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A Biotechnical Newsletter

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Epigenetics

SPECIAL EDITION

We now know it is not "Nature vs. Nurture," but it is how "Nurture affects Nature."

A wide range of environmental factors such as nutrition, stress and environmental toxicants can directly alter epigenetic programming during development to influence physiology and disease susceptibility in all organs, including the brain. If the germline (sperm or egg) epigenetics is altered then the environmental factor can promote the epigenetic transgenerational inheritance of disease susceptibility and phenotypic variation. This is an environmentally responsive non-genetic (epigenetic) form of inheritance which impacts all biological processes from disease etiology to evolutionary biology. For example, exposure of a gestating female can alter the germline of the offspring that then transmits this altered epigenetic information to subsequent generations (great-grand-offspring) to influence disease susceptibility (testis, ovary, kidney, mammary gland disease or obesity) and the behavioral stress response.

Therefore, what your great-grandmother was exposed to during pregnancy can effect your disease susceptibility and behavior, and you are going to pass this on to your grandchildren. This environmentally induced epigenetic transgenerational inheritance of disease susceptibility provides novel insights into disease etiology. These epigenetic biomarkers or diagnostics in the future may help identify ancestral or early life exposures and the associated later life disease. Use of these epigenetic biomarkers would allow early stage diagnostics, prior to disease development, such that preventative medicine could become a reality.

- Dr. Michael Skinner

Dr. Michael K. Skinner, Eastlick Distinguished Professor and Founding Director, Center for Reproductive Biology, School of Biological Sciences, Washington State University, Pullman, WA, USA.

Dr. Skinner's research is focused on the area of reproductive biology and environmental epigenetics. His current research has demonstrated the ability of environmental toxicants such as endocrine disruptors to promote epigenetic transgenerational inheritance of adult onset disease phenotypes, due to abnormal germline epigenetic programming during gonadal development. This non-genetic form of inheritance has a role in disease etiology and areas such as evolution. His research has been highlighted in BBC, Smithsonian and PBS documentaries and selected as top 100 discoveries in 2005 and 2007 by Discover. (**www.skinner.wsu.edu**)

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Contents

1 Epigenetics Overview

3 Epigenetics Research Methods

6 Featured Article
The Double Helix Epigenetic Switch: 5-methylcytosine and 5-hydroxymethylcytosine Exert Opposite Forces on Base Pairing of DNA Double Helix

8 Epigenetic Applications

11 Technology Spotlight
A Comprehensive Guide to Bisulfite-Converted DNA Amplification

18 Going Global with DNA Methylation Analysis

21 Genome-wide DNA Methylation Analysis Methods

25 Locus Specific 5-mC and 5-hmC Analysis

27 Featured Article
Forever 21? It may be Possible by Resetting your DNA Methylation Age Clock

28 Technology Spotlight
State-of-the-Art Services for DNA Methylation Analysis

35 Epigenetics Tools and Databases

36 Who Recommends Zymo Research Products?

37 Epigenetics Glossary

39 Epigenetics Product Index

Epigenetics Overview

So what is epigenetics?

Depending on whom you ask, it can be a number of things. In its most literal sense, epigenetics means "above" or "on top of" genetics. At a molecular level, it generally encompasses the study of chemical and structural modifications to DNA, or the proteins that condense DNA into compact bundles without altering the primary sequence. These epigenetic modifications play critical roles in the regulation of numerous cellular processes, including gene expression, DNA replication, and recombination. Epigenetic regulatory mechanisms include DNA methylation and hydroxymethylation, histone modifications, chromatin remodeling, RNA modifications (e.g. methylation), and regulation by small and long non-coding RNAs. When epigenetic mechanisms are misregulated, the result can be detrimental to health and can lead to cancer, neurological disorders, and developmental abnormalities. Therefore, epigenetic modifications are emerging as important diagnostic and prognostic biomarkers in many fields of medicine.

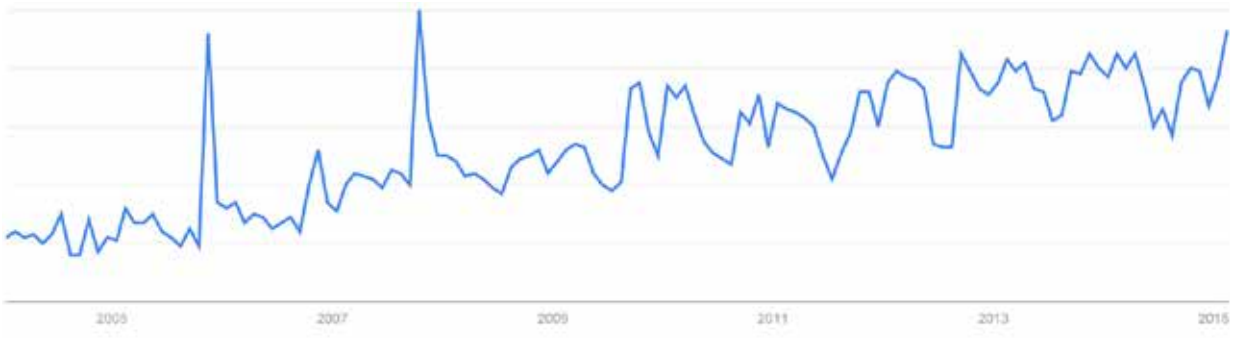
In recent years, epigenetics has exploded into one of the most exciting and rapidly expanding fields in biology. As The Epigenetics Company™, the scientists at Zymo Research understand the need for high-quality and reliable materials for epigenetics research, and offer an extensive line of products, and genome-wide services to facilitate investigations into epigenetic regulation of cellular processes. This "Special Edition" Epigenetics Newsletter is a guide for new and experienced researchers alike. It will introduce some of the more significant principles and recent research in areas like DNA methylation, histone modifications, and chromatin architecture. Zymo Research is committed to propelling the field of epigenetics forward by providing researchers of every discipline with the tools and knowledge needed to ensure experimental success.

DNA Methylation and Maintenance

In eukaryotes, methylation typically occurs at cytosine-guanine dinucleotides (CpGs). DNA methylation represses gene expression by blocking positive transcription factors and promoting the binding of repressive proteins (the palindromic CpG sequence is methylated on both DNA strands, allowing its propagation through cell division). DNA methylation is critical for regulating development and disease, and it can act as a link between the genome and environment through factors like diet and aging.

In humans, the maintenance of DNA methylation marks occurs enzymatically via DNA methyltransferases (DNMTs). DNMT1 recognizes hemi-methylated sites in post-mitotic cells and fully methylates them, while the *de novo* methyltransferases DNMT3a and DNMT3b target unmethylated DNA and are vital for early embryonic development[1].

DNA methylation was long-thought to be irreversible, but recently, active DNA demethylation has been demonstrated[2,3]. DNA demethylation occurs via a step-wise oxidation of 5-methylcytosine that is catalyzed by the Ten-Eleven Translocation (TET) enzymes. The first oxidation product in this process, 5-hydroxymethylcytosine (5-hmC), has been shown to act as a unique epigenetic mark. 5-hmC is more than just a side product of active demethylation, and it is enriched in the brain of the developing embryo and positively correlates with gene expression[4-6].



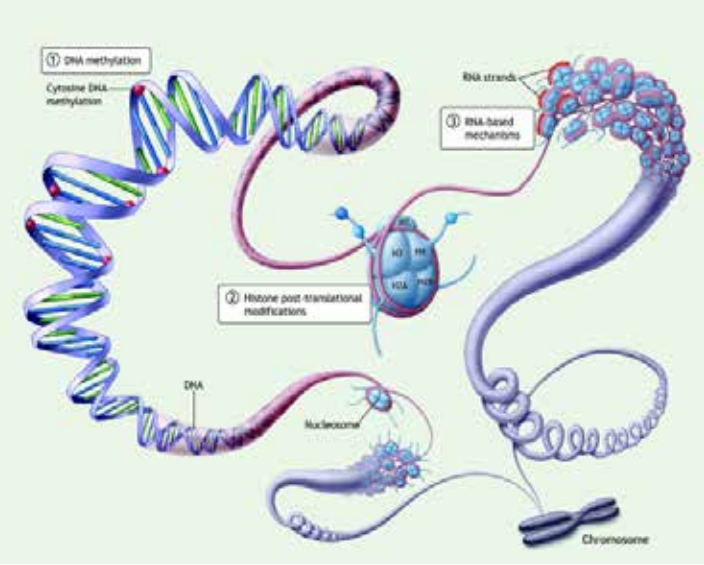
Epigenetics on the rise: A quick look at epigenetics news trending over the last few years. Source: Google Trends

Chromatin Architecture and Regulation

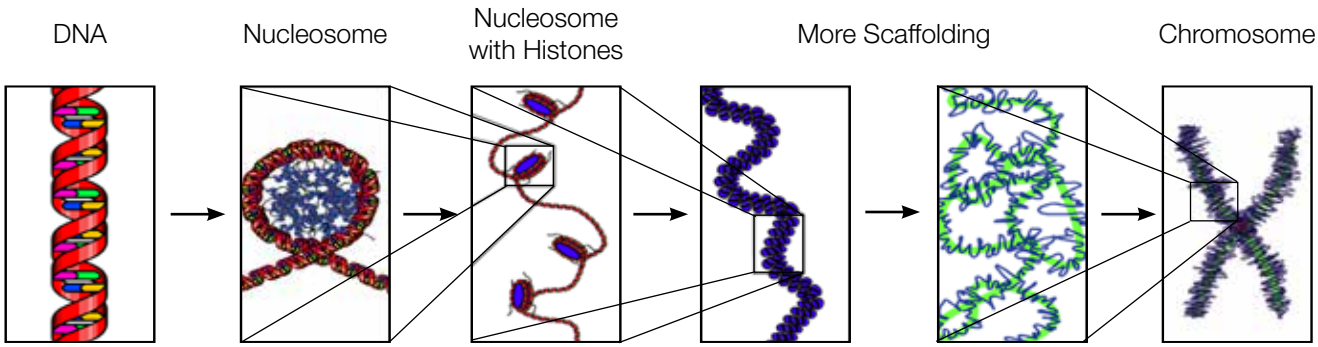
Modulation of chromatin structure is another method cells use to control gene expression. Compaction of the chromatin promotes gene silencing, while unravelling promotes gene expression. Aberrant chromatin architecture causes many diseases and disorders. For example, normal aging may be caused, in part, by a general loss of histones, altered heterochromatin structure, and changes in histone modification patterns[7]. The basic building block of chromatin is the nucleosome which is comprised of eight histone proteins. Covalent histone modifications control many aspects of chromatin structure through the recruitment of specific histone modifying proteins. Specific modifications such as acetylation, methylation and phosphorylation on specific residues in histone tails can result in diverse biological outcomes.

Histone Modifications

Chemical modifications to histone proteins, often referred to as the 'histone code', create a vast array of combinations that impact chromatin structure and gene regulation. Acetylation promotes gene expression because it reduces the positive charge of the histone and weakens its interaction with DNA. Methylation is more complex, relying on the recruitment of chromatin modifying proteins that affect chromatin structure[8]. The recent discovery of histone demethylases has shown that histone methylation is far more pliable than previously thought. It is becoming clear that complex chromatin interactions are critical for cellular function. Technological advances in genome-wide sequencing have allowed characterization of chromatin interactions in development and disease[9].



Epigenetic mechanisms of gene regulation. Source: Matouk, C et al. (2008). Epigenetic Regulation of vascular endothelial gene expression. Circulation Research 102: 873-887.



Up close and personal with DNA in different levels of compaction. Source: Wikipedia

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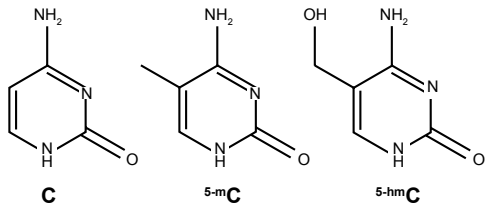
Epigenetics Research Methods

Studying epigenetic mechanisms introduces additional layers of complexity when compared to sequence- or protein-based studies alone. Epigenetic marks, like DNA methylation, or modifications to proteins like post-translational histone modifications are challenging to study directly. As a result, it's often necessary to manipulate samples to be more easily studied using common laboratory approaches like PCR and sequencing. Let's take a closer look into these areas.

DNA Methylation Analysis

DNA methylation includes several modifications to cytosine nucleotides. In mammals, methylation typically occurs at cytosine residues in CpG dinucleotides, large groups of which are termed "CpG islands". 5-mC is a critical epigenetic modification, and promoter 5-mCpG methylation is often associated with transcriptional silencing[1]. Regulation of gene expression by 5-mC is vital for multiple processes in development and disease[2]. 5-mC can also be enzymatically oxidized to restore unmethylated cytosine, and an intermediate of this process, 5-hydroxymethylcytosine (5-hmC), serves as another epigenetic mark that appears to have distinct functionality. 5-hmC is linked to transcriptional activation and appears to be critical for neuronal function[3]. The oxidation pathway also has several other intermediates, that have their own novel biological functions (i.e., formylcytosine and carboxylcytosine)[4].

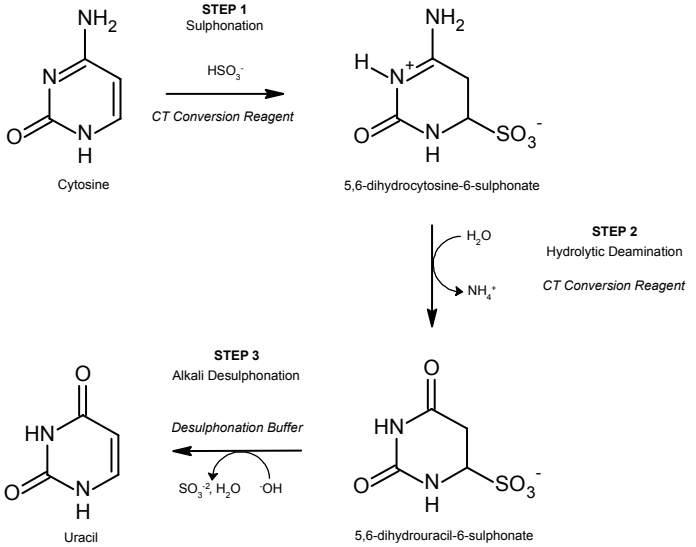


Structural differences between unmethylated cytosine, 5-methylcytosine (5-mC), and 5-hydroxymethylcytosine (5-hmC).

Challenges in DNA Methylation Analysis

Studying nucleic acid modifications like DNA methylation is inherently more challenging than just analyzing DNA sequences. PCR-based techniques do not preserve DNA methylation marks, making it impossible to "amplify" methylated DNA sequences directly from their native form. Bisulfite conversion has been the cornerstone of DNA methylation analysis since its introduction in the early 90's, and continues to be the most widely used sample preparation approach for studying DNA methylation in labs today.

The bisulfite conversion process is based on the following principle: sodium bisulfite converts unmethylated cytosine to uracil while leaving 5-mC intact[5]. This introduces specific changes to the DNA sequence that allows DNA methylation mapping at single nucleotide resolution.



Bisulfite Treatment: The gold standard for the analysis of DNA methylation, bisulfite treatment, converts unmodified cytosine to uracil while methylated cytosines are protected from this conversion (EZ DNA Methylation™ Kits, p.39). Sequence analysis post-treatment provides site specific information on DNA across the genome. This can be accomplished by PCR, hybridization, MSP, and Next-Gen sequencing.

However, the process is not without its limitations as 5-hmC and some other oxidative marks can't be discerned by traditional bisulfite conversion methods, which makes it impossible to profile both marks simultaneously[6]. While bisulfite conversion applications are useful in pinpointing DNA methylation, these classic approaches will not discern what specific DNA methylation modification is present (i.e. 5-mC or 5-hmC).

Alternative approaches for detecting DNA methylation include a variety of enrichment techniques that employ antibodies or methyl-binding proteins to bind methylated genomic DNA fragments.

An upside of using enrichment-based approaches is the ability to specifically enrich DNA based on its methylation modifications. DNA methylation enrichment can be useful in settings where researchers would like to map the specific profiles of these marks easily. But the downside to enrichment approaches is a lack of precision in their resolution. They can map a DNA methylation mark to a genomic region, but rarely to the specific base where the modification exists. The importance of the technical benefits of these approaches varies considerably as the needs of researchers can be quite different. As a result, there are a variety of DNA methylation analysis approaches in practice today. These are addressed in the sections that follow.

Standards and Controls

Specially designed DNA standards and controls may not be flashy, but they are essential for correct experimental design and successful data analysis. Without that, you're going to be struggling to nab that next big publication.

But don't worry, with the proper controls you will be able to pinpoint experimental issues with laser precision, not to mention make more sense of your data and increase the confidence in your results. Sound too good to be true? Well, the controls from Zymo Research include a suite of standards and products to help keep your DNA methylation experiments under wraps. Here is a run down of the types of standards and controls that are available, as well as some helpful advice on how to put them to good use.

Clamp-Down on Bisulfite Conversion Efficiency

When using bisulfite conversion (especially homebrew methods) anyone can have a bad day, and when your conversion is off, you need to know about it.

Zymo Research offers several products to gauge the efficiency and "completeness" of DNA/bisulfite conversion reactions. Simply include one of the appropriate DNA standards into a bisulfite reaction in parallel with your samples, and you will be able to accurately control for the conversion process. This is facilitated by each standard having a defined methylation status, which allows conversion efficiency to be evaluated in the downstream application of choice[7,8]. The standards are ideal for a broad range of analyses including:

- ✓ Checking bisulfite PCR primer design
- ✓ Optimizing bisulfite PCR and sequencing reactions
- ✓ Generating methylated DNA standard curves for quantitation[9]

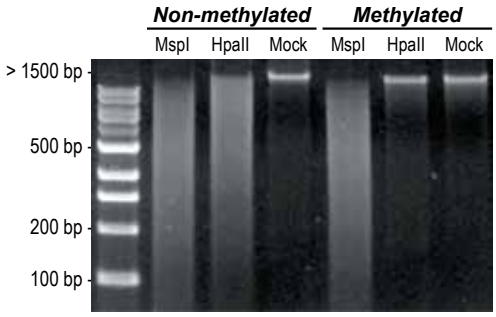
Each DNA set and standard is conveniently paired with a primer set designed to amplify a fragment of the DNA following bisulfite conversion. These standards are compatible with the EZ DNA Methylation™, EZ DNA Methylation-Gold®, EZ DNA Methylation-Direct™, and the EZ DNA Methylation-Lightning® bisulfite conversion kits (pg. 39) as well as with most homebrew methods.

Methylated & Non-Methylated Standards Derived from HCT116 DKO Cells

Methylated and non-methylated genomic DNAs are convenient for quantitating DNA methylation levels as well as for monitoring conversion efficiency in your assays. The completely methylated and non-methylated DNAs can be mixed together in various proportions to generate a standard curve for suitable quantitation of DNA methylation in your samples.

Human Methylated & Non-methylated DNA Set (Cat. No. D5014)**:** This control set contains HCT116 DKO non-methylated genomic DNA as well as enzymatically methylated (*in vitro*) DNA that has been methylated at every CpG site using CpG methylase.

Human WGA Methylated & Non-methylated DNA Set (Cat. No. D5013)**:** This control set is generated from HCT116 DKO cell genomic DNA using whole-genome amplification (WGA) technology. This standard is ideal for studying loci with ultra-low methylation levels.



Complete methylation assayed using M.SssI methylase. Non-methylated and methylated DNA was digested with restriction enzymes MspI and HpaII. MspI digests both non-methylated and methylated DNA. HpaII is sensitive to CpG methylation.

Standards Derived from Human and Mouse Tissues

Universal Methylated Human (Cat. No. D5011) **and Mouse** (Cat. No. D5012) **DNA Standards:** These universal standards provide a single completely methylated DNA from the species of interest. These genomic DNAs have been completely methylated at every CpG site, such that the methylation patterns are clearly defined. Also, Zymo Research offers a **Bisulfite-Converted Universal Methylated Human DNA Standard** (D5015). This standard is designed for use as a control for bisulfite- mediated conversion of DNA and downstream analyses including PCR, MSP, and other amplification-based assays.

