# High-throughput Analysis of DNA Modifying Enzymes by an Agilent RapidFire 360 System

## Application Note


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### Abstract

Enzymes that act on DNA are promising targets for anticancer therapeutics[1]. For example, the epigenetic enzyme DNMT1 is inhibited by 5-azacytidine, causing DNA hypomethylation and enhancing the function of tumor suppressor genes[2]. In concordance, the development of accurate and user-friendly assays to study DNA modifications has become pivotal to drug discovery. Traditional *in vitro* assays have used radioactivity, antibodies, and fluorescent substrates to relate the extent of reaction. However, each of these approaches has drawbacks that limit its practicality for large sample numbers. This application note illustrates the advantages of developing high-throughput assays for DNA-modifying enzymes using an Agilent 360 RapidFire High-throughput Mass Spectrometry System. In particular, the abilities to detect native DNA directly and measure multiple DNA species in each reaction are highlighted.


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## Introduction

An Agilent RapidFire 360 System couples microfluidic solid phase extraction with time-of-flight mass spectrometry (TOF-MS) detection, significantly increasing the throughput of traditional mass spectrometry (MS), and circumventing the need for analyte-specific method development. The platform routinely sustains a sample-to-sample cycle time of approximately 8 seconds, permitting the analysis of over 10,000 samples per day. Quenched reaction plates (96- or 384-well format) can be delivered to the system, without any offline workup, for immediate analysis.

TOF-MS detection enables the direct detection of label-free DNA species. Consequently, more native (and thus more biologically relevant) substrates can be employed and the risk of artifactual data that can result from use of modified substrates is decreased. Additionally, measuring native substrates and products can circumvent the need to synthesize and validate artificial substrates or product-specific antibodies, simplify assay design and development, and decrease time-to-screen.

TOF-MS detection also enables the concurrent measurement of multiple reaction species including substrates, intermediates, products, and internal standards. In comparison to numerous assay formats that provide only a single readout of a secondary product formation, TOF-MS data can be normalized (for example, by calculating % conversion, or dividing product signal by an internal standard), which improves the activity measurement within each sample. Measurement of all the reactants using TOF-MS can also provide unparalleled detail about the target reaction.

### Measurement of AlkB substrate, reaction intermediates, and product by RapidFire/MS

Members of the AlkB family are thought to be promising targets for prostate cancer[3] and are implicated in specific mechanisms of epigenetic gene regulation[4]. Therefore, biological assays for measuring DNA repair by AlkB may be instrumental in the development of therapeutic agents. The repair of ethenoadenine lesions in DNA by *E. coli* AlkB was investigated here.

The conversion of ethenoadenine lesions in DNA by AlkB is known to proceed through a mechanism involving two repair intermediates before the product is ultimately formed (Figure 1)[5]. First the etheno bond of the ethenoadenine substrate is catalytically epoxidized to form an epoxide intermediate. This species hydrolyzes to the glycol intermediate, which is restored to the native adenine base in DNA upon release of glyoxal. The existence of multiple intermediates along the repair pathway makes RapidFire/MS particularly suited for studying this reaction, because the system enables the high-throughput measurement of all four reaction species over time. Figure 2 shows that while the majority of converted substrate forms the adenine product, basal levels of the two repair intermediates are observed throughout the duration of the experiment. Therefore, alternative assay platforms that measure only the adenine product would not accurately relate the extent (or inhibition) of AlkB activity.

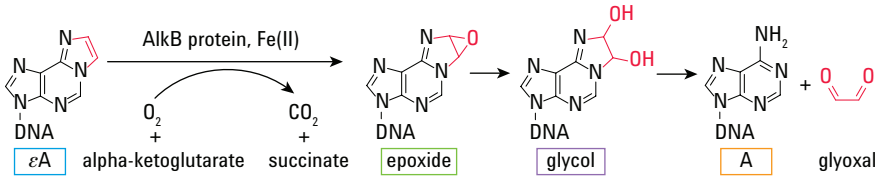


Figure 1. AlkB reaction mechanism for repair of ethenoadenine (eA) to adenine (A). Epoxidation of $\varepsilon$A at the etheno bond yields the epoxide intermediate. This species hydrolyzes to the glycol intermediate, which, upon release of glyoxal, is restored to the native adenine base in DNA.

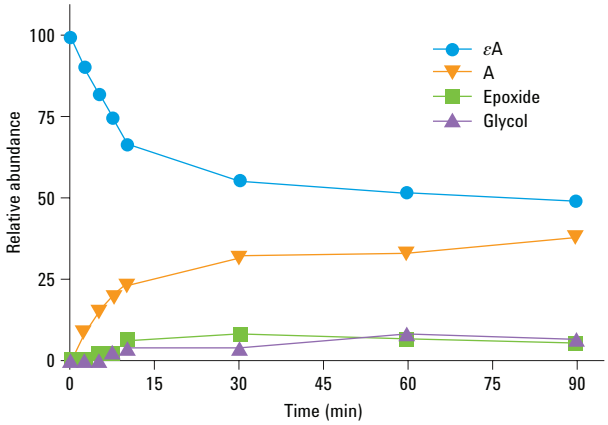


Figure 2. Conversion, over time, of the $\varepsilon$A AlkB substrate (contained within a 16-mer deoxyoligonucleotide) to the epoxide intermediate, glycol intermediate, and final product adenine.

