The Beauty of Science is to Make Things Simple

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A Biotechnical Newsletter

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Epigenetics

SPECIAL EDITION

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ABOUT THIS ISSUE

Just as the last decade of scientific research was revolutionized by genomic sequencing, particularly the Human Genome Project, the next decade will be dominated by epigenetics. This "Special Edition" Epigenetics Newsletter contains four (4) review articles from leaders in the field of study that include: 1) Epigenetics, 2) Epigenetic Regulation in Mammalian Genomes, 3) Epigenetics & Cancer, and 4) Epigenetic Regulation of Gene Expression in Insects & Plants. Also featured are the state-of-the-art epigenetic technologies from Zymo Research for DNA methylation, histone, chromatin, and small RNA analysis.

From the scientists and team at Zymo Research Corporation.

"It is not the strongest of the species that survives, nor the most intelligent that survives. It is the one that is most adaptable to change."

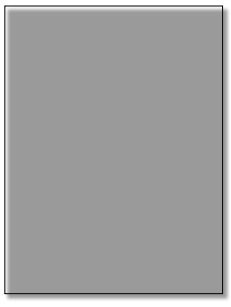
- Charles Darwin

"Habits form a second nature."

- Jean Baptise Lamarck

"[C]hanges of type must take place if the conditions of life be altered, and the species possesses the capacity of fitting itself to its new environment."

- Gregor Mendel



COVER IMAGE

Twin boys superimposed over an image of luminescent DNA sequencing clusters. High-throughput, next generation sequencing allows deep coverage to identify epigenetic signatures that distinguish differences between identical twins.

Constructed with permission from the original 1968 photograph "Ndebeli Boys" by Dr. D'Lynn Waldron (reference http://dlwaldron.com).

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REVIEW

Epigenetics

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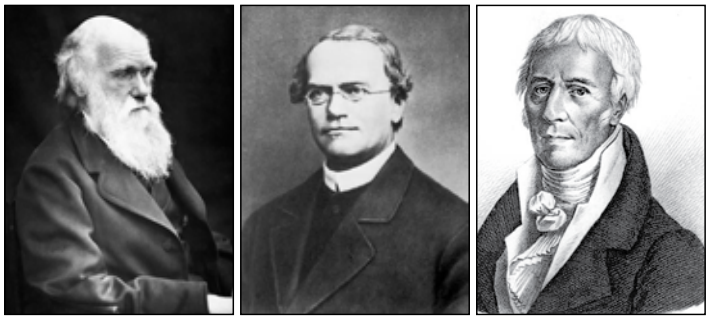
The field of epigenetics, transcending genetics, genomics, and molecular biology, is now poised to be the vanguard of biological science. The rise of epigenetics marks a maturation of the field, which only 50 years ago was given its name and a vague definition, but is now a dynamic discipline, challenging and revising traditional paradigms of inheritance. Through epigenetics the classic works of Charles Darwin, Gregor Mendel, and Jean-Baptiste Lamarck (Figure 1) are now seen in different ways. As more factors influencing heredity are discovered, today's scientists are using epigenetics to decipher the roles of DNA, RNA, proteins, and environment in inheritance. The future of epigenetics will reveal the complexities of genetic regulation, cellular differentiation, embryology, aging, cancer, and other diseases. In this review fundamental concepts of epigenetics will be described, including chromatin structure, epigenetic markers, model systems, research methods, and future directions.

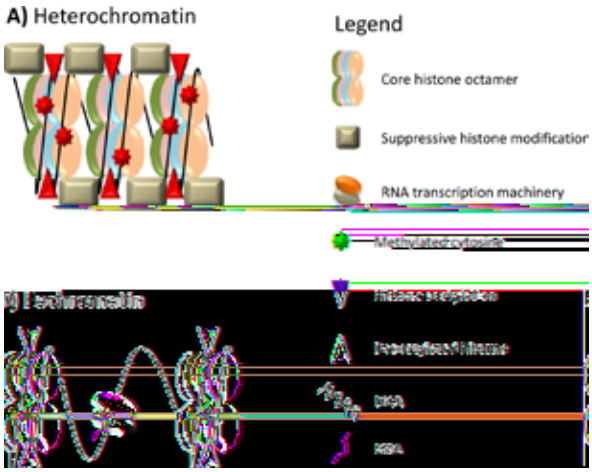


Prescient sages of biology: Darwin, Mendel, and Lamarck.