Leave No Base Unchecked

In addition to the above standards, Zymo Research offers a number of controls and standards for use in qPCR, LC-MS, HPLC, and immuno-based assays including ELISA and dot blots.

5-Methylcytosine & 5-Hydroxymethylcytosine DNA Standard Set (Cat. No. D5405)**:** This extremely versatile standard set is ideal for the development of calibration curves for a number of assays such as qPCR, LC-MS, and HPLC. A unique feature of the 5-Methylcytosine & 5-Hydroxymethylcytosine DNA Standard Set is that it is a group of three linear 897 bp long dsDNA oligos containing the same sequence, but varying in their cytosine modifications. The cytosines are either unmodified, 5-mC or 5-hmC. This allows for the comparison of 5-mC and 5-hmC levels in any DNA to be interrogated.

Human Matched DNA Set (Cat. No. D5018)**:** This standard is comprised of a set of organ-specific human genomic DNAs derived from a single individual. These DNAs serve as an ideal control for 5-mC and 5-hmC detection and quantification, since cytosine and its methylated and hydroxymethylated counterparts are present at tissue-specific loci and in biologically relevant levels.

Mouse 5-hmC & 5-mC DNA Set (Cat. No. D5019)**:** Similar to the human set, the mouse genomic DNA standards function as the perfect control for detection and quantification of 5-mC and 5-hmC in your mouse DNA sample. 5-hmC and 5-mC levels within this DNA standard set have been precisely quantified using mass spectrometry (LC/MS) and thoroughly validated by ELISA to ensure the most consistent results.

Be a Control Freak!

Now that you've seen the wide assortment of standards and controls for DNA methylation analysis, there's really no excuse for not having a finger on the pulse of your experimental performance. Select the ones that will work best in your system, or consult the experts at Zymo Research who can help point you in the right direction.

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Case Study

The Dutch Hongerwinter (Hunger Winter) was a devastating famine that took place in a German-occupied part of the Netherlands near the end of World War II. It was an extremely cold winter that was further exacerbated by a German ban on food transport. Consequently, food availability dropped significantly and caloric intake was reduced to nearly 30% of the normal daily intake. By the time food supplies were restored seven months later, nearly 20,000 people had died.

From this great tragedy came remarkable scientific studies. Even though times were tough, women conceived and gave birth to babies. It was from these children and even their future children that researchers were able to look at the effects of maternal malnutrition during different periods of gestation on health in adult life. For example, if a baby was born small due to malnutrition throughout the mothers' pregnancy, they remained small for the rest of their lives with lower rates of obesity. On the other hand, babies that were born a normal size due to malnutrition only during the early stages of pregnancy had higher obesity rates than normal. What was even more intriguing was that these effects also trickled down to the grandchildren of the mothers that were malnourished during their pregnancy. These findings suggest that health conditions found later in life can originate through adaptations by the fetus in response to malnutrition during the mother's pregnancy[10-11].

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The Double Helix Epigenetic Switch:
5-methylcytosine and 5-hydroxymethylcytosine Exert Opposite Forces on Base Pairing of DNA Double Helix

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Zymo Research Corporation

Abstract

DNA base pairing governs the fundamental function of DNA in life. Importantly, annealing and unwinding of base-paired double helical DNA strands are essential for DNA replication and transcription processes. Moreover, epigenetic DNA base modifications have become recognized to be involved in regulation of DNA at all levels in higher organisms. Our recent research into DNA base modifications has shown that 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) modifications dramatically change the properties of C:G base pairing. In contrast to the 5-mC:G pairing, which increases the base pairing stability relative to normal C:G pairing, we find that 5-hmC:G base pairing greatly decreases stability relative to both C:G and 5-mC:G base pairing. It is evident that cytosine epigenetic modifications provide another layer of hidden codes, which serve as a "lock", neutral and "unlock" mechanism on DNA beyond the canonical genetic codes. We call this the Double Helix Epigenetic Switch.

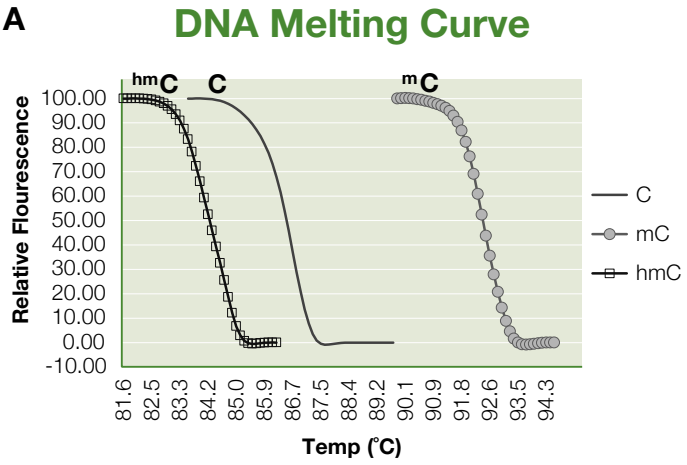
Introduction

DNA is the blueprint for life, coding all of the genes needed in each cell within each tissue in all organisms on Earth. It has been over half a century since the discovery of the DNA double helix and uncovering of genetic codes. In the last decade, the development of epigenetic understanding has further elucidated some fundamental mechanisms of how genes are organized, regulated and inherited through elaborated epigenetic regulation mechanisms. (In addition, the century old debate on nature versus nurture has finally begun to converge into a more complete picture of biology, where genetics and epigenetics are both considered. It is now clear that both nature and nurture are important).

Cytosine modifications in both 5-mC and 5-hmC are two important epigenetic markers and their involvement in gene regulation has been intensively studied in the last decade. Although fundamental A:T and C:G base pairings are well known for the DNA double helix structure, the direct biochemical effects of epigenetically modified bases of 5-mC and 5-hmC on DNA has not been thoroughly investigated. Here we report the 5-mC and 5-hmC base modification effects on C:G base pairing and the overall effects on dsDNA stability.

Results and Discussion

5-mC and 5-hmC exert opposite forces on DNA stability
High resolution melting (HRM) analysis was used to measure the dsDNA stability. This analysis directly measures DNA as either dsDNA (base-paired) or single stranded (denatured) status. This was used as a measurement of DNA stability for different cytosine modifications in a 897bp DNA fragment (5-methylcytosine & 5-hydroxymethylcytosine DNA Standard Set, D5405, Zymo Research Corp.) with relative evenly distributed G, A, T and C. The C was either 100% native C, or 100% 5-mC or 5-hmC.



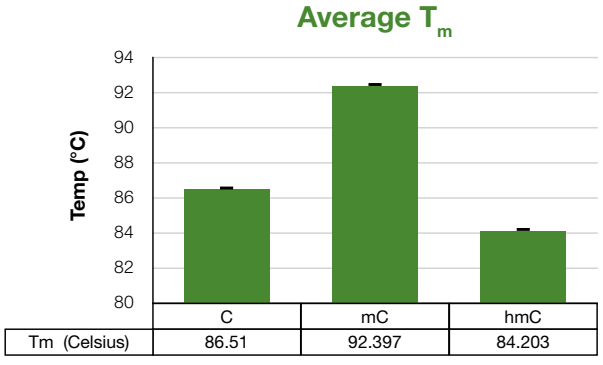


Figure 1. 5-Hydroxymethylcytosine decreases thermodynamic stability of DNA. Procedure: (**A**) Melting curves of DNA standards containing 100% of their cytosine as either unmodified cytosine (C), 5-methylcytosine (5-mC), or 5-hydroxymethylcytosine (5-hmC) were analyzed by high resolution melting (HRM). Samples were done in triplicate and averages were plotted. (**B**) Tm's were calculated by finding the 50% relative fluorescence levels.

The 5-mC containing DNA showed a dramatic increase in DNA melting temperature, on the other hand, the 5-hmC showed a dramatic decrease in DNA melting temperature (Fig 1A). When the 50% DNA melting point was used for measurement, 5-mC could increase the effective DNA denaturation temperature by 6°C while 5-hmC decreased the effective DNA denaturation temperature by over 2°C in relation to native C. When measuring 5-hmC vs 5-mC, the melting temperature difference was shown to be over 8°C for the same DNA (Fig 1B).

The above observed results were demonstrated using a relatively large DNA fragment (897bp) and represented the collective effect of the whole fragment.

Next, we measured the single cytosine base modification effect on dsDNA stability. To do this, a synthetic 52bp template was designed with a modified C in the middle (Fig 2A). In this set up, the DNA melting temperature changes will result from the effect of the single modified base. As shown in Fig 2B, the effect of the DNA melting temperature could be observed reproducibly, even on a single base modification. This demonstrates that the modifications are affecting the strength of the C:G base pairing. Clearly the 5-hmC:G bond is noticeably weaker than the 5-mC:G bond and the normal C:G bond strength is somewhere in between. This and several other experiments (data not shown here) showed similar results, all of which concluded that the 5-mC increases the dsDNA stability.

A **Template**

CACTATCATAAATAAATATTATAA →

GTGATAGTATTTATTTATAATATTTCGACTCTACAACTCTATTCACCAAACA

B

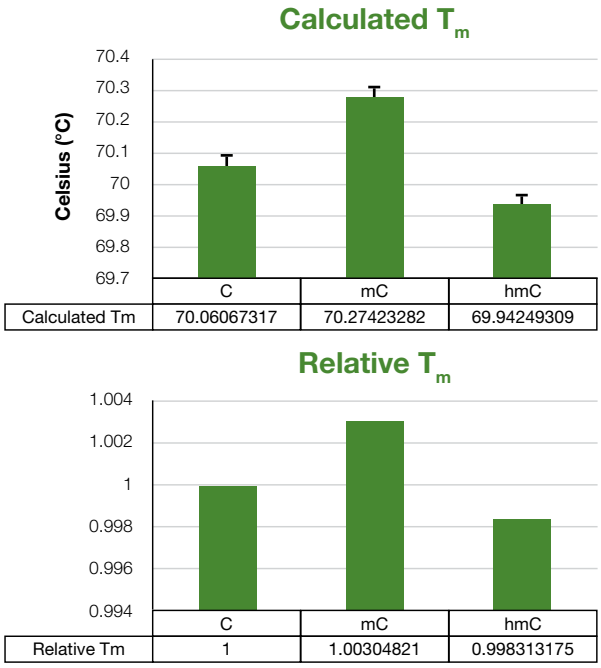
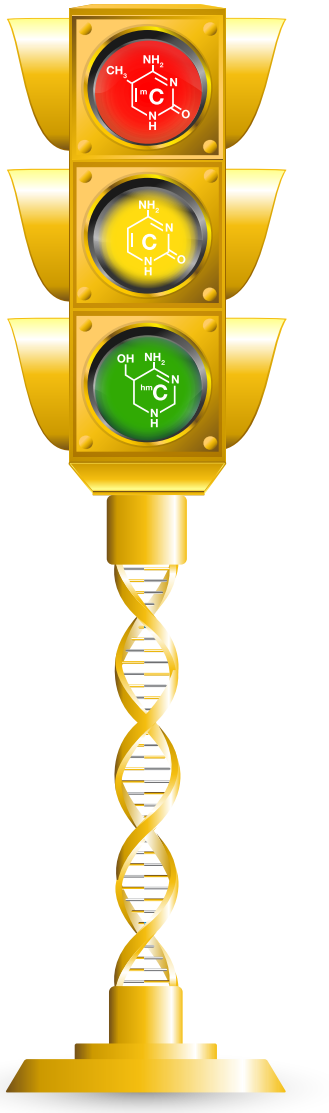


Figure 2. 5-Hydroxymethylcytosine decreases thermodynamic stability of DNA
Procedure: Template was created by primer extension with a dNTP mix containing either cytosine, 5-methylcytosine, or 5-hydroxymethylcytosine. (**A**) Templates were designed to incorporate either cytosine on the extended strand. Template strand (bottom strand 52mer) and elongation primer (italicized bold 24mer). (**B**) Melting curves were analyzed by high resolution melting (HRM). Tm's were calculated by finding the 50% relative fluorescence levels.

Conclusions

Taken together, these results present a unique view of the dynamics of epigenetic modifications. The cytosine modifications not only cause structural changes on the DNA backbone, which may affect the protein binding directly due to the changed chemical structure, but these modifications can also affect the stability of the double helix directly. It is well known that DNA unwinding is an essential step in transcription initiation and DNA replication. It is conceivable that the cytosine mC and hmC modifications also serve as a DNA intrinsic "molecular switch." We call this the Double Helix Epigenetic Switch for its potential to be in a locked, neutral and unlocked status. Thus, cytosine epigenetic modifications give dsDNA another coding dimension beyond the primary code. Together, genetic and epigenetic information render dsDNA into life's blueprint.



Like the lights on a traffic signal, 5-mC is generally associated with gene silencing whereas 5-hmC often acts as the green light for gene transcription. The Double Helix Epigenetic Switch serves as a lock, neutral and unlock mechanism giving dsDNA another coding dimension beyond the canonical genetic codes.

Epigenetic Applications

Epigenetics in Basic Research

Epigenetics has deepened the understanding of many basic cellular functions. For example, epigenetic processes guide stem cell differentiation due to the co-existence of repressive and activating histone modifications at "bivalent domains" in stem cells. These domains can switch between active or repressed states to drive differentiation into specific cell types[1]. Changes in DNA methylation are a hallmark of numerous cancers, with different tumor types displaying specific DNA methylation profiles. More and more, cancers are being viewed as epigenetic disorders, which has led to a significant increase in the investigation of the topic. In general, global 5-mC levels drop during tumor progression, while CpG island methylation increases[2,3]. Histone modifications[4] and recently even long-range chromatin interactions[5] have been implicated in some cancers.

Epigenetics in Diagnostics

Growing knowledge and technology advancements have really helped to drive the development of improved diagnostic tests, leading to enhanced predictive diagnostic indicators for epigenetic changes in numerous diseases. For example, early and accurate detection of psychiatric disorders is a difficult task that is being made easier by epigenetic biomarkers[6]. In cancers, distinguishing tumor tissue from normal tissue can be characterized by epigenetic changes. Saliva, urine, and blood are non-invasive sources of tumor DNA. A wide variety of cancers are associated with cell-free DNA circulating in blood, which can also be used for epigenetic tests[7]. The possibility of earlier, less invasive cancer diagnostics is very motivating to the field of epigenetics.

A German company, Epigenomics, was one of the first to attempt diagnosis of cancers using DNA methylation in the late 90's. In early 2000, the development of the first methylation panels for numerous cancers generated a great deal of excitement[8]. Recently, changes in DNA hydroxymethylation status have also been identified as a potential biomarker in cancer, specifically in melanoma and liver cancer[9-10]. Investigating these changes may facilitate diagnosis of post-traumatic stress disorder and autism[11-12]. Dozens of potential marker panels are now being developed for specific cancers.

Clinical Applications

DNA methylation analysis is proving to be useful for many clinical applications. Zymo Research provides products and services to cover most aspects of epigenetic analysis. Many of the products are supplied in a wide range of formats including a 96-well option. Compatible with many commercial liquid handlers, this format is ideal for automated and high-throughput applications.

Researchers from the University of Southern California used the EZ DNA Methylation™ Kit combined with pyrosequencing to analyze DNA methylation levels in the urine of patients with non-invasive urothelial carcinoma[13].

Did you know? Zymo Research provides the following products for clinical applications:

Product	Description	Size	Cat. No.
DNA PURIFICATION			
Quick-DNA™ Universal Kit	Extract high-quality DNA easily and reliably from any sample source	50 Preps., 200 Preps.	D4068, D4069
Quick-DNA™ Universal 96 Kit		2x96 Preps, 4x96 Preps	D4070, D4071
Quick-DNA™ Urine Kit	Purify cellular and/or cell-free DNA easily from up to 40 ml of urine	50 Preps.	D3061
Quick-cfDNA™ Serum and Plasma Kit	High-quality DNA is easily purified from up to 5ml of serum/plasma	50 Preps.	D4076
ZR Fecal DNA MicroPrep™ ZR Fecal DNA MiniPrep™ ZR Fecal DNA MidiPrep™	Rapid method for the isolation of inhibitor-free, PCR quality DNA from fecal samples	50 Preps. 50 Preps. 25 Preps.	D6012 D6010 D6110
Pinpoint® Slide DNA Isolation System	Streamlined method for the isolation of genomic DNA from targeted areas of fresh and FFPE tissues	50 Preps.	D3001
RNA PURIFICATION			
Quick-RNA™ MicroPrep *Quick*-RNA™ MiniPrep *Quick*-RNA™ MidiPrep ZR-96 *Quick*-RNA™	High-quality total RNA from a wide range of samples	50 Preps., 200 Preps. 50 Preps., 200 Preps. 25 Preps. 2x96 Preps., 4x96 Preps.	R1050, R1051 R1054, R1055 R1056 R1052, R1053
Direct-zol™ RNA MiniPrep Direct-zol™-96 RNA Direct-zol™-96 MagBead RNA	Quick extraction of total RNA directly from TRIzol® or TRI Reagent®. Bypasses phase separation and precipitation procedures, for non-biased recovery of miRNA	50 Preps., 200 Preps. 2x96 Preps., 4x96 Preps. 2x96 Preps., 4x96 Preps., 8x96 Preps.	R2050, R2052 R2054, R2056 R2100, R2102, R2104
ZR Whole-Blood RNA MiniPrep™	Convenient, fast method for purifying total RNA from whole blood samples	50 Preps., 100 Preps.	R1020, R1021
ZR Soil/Fecal RNA MicroPrep™	Isolate inhibitor-free RNA from soil and fecal samples	50 Preps.	R2040
ZR Viral RNA Kit™ ZR-96 Viral RNA Kit™	Quick, 5-minute recovery of viral RNA from plasma, serum and other samples	50 Preps., 200 Preps. 2x96 Preps., 4x96 Preps.	R1034, R1035 R1040, R1041
ZR Viral DNA/RNA Kit™	Quick co-purification of viral DNA/RNA from a wide range of sources	25 Preps., 100 Preps.	D7020, D7021
Pinpoint® Slide RNA Isolation System I Pinpoint® Slide RNA Isolation System II	Allows for the isolation of total RNA from fresh tissue sections	50 Preps., 50 Preps.	R1003, R1007
DNA/RNA Shield™	Preserves genetic integrity and expression profiles of samples at ambient temperatures	50 ml, 250 ml	R1100-50, R1100-250

High-Throughput/Automated Solutions

The ability to detect and quantify DNA methylation efficiently and accurately has become essential for the study of gene expression, genetic diseases, cancer, and many other important aspects of biology. A number of methods have been developed to detect and quantify DNA methylation; most of these techniques rely on bisulfite conversion of the DNA prior to analysis.

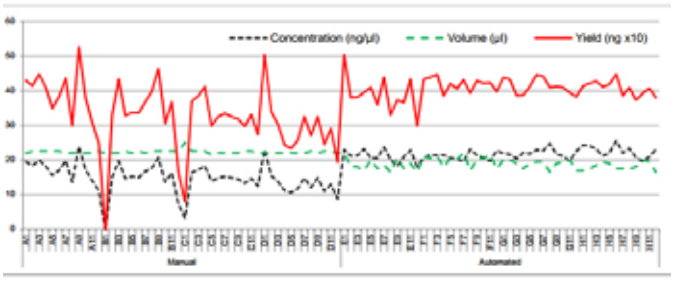
To date, nearly all bisulfite conversion products have been dependent on the manual manipulation of spin columns making them of limited throughput. However, high-throughput (96-well) automated and semi-automated bisulfite treatment of DNA is now possible with the EZ-96 DNA Methylation™ MagPrep line of kits from Zymo Research.

Automated Bisulfite-Converted DNA Purification

TECAN.

By adapting the clean-up of bisulfite-converted DNA to a magnetic bead-based procedure and coupling it to a Tecan liquid handling robot, Zymo Research has opened the door to high-throughput bisulfite conversion of samples for methylation analysis.

- ✓ Automation-ready magnetic bead format kits
- ✓ Fast, consistent clean-up and purification of bisulfite treated DNA
- ✓ DNA is ideal for bisulfite PCR, library preparation, Next-Gen sequencing, arrays, etc.



