TECHNICAL NOTE

Automated bisulfite conversion for Infinium™ Methylation BeadChips

Higher efficiency DNA processing for epigenetic studies

- Rapid bisulfite conversion shortens overall assay time
- Automated liquid handling reduces hands-on time

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Introduction

DNA methylation

DNA methylation is a biological phenomenon that regulates gene expression in response to genetic and environmental factors. In mammals, changes in the DNA methylome, primarily through methylation of cytosine bases in CpG dinucleotides, arise from events such as embryonic development, oncogenesis, infection, and environmental exposure.[1-6] Studying DNA methylation can provide important insights into how specific stimuli change genetic regulation. In terms of applications, DNA methylation is a promising biomarker, and high-accuracy models, and classifiers have been built using methylation signatures.[7-9] Bioinformatic tools have been developed for the diagnosis of cancer, classification of various Mendelian disorders, and determination of a research subject's age without a priori knowledge.[10-12]

Infinium Methylation BeadChips are microarray tools for quantitative, high-throughput measurements of DNA methylation at the single CpG site level.[13,14] Currently, there are three BeadChip configurations for DNA methylation analysis: 8×1 HD, 12×1 HD, and 24×1 HTS (Figure 1).

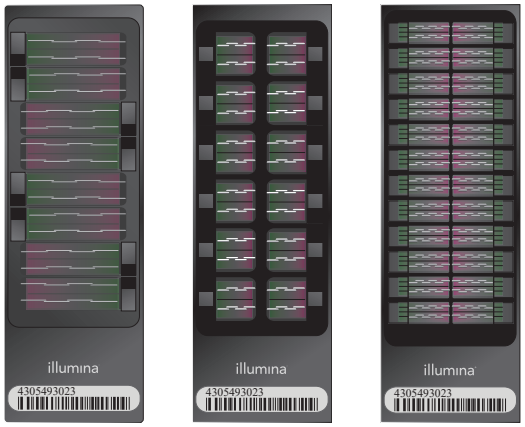

Figure 1: Methylation BeadChips are available in 8×1 HD, 12×1 HD, and 24×1 HTS configurations, shown from left to right.

Bisulfite conversion

To measure the proportions of methylated and unmethylated cytosine bases using Infinium Methylation BeadChips, the input DNA must first undergo bisulfite conversion. Under specific conditions, bisulfite reaction with DNA causes unmethylated cytosine bases to be rapidly deaminated to form uracil (Figure 2A), while methylated cytosine bases deaminate approximately two orders of magnitude more slowly (Figure 2B).[15,16] After whole-genome isothermal amplification, bisulfite-reacted cytosine bases are converted to thymine and unreacted methylcytosine bases are retained as cytosine. The bisulfite-treated DNA can be inputted into the Infinium Methylation Assay for methylation quantification using Illumina Methylation BeadChips.

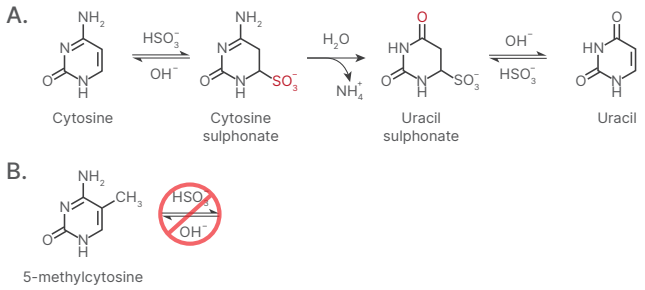

Figure 2: Depiction of bisulfite conversion of cytosine nucleotides—(A) Chemical equilibrium of bisulfite conversion of cytosine to uracil. (B) Under optimized reaction conditions, bisulfite conversion of 5-methylcytosine does not occur to an appreciable degree.

While bisulfite conversion is a robust technique, this process adds time and labor that can hinder high-throughput processing. This technical note describes workflow improvements for bisulfite conversion that can reduce the time and manual handling involved in processing samples for methylation analysis.

We tested the ability of a rapid bisulfite conversion protocol to shorten the current workflow for Infinium Methylation BeadChips from 1.5 days to approximately three hours. We also examined whether a Hamilton liquid-handling robot was able to carry out the same rapid bisulfite conversion in order to reduce the hands-on time required. The results of this study show that a rapid bisulfite conversion kit is functionally equivalent to the previously validated bisulfite conversion kit when tested with cell line DNA in Infinium Methylation Assay protocols. We also show that an automation-supported protocol can be deployed to deliver equivalent results to manual processing.