Chromatin Structure and Epigenetic Markers

To understand epigenetics requires an understanding of chromatin structure (Figure 2). Chromatin, which is organized into repeating units called nucleosomes, is the complex of DNA, protein, and RNAs that comprises chromosomes[1]. A nucleosome consists of 147 bp of double-stranded DNA wrapped around an octamer of histone proteins, usually two copies each of the core histones H2A, H2B, H3, and H4. In mammalian cells, most of the chromatin exists in a condensed, transcriptionally silent form called heterochromatin. Euchromatin is less condensed, and contains most actively transcribed genes. Histones and DNA are chemically modified with epigenetic markers that influence chromatin structure by altering the electrostatic nature of the chromatin or by altering the affinity of chromatin-binding proteins.

DNA can be modified by methylation of cytosine bases[10]. The enzymes that methylate DNA are called DNA methyltransferases. In humans the *de novo* DNA methyltransferases DNMT3A and DNMT3B methylate the genome during embryonic development, whereas the maintenance DNA methyltransferase DNMT1 methylates hemimethylated DNA following mitosis. Methylated DNA is suppressive of gene expression, as it attracts methylcytosine binding proteins that promote chromatin condensation into transcriptionally repressive conformations. In mammals, only cytosines preceding guanines (CpG dinucleotides) are known to be highly methylated. CpG dinucleotides are underrepresented and widely dispersed in the human genome. Although the majority of CpGs are located in non-coding regions and typically methylated, most remaining CpG



Heterochromatin (A) is densely compact, featuring methylated DNA and suppressive histone modifications and deacetlyated histones. By contrast, euchromatin (B) is less densely packed, having acetylated histones, lacking suppressive histone modifications, and featuring less DNA methylation. Open conformations of euchromatin favor transcription.

dinucleotides are found in clusters upstream of gene coding sequences. These clusters, called CpG islands, are typically non-methylated so as to allow gene expression.

Histones are subject to several different covalent modifications, including methylation, acetylation, phosphorylation, ubiquitylation, and sumoylation[1,8]. The hypothesis of the histone code was developed to suggest that combinations of histone modifications ultimately control gene expression. While it is not clear that this hypothesis is universal, several supporting examples have been reported. Histone modifications can have varying effects owing to the type of modification and the location of the modification on the histone. The best-characterized histone modifications are acetylation and methylation. Acetylation of histone lysine residues is associated with euchromatin because it weakens the charge attraction between histone and DNA, serving to decondense chromatin and facilitate transcription. Histone methylation can be either repressive or activating, depending upon location. For example, methylation of the lysine at the fourth residue of histone 3 (H3K4Me) promotes a transcriptionally active conformation, whereas H3K9Me promotes a transcriptionally repressive conformation. H3K36Me can be activating or repressive, depending upon proximity to a gene promoter region.

Histones are not the only proteins that interact with DNA in chromatin. Nucleosome remodeling complexes manipulate chromatin structure, thereby affecting gene silencing and expression. Chromatin remodeling proteins affect chromatin structure in various ways. They can expose DNA wrapped in nucleosomes by sliding histones along the DNA, or detach the histone octamer completely from a DNA sequence. They can also remove only the H2A-H2B subunits of the histone octamer, leaving the H3-H4 subunits, and resulting in a non-canonical structure. In recent studies, the reverse reaction has also been observed for the insertion of variant histone proteins. Not all nucleosome remodeling proteins possess the same functions. The SWI/SNF family, which is found in yeast, fly, plants, and humans, can slide nucleosomes, eject histones, and displace H2A-H2B dimers. The ISWI family, which is only found in mammals, is capable of sliding, but not histone ejection. Some ISWI family proteins can displace H2A-H2B dimers, while others cannot. The Mi-2/NuRD complex has DNA sliding activity, and, unique among chromatin remodeling complexes, also carries histone deacetylase activity[2].

RNAs are known to play several interesting roles in the control of chromatin structure. In plants a process called RNA-directed DNA methylation uses siRNAs generated by RNA Polymerase IV and the DICER LIKE 3 protein to localize the DNA methyltransferase DRM2 to its specific target sequence[7]. Another epigenetic trait dependent upon RNA is X chromosome inactivation[1]. This process occurs in female mammals to control expression dosage of the genes encoded on the X chromosome. In females, one of the two X chromosomes is inactivated in a process featuring the expression of the RNA *Xist*, which binds to the entire length of the chromosome from which it is transcribed. *Xist* recruits chromatin-remodeling proteins and blocks transcription machinery from binding to the inactivated chromosome (see page 8, Figure 2).

