides in a digested sample of DNA, yielding different retention times on the chromatographic column. Secondly, in tandem mass spectrometry, the deoxyribonucleotides are ionised by electrospray ionisation producing protonated ions with unique mass/charge ratios (m/z). The first mass analyser sequentially selects these molecules, and they are accelerated into a collision cell with sufficient kinetic energy to cause fragmentation upon collision with an inert target gas that is present in the cell. Thirdly, a unique pattern of fragment ions is produced from the selected ion when the second mass analyser is scanned. Quantification is performed with utmost sensitivity when both mass analysers are set to pass (i) the protonated molecules of interest and (ii) an abundant fragment ion respectively in what is called the 'selected reaction monitoring' mode [35].

Several variations of LC-MS/MS for global DNA methylation analysis have been published [28,34,36-38]. These methods all rely on the same basic principles of selectivity described above, but vary in terms of the solvents, HPLC columns used and type of standards used for quantification. One assay based upon the method of Quinlivan & Gregory [28] involves the in-house biosynthesis of [$^{15}N_3$]-deoxycytidine (dC) and [15N3]-5mdC internal standards from bacteria and a one-step digestion of genomic DNA into deoxyribonucleosides [39]. The digested sample is spiked with known quantities of [U-15N]-internal standards and analyzed using LC-MS/MS. Typically, 10-20 µL of the digested DNA sample (of 1-100 ng/µL) is injected onto a HPLC column whilst running a solvent (e.g. methanol) gradient which allows separation of 5mdC and dC due to their different hydrophobicity. The eluted samples are analyzed by electrospray ionisation using a triple quadrupole mass spectrometer with selected reaction monitoring (explained above) to allow monitoring of the mass to charge transitions of sample and isotopic internal standard dC and 5mdC. The chromatograms are analyzed for the area under the curve and the ratio between the sample (analyte) and internal standard are used to calculate the quantity of dC and 5mdC in the sample, and hence the percentage of 5mdC in the genome. An example of this chromatogram is shown in Figure 1.

The advantages of LC-MS/MS include its extreme sensitivity, with a limit of detection reported to be only 4 ng of genomic DNA [34]. The assay can also be used to analyze DNA from any species since it is not sequence dependent. The flexibility of the assay also allows the reliable comparison of 5mC levels obtained using variations of the LC-MS/MS method. One of the most critical advantages of HPLC and LC-MS/MS based assays is that it is independent of DNA quality, which is particularly useful when analysing DNA samples of limited quality or quantity.

More recently, the discovery of 5hmC has resulted in the development of several LC-MS/MS methods allowing simultaneous quantitation of 5mC and 5hmC content in the same sample [40-42]. These assays are ultra-sensitive, which is important since the 5hmC base is much scarcer than 5mC. Several assays for simultaneous quantitation of 5mC and 5hmC were able to detect 5hmC in 50ng of digested genomic DNA at the 0.1% level [40,41]. Examples of chromatograms showing both 5mC and 5hmC in the same sample are illustrated in these manuscripts [40,41]. The ability to quantify 5hmC and 5mC is a particular strength of LC-MS/MS since both 5hmC and 5mC are refractory to bisulfite modification and therefore cannot be distinguished using standard bisulfite-based assays. One alternative is to use oxidative bisulfite modification [43], which converts both 5hmC and unmethylated cytosine, however each sample must be analyzed using separate standard and oxidative bisulfite assays in order to extrapolate the locations of 5hmC and 5mC. Other existing assays for 5hmC such as antibody based methods are much less sensitive than LC-MS/MS [41]. In addition to 5hmC, related intermediate bases such as 5-formylcytosine and 5-carboxycytosine which can be generated by Tet proteins, are found to be present at very low levels in mouse embryonic stem cells and cancer cell lines and can be accurately measured using LC-MS/MS assays [44].

There are several limitations to LC-MS/MS relating to the highly specialised nature of the method and the need to access or purchase expensive instruments. Setting up LC-MS/MS would require a HPLC pump, HPLC column, autosampler (so that samples can be automatically injected into the HPLC column), and a mass spectrometer. Isotopic internal standards also tend to be expensive and need to be manufactured in-house [39] or custom-purchased. In addition, researchers interested in using LC-MS/MS without prior experience in the method would require several months of training in order to become familiarised with the operation, optimisation and troubleshooting of the instrument. While it is likely that LC-MS/MS will remain a highly specialised technique given the complexity of the instrument set-up, it is possible that improvements to software and user interfaces may make it more accessible to the non-expert user in the future. For example, a refinement of the LC-MS/MS assay using inexpensive and commercially readily available isotopic internal standards (from Cambridge Isotope laboratories, Andover, MA) and a shorter run-time (2.5 minutes, 4-fold less than previous assays) may make LC-MS/MS more feasible for new users [45]. For researchers interested in analysing large numbers of samples over several projects, expertise in LC-MS/MS is certainly worthwhile and transferrable to other LC-MS/MS based assays. Alternatively, for researchers interested in analysing only a small number of samples, collaboration with existing users or a mass spectrometry core facility as a fee-for-service would be more feasible. Currently the application of LC-MS/MS for global DNA methylation is primarily an interest for research laboratories rather than diagnostic laboratories. It is also important to note that LC-MS/MS measures absolute global DNA methylation levels but that it does not provide any information regarding the genomic location of this methylation.