Consistent recoveries with automated processing. Graph shows concentration, recovered volume and total yield for replicate bisulfite-converted DNA samples across a 96-well plate. Half of the samples (rows A-D) were processed manually. The other half of the samples (rows E-H) were processed using the Tecan Freedom EVO® platform and a dedicated script*.

Product	Size	Cat. No.
EZ-96 DNA Methylation™ MagPrep	4x96 Rxns., 8x96 Rxns.	D5040, D5041
EZ-96 DNA Methylation-Gold™ MagPep	4x96 Rxns., 8x96 Rxns.	D5042, D5043
EZ-96 DNA Methylation-Direct™ MagPrep	4x96 Rxns., 8x96 Rxns.	D5044, D5045
EZ-96 DNA Methylation-Lightning® MagPrep	4x96 Rxns., 8x96 Rxns.	D5046, D5047

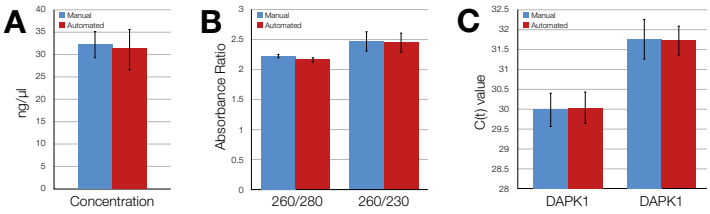
*For scripts, please contact tech@zymoresearch.com ("automation scripts" in subject heading).

Semi-Automated Bisulfite Treatment of DNA

SciGene

High-throughput (96-well) semi-automated bisulfite treatment and clean-up of DNA is now possible using EZ-96 DNA Methylation™ Kits together with the SciGene ThermoPrep® Workstation.

The ThermoPrep® Workstation is an open platform liquid handler with three built-in Peltier thermal cyclers and a universal head for moving plates and for single or eight channel tip dispensing. Precise, low-volume dispensing protocols can be easily programmed using a Visual Basic interface. Using the ThermoPrep® Workstation to perform the liquid handling and incubation tasks associated with bisulfite conversion allows increased throughput of samples while limiting the possibilities for pipetting errors in a 96-well format. Up to two 96-well plates can be processed at one time with only limited operator attention required.



Comparison of Manual vs. Automated Bisulfite Conversion. Graphs show average A) concentration (ng/µl), B) absorbance ratios (Ab260/280nm, Ab260/230nm), and C) Ct values with two different primer sets (DAPK1, RASS) for samples processed using a 96-well format kit (EZ-96 DNA Methylation-Lightning® Kit). Half the samples were processed manually (Manual) and the other half were processed using the ThermoPrep® Workstation with a dedicated script (Automated).

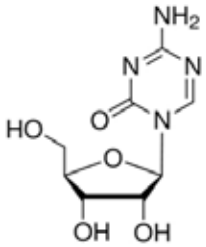
Product	Size	Cat. No.
EZ-96 DNA Methylation™ Kit	2x96 Rxns.	D5003, D5004
EZ-96 DNA Methylation-Gold™ Kit	2x96 Rxns.	D5007, D5008
EZ-96 DNA Methylation-Direct™ Kit	2x96 Rxns.	D5022, D5023
EZ-96 DNA Methylation-Lightning™ Kit	2x96 Rxns.	D5032, D5033

SciGene Product (www.scigene.com)	Size	Cat. No.
ThermoPrep® Workstation	1 unit	2000-00-1

Epigenetic-Based Therapies

Beyond being markers for disease, epigenetic changes are often causal. Over the last two decades there has been intense research into therapeutic drugs to prevent aberrant epigenetic changes. Epigenetic drugs are generally either nucleoside analogues that mimic cytosine in DNA but cannot be methylated, or enzymatic inhibitors. Histone deacetylase (HDAC) inhibitors are the most common of these drugs, but inhibitors of histone acetyltransferases, methyltransferases, and demethylases are also in development[14].

The first FDA-approved epigenetic drug was Vidaza (5-azacitidine), a nucleoside analog developed by Celgene to treat myelodysplastic syndrome (MDS). In 2006 Merck released Zolinza (vorinostat), an HDAC inhibitor used to treat cutaneous T cell lymphoma (CTCL). Celgene and Merck have remained strong players in various epigenetic cancer therapies.

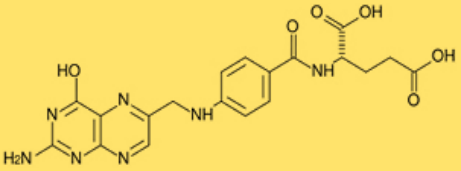


5-azacitidine

More recently, Constellation Pharmaceuticals has focused on targeting chromatin-modifying proteins in cancers. Intensified research by these and other companies has already accelerated the pace of interesting findings, which suggest the coming years will be an exciting time for epigenetic-based therapies.

Did You Know?

The environment has a significant impact on our genetic legacy; everything from stress to what we eat to air pollution can alter our genes. Recent studies have demonstrated that a woman's diet during pregnancy could have serious implications to her baby's epigenetic profile. Prenatal diets that are low in nutrients containing "methyl groups" such as folic acid and vitamin B-12 have been linked to asthma and developmental defects in infants[15].



In a recent article published in *Epigenetics & Chromatin*, Barua et al. demonstrated that the offspring of pregnant mice fed a diet high in folic acid exhibit significant changes in DNA methylation patterns of their neuronal tissue[16]. This study features the use of the Methyl-MiniSeq™ service for genome-wide DNA methylation analysis provided by Zymo Research (p.29).

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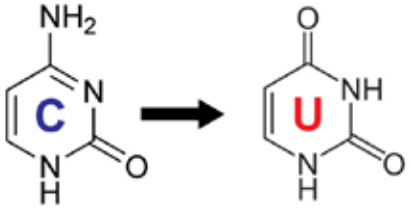
A Comprehensive Guide to Bisulfite-Converted DNA Amplification

Over the years, bisulfite conversion has become the most widely used method for DNA methylation analysis. It is the most convenient and effective way to map DNA methylation to individual bases. As the first step in numerous downstream analysis techniques, it is absolutely critical that the bisulfite conversion process be understood in order to be performed well.

The scientists at Zymo Research have spent an extraordinary amount of time studying and optimizing bisulfite conversion in support of their customers. Today there are fewer questions regarding the bisulfite conversion process, but more questions related to analyzing bisulfite-converted DNA in the context of diverse downstream applications. In this guide, there are key points and considerations that will aid researchers of any experience level to better understand key factors that can impact downstream analysis.



Tips for Succeeding Downstream of Bisulfite Conversion

Since DNA methylation analyses are just getting started after bisulfite conversion, it's useful to first understand what just happened to a DNA sample during the conversion.

Bisulfite conversion is a relatively harsh procedure that will dramatically change the chemical and physical properties of a DNA sample. During the process, genomic DNA transitions from a large, stable double-stranded molecule into an assortment of randomly fragmented, single-stranded ones. Additionally, non-methylated cytosine bases have been converted to uracil during the process. Basically, DNA does not resemble DNA anymore and has gone through a major overhaul, so you will have to make some adjustments to your subsequent experimental procedures.

Checking the Quality of Bisulfite-Converted DNA

Just as with any other experiment, you will want to assess the quality and quantity of your bisulfite-converted DNA sample before moving forward. A couple of common ways to accomplish this include:

- ✓ UV Spectrophotometry
- ✓ Agarose Gel Electrophoresis

Keep in mind that it's necessary to make a few adjustments to each of these methods when working with bisulfite-converted material as highlighted below.

UV-Quantification of Bisulfite-Converted DNA

When using a spectrophotometer, use a value of 40 ng/µl for Ab260nm = 1.0, as the converted DNA will now closely resemble RNA. At first, many researchers will notice their recovery may seem low. There are two possible reasons that can account for this:

1) Sample could have been lost during the bisulfite treatment process, particularly if the DNA input used was degraded and of poor quality.

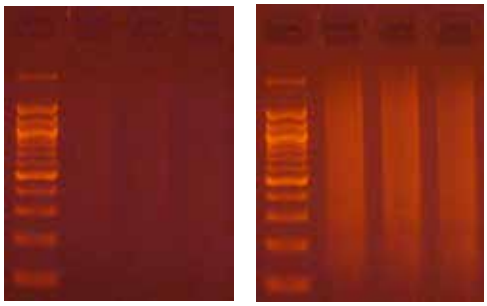
2) RNA contamination biased the initial quantification of DNA input. During the bisulfite conversion and clean-up processes, RNA is removed so your follow up quantification will appear lower. In either case, do not fear; the recovered material is generally still sufficient, particularly if a PCR step is planned downstream. Input DNA that is RNA-free and intact will typically yield the best results.

DNA

Having high-quality input DNA is a great start to successful bisulfite conversion. Zymo Research's *Quick*-DNA™ Universal Kits are an easy and reliable way to extract ultra-pure DNA from any sample source. See pg. 8 for ordering information.

Gel Analysis of Bisulfite-Converted DNA

When using an agarose gel to analyze the converted DNA, use a 2% gel with a 100 bp marker. Also, it can be necessary to load up to 100 ng of sample in order to visualize the DNA. Many researchers worry when at first they can't see any bands in their gel. This is normal as most of the DNA will be single stranded. To remedy this, cool the gel for a few minutes in an ice bath. This will drive some base pairing between the single-stranded molecules so the ethidium bromide can intercalate and illuminate your recovered material. The recovered DNA will usually appear as a smear between 1,500 bp down to 100 bp.



The Challenges of Bisulfite-Converted DNA. DNA fragmentation and limited base pairing make it difficult to visualize the DNA following bisulfite conversion. Samples of bisulfite-converted human genomic DNA were analyzed in a 2% agarose gel with a 100 bp marker visualized before (left panel) and after chilling in an ice bath (right panel).

PCR-Based Analysis of Bisulfite-Converted DNA

There is a tremendous range of bisulfite-based methods out there, but we'll focus on some of the most widely used in labs today (i.e., PCR-based).

Numerous bisulfite-based analysis approaches employ PCR amplification, and some of the more widely used include:

- ✓ Methylation Specific PCR (MSP)
- ✓ Bisulfite Sequencing
- ✓ Bisulfite Pyrosequencing

The PCR step in these methods is also the most prone to error. Again, most of the challenges of amplifying bisulfite-converted DNA relate to the fact that the sample's physical traits have changed, including:

- ✓ The converted DNA has become highly fragmented
- ✓ The strands are no longer complementary
- ✓ The molecules have lost most of their cytosine composition, resembling more of a three-base containing nucleic acid

Although, these conditions do not make for an easy PCR experience, a few practices can mitigate the impact posed from this challenging template including:

- ✓ Designing optimized primers
- ✓ Using a "hot start" polymerase
- ✓ Optimizing annealing conditions

Optimizing Bisulfite Primer Design

Optimizing primers for bisulfite amplification can be challenging at first, but if you follow a few guidelines, you should be on your way to successful results relatively quickly. There are two types of primer design approaches depending on your application: 1) bisulfite PCR primers, which will amplify a region regardless of methylation status, and 2) Methylation Specific PCR (MSP) primers, which are designed to amplify only when a target locus is either methylated or unmethylated.

Featured Tool: Bisulfite Primer Seeker

With over a decade of experience in bisulfite conversion technology, Zymo Research has simplified the tedious process of bisulfite primer design with the **Bisulfite Primer Seeker**. What sets this program apart from other available options is its ability to design primers for particularly CG-rich templates. The Bisulfite Primer Seeker also stands out by supplying multiple primer options for amplicons that span different regions within your sequence of interest.

www.zymoresearch.com/tools/bisulfite-primer-seeker

Primer Design for Bisulfite PCR

In this scenario, primers are used as they are in other PCR settings, to amplify the template. Most often the samples will be analyzed by another downstream methodology (e.g., bisulfite sequencing, mass spectrophotometry) that will determine the DNA methylation status.

Unlike normal PCR where the template is typically comprised of 4 nucleotide bases, bisulfite PCR primers need to be longer to contend with the loss of most of the cytosines. Aim for primers of at least 26-30 bases of sufficient specificity. Also, since the template will be highly fragmented, targeting an amplicon size between 150-300 bp is recommended.

Next, you will want to make sure that the primers avoid CpG sites, or if that is not possible; try to locate them at the 5' end of the primer with a mixed base at the cytosine position.

When designing the primers, it is a good exercise to 'convert' the DNA first – changing all of the non-CpG cytosines to uracils. It is also important to note that only one strand of the template will be amplified by a given primer set. Because the DNA strands are no longer complementary, only the reverse primer will actually bind to the target DNA. The forward primer will only have a complementary template once generated from the reverse primer. Usually, 35 to 40 cycles are required for successful amplification.

Primer Design for Methylation Specific PCR (MSP)

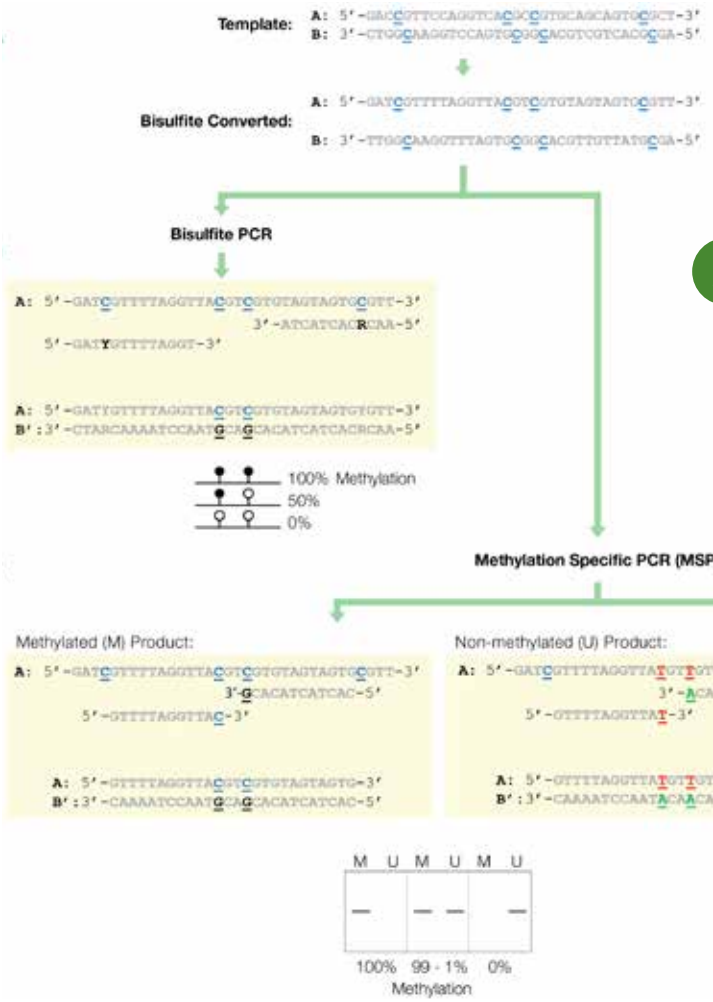
Unlike primers for bisulfite PCR, MSP primers serve the dual functions of amplifying bisulfite-converted DNA and interrogating the DNA methylation status at specific CpG sites.

In addition to observing the above practices for standard bisulfite amplification, MSP primers should be designed taking the following guidelines into account:

- ✓ CpG site(s) of interest must be included in the primers.
- ✓ The CpG(s) should be located at 3' end of the primers.
- ✓ Two primer sets are required for each amplicon that will be interrogated:

  - A "Methylated primer set that contains cytosine (C) at CpG sites.
  - A "Non-methylated" primer set that uses thymine (T) in place of C at CpG locations.

Flowchart of Primer Design for Bisulfite PCR and Methylation Specific PCR (MSP)



A — Following bisulfite treatment, the two converted strands of the DNA template are no longer complementary.

B — Primers for Bisulfite PCR are designed for subsequent sequencing and analysis of cytosines within the amplicon. CpG sites within the primers should be avoided or located at the 5'-end with a mixed base at the cytosine position (Y= C/T, R= G/A). Sequencing data is commonly represented by a "lollipop" plot where closed circles represent methylated cytosine positions and open circles non-methylated ones.

C — Primers for Methylation Specific PCR (MSP) are designed to target and assess the methylation status at specific CpG sites. CpG sites within the primers must be located at the 3'-end to increase their specificity to methylated (M) or non-methylated (U) templates. Completely methylated or non-methylated templates will generate a single amplicon from only their representative primer set following MSP. Samples with mixed methythation, will be amplified by both primer sets.

Use a Hot Start Polymerase

When amplifying bisulfite-converted DNA, it is ideal to use a hot start polymerase. Non-specific amplification is a relatively common phenomenom with bisulfite-treated DNA due to its AT-rich nature.

A hot start polymerase will minimize primer dimerization and the non-specific amplification products that go along with it. A hot start polymerase will do this better than regular *Taq* polymerase.

Product	Size	Cat. No.
Zymo*Taq*™ DNA Polymerase	50 Rxns.	E2001
Zymo*Taq*™ DNA Polymerase	200 Rxns.	E2002
Zymo*Taq*™ PreMix	50 Rxns.	E2003
Zymo*Taq*™ PreMix	200 Rxns.	E2004

Zymo*Taq*™ DNA Polymerase is a hot start polymerase that is ideal for amplification of bisulfite-converted DNA. Zymo*Taq*™ reduces primer dimer and non-specific product formation, whereas conventional polymerases typically exhibit these problems with bisulfite-converted DNA templates.

Turn up the Heat with Higher Annealing Temperatures

Typically, higher annealing temperatures are key for good amplification. Work by M.D. Anderson's Lanlan Shen, confirmed that raising the annealing temperatures was instrumental in improving PCR efficiency from bisulfite-converted templates[1].

The good news is that longer primers for bisulfite primer design, not only help with specificity, but also bring your melting temperature up. It's also best to include as many guanines as possible in the primer regions to increase the melting temperature of the primer to a recommended 55-60 °C range.

When first trying out a new primer set, run an annealing temperature gradient. This will help you identify the ideal annealing temperature to use in your experiments. Troubleshooting your primers before starting your PCR analysis will save you time and eliminate any non-specific results.

Choose Wisely: Which Bisulfite Conversion Approach is Right for You?

A few years ago, there wasn't much to choose from in terms of bisulfite conversion technologies. Today, there are many options that can also present some challenges to choosing what is best for your application.

Let's run through a few considerations and research scenarios that will impact which type of bisulfite-treatment kit you'll want to use:

- Researchers New to Bisulfite Conversion
- Researchers Seeking Speed & Convenience
- Researchers Working with Cell and Tissue Samples
- Researchers Working with Small Amounts of Precious Samples
- Researchers Seeking Validated Kits for Use in Commercial and Academic Protocols

Available Formats

Zymo Research's bisulfite conversion kits are available in the indicated formats, with the indicated elution volumes.

Spin Column

≥ 10 µl

Single columns are ideal for routine microcentrifuge-based processing of relatively few samples.

96-Well

≥ 15 µl

96-well spin plates allow increased throughput for larger studies and sample numbers.

MagBead

≥ 25 µl

Magnetic beads are suited for high-throughput automated handling of large sample sets.

Did You Know?

Sodium bisulfite is also very helpful in preserving wine during storage. Excessive oxygen is wine's major enemy during the time between fermentation and consumption. Excessive oxygen can cause a wine to turn brown or slightly orange and it can also bring out a flavor in wine similar to raisins or, in more extreme cases, old fashioned cough syrup.

Adding sodium bisulfite to wine before bottling helps to eliminate these oxidative side effects and will help to preserve the wine's overall character. It does so by driving out excess oxygen that may be saturated in the wine. The sulfur gases from the sodium bisulfite displace the oxygen and later permeate the air space in the wine bottle as well.

The recommended dose of sodium bisulfite is 1/16 teaspoon per gallon of wine to be added before bottling.