Model Systems and the Diversity of Epigenetics Research

The current concept of epigenetics is derived from observations of several model species ranging from unicellular fungi to mammals[1]. Yeast have been used as model systems to study chromatin structure. Work on the budding yeast *Saccharomyces cerevisiae* has helped elucidate chromosome structure and telomere silencing. The Sir family of proteins, all but one of which are unique to budding yeast, maintains chromatin silencing in *S. cerevisiae*. Perhaps the best known example of epigenetic gene silencing in *S. cerevisiae* is mating-type switching, which features the translocation of alleles between transcriptionally active and silent regions of a chromosome. The fission yeast, *Schizosaccharomyces pombe*, is also a model for chromatin structure, but differs significantly from budding yeast. Instead of using the Sir proteins to control chromatin structure, *S. pombe* is more similar to higher eukaryotes, using histone modification and siRNA. Not surprisingly, SWI/SNF chromatin remodeling proteins of *S. pombe* are also more similar to those of higher eukaryotes than the divergent *S. cerevisiae*.

Although DNA methylation is not observed in yeast, it is present in the fungus *Neurospora crassa*, making it a model organism for DNA methylation studies[1]. A phenomenon called repeat-induced point mutation (RIP) was discovered in *N. crassa*. RIP is a genome defense mechanism in which repeated sequences are prone to cytosine methylation and deamination to induce G:C to A:T mutations. Another model organism that has contributed to epigenetics is the protozoan *Tetrahymena thermophila*. Epigenetic mechanisms are used to regulate gene expression on the two nuclei of ciliates. Ciliates have a micronucleus that is dormant during most of the life cycle, but active during reproduction. The partition of active and suppressed nuclei in *T. thermophila* enabled the identification of histone variants, the first histone acetyltransferase, histone lysine methylation, and histone phosphorylation. *T. thermophila* is also an interesting organism for the study of RNAi. It has one RNAi pathway that functions in gene silencing and a second pathway that functions in DNA rearrangement and deletion during sexual reproduction[1].

The fly *Drosophila melanogaster* is a classic genetic model organism that is also a model for epigenetic research. A review of epigenetics in flies and other insects can be found in a separate article in this Newsletter (see page 12). Observations of epigenetic phenomena in *Drosophila* were made decades before the term epigenetics was devised[3]. Position effect variegation - the change in phenotype due to the change of a gene's position in the genome - was first described in the 1930s through observations of eye color (Figure 3). *Drosophila* normally have red eyes, but mutations in the *white* gene cause *white* eyes. However, some flies have patchy red and white eyes, but not because of the *white* mutant allele. Instead, some cells have undergone a chromosomal inversion that moved the wild type *white* gene in close proximity to pericentromeric heterochromatin, suppressing expression. Further research of suppressors and enhancers of position effect variegation have lead to the discovery of more epigenetic factors such as chromatin remodeling proteins and histone modifying proteins. The Polycomb group (PcG) and Trithorax group (TrxG) proteins were originally discovered in *Drosophila*, but are the subject of research in species ranging from yeast to human[12]. PcG proteins repress transcription by binding to Polycomb response elements (PRE). Different PcG protein complexes have been shown to methylate histones or bind to modified histones, making transcriptionally suppressive architectural changes. Conversely, the TrxG proteins bind to PREs, but serve to activate transcription via histone modification and chromatin remodeling.

Drosophila Position effect variegation causes patchy eye coloration due to differential expression of the *white* gene. Photo credit to Elena Gracheva.

Three other model organisms well-established in genetic research are also important for epigenetic research. Plants, such as *Arabidopsis thaliana*, have epigenetic mechanisms as sophisticated as mammals, including RNAi pathways, DNA methylation, histone modification, and chromosome remodeling complexes[7]. Plant epigenetics is reviewed in greater detail in another article within this Newsletter (see page 12). The worm, *Caenorhabditis elegans*, long used to study development, has been used to study cellular differentiation, X-linked dosage compensation, and RNAi[1]. Finally, mice, as mammals, are more similar to humans than any of these model systems, and are used as models for epigenetic research, particularly in embryology and stem cell research, but also in environmental studies, including effects of behavior and nutrition.