## Measurement of cytosine methylation in DNA by RapidFire/MS

Methylation of cytosine residues in DNA plays an important role in epigenetic gene regulation. The addition of methyl groups to DNA is carried out by a number of DNA methyltransferase (MTase) enzymes. As a model system we chose the SssI MTase, which methylates cytosine bases in CpG sequences, and a 24-mer DNA duplex substrate (Figure 3). The substrate was designed to have eight possible sites of methylation on the top DNA strand. To stimulate enzymatic activity, the cytosine bases in the bottom DNA strand were premethylated before reaction.

Following a 1 hour incubation between the DNA MTase and the model substrate, all nine possible methylation states of the top strand (0-methyl to 8-methyl) were measured by RapidFire/MS (Figure 4). While the abundances of the possible products were distributed, the predominant number of methylations observed was five. Longer reaction times did not produce products that were methylated further (data not shown), indicating the MTase has differential activity on the eight possible sites of methylation.

To simplify the calculations of substrate conversion over time, the abundances of the 1-methyl to the 6-methyl products were added together to produce a total products value. Percent conversion was then calculated by multiplying the total product value by 100 and dividing that number by substrate-plus-products. This representation of the data allowed the determination of reaction conditions that yielded linear substrate methylation over time (Figure 5).

5′– CGGCCG CGGCCG CGGCCG CGGCCG–3′

3′– GCCGGC GCCGGC GCCGGC GCCGGC–5′

Figure 3. Model substrate for measuring multiple methylations of DNA. The top DNA strand contains eight potential sites for cytosine methylation (marked in blue). The cytosine bases in the bottom DNA strand were pre-methylated before reaction.

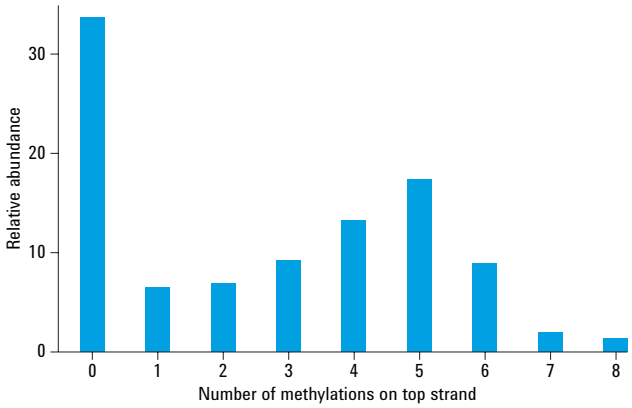


Figure 4. Measurement of all possible methylation products of the top DNA strand (Figure 3) after a 1 hour reaction.

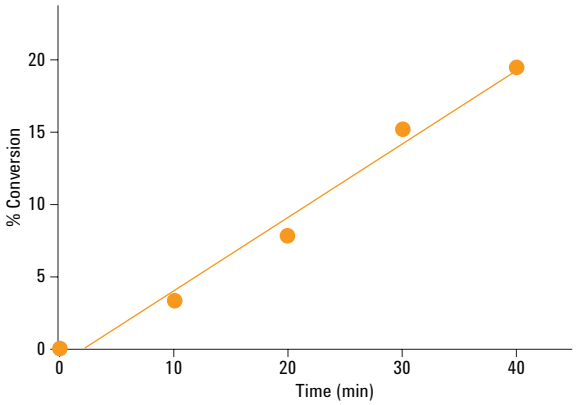


Figure 5. Methylation of DNA over time. The abundances of the 1-methyl to the 6-methyl products were added together to produce a total products value. Percent conversion was calculated by multiplying the total product value by 100 and dividing that number by substrate-plus-products.

In conjunction with validating an assay suitable for screening, a concentration response experiment was conducted with S-adenosylhomocysteine (SAH), a known DNA MTase inhibitor. Results demonstrated a concentration-specific inhibition of MTase activity by SAH, and the calculated $IC_{50}$ value (approximately 14 µM, Figure 6) was within the expected range for the reaction conditions used.

Further, a set of 384 known bio-actives was screened at 10 µM against the DNA MTase, and the results were ordered by frequency of percent inhibition observed (Figure 7). Hits were designated by inhibition values greater than three standard deviations from the mean of the set. Eight hits were identified and comparison of the structures revealed a number of substituted quinolones, which have previously been identified as capable of inhibiting MTase enzymes[6].