Researchers New to Bisulfite Conversion

If you are a new user and starting a bisulfite conversion project from scratch, you will need a tried and true bisulfite conversion kit that's ready to go right out of the box. The EZ DNA Methylation-Lightning® Kit sports a fast, highly robust and simple protocol, along with pre-mixed reagents making it a great all-around performer. The ease and efficiency of the Lightning Kit is ideal for scientists just entering the realm of DNA methylation.

Research Situation: New Bisulfite Users
Kit Recommendation: EZ DNA Methylation-Lightning® Kit
(Cat. Nos. D5030, D5031, D5032, D5033, D5046, D5047)

Researchers Seeking Speed and Convenience

Many bisulfite conversion techniques feature an incubation time of several hours, or even overnight, and require reagent preparation and set up ahead of time. The EZ DNA Methylation-Lightning® Kit is designed for researchers who crave simplicity and don't have time to spare.

The Lightning Kit features a pre-mixed conversion reagent that is ready-to-use immediately out of the box and it has been optimized to completely convert DNA in about 1 hour.

Research Situation: Seeking Speed and Convenience
Kit Recommendation: EZ DNA Methylation-Lightning® Kit
(Cat. Nos. D5030, D5031, D5032, D5033, D5046, D5047)

Researchers Working with Cell and Tissue Samples

Most bisulfite conversion protocols will generally require you to first isolate the DNA from your sample prior to bisulfite conversion. This is because cellular debris can reduce the efficiency of the conversion reaction. The downside to any purification step though, is that recovery is never 100%, so you can lose precious sample.

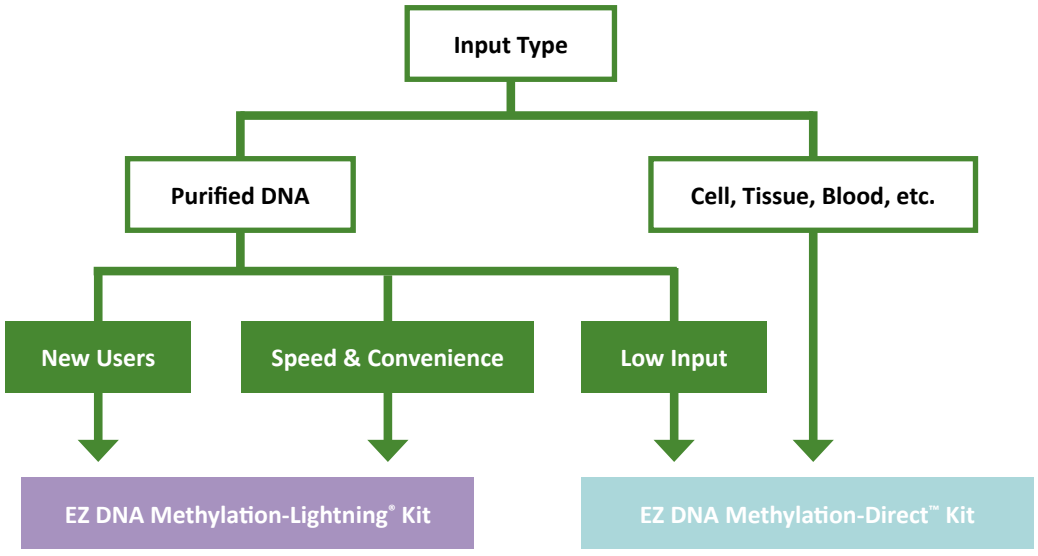
To address this, the EZ DNA Methylation-Direct™ Kit combines a unique proteinase digestion step with the conversion reaction enabling robust conversion without having to first perform a DNA purification step. This maximizes the recovery of the DNA template for bisulfite conversion.

Research Situation: Direct Input of Cellular and Tissue Samples
Kit Recommendation: EZ DNA Methylation-Direct™ Kit
(Cat. Nos. D5020, D5021, D5022, D5023, D5044, D5045)

Researchers Working with Small Amounts of Precious Samples

Another challenging situation researchers often face is having very little sample to work with (e.g., FFPE, LCM, Needle core biopsies). Moreover, Zymo Research has developed a specialized conversion reagent in the EZ DNA Methylation-Direct™ Kit for ultra-low sample inputs, whether the DNA has been purified or not.

Research Situation: Small Sample Inputs
Kit Recommendation: EZ DNA Methylation-Direct™ Kit
(Cat. Nos. D5020, D5021, D5022, D5023, D5044, D5045)



Researchers Seeking Validated Kits for Use with Commercial Platforms

Bisulfite conversion technologies from Zymo Research are recommended by a number of third parties due to their reliable performance.

Illumina Infinium®

(http://www.illumina.com/products/methylation_450_beadchip_kits.html)

The HumanMethylation450 BeadChip array allows interrogation of DNA methylation status at about 450,000 unique CpGs in a single experiment. The scientists at Illumina® validated several commercially available kits and found the EZ DNA Methylation™ Kit to be highly compatible with their protocol.

Kit Recommendation: EZ DNA Methylation™ Kit
(Cat. Nos. D5001, D5002, D5004, D5040, D5041)

Agena MassARRAY® EpiTYPER® DNA Methylation

(http://agenabio.com/epityper-dna-methylation)

MassARRAY® is a MALDI-TOF based system that can measure individual methylation ratios for CpGs within a target sequence to determine relative methylation status of up to several hundred CpGs in multiple samples. The team at Sequenom found that the EZ DNA Methylation™ Kit enabled reliable and consistent results on the MassARRAY® EpiTYPER® platform.

Kit Recommendation: EZ DNA Methylation™ Kit
(Cat. Nos. D5001, D5002, D5003, D5004, D5040, D5041)

Agilent SureSelect™ Target Enrichment

(http://www.genomics.agilent.com/article.jsp?pageId=2094)

SureSelect XT™ Methyl-seq uses sample enrichment techniques combined with bisulfite sequencing for the analysis of DNA methylation status of 3.7 million individual CpG sites at once. The system targets promoters, CpG islands, and known differentially methylated regions (DMRs). Scientists at Agilent have shown high performance with the EZ DNA Methylation-Gold™ Kits in this system.

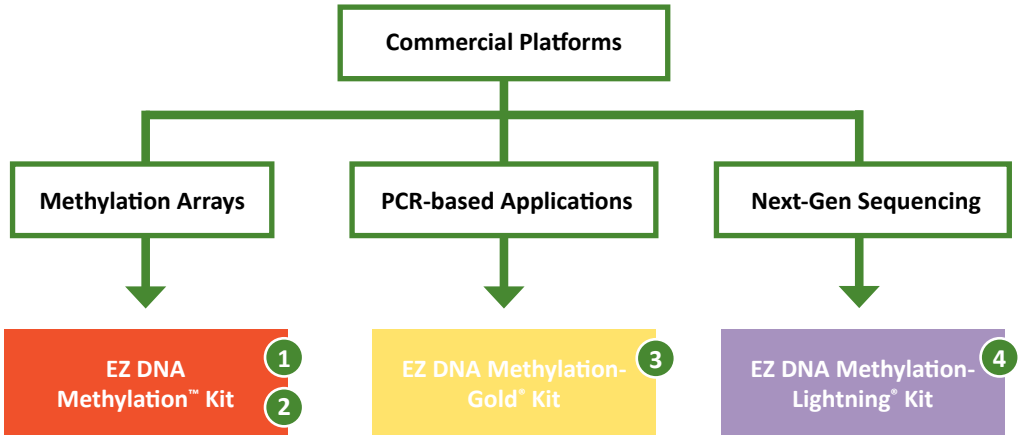
Kit Recommendation: EZ DNA Methylation-Gold™ Kit
(Cat. Nos. D5005, D5006, D5007, D5008, D5042, D5043)

Roche NimbleGen SeqCap™ Epi Enrichment System

(http://www.nimblegen.com/products/seqcap/epi-system/)

SeqCap Epi Enrichment enables the targeting of selected genomic regions from bisulfite-treated genomic DNA, which can act as a screening tool to identify specific regions in the genome for methylation variation assessment. Scientists at Roche NimbleGen have shown the EZ DNA Methylation-Lightning® Kit works well with this system.

Kit Recommendation: EZ DNA Methylation-Lightning® Kit
(Cat. Nos. D5030, D5031, D5032, D5033, D5046, D5047)



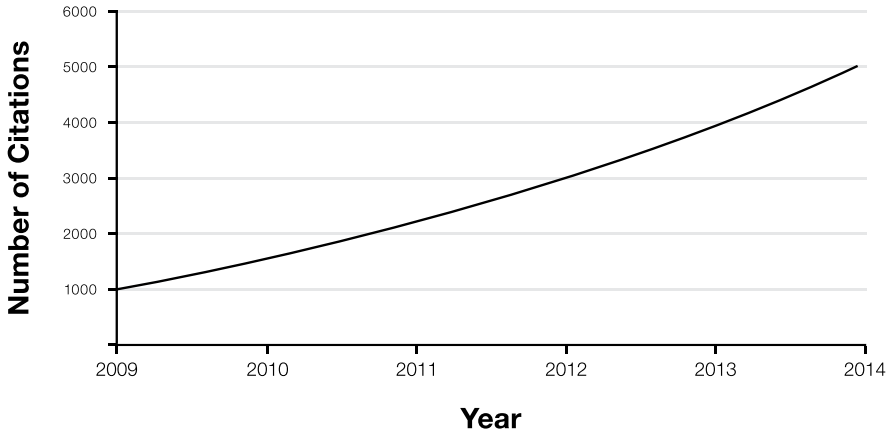
EZ DNA Methylation™ Kits are recommended by:

1. Illumina Infinium® and GoldenGate® assay systems
2. Agena MassARRAY® EpiTYPER®
3. Agilent Technologies SureSelect™ Methyl-seq Target Enrichment System
4. Roche NimbleGen SeqCap™ Epi Enrichment System

96% of researchers
were satisfied with the overall performance
of their EZ DNA Methylation™ kit

95% of researchers
would recommend our bisulfite conversion technologies
to a colleague

Most-cited Technologies for DNA Methylation Analysis & Detection



Bisulfite Conversion Kits

Product		Size	Cat. No.
EZ DNA Methylation™ Kit	Streamlined, proven procedure for bisulfite-converted DNA	50 Rxns., 200 Rxns.	D5001, D5002
EZ-96 DNA Methylation™ Kit (Shallow Well Format) EZ-96 DNA Methylation™ Kit (Deep Well Format)		2x96 Rxns. 2x96 Rxns.	D5003 D5004
EZ-96 DNA Methylation™ MagPrep		4x96 Rxns., 8x96 Rxns.	D5040, D5041
EZ DNA Methylation-Gold® Kit	Complete bisulfite conversion of GC-rich DNA in less than 3 hours	50 Rxns., 200 Rxns.	D5005, D5006
EZ-96 DNA Methylation-Gold® Kit (Shallow Well Format) EZ-96 DNA Methylation-Gold® Kit (Deep Well Format)		2x96 Rxns. 2x96 Rxns.	D5007 D5008
EZ-96 DNA Methylation-Gold® MagPrep		4x96 Rxns., 8x96 Rxns.	D5042, D5043
EZ DNA Methylation-Direct™ Kit	Complete bisulfite conversion of DNA directly from blood, tissue, cells FFPE and low-input samples	50 Rxns. 200 Rxns.	D5020 D5021
EZ-96 DNA Methylation-Direct™ Kit (Shallow Well Format) EZ-96 DNA Methylation-Direct™ Kit (Deep Well Format)		2x96 Rxns. 2x96 Rxns.	D5022 D5023
EZ-96 DNA Methylation-Direct™ MagPrep		4x96 Rxns., 8x96 Rxns.	D5044, D5045
EZ DNA Methylation-Lightning® Kit	Fastest method for complete bisulfite conversion of DNA	50 Rxns., 200 Rxns.	D5030, D5031
EZ-96 DNA Methylation-Lightning® Kit (Shallow Well Format) EZ-96 DNA Methylation-Lightning® Kit (Deep Well Format)		2x96 Rxns. 2x96 Rxns.	D5032 D5033
EZ-96 DNA Methylation-Lightning® MagPrep		4x96 Rxns., 8x96 Rxns.	D5046, D5047

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Going Global with DNA Methylation Analysis

DNA Methylation Analysis Methods

Several DNA methylation analysis techniques have emerged over the years, each with their own specific advantages and limitations. The following sections will take a closer look at the various techniques, how they work, and when they are best applied in the following settings:

- ✓ Global DNA Methylation Analysis
- ✓ Genome-wide DNA Methylation Analysis
- ✓ Locus-specific DNA Methylation Analysis

Global Quantification of DNA Methylation

Global DNA methylation detection methods can be used to determine the overall amount of methylation in a cell population. Two widely used methods for global quantification are Enzyme-Linked Immunosorbent Assays (ELISAs) and Liquid Chromatography–Mass Spectrometry (LC-MS). These methods employ different approaches to quantify global DNA methylation in samples.

While ELISAs rely on antibody recognition of DNA methylation, LC-MS measures the abundance of DNA methylation using the separation and mass analysis capabilities of liquid chromatography–mass spectrometry.

Global DNA methylation detection is well suited to the preliminary stages of studies when researchers might need to quickly check for the general presence, absence, or relative abundance of DNA methylation in different samples. Once established at the global level, researchers can follow-up with genome-wide or locus-specific methods.

Many researchers have opted to develop global DNA methylation screens based on ELISAs and LC-MS for a cost-effective and rapid means to monitor whole-genome DNA methylation without the need for sequence-specific context.

ELISA Analysis of DNA Methylation

The ELISA is a popular method for quantifying various molecular targets[1]. In an ELISA, the target sample is immobilized on a solid surface and is then probed with a specific antibody against the target molecule. The primary antibody is then usually detected with a secondary antibody attached to an enzyme. After cleaning, an enzyme substrate wash produces a measurable, visible signal.

ELISAs can be used to detect global 5-mC and/or 5-hmC levels in a sample when an antibody specific to these marks is used[2]. The protocol is relatively fast and cost effective. Moreover, the streamlined nature of ELISAs makes them well suited for high-throughput experimental scenarios as a large number of samples are easily accommodated by the assay.

Global Quantification: Overall levels of 5-mC and 5-hmC in DNA samples can be rapidly and accurately determined with specifically designed ELISAs from Zymo Research (5-mC DNA ELISA Kit and Quest 5-hmC™ DNA ELISA Kit). See pg. 39 for ordering information.

Product	Size	Cat. No.
5-mC DNA ELISA Kit	1x96, 2x96	D5325, D5326
Quest 5-hmC™ DNA Elisa Kit	1x96, 2x96	D5425, D5426

LC-MS Analysis of DNA Methylation

The LC-MS approach is an alternative to an ELISA. While ELISAs rely on antibody interactions to quantify 5-mC or 5-hmC, LC-MS uses a more accurate mass spectrometry technology[3].

In this approach, the DNA sample of interest is first digested (DNA Degradase Plus™, Cat. No. E2020) down to single nucleosides. This nucleoside mix contains the methylated and unmethylated cytosines in a particular ratio. Using LC-MS, the level of different bases (5-mC and 5-hmC) can be determined in a DNA sample.

A nice feature of this technique is that it can be used for global 5-mC and 5-hmC detection simultaneously. Additionally, the LC-MS approach is a very accurate technique for quantifying global methylation levels. It is simple and cost-effective, requiring only a one-step digestion prior to LC-MS[4]. The downside of this method is that it requires access to a LC-MS platform.

Global DNA methylation analyses can be useful for measuring the total amount of DNA methylation present in a sample, but they lack the ability to map the coordinates of the DNA methylation mark itself. When this is important, researchers will generally opt for methods that allow sequence-specific profiling of DNA methylation, either in a genome-wide or targeted context.

Zymo Research's Epigenetic Services for global analysis of DNA methylation and hydroxymethylation feature a unique LC/MS (MRM) platform. Please refer to pg. 33 for more information.

Comparative DNA Methylation Profiling

It is highly valuable for researchers to have the ability to quickly and efficiently assess differential DNA methylation between two groups. There are multiple scenarios where global DNA methylation screens help investigators to zero-in where it matters most.

Let's take a look at some of the more prominent research settings where DNA methylation screening is playing a key role:

Drug Response

ELISA assays, like the 5-mC DNA ELISA and the Quest 5-hmC™ DNA ELISA Kits from Zymo Research, have made it relatively simple to see the effects that certain drug treatments have on cell populations. Here are some examples where researchers were able to monitor the effects of compound exposures using DNA methylation screens:

- Scientists from the University of Turin observed that 5-mC levels in glioblastoma stem cells rose nearly 25% when treated with temozolomide, an oral chemotherapy agent, which chemosensitized the stem cells[5].

- Investigators at Loma Linda University School of Medicine found that the exposure to endothelin-1 (ET-1), which is expressed during hypoxia, increased 5-mC in fetal cardiomyocytes, but 5-aza-2-deoxycytidine could block the effect[6].

Environmental Response

5-mC and 5-hmC ELISAs have also played a significant role in tracking DNA methylation alterations in response to environmental stimuli such as diet and air quality. These recent publications detail how DNA methylation screens measure the effects of environmental factors:

- University of Louisville researchers set out to study the neuroprotective properties of folic acid. They observed no 5-mC changes in mice fed folic acid in their diets. However, the researchers did notice 5-mC levels increased in mouse brain tissue when cystathionine-ß-synthase (a hyperhomocysteinemia related enzyme) was included in the diet[7].

- Researchers at the National Institutes of Health investigated the role of epigenetics in asthma and allergic responses. The group showed that exposure to house dust mites (HDM) lowered 5-mC in mouse lungs, but also increased the percentage of 5-hmC in comparison to control mice treated with saline[8].

Differential Disease States and Tissue Types

Rapid assays, like ELISAs, are extremely helpful in initial screens for potential DNA methylation shifts between tissue types or disease conditions. The studies below used this approach to identify differential DNA methylation levels between healthy and diseased cells, in order to conduct more locus-specific experiments later on:

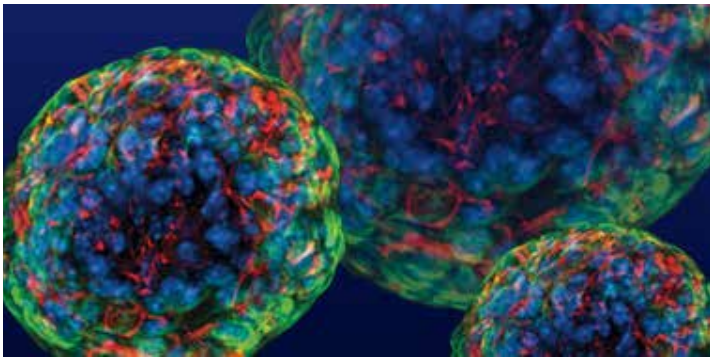
- A research team at Johns Hopkins University determined the 5-mC and 5-hmC levels in cardiovascular disease-free men. The authors then went on to correlate their data with patients showing abnormal metabolic conditions or low levels of atherosclerosis to generate an initial dataset for future studies[9].

- Scientists from the University of Louisville studied 5-mC levels in cardiomyocytes that were undergoing hyperhomocysteinemia (HHcy), which is associated with neurovascular diseases via aberrant DNA methylation and histone acetylation. They observed that HHcy cardiomyocytes indeed, had elevated 5-mC levels compared to wild type cells[10].

- While looking into Autoimmune Addison's Disease (AAD), investigators at the Universities of Bergen and Oslo quantified the 5-mC levels of CD4+ T cells. The team showed that AAD patients had much lower 5-mC levels than healthy subjects[11].

Did You Know?

DNA methylation ELISAs can efficiently pre-screen samples prior to NGS. High-throughput sequencing based studies require a big investment in time, capital, equipment and resources. So, it makes sense to be judicious about what you choose to throw into each sequencing run. With a quick ELISA DNA methylation assay, you can decide exactly what deserves more in-depth, locus-specific attention, and which samples are better left out…all at a fraction of the cost and effort. It's hard to argue with efficiency. Jump ahead to pg. 39 for Zymo Research's ELISA detection kits.



Stem Cell Monitoring

Another emerging application of DNA methylation screening is the monitoring of cultured stem cells. Several recent reports have demonstrated that epigenetic properties of stem cells can shift significantly over time, depending on variables like culture conditions and passage number, greatly impacting their experimental behavior and clinical utility[12,13].