Methods

DNA samples

Experiments with 8×1 HD BeadChips used 500 ng of HeLa (BioChain Institute, Catalog no. D1255811), Raji (BioChain Institute, Catalog no. D1255840), MCF7 (BioChain Institute, Catalog no. D1255830), or Jurkat (BioChain Institute, Catalog no. D1255815) cell line DNA input. Experiments with 24×1 HTS BeadChips used 500 ng or 1000 ng of the same DNA samples. The following kits were used for manual bisulfite conversion of DNA samples: EZ-96 DNA Methylation (Zymo Research, Catalog no. D5004), and EZ-96 DNA Methylation-Lightning MagPrep (Zymo Research, Catalog no. D5046). EZ-96 DNA Methylation-Lightning MagPrep (Zymo Research, Catalog no. D5046) was used for automated bisulfite conversion with the Hamilton Microlab STAR Liquid Handling System. The deck layout for automated procedures using the EZ-96 DNA Methylation-Lightning MagPrep Kit is shown in Figure 3.

Infinium Methylation Assays

For tests using 8×1 HD BeadChips, the standard Infinium HD Methylation assay procedure was used (available on the Infinium MethylationEPIC BeadChip support site). For tests using 24×1 HTS BeadChips, an updated Infinium HTS Methylation Assay was followed, available on the iSelect HTS Methyl Custom BeadChip Support Site. The following BeadChips were used for testing: 8×1 HD containing Human MethylationEPIC bead pools 1, 2, 3, 4, 5, and 6; 8×1 HD containing Human MethylationEPIC bead pools 1, 2, and 3; and 24×1 HTS containing Human MethylationEPIC bead pools 1, 2, 3, and 4.

Results

We compared bisulfite conversion performance between the EZ-96 DNA Methylation Kit, a kit previously validated for use with the Infinium Methylation Assay, and the EZ-96 DNA Methylation-Lightning MagPrep Kit with manual and liquid-handling automated protocols. As one metric, we tested whether a rapid bisulfite conversion kit, carried out manually or using liquid-handling automation, would yield the same degree of probes passing detection over background as with the EZ-96 DNA Methylation Kit. When comparing samples processed manually with both the EZ-96 DNA Methylation Kit and the EZ-96 DNA Methylation-Lightning MagPrep Kit using 8×1 HD BeadChips containing MethylationEPIC bead pools 1, 2, and 3, we found that both kits were able to provide > 98% of probes passing detection over background using a p-value < 0.01 (Table 1, rows 1 and 2). The percentage of probes passing detection was within error for both kits.