Epigenetics is a prominent theme in the study of human development from fertilization through aging and to death[11,13]. Epigenetic markers control the expression of genes that function in embryonic development, but other epigenetic programming events occur concurrently. These include the erasure and re-establishment of DNA methylation markers, genetic imprinting, X-chromosome inactivation, the development of pluripotent stem cells, and the differentiation of somatic cells. Although the most dramatic epigenetic events, such as the establishment of the epigenome, take place during embryonic development, the maintenance of the epigenetic state is important throughout life for the production of differentiated cells from adult stem cells and proper gene expression in specific cell types. A review of epigenetics and embryonic development can be found in a separate article in this Newsletter (see Epigenetic Regulation in

Mammalian Genomes). However, the epigenome is dynamic, as it exhibits changes during the aging process. For example, gene promoters become hypermethylated as an individual ages, whereas the CpG sequences of non-coding centromeric repeat regions become hypomethylated. Interestingly, many of the known age-related DNA methylation markers are also associated with disease.

In the last several years scientists have discovered numerous DNA methylation markers that are correlated with cancer. In a variety of cancers tumor suppressor genes such as *p16*, *p14*, and *MGMT* exhibit hypermethylation in the CpG islands upstream of the coding regions, repressing their expression[4]. Conversely, other genes such as *MAGE* and *uPA*, are usually methylated and repressed, but are hypomethylated and expressed in some cancers. The role of DNA methylation in cancer is reviewed in greater detail in another article in this Newsletter (page 9). Furthermore, a host of other diseases have epigenetic etiologies[6,15]. Prader-Willi syndrome, Angelman syndrome and pseudohypoparathyroidism, are all the result of uniparental disomy (UDP), a condition in which a person inherits both homologous chromosomes (or segments of chromosomes) from the same parent. UDP can be the result of gene deletion, translocation, or a defect in imprinting. Other epigenetic diseases are caused by mutations in genes necessary for chromatin structure. Rett Syndrome, for example, is caused by a genetic defect in MECP2, a methyl-CpG-binding protein that functions in gene repression.

Epigenetics Research Techniques

The methylation of cytosine in DNA introduces biochemical properties that distinguish methylated DNA from non-methylated DNA. Most importantly, sodium bisulfite deaminates cytosine into uracil, but 5-methylcytosine is resistant to this conversion, meaning a base change can be used to indicate methylation status. Additionally, there are several methylation-sensitive restriction endonucleases - which have the ability to cut DNA at a specific sequence depends upon the methylation state of the sequence. These features, when coupled with other common molecular biological methods, such as DNA sequencing, PCR, real-time PCR, Southern blotting, primer extension, HPLC, and MALDI-TOF MS, provide the epigenetic scientist with a variety of tools[5,14]. As DNA sequencing becomes more affordable and capable of higher throughput, it will be feasible for whole genome sequencing of bisulfite converted DNA to detect genome-wide DNA methylation profiles. While this is still costly, methylated DNA immunoprecipitation (MeDIP) can be used to identify methylated DNA sequences probed against a genomic array, and differential methylation hybridization (DMH) is used to compare methylation signatures of two different genomic samples by hybridization to a microarray. Although there are fewer methods for identifying histone modifications, antibodies to specific histone modifications can be used to detect those modifications by chromatin immunoprecipitation (ChIP), and the technique has been adapted for genome wide queries by hybridizing DNA identified by ChIP to a micro array (ChIP-on-chip). Furthermore, the DNA sequences associated with a protein of interest can be derived by ChIP-Seq. Several products for use in epigenetics research are described in greater detail in this Newsletter (see pages 20-40).

Epigenetics Research in the Future

The wealth of new data and knowledge relating to epigenetics obtained in recent years prefigures a vibrant future for epigenetics research. In 2007, the NIH announced funding of the Epigenomics Roadmap Initiative, which is investing heavily in identifying more epigenetic markers and developing new technologies for epigenetic research. Of great importance for the future are the integration of high-throughput sequencing technologies and the means to maintain and manipulate the glut of data that will be produced by sequencing epigenomes. The influx of epigenomic data will augment the growing database of known epigenetic markers and encourage more research to describe the functions of these markers in various tissues, stages of development, and disease states. As more epigenetic markers are associated with specific diseases, tools can be developed to diagnose patients and gauge the severity of disease. There is also great interest in therapeutic epigenetics. Several drugs, such as DNA methyltransferase inhibitors and histone deacetylase inhibitors, are already used in cancer treatment[4]. There are issues with specificity and efficacy of these drugs, so further research into their mechanisms is needed to develop better therapeutic agents. Likewise, better understanding of the various epigenetic diseases and syndromes may lead to effective drugs designed to overcome epigenetic defects.