Researchers have determined that keeping track of epigenetic factors in stem cells, like DNA methylation, with the help of relatively fast and simple screening assays can indicate the state of a stem cell population. This can be readily accomplished by the following:

1. Global DNA methylation screens including ELISAs and LC-MS
2. A focused approach; for example the *OneStep* qMethyl™ Kit from Zymo Research (Cat. No. D5310)

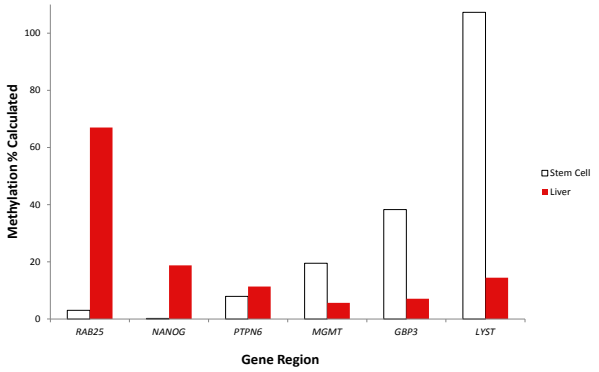
Here are a few examples where DNA methylation has been used to assess stem cell differentiation:

- Investigators from the University of Aachen studied the effects of long-term culture in human mesenchymal stem cells (MSC). They compared DNA methylation profiles and noted a substantial difference between MSCs derived from adipose tissue and bone marrow. All MSCs had highly consistent senescence-associated DNA methylation modifications that also correlated with specific histone marks including H3K9me3, H3K27me3 and EZH2. Their observations led the team to conclude that replicative senescence is epigenetically controlled[12].

- The same scientific team at the University of Aachen developed an Epigenetic-Senescence-Signature to predict cellular aging in MSCs by analysis of DNA methylation at six CpG sites (associated with the genes GRM7, CASR, PRAMEF2, SELP, CASP14 and KRTAP13-3). The group used this project as proof of principle, and intends to further validate this approach for use as a quality control of therapeutic cell products[13].

- Researchers from the University of Milan-Bicocca found that the large-scale expansion of bone marrow mesenchymal stem cells (BM-MSCs) used in a therapeutic context can initiate replicative senescence. This was linked to a significant reduction in BM-MSC 5-mC levels during long-term culture[14].

Product	Size	Cat. No.
OneStep qMethyl™ Kit	1x96	D5310



Cell Population	RAB25	NANOG	PTPN6	MGMT	GBP3	LYST
Differentiated	+	+	+	-	-	-
Pluripotent	-	-	-	+	+	+

Unique Pluripotent Stem Cell DNA Methylation Signatures are Determined with the *OneStep* qMethyl™ Kit. Human differentiated DNA (red bars) and human stem cell DNA (white bars) show different DNA methylation percentages for RAB25, NANOG, PTPN6, MGMT, GBP3, and LYST.

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Genome-wide DNA Methylation Analysis Methods

5-mC Analysis

Genome-wide analysis methods are used to examine DNA methylation broadly across a genome of interest. Genome-wide methods differ from global methods in that they also allow the characterization of changes in specific regions (loci). However, they may not necessarily capture the entire genome at this resolution.

Next-Generation Sequencing (NGS) has revolutionized the study of the epigenome, allowing the finest resolution at the highest output. However, this resolution and scale are not always necessary for researchers, and they generally introduce significant experimental costs. As a result, researchers have developed a wide variety of methods to enrich, fractionate, or view a subset of the DNA methylome to achieve high resolution and broad coverage, keeping costs manageable.

5-mC

Zymo Research's Epigenetic Services provide a variety of platforms for single nucleotide resolution DNA methylation analysis in any species. Please refer to pg. 29 for more details.

5-mC Enrichment Analysis

Antibodies with an affinity for 5-mC are frequently used to enrich DNA fragments that contain DNA methylation. There are two main classes of reagents that accomplish this: 5-mC antibodies, and methyl-binding protein-based enrichment. These approaches allow the researcher to obtain information about the genomic location of DNA methylation, and focus investigations solely on those regions of interest.

Methylated DNA Immunoprecipitation Sequencing (MeDIP-seq)

In MeDIP assays an antibody against 5-mC is used to enrich for methylated regions of the genome[1]. DNA enrichment using a methylation-specific antibody followed by microarray or sequencing analysis is a common method for mapping DNA methylation distribution across the genome.

MeDIP-seq employs quantitative next-generation sequencing to characterize an enriched sample. The use of MeDIP not only identifies the methylated regions, but also quantifies their enrichment level, which is directly related to the methylation level. Data output represents differentially methylated regions (DMRs), which are about 200 bp in size, and are given a methylation score based on the enrichment level. Thus, comparing DMRs between experimental samples can be used to identify differences in methylation[2].

MeDIP sequencing of an enriched sample is less rigorous and costly than whole-genome bisulfite sequencing (WGBS), since only a fraction of the genome is represented within the sample. Antibody-based techniques provide relative methylation information between experimental samples but are less suited for absolute methylation profiling within a single sample.

MeDIP: Zymo Research offers a Methylated-DNA IP Kit for large-scale DNA methylation analysis .

Product	Size	Cat. No.
Methylated-DNA IP Kit	10 Rxns.	D5101

Protein-Based DNA Methylation Capture Sequencing (CAP-seq)

Methyl-CpG-Binding Domain (MBD) capture is an affinity-based approach similar to antibody techniques. However, instead of an antibody, the methyl-CpG binding protein (MBP) is used to bind 5-mC. MBP-coated beads are used to enrich for 5-mC-containing DNA[3]. This enriched sample is then sequenced on a Next-Gen platform to identify and quantify the genomic regions present.

In a recent study published in the *Journal of Clinical Investigation*, researchers demonstrated that mechanical changes disturbing normal blood flow (d-flow), can also affect genome-wide DNA methylation patterns of arterial endothelial cells. The group found that DNA methylation changes were dependent upon DNA methyltransferase (DNMT) activity, and treatment with the DNMT inhibitor 5-azacytidine sufficiently restored normal DNA methylation patterns in test samples. The report suggests a mechanism linking DNA methylation changes with differential gene expression characteristic of endothelial cell dysfunction and atherosclerosis – plaque buildup and an accompanying hardening of the arteries, a major known contributor to heart disease and stroke[4]. The genome-wide DNA methylation analysis techniques featured in this study were based on the Methyl-MiniSeq™ service offered from Zymo Research (see pg. 29).

Chromatin Immunoprecipitation (ChIP)

Chromatin analysis technologies have developed at a rapid pace, evolving from techniques used for DNA methylation and protein analysis. Histone modifications and chromatin-binding proteins are most commonly investigated, and antibodies remain the most critical tool for accurate analysis. Chromatin immunoprecipitation (ChIP) is the technique used for enrichment of DNA regions that react with histones or other proteins (e.g., transcription factors). DNA enriched by ChIP can be further characterized using qPCR, microarray, or sequencing (ChIP-seq). ChIP has been a hallmark for chromatin analysis for years, but it was not until 2001 that ChIP was first used in conjunction with microarrays for broad-coverage profiling of protein and histone modifications in DNA[5].

Today, ChIP-validated antibodies can be combined with microarray (ChIP-chip) allowing researchers to customize their analysis. ChIP-chip offers a convenient low-cost method for the analysis of protein-DNA interactions. However, one drawback of ChIP-chip is that microarrays are limited to the content of the microarray. This limits the analysis to only a subset of the genome and requires researchers to have some prior knowledge of the areas of interrogation.

ChIP-seq couples antibody-based IP with the quantitative power and genome-wide coverage of Next-Gen sequencing. ChIP-seq has truly changed the field of chromatin mapping, allowing researchers unprecedented glimpses into protein-DNA interactions with unsurpassed resolution. Additionally, ChIP-seq offers a more precise analysis with a lower signal-to-noise ratio, than ChIP-chip [6-7].

ChIP

Zymo Research's Epigenetic Services offer fully customizable services for ChIP analysis. Please refer to pg. 32 for more information. For a complete list of Zymo Research products related to chromatin analysis turn to pg. 40.

DNA Methylation Analysis Using Microarrays

DNA methylation microarrays are still used for obtaining targeted, reliable and low-cost data. All methylated or potentially methylated regions can be arrayed and interrogated together[8]. The low-cost, high throughput nature of microarrays makes them well suited to large-scale exploratory studies or as an alternative to sequencing. DNA methylation microarray workflows generally follow a MeDIP or bisulfite treatment prior to the microarray readout.

One of the most common arrays, or "chips", used in human methylation research is the Illumina 450k Infinium™ methylation beadchip array. This chip includes 96% of CpG islands, >99% promoters, miRNA promoters, and non-CpG methylated sites[9].

Reduced Representation Bisulfite Sequencing (RRBS)

RRBS is a variation of bisulfite sequencing that offers an alternative to enrichment-based procedures for genome-wide DNA methylation analysis. Instead of bisulfite treating and sequencing an entire genome (i.e. WGBS), a "reduced" DNA sample is created using restriction enzymes (e.g., MspI) to eliminate unmethylated regions. The remaining sample (enriched for 5-mC) is then bisulfite-converted and sequenced[10].

In the human genome, RRBS captures approximately 85% of CpG islands and 60% of promoters, and costs significantly less than whole-genome bisulfite sequencing[11]. The method requires very little input DNA, making it ideal for precious or rare samples. Also, RRBS is well suited for high-throughput, low cost applications including clinical tests[12].

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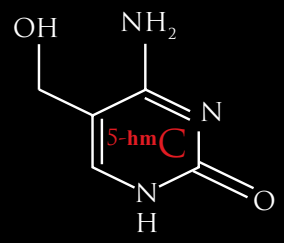
Decode the Mystery of the Sixth Base



5-Hydroxymethylcytosine

5-hmC Analysis

The recent surge in knowledge concerning 5-hydroxymethylcytosine (5-hmC), or the "6th base" as many refer to it, has greatly influenced the current understanding of epigenetics. 5-hmC is an oxidative by-product in the active demethylation pathway of 5-mC. Three TET enzymes (TET1-3) catalyze each step in the demethylation of 5-mC, first converting it into 5-hmC, followed by 5-formylcytosine (5-fC) and then 5-carboxylcytosine (5-caC)[1].

5-hmC is found at high levels in embryonic stem cells and brain tissues. The stem cell self-renewal process is hampered when TET1 levels are low, thereby reducing 5-hmC levels[2]. In the brain and embryonic stem cells, 5-hmC is prevalent in promoters, gene bodies, and intergenic areas near genes and demonstrates a positive correlation with gene expression at those loci[3-5].

5-hmC has a functional role in promoting gene expression during active demethylation, where conversion of 5-mC to 5-hmC by the TETs inhibits the repressive MBD-domain complexes and DNMT proteins that would typically be recruited to 5-mC[6]. The promotion of gene expression by active demethylation is particularly important for genes involved in brain function. 5-hmC is enriched in genes linked to synaptic function in both mouse and human brains[7].

The "gold standard" for DNA methylation, bisulfite conversion, is unable to discriminate between 5-mC and 5-hmC, thus indicating that previous studies using bisulfite methods may have been simultaneously examining 5-mC and 5-hmC. Based on the rapidly increasing interest in the epigenomic role of 5-hmC, and its chemical similarity to 5-mC, new and specific 5-hmC analysis schemes have been developed. The following are some of the latest advancements employed in today's labs.

5-hmC Enrichment Analysis

5-hmC Immunoprecipitation (hMeDIP)

Given the recent interest in hydroxymethylated DNA, specific antibodies against this mark have been developed to enrich it. hMeDIP is the technique by which genome-wide hydroxymethylation levels are detected using antibodies[8]. Like other IP assays, antibodies are used to enrich for 5-hmC containing regions which can then be detected by qPCR, microarray, or sequencing.

5-hmC Protein Capture

J-base binding protein (JBP) from T4 phage has an innate specificity for glucosylated 5-hmC. Therefore, *in vitro* glucosylation of 5-hmC-containing DNA with hydroxymethylcytosine glucosyltransferase is necessary prior to enrichment of the DNA using immobilized JBP.

The Quest-5hmC™ DNA Enrichment Kit (Cat. No. D5420) from Zymo Research features JBP for the specific enrichment of 5-hmC containing DNA. The enriched DNA is suitable for PCR, qPCR, Next-Gen sequencing, whole genome amplification, and arrays.

5-hmC

Zymo Research's Epigenetic Services offers unique platforms for genome-wide 5-hmC analysis. Please refer to pg. 31 for more details. For a full list of 5-hmC products from Zymo Research, turn to pgs. 39-40.

Case Study: You are what you eat...and what your grandparents ate!

According to researchers in Sweden, grandparent's nutrition has a direct link to their grandchild's well-being. The Overkalix study sought to identify the physiological effects of various environmental factors on transgenerational epigenetic inheritance. Researchers followed historical data and records from birth through to mortality for three cohorts in a small municipality in northern Sweden. Historical data consisted of food availability, food prices, and regional harvest statistics. This intriguing research showed that when a grandfather experienced a poor availability of food or famine, his grandchildren had a tendency to be protected against cardiovascular death and diabetes. When the effects of possible overeating among parents and grandparents during their pre-pubescent growth phase were assessed, descendants showed an increased risk of death by diabetes[9].

Oxidative Bisulfite Sequencing (OxBS-seq) and Tet-assisted Bisulfite Sequencing (TAB-seq)

Two early methods introduced to analyze both 5-hmC and 5-mC at single nucleotide resolution are Ox-BS-seq and TAB-seq. These methods both rely on oxidation to discriminate 5-hmC from 5-mC at single-base resolution, but take different approaches to do so.

OxBS-seq treats DNA with a chemical, potassium perruthenate ($KRuO_4$), to add an oxidative step to bisulfite analysis. 5-hmC is oxidized to 5-formylcytosine (5-fC). 5-fC becomes deaminated by bisulfite; therefore, subsequent bisulfite treatment converts 5-fC and unmodified C to uracil but leaves 5-mC intact[10]. A standard bisulfite run performed in parallel is used to identify 5-mC and 5-hmC. One data set is "subtracted" from the other to infer 5-hmC locations.

TAB-seq requires the use of a TET enzyme to oxidize 5-mC to 5-carboxylcytosine (5-caC)[11]. 5-hmC bases are protected from this enzyme by an initial glucosylation. After TET oxidation, bisulfite converts 5-caC to U while leaving the 5-hmC intact. Thus, TAB-seq uses a method that directly analyzes the presence of 5-hmC in DNA whereas OxBS uses an indirect method. This indirect analysis can lead to increased error within OxBS-seq experiments[12]. However, TAB-seq also has its own drawbacks; TET-mediated conversion is expensive and only about 95% efficient[11].

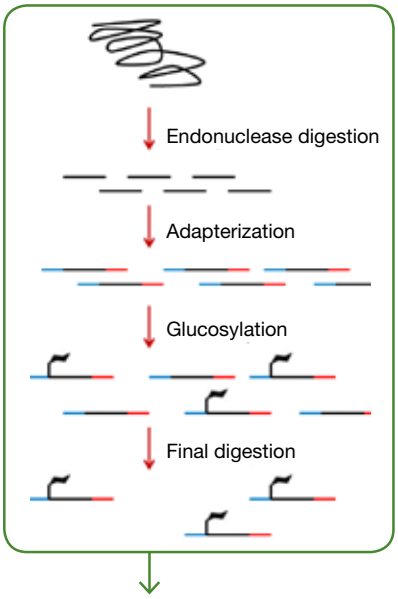
If detailed analysis of both 5-mC and 5-hmC is required, OxBS-seq is the more efficient technology. Both OxBS-seq and TAB-seq methods are suited for genome-wide, high-resolution experiments, but depending on your sample constraints and budget, these methods may not be optimal.

Reduced Representation Hydroxymethylation Profiling (RRHP)

The scientists at Zymo Research have developed a unique RRHP method for strand-specific, single-nucleotide resolution profiling of 5-hmC in genomic DNA. RRHP shares the same concept as RRBS in that the genomic sample is first enriched for 5-hmC-containing regions. Genomic DNA is digested with MspI, followed by adapter ligation, 5-hmC glucosylation, and a second MspI digestion. The first digestion enriches for CpG regions while the second digests any non-glucosylated fragments, preventing their amplification by PCR.

Unlike earlier methods developed for mapping 5-hmC (e.g. Ox-BS-seq and TAB-seq), RRHP does not require bisulfite conversion, so the DNA is not fragmented or damaged. This is a key feature that enables researchers to use minimal inputs of DNA (as low as 100 ng) to perform in-depth analyses of 5-hmC. Additionally, the RRHP approach can be aligned against RRBS data to calculate and determine the location of both 5-hmC and 5-mC.

Overview of the RRHP Process



Glucosylated fragments are amplified and sequenced

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Locus Specific 5-mC and 5-hmC Analysis

When research is focused on the DNA methylation status of individual genomic regions or loci (e.g., epi-biomarkers), high-throughput sequencing can often be overkill. For these cases, it may make more sense to use methods that will provide only the information you need. Several tried and true technologies offer the capability to deliver single nucleotide DNA methylation analysis at the regions of interest.

Zymo Research's Epigenetic Services offer Targeted DNA Methylation Sequencing for locus-specific analysis of DNA methylation. Please refer to pg. 30 for more information.

Targeted DNA Methylation Sequencing

The targeted bisulfite sequencing service from Zymo Research (pg. 30) combines the well-established bisulfite conversion technologies with the power of next-generation sequencing to provide the most comprehensive, unique, and locus-specific DNA methylation analysis service available. By harnessing the power of next-generation sequencing, each DNA methylation site of interest receives high sequencing depth of coverage for accurate, quantitative, and single-base resolution data output. Zymo Research routinely sees sequencing depths >1000x assuring ample coverage of gene loci across all samples. Furthermore, by incorporating the Fluidigm Access Array™ microfluidics PCR amplification instrument into this service platform, Zymo Research is able to simultaneously analyze dozens or even hundreds of samples in a short period of time.

DNA Methylation Analysis by Pyrosequencing and Sanger Sequencing

Several other sequencing technologies are available for targeted methylation analysis. For instance, pyrosequencing has become popular for targeted bisulfite sequencing as well as for the confirmation of array and sequencing experiments. Pyrosequencing works by quantifying the incorporation of each new nucleotide in the sequencing reaction using an ATP sulfurylase, luciferase, and apyrase system. In combination with bisulfite conversion, pyrosequencing measures the percent methylation of single cytosines[1]. After bisulfite treatment and PCR, interrogated cytosines are a mixture of C and T that represent methylated and unmethylated nucleotides, respectively. By measuring the amount of light emitted following G and A incorporation, the percentage of methylated nucleotides are quantified. Traditional Sanger sequencing can also be used to detect DNA methylation in a similar way[2]. It is not as accurate, but it is much less expensive. Primer design is challenging in any locus-specific, bisulfite experiment, so use of a bisulfite primer design software is an absolute necessity (pg. 12).

MSRE-qPCR Approaches for 5-mC and 5-hmC Detection

Real-time PCR (qPCR) is versatile and can be used to study methylation in numerous applications, where it can be combined with methylation-specific restriction endonuclease (MSRE) digestion. This method can be fast and economical and can yield relative methylation status at specific cytosines. Importantly, each primer pair can be used to assay only one cytosine at a time. Zymo Research offers a bisulfite-free *OneStep* qMethyl™ Kit (D5310) that is used for the detection of region-specific DNA methylation via the selective amplification of methylated cytosines in a CpG dinucleotide context. Following MSRE digestion, DNA is amplified via real-time PCR in the presence of a fluorescent dye and quantitated on the basis of methylation content.

qPCR can also be utilized for 5-hmC analysis. For instance, in Zymo Research's Quest 5-hmC Detection Kit™, 5-hmC in DNA is first glucosylated, digested with MspI, and then amplified via qPCR prior to analysis. Only the intact DNA fragments containing 5-hmC will be amplified. This method can be used to quantify the amount of 5-hmC in specific regions of DNA between many samples, and 5-hmC can be assayed with relatively high-throughput using this method.

qPCR approaches for 5-mC & 5-hmC: Zymo Research offers a *OneStep* qMethyl™ Kit as a bisulfite-free qPCR approach to 5-mC analysis. In addition, the Quest 5-hmC Detection Kit™ is offered for qPCR detection of 5-hmC.