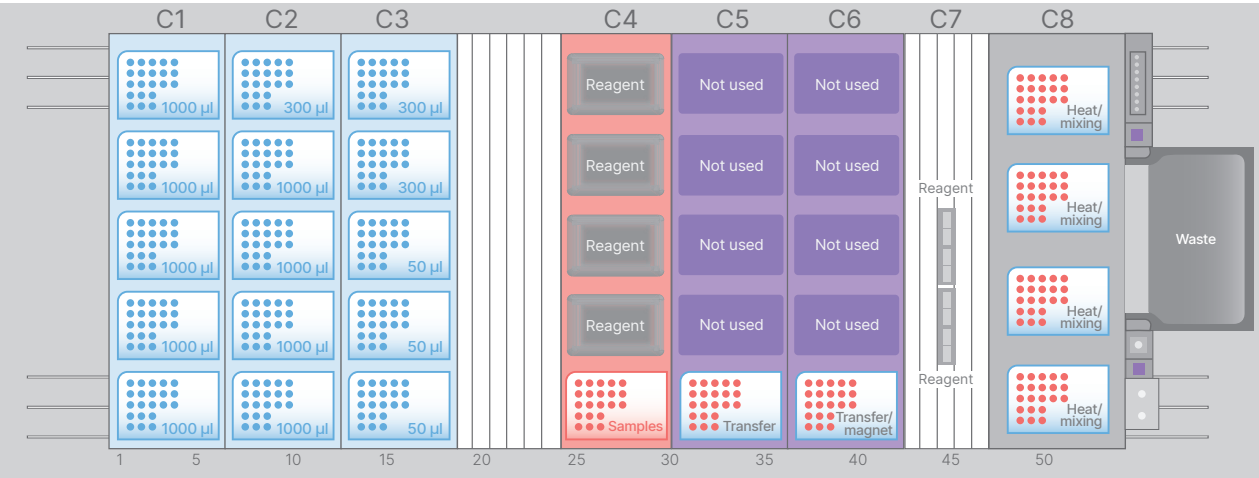

Figure 3: Hamilton Microlab STAR liquid handler deck layout used for 96-well bisulfite conversion of samples with EZ-96 DNA Methylation-Lightning MagPrep Kit—Carriers C1, C2 and C3 contain pipette tips. The top 4 positions of C4 contain bisulfite conversion reagents. The bottom rows of carrier C4, C5, and C6 are used for sample transfer steps. The bottom row of carrier C6 contains the magnet for bead capture. Carrier C7 contains additional bisulfite conversion reagents. Carrier position C8 contains on-deck heating and mixing elements.

Table 1: Bisulfite conversion experimental summary

Kit (Catalog no.)	BeadChip format (bead pools used)	Probes	Preparation	Probes passing detection (%)[a]	No. of samples	Cell line
EZ DNA Methylation (D5004)	8×1 HD (MethylationEPIC 1, 2, 3)	451,687	Manual	99.89 ± 0.01	16	Raji, MCF7
EZ DNA Methylation Lightning MagPrep (D5046)	8×1 HD (MethylationEPIC 1, 2, 3)	451,687	Manual	99.91 ± 0.04	15	Raji, MCF7
EZ DNA Methylation (D5004)	8×1 HD (MethylationEPIC, 1, 2, 3, 4, 5, 6)	865,918	Manual	99.84 ± 0.05	20	HeLa, Jurkat, Raji, MCF7
EZ DNA Methylation Lightning MagPrep (D5046)	8×1 HD (MethylationEPIC, 1, 2, 3, 4, 5, 6)	865,918	Automated	99.46 ± 0.38	24	HeLa, Jurkat, Raji, MCF7
EZ DNA Methylation (D5004)	24×1 HTS (MethylationEPIC 1, 2, 3, 4)	615,320	Manual	99.61 ± 0.18	48	HeLa, Jurkat, Raji, MCF7
EZ DNA Methylation Lightning MagPrep (D5046)	24×1 HTS (MethylationEPIC 1, 2, 3, 4)	615,320	Automated	99.20 ± 0.38	30	HeLa, Jurkat, Raji, MCF7

a. Detection at p-value < 0.01

This observation suggests that the manually performed EZ-96 DNA Methylation-Lightning MagPrep Kit converts DNA to an equivalent degree to the legacy EZ-96 DNA Methylation Kit and can be used in Infinium Methylation Assay manual workflows.

Next, we examined the capability of the EZ-96 DNA Methylation-Lightning MagPrep Kit, carried out with liquid-handling automation, to provide the same level of probes passing the detection threshold as observed with manual preparation using the EZ-96 DNA Methylation Kit (Table 1, rows 3 through 6). Using 8×1 HD BeadChips containing MethylationEPIC bead pools 1, 2, 3, 4, 5, and 6 and 24×1 HTS BeadChips containing MethylationEPIC bead pools 1, 2, 3, and 4, we found that both configurations supported > 98% of probes passing detection with a p-value < 0.01. Additionally, the number of probes passing the detection threshold when using manual and automated procedures, for both BeadChip configurations, was within error. These findings support the use of the EZ-96 DNA Methylation-Lightning MagPrep Kit in an automated configuration with 8×1 HD and 24×1 HTS BeadChips.

We also analyzed the degree of concordance for identical DNA samples that were bisulfite converted using different methods. The concordance plot for two replicate samples run on Human MethylationEPIC typically yields an $r^2 > 0.96$, providing a useful benchmark for evaluating performance between manual and automated protocols, or between different bisulfite conversion kits. If technical replicates generated using a new bisulfite conversion technique do not exhibit a reduced r^2 value compared to technical replicates generated using the previously validated method, then the new bisulfite conversion method can be substituted into the Infinium Methylation Assay protocol.

We compared the manually performed EZ-96 DNA Methylation Kit with the manually performed EZ-96 DNA Methylation-Lightning MagPrep Kit to check compatibility of the latter kit with manual processing. Samples were run on an 8×1 HD BeadChip containing MethylationEPIC bead pools 1, 2, and 3. In a comparison between the two kits, the beta value concordance plots obtained for bisulfite-converted MCF7 DNA samples showed an $r^2 > 0.96$ with no effect due to sample positions on the BeadChips (Figure 4). This supports the conclusion that the EZ-96 DNA Methylation-Lightning Kit can be used in manual protocols for bisulfite conversion upstream to Infinium Methylation Assays.