Applications of LC-MS/MS Assays for Global DNA Methylation

Interest in the measurement of global DNA methylation has arisen in the setting of both clinical and pre-clinical studies seeking

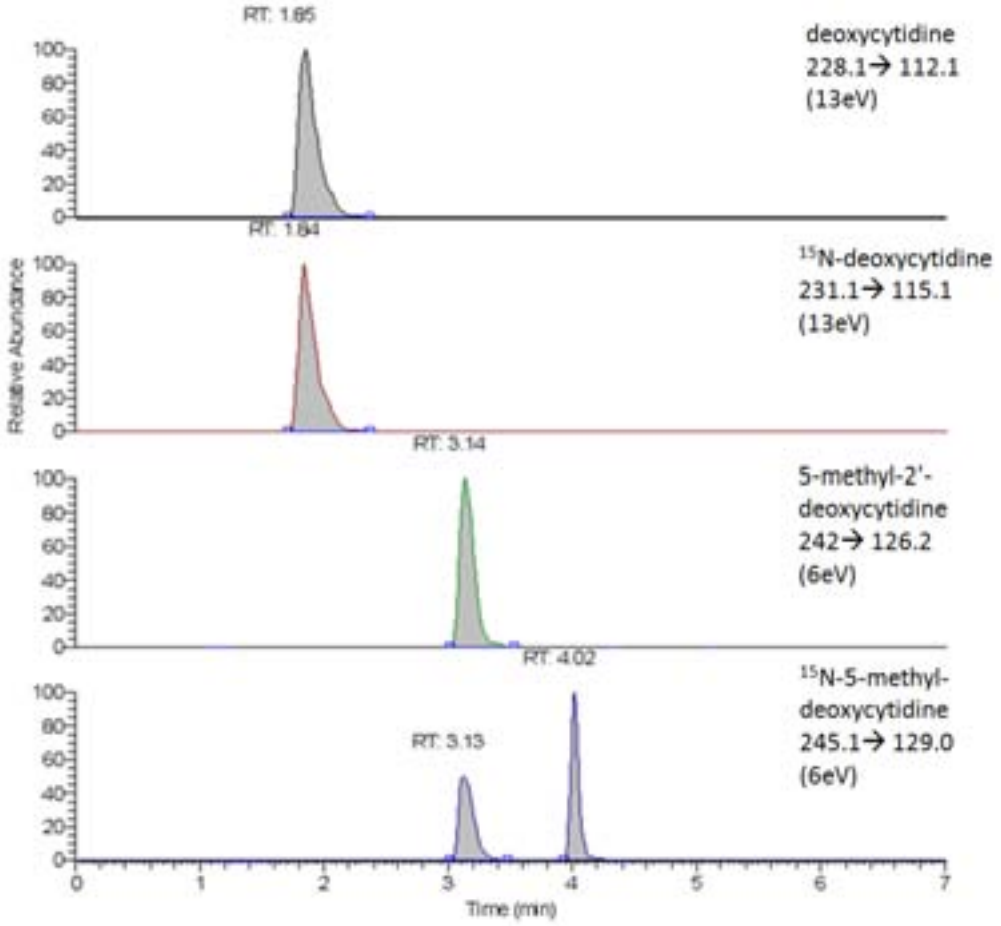

Figure 1: Chromatogram of a typical injection of digested DNA for global methylation using LC-MS/MS. Selected reaction monitoring chromatograms obtained from the LC-MS/MS analysis of human genomic DNA showing deoxycytidine (retention time 1.85 min) and 5-methyl-2'-deoxycytidine (retention time 3.14 min) and their corresponding internal standard peaks. A second peak is observed with identical precursor-product transition as ^{15}N 5-methyl-2'-deoxycytidine which is likely to be uridine (245.1→ 129.1 RT 4.02 min). However this does not interfere with quantitation of 5-methyl-2'-deoxycytidine due to the different retention time. The mass to charge transitions for each analyte are shown alongside the collision energy (in brackets).
Abbreviations: RT: Retention Time.

to investigate the mechanisms and consequences of global DNA hypomethylation in physiological and disease states. These include studying the changes in global DNA methylation during normal embryonic development or cellular differentiation and the effects of environmental and toxin exposures on global DNA methylation and correlation with disease risk. Two specific applications of LC-MS/MS assays for quantifying global DNA methylation will be described in further detail below.