Product	Size	Cat. No.
OneStep qMethyl™	1x96 well	D5310
OneStep qMethyl™-Lite	1x96 well	D5311
Quest 5-hmC Detection Kit™	25 Preps., 50 Preps.	D5410, D5411
Quest 5-hmC Detection Kit™-Lite	25 Preps., 50 Preps.	D5415, D5416

A Diverse Tool Set

Unlike most other conventional DNA and RNA analysis methods, epigenetics research exists at the convergence of nucleic acids and their modifications, and the proteins that interact with them. As a result, studies targeting epigenetic mechanisms present many new challenges. Several innovative methods have been introduced over the years that enable efficient analysis of epigenetic marks and processes, but no one method is perfectly suited for every scenario. This results in a wide variety of techniques being employed in labs today and choosing the right approach is not easy. Researchers must take into account many factors, including resolution, coverage, accuracy, sensitivity, throughput, budget, ease of use, and the informatics required for the analysis - just to name a few!

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Chromatin Analysis *Made Simple*™

Zymo-Spin™ ChIP Kit

- ✓ Features a unique workflow using a micro-elution spin column for purification of ChIP DNA. High quality ChIP DNA is ideal for ChIP-qPCR, ChIP-seq and other molecular applications.

ChIP DNA Clean & Concentrator®

- ✓ Provides a simple, two (2) minute DNA clean-up from any step in a standard ChIP protocol.

EZ Nucleosomal DNA Prep Kit

- ✓ Used for the isolation of nucleosome-associated DNA from mammalian and yeast cells and is ideal for nucleosome mapping studies.

Product	Size	Cat. No.
Zymo-Spin™ ChIP Kit	10 Preps., 25 Preps.	D5209, D5210
ChIP DNA Clean & Concentrator®	50 Preps.	D5205
ZR-96 ChIP DNA Clean & Concentrator®	2x96 Preps., 4x96 Preps.	D5206, D5207
EZ Nucleosomal DNA Prep Kit	20 Preps.	D5220

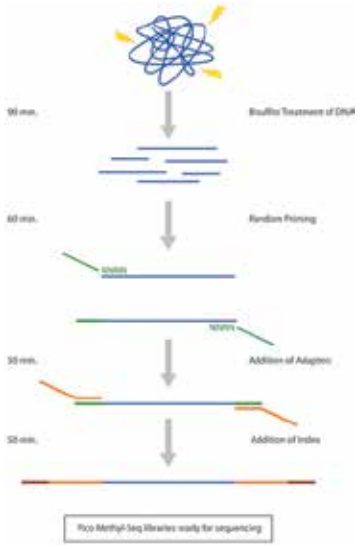
NGS Library Prep Kits

Pico Methyl-seq™ Library Prep Kit

The Pico Methyl-seq™ Library Prep Kit provides a streamlined workflow for making Whole Genome Bisulfite Sequencing (WGBS) libraries.

- ✓ Post-bisulfite library preparation for WGBS
- ✓ Accommodates ultra-low DNA input and compatible with FFPE samples
- ✓ Simple, ligation and gel-free workflow can be completed in a few hours
- ✓ Illumina® compatible

Product	Size	Cat. No.
Pico Methyl-seq™ Library Prep Kit	10 Preps., 25 Preps.	D5455, D5456

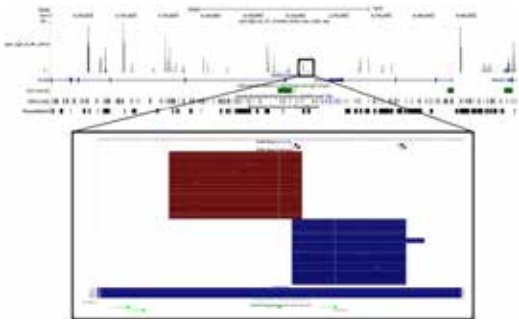


RRHP™ 5-hmC Library Prep Kit

The RRHP™ 5-hmC Library Prep Kit is the first comprehensive solution for quantitative analysis of genome-wide 5-hmC positions at single base resolution.

- ✓ Innovative library preparation for strand-specific mapping of 5-hmC in DNA
- ✓ Streamlined workflow accommodates low DNA inputs
- ✓ Libraries are ideal for Next-Gen sequencing (Illumina®) or array

Product	Size	Cat. No.
RRHP™ 5-hmC Library Prep Kit	12 Preps., 25 Preps.	D5450, D5451



Single-locus resolution analysis of 5-hmC in human brain DNA can be achieved using the RRHP™ 5-hmC Library Prep Kit from Zymo Research. DNA was prepared according to the protocol and then sequenced with an Illumina HiSeq™ 2000. Tracks (bottom figure) display relative quantitation of 5-hmC strand distribution (blue = sense, red = antisense) as well as SNP positions within the fragments at a single locus.

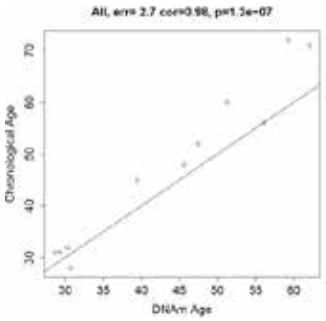
Forever 21? It May Be Possible by Resetting Your DNA Methylation Age Clock

Wei Guo
Zymo Research Corporation

As an organism ages, cells and tissues differentiate and change. These changes can include accumulation of DNA mutations, alterations to telomeres, decay of cellular and tissue structures, and changes in gene expression. Both differentiation of tissues and age effects are at least partially caused by epigenetic modifications of the genome, including DNA methylation, which is mainly in the form of 5-methylcytosine in the dinucleotide CpG. Age-associated differential methylation patterns have been previously observed, with hypomethylation in regions mostly outside of CpG islands (CGIs) and hypermethylation within CGIs. These results suggested that there may be an epigenetic clock inside our body to accurately track age. Recently, Steven Horvath of The University of California Los Angeles reported development of an epigenetic predictor of age that can determine an individual's so-called DNA methylation age, which was usually accurate to within <5 years compared to the actual chronological age[1].

Horvath created the epigenetic age predictor utilizing an unprecedented collection of 8000 samples from 82 publicly available DNA methylation array datasets, consisting of 51 healthy tissues and cell types. The predictor is not tissue-specific and can estimate DNA methylation age of most tissues and cell types. In this study, Horvath used 39 of the datasets to create a training set which represented a wide spectrum of tissues and cell types and had a mean age (43 years) similar to that of the validation set of 32 datasets. The training data were pre-processed and normalized, retaining 21,369 CpGs for further analysis that a) were present on both the Illumina 27K and 450K bead-array platforms and b) had fewer than 10 missing values. A panel of 353 "clock" CpGs was selected by using statistical analyses for prediction of DNA methylation age. The performance of the age predictor was remarkably accurate, with an age correlation coefficient of 0.97, and error rate of 2.9 years in the training set and a correlation coefficient of 0.96, and error rate of 3.6 years in the validation set.

The 353 clock CpGs are located in genes significantly enriched for cell death/survival, cellular growth/proliferation, organismal/tissue development, and cancer. Of these sites, methylation at 193 and 160 CpGs positively and negatively correlated with increasing age, respectively. The age-related change in methylation (beta value) was generally very small (the average absolute difference in DNA methylation levels across the 353 CpGs was only 0.032 between subjects <35 years old and >55 years old). The rate of change, or the ticking rate of the epigenetic clock, was observed to be high before adulthood and slows down to a constant linear rate after adulthood. Horvath proposed that the DNA methylation age measures the cumulative work done by an epigenetic maintenance system. Using the epigenetic age predictor developed by Horvath and real methylation data, the bioinformatics team at Zymo Research created the figure below which shows the high correlation between the predicted DNA methylation age and the actual chronological age of healthy subjects.



This plot, created by the bioinformatics team at Zymo Research, shows the high correlation between the predicted DNA methylation (DNAm) age and the actual chronological age of healthy subjects using the epigenetic age predictor developed by Horvath and real methylation data. err, error rate in years; cor, correlation coefficient; p, p-value.

The author also looked into DNA methylation age in cancer. Across all 32 cancer datasets (6000 cancer samples) there was only a weak correlation between DNA methylation age and chronological patient age (correlation coefficient of 0.16, p=2.5e-33), indicating that the epigenetic clock loses track of time in most cancers. While some cancer types (bone marrow, brain cancer) show positive age acceleration, which is defined as difference between DNA methylation age and chronological age, others (colorectal, uterine cancer) show negative age acceleration. Interestingly, the difference in DNA methylation age and chronological age in cancer may be useful in the diagnostics, prognostics, and treatment of malignancies.

Horvath's study additionally demonstrated that the DNA methylation age is a highly heritable measure of age acceleration, is close to zero for embryonic (ES) and induced pluripotent stem (iPS) cells, correlates with the number of passages for ES and iPS cells, and is also applicable in chimpanzee tissues. Determining the DNA methylation age should be useful for studies in development, cancer, aging and forensics. For example, the predicted age of a criminal suspect can be estimated by analyzing DNA methylation patterns in biological traces left at a crime scene. In the future, DNA methylation age could be used to assess an individual's risk for one or more age-related diseases, and intervention and treatment could be tailored based on his or her biological DNA methylation age instead of the actual chronological age. Since methylation is usually reversible, it may be possible to purposely biochemically modify your age-related CpGs and reset the epigenetic clock in your body back to more youthful levels. Do you believe that there is an epigenetic clock in our body to track age? And if so, what do you think of the idea of intentionally rewinding our DNA methylation age clock to stay forever 21?

REFERENCE

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Technology Spotlight:

Apple
(Malus domestica)

Alligator
(Alligator mississippiensis)

Cow
(Bos taurus)

Baboon
(Papio anubis)

Bean
(Phaseolus vulgaris)

Pig
(Sus scrofa domesticus)

Barrel Clover
(Medicago truncatula)

Mouse
(Mus musculus)

Explore Epigenomics

with Next-Gen sequencing services

Shown here are some of the diverse species analyzed by our team

Zebra Finch
(Taeniopygia guttata)

Human
(Homo sapien)

Fruit Fly
(Drosophila melanogaster)

Wine Grape
(Vitis vinifera)

Salmon
(Salmo salar)

Platypus
(Ornithorhynchus anatinus)

Oppossum
(Didelphimorphia)

Chicken
(Gallus gallus domesticus)

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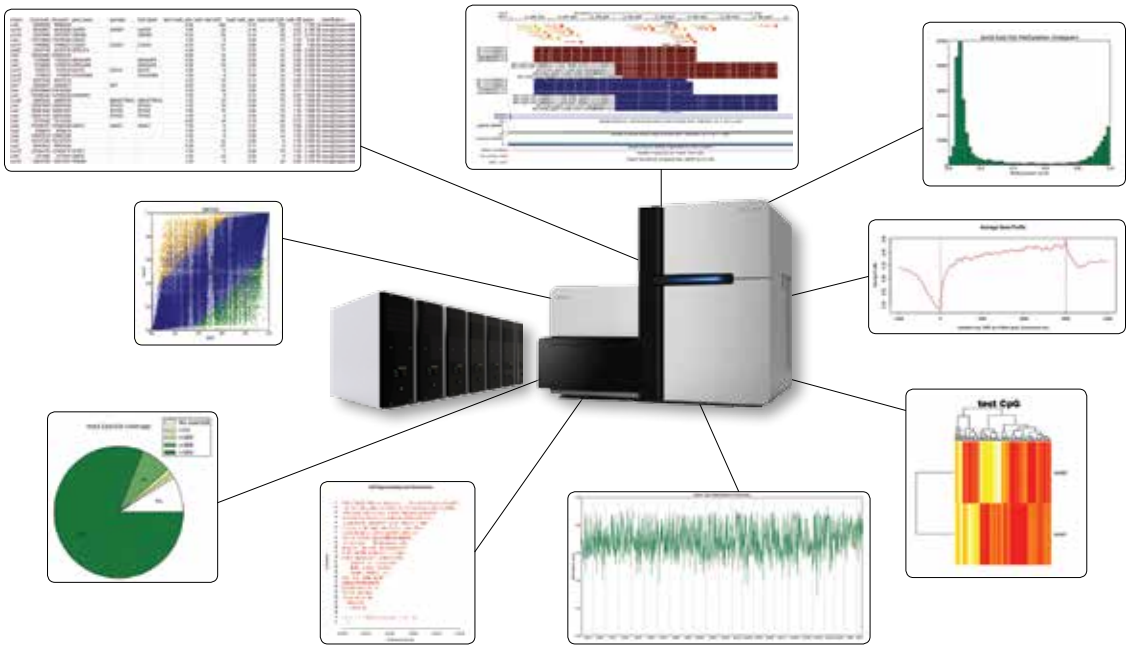
Just send us your samples, and we will return the genome-wide analyses as customizable publication-ready graphs and figures. Now that's...*epigenetics made simple!*

Zymo Research makes genome-wide epigenetic analyses available to every researcher with its comprehensive repertoire of services. All Next-Gen epigenetic sequencing services feature state-of-the-art sample prep technologies and workflows, cutting-edge data processing, and all services are offered at competitive pricing. *We also do the bioinformatics so you don't have to!*

Simply send us your samples... and let us do the rest!



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illumina CSPro® CERTIFIED SERVICE PROVIDER

Genome-wide DNA Methylation Analysis Services

5-mC

- ✓ Powered by Zymo Research's industry-leading bisulfite conversion chemistries combined with the latest Next-Gen sequencing technologies
- ✓ Compatible with low DNA inputs including FFPE, LCM, FACS samples, and others
- ✓ Compatible with samples from any organism (human, mouse, rat, plant, and hundreds more!)
- ✓ Comprehensive bioinformatics analysis includes simultaneous SNP detection

Zymo Research offers three individual platform technologies for single nucleotide resolution DNA methylation (5-mC) analysis:

The **Methyl-MiniSeq™ Service** covers ~10% of the methylome

The **Methyl-MidiSeq™ Service** covers ~30% of the methylome

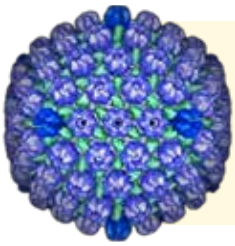
The **Methyl-MaxiSeq™ Service** profiles the entire methylome

Comparison of Genome-Wide DNA Methylation Analysis Technologies

	Zymo Research			Other Technologies		
	Methyl-MiniSeq™	Methyl-MidiSeq™	Methyl-MaxiSeq™	MethylCap-seq/ MBD-seq	MeDIP-seq	Infinium® 450k Array
Overview	Economical methylation analysis	Expanded methylation analysis	Complete methylation analysis	Identifies regions, not individual CpGs	Limited by antibody specificity	Analysis of human samples only
Compatible with Low DNA Inputs	Yes	Yes	Yes	No	No	No
Single-Base Resolution	Yes	Yes	Yes	No	No	Yes
Methylome Coverage*	3-4 million sites	8-9 million sites	Entire methylome	Variable**	Variable**	< 0.5 million sites
Quantitative Analysis	Yes	Yes	Yes	No	No	Semi-quantitative
SNP Detection	Yes	Yes	Yes	Yes	Yes	No
Genomic Regions Covered	Nearly all CpG islands and gene promoters	Nearly all CpG islands, gene promoters, gene bodies, and regulatory regions	Entire methylome	Densely methylated regions only	Densely methylated regions only	Limited to pre-designed regions

*calculations based on human genome

**dependent on capture efficiency and methylation levels



In a recent study published in the *Journal of Virology*, Birdwell and colleagues examined epigenetic modifications in oral keratinocytes following Epstein-Barr virus (EBV) infection. Using the Methyl-MiniSeq™ Service from Zymo Research, the group found that EBV infection induced long-lasting DNA methylation changes at CpG islands, promoters, and within gene bodies when compared to uninfected cells. Furthermore, the DNA methylation changes correlated with gains and losses in expression of a subset of affected genes[1]. These findings provide mechanistic insights as to how EBV infection contributes to DNA methylation changes commonly observed in EBV-associated carcinomas and hints at the potential for future epigenetics-based therapies.

Targeted DNA Methylation Analysis Services

Targeted DNA Methylation Sequencing

- ✓ Includes primer design and assay validation for amplification of bisulfite-converted DNA at the region(s) of interest
- ✓ Quantitative analysis of even extremely low DNA methylation levels
- ✓ Compatible with FFPE samples

This service allows for focused DNA methylation sequencing of a few to hundreds of specific regions of interest across the genome. Targeted DNA Methylation analysis allows for parallel PCR amplification of multiple loci from the same sample source with only 500 ng of input DNA.

Comparison of Targeted DNA Methylation Analysis Technologies

	Zymo Research	Pyrosequencing*
Overview	Parallel PCR amplification of multiple loci from the same sample source with only 500 ng input DNA	One locus at a time; requires 1 µg input DNA. Difficulty distinguishing the homopolymeric sequences in bisulfite-converted DNA
Saves Time & Money Compared to Genome-Wide Sequencing	Yes	Yes
>100-1,000-Fold Coverage at each CpG Site of Interest	Yes	No
1,000s of Loci can be Analyzed in a Single Sequencing Run	Yes	No
Multiplex Compatibility	Yes	No

*Colony Pyrosequencing

Researchers used the Methyl-MiniSeq™ and Targeted DNA Methylation Sequencing services from Zymo Research to identify and validate methylation markers linked to colorectal cancer (CRC) in African Americans. Genome-wide methylation analysis with Methyl-MiniSeq™ revealed 355 differentially methylated CpG sites in 13 genes in addition to hypomethylated Long INterspersed Elements (LINEs) in CRC samples. Furthermore, six of these genes contained the 50 CpGs with the highest degree of differential methylation and were then studied further using 42 samples and Targeted DNA Methylation Sequencing[2]. With the larger sample set and more focused sequencing, the authors were able to validate four genes, EID3, BMP3, GAS7, and GPR75, as novel CRC methylation markers in African American patients.

Did You Know?

Advances in whole genome sequencing technologies and epigenetics have recently re-invented the wheel when it comes to forensic DNA analysis. While current methods are mainly presumptive and not as sensitive or specific as they could be, epigenetic analyses can probe even deeper by distinguishing tissue or body fluid origin, providing age identification as well as discriminating between identical twins. Through the use of bisulfite sequencing technologies, researchers in Florida have recently defined epigenetic markers that display differential methylation patterns between blood, saliva, semen and epithelial tissue[3]. These findings can be instrumental in identifying an individual suspect from a pool of samples, exonerating innocent individuals and assisting with cold cases.

Genome-wide DNA Hydroxymethylation Analysis Services

Reduced Representation Hydroxymethylation Profiling (RRHP)

5-hmC

- ✓ For single-base resolution, semi-quantitative, and strand-specific identification of 5-hmC in DNA across the entire genome
- ✓ Capable of distinguishing between methylcytosine and hydroxymethylcytosine in DNA when used in conjunction with Methyl-MiniSeq™ (p.29)
- ✓ Comprehensive bioinformatics analysis includes simultaneous SNP detection

The scientists at Zymo Research have pioneered this unique approach to enable single-nucleotide 5-hmC profiling on a genome-wide scale. The RRHP workflow allows for DNA to be specifically fragmented and then size selected to focus the analysis on areas with the most hydroxymethylation information. Following library preparation, 5-hmC is glucosylated and the library is digested once more with restriction enzymes sensitive to glucosylation.