Next, using an 8×1 BeadChip containing Methylation EPIC bead pools 1, 2, 3, 4, 5, and 6, we compared data from HeLa DNA samples processed manually with the EZ-96 DNA Methylation Kit, and in an automated protocol using the EZ-96 DNA Methylation-Lightning MagPrep Kit. Replicate samples in this comparison produced an

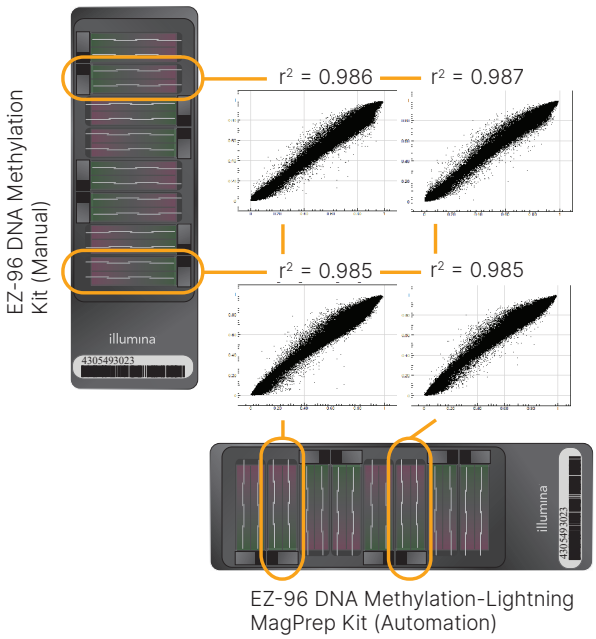

Figure 4: Beta value concordance plot comparison of MCF7 cell line DNA methylation run on 8×1 HD BeadChips—DNA was manually processed with either the EZ-96 DNA Methylation Kit or the EZ-96 DNA Methylation-Lightning MagPrep Kit and run on 8×1 HD BeadChips containing MethylationEPIC bead pools 1, 2, and 3.

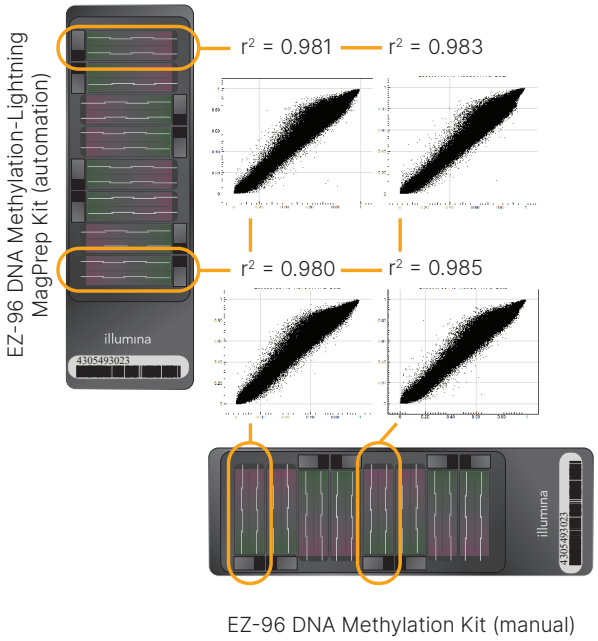

Figure 5: Beta value concordance plot comparison of HeLa cell line DNA methylation run on 8×1 HD BeadChips—DNA was manually processed with the EZ-96 DNA Methylation Kit or processed with liquid-handling automation using the EZ-96 DNA Methylation-Lightning MagPrep Kit. Processed DNA was run on 8×1 HD BeadChips containing MethylationEPIC bead pools 1, 2, 3, 4, 5, and 6.

r^2 > 0.96 regardless of the location of samples on the BeadChips, demonstrating concordance of methylation levels between the manual and automated bisulfite conversion protocols (Figure 5). These data show that the EZ-96 DNA Methylation-Lightning MagPrep kit used with liquid-handling automation meets the criteria established for compatibility with the 8×1 HD methylation BeadChips.