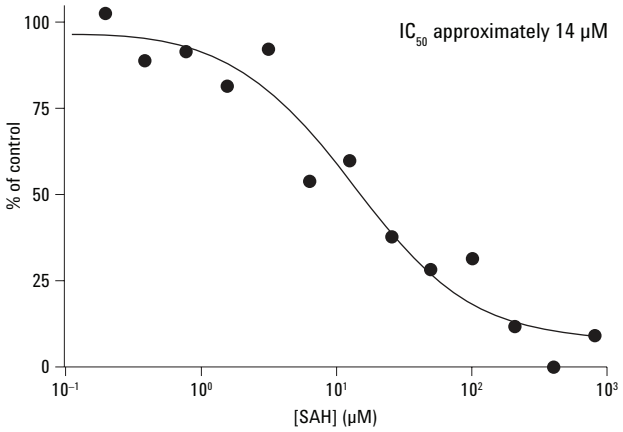


Figure 6. Concentration response curve for S-adenosylhomocysteine (SAH).

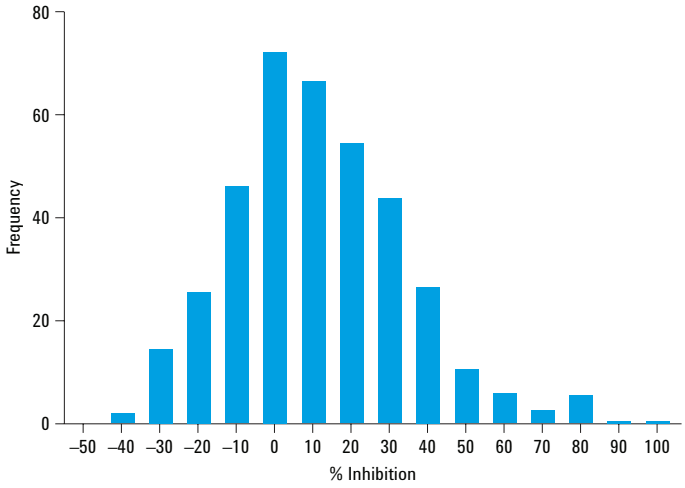


Figure 7. Histogram of results from screening 384 known bio-actives against cytosine methylation in DNA.

## Conclusions

The Agilent RapidFire Mass spectrometry System has multiple advantages for measuring epigenetic modifications and repair events on DNA. Direct detection of native DNA species by TOF-MS circumvents the need for substrate labels, avoiding any adverse effect these labels may have on the reaction. Additionally, direct measurement of reactants bypasses the time and trouble associated with the development of artificial substrates or product-specific antibodies necessary with other assay formats. TOF-MS detection also enables the measurement of multiple species from each reaction. Consequently, reaction data can be normalized to improve quality, and provide unparalleled detail about the target reaction. Finally, the RapidFire system enables the quality of TOF-MS data to be achieved with little sacrifice to throughput. Microscale online sample preparation is achieved in approximately 8 seconds, enabling the analysis of over 10,000 samples per day.

## References


1. Kelley M.R. and Fishel M.L., DNA repair proteins as molecular targets for cancer therapeutics. *Anticancer Agents Med Chem*., **2008** May;8(4):417-25.

2. Mishra M.V., Bisht K.S., Sun L., Muldoon-Jacobs K., Awwad R., Kaushal A., Nguyen P., Huang L., Pennington J.D., Markovina S., Bradbury C.M., and Gius D., DNMT1 as a molecular target in a multimodality-resistant phenotype in tumor cells. *Mol Cancer Res*., **2008** Feb;6(2):243-9.

3. Koike K., Ueda Y., Hase H., Kitae K., Fusamae Y., Masai S., Inagaki T., Saigo Y., Hirasawa S., Nakajima K., Ohshio I., Makino Y., Konishi N., Yamamoto H., and Tsujikawa K., Anti-tumor effect of AlkB homolog 3 knockdown in hormone-independent prostate cancer cells. *Curr Cancer Drug Targets.*, **2012** Sep;12(7):847-56.

4. Pan Z., Sikandar S., Witherspoon M., Dizon D., Nguyen T., Benirschke K., Wiley C., Vrana P., and Lipkin S.M., Impaired placental trophoblast lineage differentiation in Alkbh1(-/-) mice. *Dev Dyn*., **2008** Feb;237(2):316-27.

5. Delaney J.C., Smeester L., Wong C., Frick L.E., Taghizadeh K., Wishnok J.S., Drennan C.L., Samson L.D., and Essigmann J.M., AlkB reverses etheno DNA lesions caused by lipid oxidation *in vitro* and *in vivo*. *Nat Struct Mol Biol*., **2005** Oct;12(10):855-60.

6. Phiasivongsa P., Redkar S.G., Gamage S., Brooke D., Denny W., Bearss D.J., Vankayalapati H., Xu Y., and Swierczek K., Quinoline Derivatives for Modulating DNA Methylation. Patent application number: 20110256092. Oct 20, **2011**.

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