Comparison of Genome-wide, 5-hmC Analysis Technologies

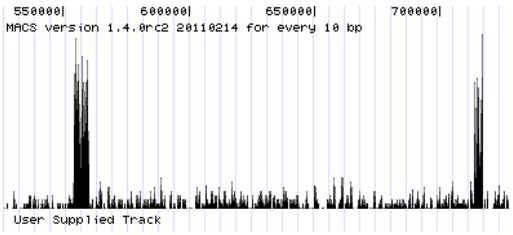
	Zymo Research	Other Technologies	
	RRHP	OxBS-seq	TAB-seq
Available as a Service	Yes	No	No
Compatible with Low DNA Inputs	Yes	No	No
Detection Sensitivity Below 5%	Yes	No	No
Bisulfite Conversion-free	Yes	No	No
Allows for Simultaneous SNP Detection	Yes	No	No
Native Sequence Display	Yes	No	No
Single-base Resolution	Yes	Yes	Yes
Reproducibility	High	Low	Medium

In a recent article published in the *Journal of Biological Chemistry*, scientists demonstrated that pro-inflammatory cytokines IL-1ß and TNF-a can modulate the TET-1 oxidation pathway in cartilage cells and play a major role in arthritis pathogenesis. Using Zymo Research's Reduced Representation Hydroxymethylation Profiling (RRHP) Services, they analyzed 5-hydroxymethylation (5-hmC) levels genome-wide and at the promoter regions of select IL-1ß-regulated genes in primary chondrocytes treated with the cytokines. 5-hmC was found to correlate with gene expression changes indicative of an inflammatory response commonly seen in arthritis[4].

5-hmC Capture Sequencing (CapSeq)/JBP1-seq

5-hmC

- ✓ An improved J-Binding Protein (JBP)-based 5-hmC capture procedure for unbiased, highly-specific enrichment of hydroxymethylated DNA
- ✓ Following sequencing, 5-hmC are identified by peaks in the genome browser tracks
- ✓ Unparalleled sensitivity and specificity compared to antibody-based enrichment methods having high backround noise



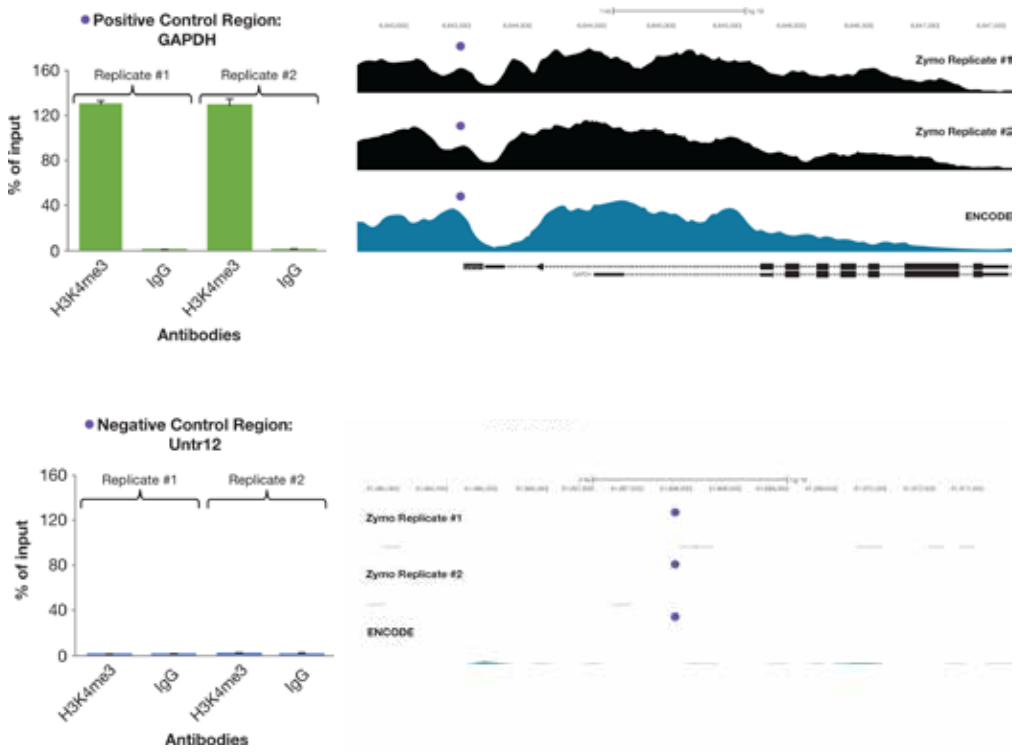
Genome-wide 5-hmC CapSeq is an extremely useful approach for researchers looking to better understand 5-hmC. Following glucosylation, all hydroxymethylated DNA can then be enriched using JBP1 which has a strong affinity and extremely high specificity for g-5-hmC and for subsequent 5-hmC analysis following NGS.

Additional Services

ChIP-seq

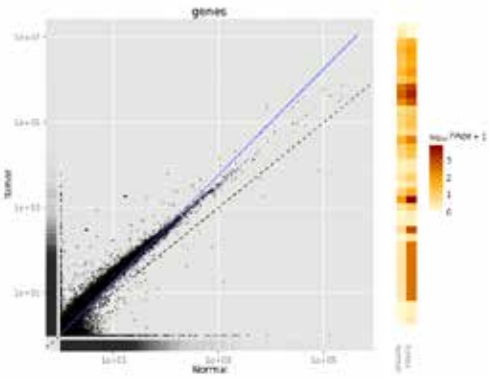
- ✓ Full service analysis including the ChIP assay, library prep, Next-Gen sequencing and bioinformatics
- ✓ Optimized pipeline for most histone modifications
- ✓ ChIP-seq antibody validation service also available to identify the best antibodies for your ChIP-seq assay
- ✓ Rapid turnaround at an economical price

ChIP-seq is instrumental for understanding histone modifications and investigating protein-DNA interactions. Zymo Research makes it easy and economical to perform and analyze chromatin immunoprecipitation assays on a genome-wide scale.



RNA-seq

- ✓ Next-Gen transcriptome-wide analysis at your fingertips
- ✓ Transcriptome-wide coverage of total RNA and/or small RNAs
- ✓ Complete workflow available from RNA purification to bioinformatics



Achieve transcriptome-wide coverage of total RNA or small RNAs using the latest Next-Gen sequencing technologies. Let Zymo Research do the work for you starting from RNA purification, sample prep, Next-Gen sequening and bioinformatic analysis. There are a variety of services to choose from including mRNA-seq, total RNA-seq as well as small RNA-seq.

Mass Spectrometry

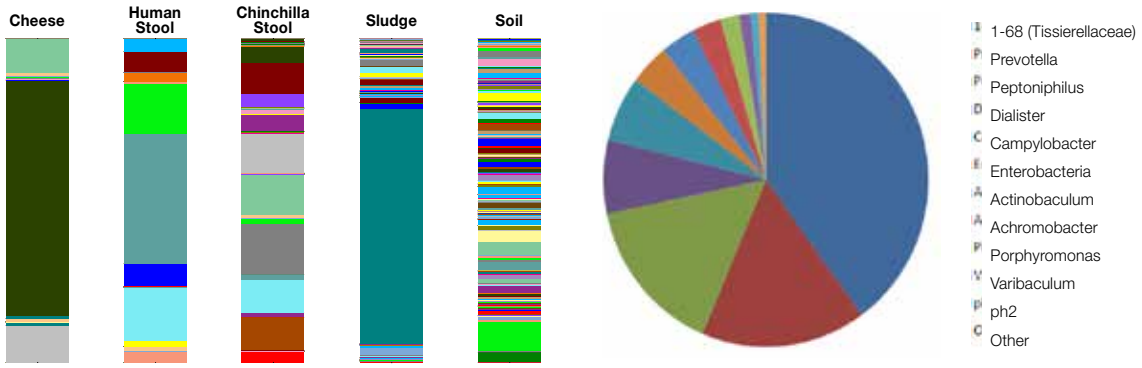
- ✓ LC/MS (MRM) global analysis of DNA methylation and hydroxymethylation
- ✓ Sensitive, accurate and quantitative analysis of 5-mC and 5-hmC modifications in DNA

Metagenomic Services

- ✓ **Microbial Composition Profiling**
  - 16s rRNA gene, 18s rRNA gene, and/or fungal ITS sequencing for genus-level determination
  - Shotgun Metagenomic Sequencing for species-level determination
- ✓ **Novel Microbe Identification:** High-quality, paired-end and mate-pair reads ensure superior de novo genome assembly from metagenomes
- ✓ **Biological Function Analysis:** The full gene complement and metabolic pathway analysis in a microbial community (e.g., antibiotic resistance)

MICROBIOMICS

NO MICROBE LEFT BEHIND...



Custom Bioinformatics Analysis

Do you have Next-Gen sequencing data that you need analyzed? Zymo Research offers complete bioinformatics solutions to fulfill your needs. Whether it is whole-genome bisulfite sequencing data or ChIP-seq data, we can help make sense of your overwhelming data sets. We use established as well as customizable bioinformatic pipelines to transform raw sequence data into manageable and interpretable figures and data sets. Simply provide the raw (FASTQ) or aligned (SAM or BAM) data and we will provide you with your desired downstream analyses.

All services can be customized and combined to meet your needs!

www.zymoresearch.com/services | services@zymoresearch.com | Phone: (949) 679-1190 | Toll-free: (888) 882-9682

REFERENCES

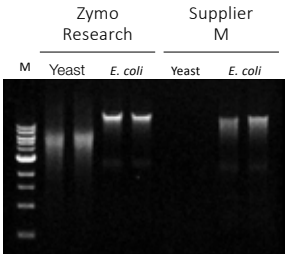
1. Birdwell, et al., (2014). Genome-wide DNA methylation as an epigenetic consequence of Epstein-Barr virus infection of immortalized keratinocytes.J Virol. Oct 1;88(19): 11442-58.
2. Ashktorab, H, et al., (2014). DNA methylome profiling identifies novel methylated genes in African American patients with colorectal neoplasia. Epigenetics. 9(4): 503-512.
3. Madi T, Balamurugan K, Bombardi R, Duncan G, McCord B. (2012). The determination of tissue-specific DNA methylation patterns in forensic biofluids using bisulfite modification and pyrosequencing. Electrophoresis. 33, 1736-1745.
4. Haseeb A, Makki MS, Haggi TM. (2014). Modulation of ten-eleven translocation 1 (TET1), Isocitrate Dehydrogenase (IDH) expression, α-Ketoglutarate (α-KG), and DNA hydroxymethylation levels by interleukin-1β in primary human chondrocytes. J Biol Chem. Mar 7;289(10):6877-85.

Isolate Quality DNA for your Metagenomic Analyses

- ✓ Complete, unbiased lysis of all microbes, including tough-to-lyse organisms
- ✓ Complete removal of PCR-inhibitors
- ✓ Clean-Spin™ technologies ensure ultra-pure DNA is ready for all downstream applications including Next-Gen sequencing, PCR, etc.

Fungal/ Bacterial DNA

Reliably isolate total DNA in just minutes from microbial samples such as biofilms, cultures, swabs, filters, etc.



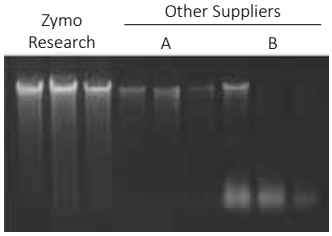
High-yield DNA is successfully isolated from *S. cerevisiae* (spores) and *E. coli* cells using the ZR Fungal/Bacterial DNA Kit™. Equivalent amounts of yeast or bacteria were processed using the kits from Zymo Research or Supplier M.

Lengerova, M. et al. (2012). J. Clin. Microbiol, 50 (3), 602-608.

ZR Fungal/Bacterial DNA Kits™	Size (Cat. No.)
MiniPrep	50 Preps. (D6005)
96-well	2x96 Preps. (D6006)

Fecal DNA

Ensure purification of inhibitor-free DNA from stool samples (birds, rats, mice, cattle, human, etc.).



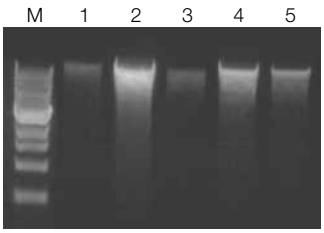
Comparison of DNA yields from rat stool samples using the ZR Fecal DNA MiniPrep™ and kits from Suppliers A and B. Equivalent amounts of stool was processed using each kit and eluted with equal volumes.

Yoshikawa, H. et al. (2011) Parasitology Research, 109 (4), 1045-1050.

ZR Fecal DNA Kits™	Size (Cat. No.)
MiniPrep	50 Preps. (D6010)
96-well	2x96 Preps. (D6011)

Soil Microbe DNA

Rapid isolation of inhibitor-free DNA from a variety of microbial communities in clay, sandy, silty, peaty, chalky, and loamy soils.



Metagenomic DNA isolated from various soil samples (1 – 5) using the ZR Soil Microbe™ DNA Kit. M: 1 kb marker (NEB); 1-5: soil samples (sand, sandy clay loam, hydrophobic sandy loam, course sandy loam, fine gravel).

Aerts, S. et al. (2009) BNRC, ER-61; CCHO-2004-2470/00/00 DS 251-B62).- ISSN 1782-2335

ZR Soil Microbe DNA Kits™	Size (Cat. No.)
MiniPrep	50 Preps. (D6001)
96-well	2x96 Preps. (D6002)

Epigenetics Tools and Databases

Epigenetic tools and databases can make a researcher's life much easier. A wide-variety of free, online utilities are available that allow you to tap into specialized information and expertise right from your own lab. Here are a handful of useful tools and databases that can elevate any epigenetics experiment.

Data Visualization: EpiExplorer

http://epiexplorer.mpi-inf.mpg.de/

EpiExplorer is a web tool that allows you to upload your own datasets, and then compare them to large reference epigenomes, all without the need for a ton of computing power.

Data Browser: Roadmap Epigenomics Visualization Hub (VizHub)

http://vizhub.wustl.edu/

This user-friendly browser incorporates a GoogleMaps style interface that allows you to search, pan and zoom across entire reference genomes compiled by the NIH Roadmap Epigenomics Consortium.

Data Analysis: DMAP (Differential Methylation Analysis Package)

http://biochem.otago.ac.nz/research/databases-software/

The DMAP suite aids in large-scale genomic DNA methylation analysis. DMAP filters and processes bisulfite-sequencing data to create reference methylomes for any genome, and map differentially methylated regions to genes and CpG features.

Epigenetic Repository: Human Epigenome Atlas

http://www.genboree.org/epigenomeatlas/index.rhtml

The Human Epigenome Atlas is a collection of human reference epigenomes and includes details about their integrative and comparative analyses.

Epigenetics Resource & Community: EpiBeat

http://epibeat.com

EpiBeat is the epigenetics resource and community for you to stay up-to-date and join the conversation on the rapidly expanding field of epigenetics. It's a simple way to keep your finger on the pulse of epigenetic discovery!

Bisulfite Primer Design: Zymo Research's Bisulfite Primer Seeker

http://www.zymoresearch.com/tools/bisulfite-primer-seeker

Zymo Research's online tool simplifies the tedious bisulfite primer design process.

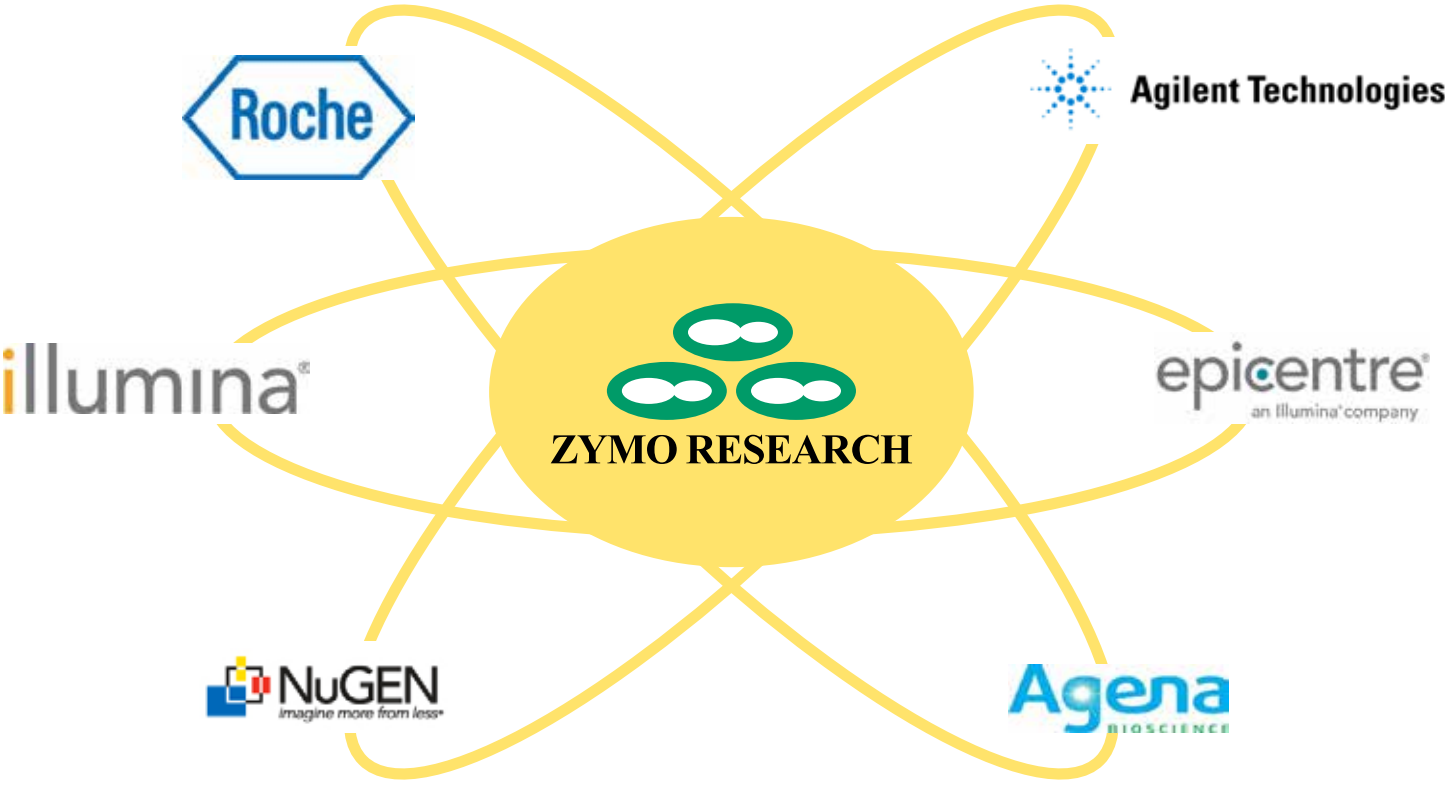
All Things Epigenetics: EpiGenie

http://epigenie.com

EpiGenie is a team of folks who scour through PubMed, watch out for interesting epigenetics press releases, conduct interviews with epigenetics researchers, and work closely with the technology providers to stay on top of the most applicable epigenetics-related research.

Who Recommends Zymo Research's Products?

Zymo Research products are part of many third party applications including those from Illumina®, Roche®, Nugen®, and more!



Zymo Research Product	Cat. No.	Company	Application
DNA Clean & Concentrator®-5	D4013 D4023	Illumina®	Nextera DNA Sample Prep Kit
DNA Clean & Concentrator®-25	D4005	NuGen® Roche NimbleGen®	WT-Ovation® Pico RNA Amplification System Sequence Capture Arrays (2.1 M and 385 K arrays)
Genomic DNA Clean & Concentrator®	D4010	Illumina®	Nextera DNA Sample Prep Kit Infinium HD FFPE Restore
EZ DNA Methylation™ Kit	D5001 D5002 D5004	Illumina® Agena	Goldengate and Infinium Assays MassARRAY® EpiTYPER®
EZ DNA Methylation-Gold® Kit	D5006	Agilent Technologies®	SureSelect XT™ Methyl-seq Target Enrichment System
EZ DNA Methylation-Lightning® Kit	D5030 D5031	Illumina® Roche NimbleGen®	EpiGnome™/TruSeq DNA Methylation SeqCap Epi Enrichment System
RNA Clean and Concentrator®-5	R1015	Illumina® Epicentre®	Ribo-Zero™ rRNA Removal Kit Message*BOOSTER*™ Whole Transcriptome cDNA Synthesis Kit

Epigenetics Glossary

5-hydroxymethylcytosine (5-hmC): an epigenetic modification of DNA; can result from the oxidation of 5-methylcytosine modification by the Tet family of enzymes.