Recent advances in embryology have posed more questions related to epigenetics, particularly to the mechanics of genome demethylation and the re-establishment of methylation in early embryonic development[11]. The epigenetic markers associated with the production of pluripotent embryonic stem cells is also of high interest for its relevance in reprogramming differentiated cells to make induced pluripotent stem cells[9]. Beyond embryonic development, phenomena relating to the acquisition of epigenetic markers during an organism's life span and their passage to offspring is a tantalizing area of research with many questions to be answered regarding mechanisms, and environmental influences. *The Golden Age of Epigenetics is Now!*

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REVIEW

Epigenetic Regulation in Mammalian Genomes

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Abstract

DNA methylation, histone modifications and higher order chromatin structure play a central role in the regulation of mammalian genome organization. The epigenetic signature of any cell provides valuable information about its cellular state, its developmental potential, and its health. A detailed description and understanding of the epigenome will notably expand our understanding of human health and disease. The aim of this review is to provide a broad overview of epigenetic modifications and mechanisms in large mammalian genomes.

Introduction

It has been over 30 years since DNA methylation was first postulated to be a heritable modification capable of influencing gene expression[35,70]. The addition of a methyl-group to the cytosine base does not change the primary DNA sequence and is therefore considered to be an epigenetic modification, literally meaning to act "on top of" or "in addition" to genetics. The Human Epigenome Project was initiated as a mixed academic and industrial consortium in Europe, aiming to "identify, catalogue and interpret genome-wide DNA methylation patterns of all human genes in all major tissues" (for more information go to: http://www.epigenome.org/index.php). While DNA methylation is commonly agreed to be an epigenetic mark, other modifications of the chromatin structure remain more controversial[44]. Part of this discussion may have been sparked by the recent support for epigenetics from the NIH Roadmap Initiative. The NIH project definition includes: "Epigenetics is an emerging frontier of science that involves the study of changes in the regulation of gene activity and expression that are not dependent on gene sequence. For purposes of this program, epigenetics refers to both heritable changes in gene activity and expression (in the progeny of cells or of individuals) and also stable, long-term alterations in the transcriptional potential of a cell that are not necessarily heritable" (for more information go to http://nihroadmap.nih.gov/epigenomics/).

Looking at the controversy and concerns raised[50] it becomes rapidly clear that arguments over the relevance of such a project are largely semantic[34,44]. In this review we focus on mammalian epigenetic modifications in the broader sense, including DNA methylation, histone modifications and their connection with non-coding RNAs. In principle, any mechanism that provides regulatory information to a genome without altering its primary nucleotide sequence could be considered epi- (on top of) genetic. We begin by providing a general overview of the modifications and responsible enzymes, followed by a more detailed discussion of various epigenetic mechanisms that exist in complex genomes and some concluding remarks about the future of the human epigenome project.

Epigenetic Modifications

In mammalian genomes DNA methylation exists primarily within the context of the CpG dinucleotide. Three catalytically active enzymes, DNA methyltransferase 1 (Dnmt1), Dnmt3a and Dnmt3b are required for the establishment and maintenance of DNA methylation patterns[8]. Two additional enzymes, Dnmt2 and Dnmt3L, display high homology and are expressed in several cell types including embryonic stem cells[7]. However, deletion of the Dnmt2 gene has no apparent effect[62] and its role as a DNA methyltransferase has been questioned[28].

The Dnmt3L protein lacks the catalytic domain, but is highly expressed in the early embryo, ES cells and germ cells. Dnmt3L deficient mice are viable, but male mice are sterile and heterozygous offspring of homozygous females die due to imprinting defects[9,10]. In the presence of the *de novo* methyltransferases, Dnmt3a and Dnmt3b, low levels of non-CpG methylation have been reported[20,30,54,69]. However, in contrast to plants, the role of non-CpG methylation has not been extensively studied in higher organisms, though it has been suggested that CpA methylation is involved in the regulation of enhancers that are required for olfactory receptor choice in the mouse brain[48]. Features of DNA methylation in plants, including CG, CHG and CHH (H being A, T or C) methylation, are discussed in the review "Epigenetic Regulation of Gene Expression in Insects and Plants," page 12.