Applications in Evaluating Environmental Factors Influencing DNA Methylation

Deficiency in folate and polymorphisms in folate metabolism enzymes are associated with global DNA methylation changes, which may lead to an increased risk of epithelial cancers and vascular disease [46,47]. LC-MS/MS has been used extensively in this context to measure global DNA methylation in human peripheral blood mononuclear cells. For example, elevated homocysteine (an inflammatory marker which accumulates in the setting of methyl donor deficiency) was associated with global DNA hypomethylation in patients with vascular disease [48]. Peripheral blood global DNA methylation was significantly lower in older subjects homozygous for the C677T polymorphism within the gene encoding the folate metabolism enzyme MTHFR [37]. Interestingly, DNA methylation was significantly lower in MTHFR TT subjects with low plasma folate, but not MTHFR TT subjects with high plasma folate [49], suggesting a gene-nutrient interaction exists that influences peripheral blood DNA methylation. Folate form distribution was also significantly related to global DNA methylation levels in the blood and normal bowel samples of patients with colorectal cancer [25]. A recent retrospective analysis compared peripheral blood DNA methylation in 68 subjects with a history of cancer at enrolment and 62 subjects who developed cancer during an 8-year follow-up with age and sex-matched controls for prevalent and incident cancer cases. The investigators reported significant global DNA hypomethylation in cancer patients (4.39%, 95% CI 4.25-4.53 vs 5.13%, 95% CI 5.03-5.21, P<.0001) as well as in the baseline DNA methylation level of subjects with cancer on follow-up (4.34%, 95% CI 4.24-4.51 vs 5.08%, 95% CI 5.02-5.22, P<.0001) [50]. This study was performed in Italy, where mandatory folate fortification has not been introduced. Although further studies with larger sample sizes are required, this striking finding suggests that global DNA

methylation may be a useful epigenetic biomarker for cancer risk estimation in certain populations. An important meta-analysis of 34 population-based case control studies investigating blood genomic DNA methylation in relation to cancer risk found an overall significant inverse relationship between genomic DNA methylation and cancer risk (95% CI 1.2-6.1). However no overall risk association was found for studies using surrogates for genomic methylation including LINE-1 methylation (95% CI 0.8-1.7) [51]. Taken together, the use of LC-MS/MS to accurately quantify global DNA methylation have helped to elucidate gene-environment interactions that affect global DNA methylation content and associations with disease.

In addition to folate, biochemical and hormonal factors may also influence global DNA methylation. For example, LINE-1 methylation was found to be directly correlated with serum glucose and inversely correlated with serum sex hormones among postmenopausal women with low serum folate [52]. Whether this relationship is specific to LINE-1 repeats or reflects global DNA methylation levels is uncertain.

Applications in Monitoring the Effect of Demethylating Drugs

The nucleoside analog and DNMT inhibitor 5-aza-2'-deoxycytidine is currently a front line therapy for patients with myelodysplastic syndrome, and has also been approved for the treatment of myeloid malignancies such as acute myelogenous leukemia and chronic myelomonocytic leukemia [53]. By inhibiting DNMTs, this drug results in genome-wide DNA hypomethylation, which is thought to reactivate critical genes silenced by hypermethylation. LC-MS/MS assays have been used extensively in clinical and cell line studies to monitor the response to demethylating drugs such as 5-aza-2'-deoxycytidine. In clinical studies the assay has also been used to monitor dose-dependent effects on global methylation following 5-aza-2'-deoxycytidine therapy in patients with leukemia [54]. Furthermore in a study of the effects of 5-aza-2'-deoxycytidine on colorectal cancer cell lines, LC-MS/MS allowed the monitoring of global methylation changes before, during and after treatment [55]. LC-MS/MS may also provide a useful screening tool to stratify samples according to global DNA methylation status for further analysis using more comprehensive techniques such as genome-wide methylation profiling.

Finally, it is anticipated that LC-MS/MS assays will play an increasing role in elucidating the tissue distribution and function of 5hmC in different developmental states and disease conditions. Several studies have confirmed higher levels of 5hmC in embryonic stem cells and neurons and a reduction of 5hmC occurs in some tumors including hepatocellular [42], lung and brain cancer [56].

Conclusions

The measurement of global DNA methylation and hydroxymethylation is challenging due to the high degree of inter-individual variability and background 'noise' which entails a need for highly accurate and sensitive assays. Of the many assays for assessing global DNA methylation levels, LC-MS/MS has consistently been reported as the most robust and sensitive. Potential applications of LC-MS/MS include the examination of environmental factors that affect global DNA methylation or hydroxymethylation and its associations with disease, as well as the effects of demethylating drugs in both clinical and preclinical samples. While LC-MS/MS is not suitable for determining the precise genomic location of methylation, it may be used as a screening tool to select the most interesting samples for further analysis or as an analytical tool for the measurement of environmental, dietary or pharmacologically-induced changes in DNA methylation.

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