5-methylcytosine (5-mC): an epigenetic modification of DNA that usually occurs at CpG dinucleotides; the 5-mC modification usually correlates with repressed gene expression.

Adult stem cell: multipotent stem cells present in differentiated tissue; also known as tissue-specific stem cells.

Bisulfite conversion: the deamination of non-methylated cytosine bases to uracil by treatment with sodium bisulfite ($NaHSO_3$); 5-mC bases are resistant to bisulfite conversion.

Bisulfite sequencing: determining the sequence of bisulfite-converted DNA; considered the "gold standard" of DNA methylation analysis.

Blastocyst: an early embryonic structure consisting of distinct outer trophectoderm cells, which develop into the placenta, and the inner cell mass, which develops into the fetus.

Body methylation: methylation of DNA bases within coding sequences of actively transcribed genes found within euchromatin.

Bromodomain: a protein motif that binds acetylated lysine residues; commonly present in proteins that recognize acetylated histones, such as chromatin-remodeling factors.

ChIP-on-chip: a combination of chromatin immunoprecipitation and DNA hybridization to genomic microarrays (also known as ChIP-chip).

ChIP-seq: a method combining chromatin immunoprecipitation and DNA sequencing to analyze specific DNA-protein interactions. Next-gen DNA sequencing is often performed, resulting in a genome-wide analysis of protein-chromatin interactions.

Chromatin: the complex of DNA, histones, RNA, and other proteins that comprise the structural basis of chromosomes.

Chromatin immunoprecipitation (ChIP): a method used to identify proteins bound to DNA and the sequence to which they bind using an antibody to specifically immunoprecipitate the protein of interest; the DNA sequence that co-precipitates with the protein can be identified by PCR, hybridization, or sequencing.

Chromodomain: a motif of 40-50 amino acids common to proteins that function in chromatin remodeling; may function in binding DNA, RNA, and protein; often binds methylated histones.

Combined bisulfite restriction analysis (COBRA): a quantitative technique for the detection of methylated DNA in which DNA is subjected to bisulfite conversion and digestion with restriction endonucleases that are specific for sequences containing CpG sites (and thus are subject to methylation); the digestion products are a direct reflection of DNA methylation at the restriction sites.

Constitutive heterochromatin: heterochromatin, often located near centromeres (also known as pericentric heterochromatin), that is irreversibly silenced; DNA within constitutive heterochromatin is typically AT-rich.

CpG islands: regions of DNA enriched for CG dinucleotides; CpG islands are typically 300-3000 bp long, located upstream of gene coding regions, and usually protected from DNA methylation.

De novo methylation: the establishment of genomic DNA methylation during embryonic development; in mammals, after genomic DNA is demethylated in the zygote, the methyltransferases DNMT3a and DNMT3b methylate DNA between embryonic implantation and gastrulation.

Differentially DNA-methylated region (DMR): a region of DNA that is methylated differentially in the two chromosomes of a diploid cell; often associated with genomic imprinting.

DNA methylation: a heritable, reversible epigenetic modification in which a methyl group is covalently added to a DNA sequence, usually the 5th carbon of the cytosine pyrimidine ring in a CpG dinucleotide, although CpHpG and CpHpH sequences can be methylated in plants.

DNA methyltransferase: an enzyme that catalyzes the addition of a methyl group to a DNA nitrogenous base; the 5-mC class adds a methyl group to the 5-carbon position of cytosine bases; humans produce DNMT1, the maintenance methyltransferase, which is active at hemimethylated sites, and the methyltransferases DNMT3a and DNMT3b, which function during embryonic development and shortly after birth.

Dosage compensation: mechanisms involved in equalizing the expression of genes encoded on the X-chromosome between the two sexes; some examples are X chromosome inactivation in female mammals, X chromosome upregulation in male *Drosophila* flies, and partial repression of both X chromosomes in hermaphrodite *C. elegans* worms.

Embryo: an individual organism between the onset of multicellularity through birth; alternatively defined as beginning with implantation of the blastocyst in the uterus; in human development the term is usually used until the 8th week of pregnancy, from which point the term fetus is used.

Embryonic stem cell (ES cell): pluripotent stem cells found in the blastocyst, inner cell mass, and embryo.

Epiallele: variations in the epigenetic status of a gene or locus; often associated with differential methylation.

Epigenetic mark: a modifying moiety that carries an epigenetic signal; examples include methylation of DNA, methylation, acetylation, phosphorylation, ubiquitination, and sumoylation of histones.

Epigenetic silencing: the suppression of gene transcription or expression because of epigenetic factors such as RNAi, DNA methylation, histone modification, or chromatin remodeling.

Epigenetic therapy: application of chemical compounds, such as DNA methyltransferase inhibitors (e.g. 5-azacytidine, 5-aza-2'-deoxycytidine), to target epigenetically regulated mechanisms in patients.

Epigenetics: heritable traits that can be maintained through cell division and sexual reproduction that are not the result of a change in DNA sequence; epigenetic factors include chromatin conformation, DNA methylation, histone modification, and RNAi.

Epigenome: all of the epigenetic marks present throughout the genome of a cell.

Euchromatin: decondensed chromatin that is conformationally favorable for transcription; euchromatin typically has less DNA methylation than heterochromatin, and its associated histones have modifications that favor gene transcription.

Facultative heterochromatin: heterochromatin that may become transcriptionally active in specific cell development fates.

Hemimethylated: the status of a symmetrical DNA sequence (such as CG or CHG) that is methylated on only one strand.

Heterochromatin: condensed chromatin that is conformationally unfavorable for transcription; heterochromatin typically has more DNA methylation than euchromatin, is associated with histones containing repressive modifications, and can be associated with repressive non-coding RNAs.

Histone acetyltransferase (HAT): enzyme that acetylates histones at specific lysine residues.

Histone deacetylase (HDAC): enzyme that removes acetyl groups from N(6)-acetyl-lysine residues on a histone.

Histone: chromosomal architectural proteins that bind DNA within nucleosomes; in eukaryotes there are 4 core histones: H2A, H2B, H3, and H4, the non-nucleosomal linker histone H1, and variant histones.

Histone code: the hypothesis that the locations and types of histone modifications, through chromatin remodeling and/or recruitment of transcription factors, predicts the effects of those modifications on gene expression.

Histone methyltransferase: a class of enzymes that add methyl groups to specific histone residues; members include histone-lysine N-methyltransferase and histone-arginine N-methyltransferase.

Histone modification: posttranslational addition or removal of epigenetic marks from histones; includes methylation, acetylation, phosphorylation, ubiquitination, sumoylation, and the removal of these marks.

Histone variants: paralogous histones that can replace the major core histone proteins and may have distinct gene regulatory functions; also known as replacement histones.

Hypermethylation: increase in the level of DNA methylation in a population of cells relative to a reference or normal sample; may be used to describe a specific nucleotide or a group of nucleotides.

Hypomethylation: decrease in the level of DNA methylation in a population of cells relative to a reference or normal sample; may be used to describe a specific nucleotide or a group of nucleotides.

Imprinting: epigenetic regulation in which maternally and paternally inherited alleles are differentially expressed owing to cis-acting modifications of DNA or histones inherited from parental chromosomes.

Inner cell mass (ICM): pluripotent cells located in the interior of the blastocyst that develop into the fetus.

Induced pluripotent stem (iPS) cells: differentiated cells reprogrammed to pluripotency by ectopic expression of reprogramming factors such as Oct3/4, Sox2, Klf4, and c-Myc.

Large non-coding RNA: non-coding RNA larger than 200 nucleotides; can have roles in epigenetic regulation of gene expression.

Loss of imprinting (LOI): activation of an allele normally silenced by genomic imprinting; LOI causes excess gene product to be produced and is often associated with tumorigenesis.

Methylation specific PCR (MSP): a technique used to determine the methylation status of specific DNA sequences by PCR amplification of a bisulfite-converted template with different primer sets that distinguish methylated DNA and non-methylated (C-T converted) DNA.

Methylated DNA immunoprecipitation (Methyl-DIP or MeDIP): a technique used to identify methylated DNA by precipitation with an antibody specific for 5-mC and followed by detection of precipitated DNA by PCR, hybridization to a genomic microarray, or sequencing.

Methylation-sensitive Single-Nucleotide Primer Extension (Ms-SNuPE): a technique used to query methylation status of a targeted base bisulfite conversion followed by primer extension with labeled dCTP or dTTP to distinguish methylated and non-methylated DNA.

microRNA (miRNA): Small RNA molecules (usually 21-23 nucleotides) that play a role in regulating gene expression by transiently suppressing translation of an mRNA molecule or by directing its cleavage.

Multipotency: the property of stem cells describing their ability to differentiate into cells of a specific lineage, but not other lineages; example: hematopoietic stem cells can differentiate into multiple types of blood cells, but not into muscle cells, skin cells, or cells of any other lineage.

Non-coding RNA (ncRNA): RNA molecules that do not contain protein-coding potential; ncRNAs can be highly abundant and functionally important RNA molecules that can epigenetically regulated gene expression or other cellular processes, such as nuclear organization and splicing; the majority of most genomes are transcribed as non-coding RNA; examples include microRNAs (miRNAs), small interfering RNAs (siRNAs), Piwi-interacting RNAs (piRNAs), large non-coding RNAs (lncRNAs), natural antisense transcripts (NATs) and large intergenic RNAs (lincRNAs).

Nucleosome: the repeating unit of chromatin structure; one nucleosome is comprised of 147 bp of DNA wrapped around a protein octamer including two molecules each of the core histones H2A, histone H2B, histone H3, and histone H4.

Piwi-interacting RNA (piRNA): the largest class of small non-coding RNA molecules expressed in animal cells; piRNAs are 26-34 nucleotides in length and form RNA-protein complexes through interactions with piwi proteins; piRNAs differ from miRNAs and siRNA in both methods of biogenesis and function, but are known to play a role in silencing retrotransposons in germ cells.

Pluripotency: the property of embryonic stem cells to differentiate into cells of any three germ layers (endoderm, mesoderm, ectoderm); pluripotent cells are more differentiated than totipotent cells and less differentiated than multipotent cells.

Polycomb-group (PcG): a group of proteins functioning in histone modification, histone binding, or DNA binding that facilitate gene repression; named for the *Drosophila melanogaster* Polycomb gene.

Position effect variegation (PEV): the variable silencing of a gene because of its proximity to heterochromatin.

RNA interference (RNAi): posttranscriptional gene silencing mediated by small RNA sequences that are capable of hybridizing to a target mRNA sequences.

Small activating RNA (saRNA): miRNAs that can activate gene expression by binding to promoter sequences.

Small interfering RNAs (siRNA): small RNAs (21-24 nt) that function in gene silencing, heterochromatin assembly, and RNA directed DNA methylation.

Somatic cell nuclear transfer (SCNT): transplantation of a diploid nucleus from a somatic cell to an enucleated egg cell, artificially mimicking fertilization and potentiating development; SCNT is used for reproductive cloning.

Stem cell: an undifferentiated cell that is capable of producing daughter stem cells by mitosis or differentiating into specialized cell types.

Totipotency: the property of fertilized egg cells and early zygotic cells to differentiate into embryonic and extraembryonic cells.

Trithorax-group (trxG): a group of proteins functioning in transcriptional regulation, chromatin remodeling, and histone lysine methyltransferase activity that facilitate gene expression; named for the *Drosophila melanogaster* trithorax gene.

Tumor suppressor gene: a gene that functions in regulation of cell cycle and/or promotes apoptosis, protecting the individual from the development of cancer; tumor suppressor genes are often mutated in cancer.

Uniparental disomy: the condition in which an offspring inherits both copies of a chromosome (or a segment thereof) from the same parent. Genomic imprinting under such conditions can cause loss of expression or aberrant expression of alleles.

X-inactivation: a dosage compensation mechanism in which one of two X-chromosomes in the cells of female mammals is epigenetically silenced.

Xist: X inactive specific transcript; the non-coding RNA transcribed from the X-inactivation center (Xic) that binds along the entire chromosome from which it is transcribed to mediate X chromosome inactivation in placental mammals.

Zygote: the totipotent cell that results from the union of the oocyte and sperm gametes.

Epigenetics Product Index

Bisulfite Conversion

Product		Size	Cat. No.
EZ DNA Methylation™ Kit	Streamlined proven procedure for bisulfite-converted DNA	50 Rxns. 200 Rxns.	D5001 D5002
EZ-96 DNA Methylation™ Kit (Shallow Well Format) EZ-96 DNA Methylation™ Kit (Deep Well Format)		2x96 Rxns. 2x96 Rxns.	D5003 D5004
EZ-96 DNA Methylation™ MagPrep		4x96 Rxns. 8x96 Rxns.	D5040 D5041
EZ DNA Methylation-Gold® Kit	Complete bisulfite conversion of GC-rich DNA in less than 3 hours	50 Rxns. 200 Rxns.	D5005 D5006
EZ-96 DNA Methylation-Gold® Kit (Shallow Well Format) EZ-96 DNA Methylation-Gold® Kit (Deep Well Format)		2x96 Rxns. 2x96 Rxns.	D5007 D5008
EZ-96 DNA Methylation-Gold® MagPrep		4x96 Rxns. 8x96 Rxns.	D5042 D5043
EZ DNA Methylation-Direct™ Kit	Complete bisulfite conversion of DNA directly from blood, tissue, cells FFPE and low-input samples	50 Rxns. 200 Rxns.	D5020 D5021
EZ-96 DNA Methylation-Direct™ Kit (Shallow Well Format) EZ-96 DNA Methylation-Direct™ Kit (Deep Well Format)		2x96 Rxns. 2x96 Rxns.	D5022 D5023
EZ-96 DNA Methylation-Direct™ MagPrep		4x96 Rxns. 8x96 Rxns.	D5044 D5045
EZ DNA Methylation-Lightning® Kit	Fastest method for complete bisulfite conversion of DNA	50 Rxns. 200 Rxns.	D5030 D5031
EZ-96 DNA Methylation-Lightning® Kit (Shallow Well Format) EZ-96 DNA Methylation-Lightning® Kit (Deep Well Format)		2x96 Rxns. 2x96 Rxns.	D5032 D5033
EZ-96 DNA Methylation-Lightning® MagPrep		4x96 Rxns. 8x96 Rxns.	D5046 D5047

5-mC and 5-hmC Analysis

Product	Description	Size	Cat. No.
5-mC DNA ELISA Kit	For high-throughput, detection of global 5-mC in DNA	1x96 Rxns. 2x96 Rxns.	D5325 D5326
Methylated-DNA IP Kit	Methylated DNA enrichment for large-scale DNA methylation analysis	10 Rxns.	D5101
Anti-5-Methylcytosine Antibody	Specifically binds to 5-mC in ssDNA context	50 µg 200 µg	A3001-50 A3001-200
Anti-5-Hydroxymethylcytosine Polyclonal Antibody	Robustly distinguish between 5-hmC and 5-mC	25 ug 50 ug 200 ug	A4001-25 A4001-50 A4001-200
OneStep qMethyl™ Kit	Single step, bisulfite-free DNA methylation analysis	44 Tests	D5310
OneStep qMethyl™ - *Lite*	Like the *OneStep* qMethyl™ Kit but omits the inclusion of SYTO® 9 dye	44 Tests	D5311
OneStep qMethyl™ Arrays	Premade 96-well assays ideal for rapid high-throughput screening of methylation status	1x96 Well	D5312
Quest 5-hmC™ DNA ELISA Kit	Sensitive and specific quantitation of 5-hmC DNA from a variety of samples	1x96 Rxns. 2x96 Rxns.	D5425 D5426
Quest 5-hmC™ DNA Enrichment Kit	Clean and uniform enrichment of 5-hmC DNA by J-Binding Protein	25 Rxns. 50 Rxns	D5420 D5421
Quest 5-hmC Detection Kit™	Distinguish 5-hmC in sequence- and locus-specific context within DNA	25 Preps. 50 Preps.	D5410 D5411
DNA Degradase™	Fast single-enzyme digest for completely degrading DNA into its individual nucelotide.	500 U 2,000 U	E2016 E2017
DNA Degradase Plus™	Fast single-enzyme digest for completely degrading DNA into its individual nucleoside	250 U 1,000 U	E2020 E2021

5-mC and 5-hmC Analysis Continued

Product	Description	Size	Cat. No.
CpG Methylase (M.SssI)	Completely methylates all cytosines within a 5'...CpG...3' context in double-stranded, non-methylated and hemimethylated DNA	200 U 400 U	E2010 E2011
GpC Methylase (M.CviPI)	Completely methylates all cytosines within a 5'...GpC...3' context in double-stranded DNA	200 U 1000 U	E2014 E2015
5-hmC Glucosyltransferase	For specific modification of 5-hmC with a glucose moiety	100 U 200 U	E2026 E2027
Hydroxy/Methylated Nucleotides	Ready to use 5-hmC and 5-mC dNTP mixes to incorporate into PCR amplicons	10 mM	D1030 D1040
ZymoTaq™ PreMix	Hot start DNA Polymerase for robust product formation from difficult templates (e.g., bisulfite-converted DNA)	50 Rxns. 200 Rxns.	E2003 E2004
ZymoTaq™ qPCR PreMix	Similar to ZymoTaq™ PreMix but includes SYTO®9 fluorescent dye	50 Rxns. 200 Rxns.	E2054 E2055
QuestTaq™ PreMix	Ideal for robust, non-biased amplification of 5-mC, 5-hmC, and g5-hmC modified DNA	50 Rxns. 200 Rxns.	E2050 E2051
QuestTaq™ qPCR PreMix	Similar to QuestTaq™ PreMix but includes SYTO®9 fluorescent dye	50 Rxns. 200 Rxns.	E2052 E2053

5-mC and 5-hmC DNA Standards and Controls

Product	Description	Size	Cat. No.
Human Methylated & Non-methylated DNA Set	DNA that has been methylated at every CpG site	1 Set	D5014
Human WGA Methylated & Non-methylated DNA Set	Ideal for studying loci with ultra-low methylation levels	1 Set	D5013
Universal Methylated Human DNA Standard	Provides a single completely methylated human DNA	20 Rxns. (5 µg)	D5011
Universal Methylated Mouse DNA Standard	Provides a single completely methylated mouse DNA	20 Rxns. (5 µg)	D5012
Human Matched DNA Set	Organ-specific human genomic DNAs derived from a single individual	1 Set	D5018
5-mC & 5-hmC DNA Standard Set	Allows for the comparison of 5-mC and 5-hmC levels in any DNA to be interrogated	1 Set	D5405
Mouse 5-hmC & 5-mC DNA Set	Organ-specific mouse genomic DNAs derived from a single mouse	1 Set	D5019

Chromatin Analysis

Product	Description	Size	Cat. No.
Zymo-Spin™ ChIP Kit	Unique workflow for efficient cross-linking, shearing and immunoprecipitation	10 Preps. 25 Preps.	D5209 D5210
ChIP DNA Clean & Concentrator®-Capped Column	Two-minute DNA clean-up from any step in a standard ChIP protocol	50 Preps.	D5205
ZR-96 ChIP DNA Clean & Concentrator®		2x96 Preps. 4x96 Preps.	D5206 D5207
EZ Nucleosomal DNA Prep Kit	For the isolation of nucelosome-associated DNA from fresh or frozen cells	20 Preps.	D5220

NGS Library Prep Kits

Product	Description	Size	Cat. No.
Pico Methyl-seq™ Library Prep Kit	Post-bisulfite library preparation for whole genome bisulfite sequencing	10 Preps. 25 Preps.	D5455 D5456
RRHP™ 5-hmC Library Prep Kit	Innovative library preparation for strand-specific mapping of 5-hmC in DNA	12 Preps. 25 Preps.	D5450 D5451

RNA Methylation Analysis

Product	Description	Size	Cat. No.
EZ RNA Methylation™ Kit	Fast and reliable bisulfite conversion of RNA for methylation analysis	50 Rxns. 200 Rxns.	R5001 R5002

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We now know it is not "Nature vs. Nurture," but it is how "Nurture affects Nature."

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