We finally compared data from HeLa DNA samples processed manually using the EZ-96 DNA Methylation Kit and processed using liquid-handling automation with the EZ-96 DNA Methylation-Lightning MagPrep Kit, prior to running on 24×1 HTS BeadChips configured with Human MethylationEPIC bead pools 1, 2, 3, and 4. Concordance plots for these samples showed r^2 > 0.96 for the two bisulfite conversion methodologies without any effect of BeadChip sample positions on the data. These results provide support for the use of liquid-handling automation and the EZ-96 DNA Methylation-Lightning Kit for bisulfite conversion when using Infinium 24×1 HTS Methylation BeadChips (Figure 6).

Conclusions

The results shown in this technical note support the use of the Zymo Research EZ-96 DNA Methylation-Lightning MagPrep Kit, in manual and automated formats, upstream to Infinium Methylation Assays. Minimal differences can be expected in terms of probes passing detection metrics or final measured DNA methylation levels when using either the EZ-96 DNA Methylation Kit, or the EZ-96 DNA Methylation-Lightning MagPrep Kit.

Until recently, researchers have not had a rapid or automated solution for bisulfite conversion of DNA in preparation for Infinium Methylation Assay analysis. We anticipate that users will benefit from these findings through decreased overall processing time and reduced hands-on time for bisulfite conversion. As a result, this will help laboratories scale up the number of Infinium methylation BeadChips they are able to process.

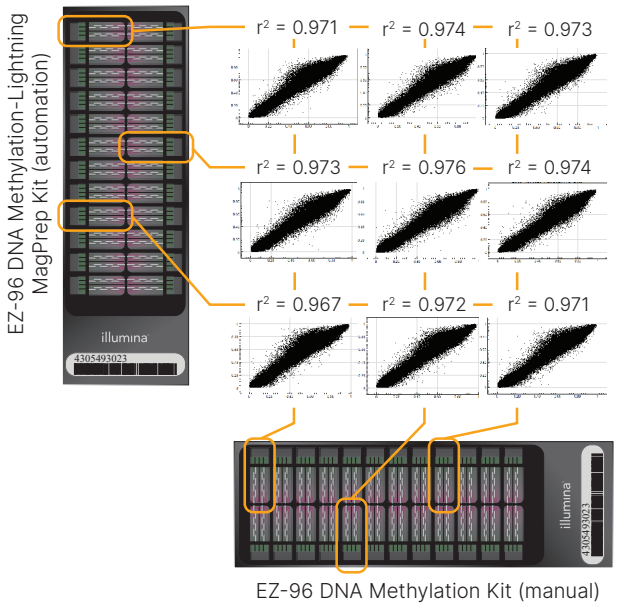

Figure 6: Beta value concordance plot comparison of HeLa cell line DNA methylation run on 24×1 HTS BeadChips—DNA was manually processed with the EZ-96 DNA Methylation Kit or processed using liquid-handling automation with the EZ-96 DNA Methylation-Lightning MagPrep Kit and then ran on 24×1 HTS BeadChips containing MethylationEPIC bead pools 1, 2, 3, and 4.

Deployment of rapid bisulfite conversion in service laboratories can be achieved with manual and automation-compatible kit configurations that are available from Zymo Research. For manual rapid bisulfite conversion, Illumina recommends using the EZ DNA Methylation-Lightning MagPrep Kit, (Catalog no. D5046). For automated rapid bisulfite conversion, Zymo Research has developed a kit configuration (Catalog no. D5049) that is equivalent to the EZ-96 DNA Methylation-Lightning MagPrep Kit, but contains buffer and reagent volumes needed for procedures using liquid-handling automation.

Manual or automated rapid bisulfite conversion procedures should require 3 to 3.5 hours to process up to 96 samples. The automation-compatible bisulfite conversion kit, liquid-handling automation scripts, and setup support are available through Zymo Research. The Microlab STAR liquid-handling automation device can be obtained from Hamilton Robotics, Inc. Other automation devices and configurations are available and supported by Zymo Research. For information about plasticware that can be used in automated procedures, please refer to the Infinium 24×1 HTS Methyl Custom BeadChip support site or contact Zymo Research technical support.

With regards to sample quality, it must be noted that the described rapid manual and automated bisulfite procedures were carried out with fresh DNA samples. Formalin-fixed, paraffin-embedded (FFPE) samples remain untested. While we do not currently have a validated protocol for FFPE samples, it is anticipated that this type of method would be useful in clinical research applications. We anticipate testing these samples in the future. Finally, while experimental tests for 12×1 HD BeadChips were not shown in this technical note, similar performance was observed for this format compared to 8×1 HD and 24×1 HTS formats.

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