Genomic DNA is packaged in higher structures (see page 8, Figure 2). Within a nucleosome 147bp of DNA are wrapped around a histone octamer (H3, H4, H2A and H2B), the terminal tails of which are subject to many modifications, including methylation, acetylation, phosphorylation, and ubiquitination[5,51]. Some of these marks (H3K27me3 and H3K9me3) clearly demonstrate mitotic inheritance[14,31] while others, including H3K36me3 and acetylation marks, have known regulatory functions, but have unknown heritability[5,56]. Many of the enzymes regulating such chromatin modifications are known and have been extensively studied including histone acetyltransferases, deacetylases, methyltransferases and more recently histone demethylases[75]. Two important and well-conserved families, the Trithorax and Polycomb, have SET domain containing histone methyltransferases specific for H3K4me3 and H3K27me3, respectively. The polycomb repressive complex 2 (PRC2) consists of three subunits Suz12, Ezh2 and Eed. The function of Eed and Suz12 remain unknown, whereas Ezh2, is the histone methyltransferase that contains the catalytic SET domain[5]. PRC2 establishes and binds H3K27 methylation patterns, which are believed to serve as a "docking site" for PRC1 (Ring1, Bmi1, Mel-18 and Cbx family proteins)[5].

The Role of Epigenetic Marks During Development

DNA methylation is generally considered a repressive modification and associated with gene silencing[8]. It is essential for normal mammalian development and required in all somatic cells (Figure 1). In mice, loss of the maintenance methyltransferase Dnmt1 results in embryonic lethality around day E8.5-9[46] and Dnmt1 mutant embryos display only ~1/3 of the normal levels of DNA methylation. Dnmt1 deficient embryos show rudiments of the major organs, but they are smaller and the embryo itself appears developmentally delayed[46]. Dnmt3b mutant embryos appear to develop normally before E9.5, but show multiple developmental defects later and no viable term embryos are recovered[61]. In contrast, Dnmt3a deficient mice can develop until term, but become runted and die about one month postnatally[61]. Using antibodies against methylcytosine as well as several locus specific DNA methylation assays, it has been established that the paternal genome is actively demethylated prior to the first cleavage division[41,52,64,73]. The maternal genome, presumably through a passive mechanism, is subsequently demethylated[73]. Several exceptions to this simplified model exist, including imprinted genes and repetitive elements[43]. DNA methylation levels are low in the preimplantation

The paternal genome is demethylated prior to the first cell division of the zygote; maternally derived DNA is demethylated after several cleavage divisions. *De novo* methylation occurs in the inner cell mass (ICM) cells, which later differentiate into the embryo. Maintenance DNA methylation retains methylation patterns as differentiated cells undergo mitosis.

embryo[73] and are unlikely to change notably in the absence of Dnmt1. It is therefore not clear why the Dnmt1 mutant embryos are able to initiate development and commit to organogenesis before around day E8.5.

Conditional deletion of Dnmt3a results in imprinting defects in the germline and mimics the phenotype of Dnmt3L mutants, suggesting an involvement of both enzymes in the process[42]. The direct interaction of both Dnmt3a and Dnmt3L has recently been demonstrated[63]. In this model, Dnmt3L guides *de novo* methylation to genomic sites that lack H3K4 (me1, me2 and me3) methylation. However, it is unclear whether this mechanism is applicable outside of germ cells.

Loss of any one of the PRC2 subunits results in severe gastrulation defects, suggesting an essential role in normal development[26,60,67]. Similar to PRC2 mutant mice, loss of PRC1 components, such as Ring1b (Rnf2), causes an early embryonic lethal phenotype[32]. Bmi-1 null mice display several hematopoietic and neurological abnormalities[81]. Taken together, these data clearly establish the essential role that DNA methylation and Polycomb mediated epigenetic marks play in normal development.

Epigenetic Marks in Differentiated Cells

It has been shown, using conditional alleles, that loss of Dnmt3a in mouse embryonic fibroblasts (MEFs) has no effect, while loss of Dnmt3b results in premature senescence or chromosomal instability as well as spontaneous transformation[20]. Conditional deletion of Dnmt1 in MEFs causes global loss of DNA methylation and p53-dependent apoptosis[38]. The global hypomethylation leads to widespread gene expression changes (>600 genes were altered using an array covering 10% of all mouse genes) including imprinted genes and germline specific genes[38], the latter of which tends to remain active and unmethylated in ES cells, but methylated in somatic cells[55,82]. Furthermore, conditional depletion of Dnmt3a and Dnmt3b in hematopoietic stem cells (HSCs; CD34- LSK-) suggested a role of *de novo* methylation in HSC self-renewal, but not differentiation[79]. Dnmt1 has been conditionally depleted in the developing nervous system and postmitotic neurons. While the postmitotic neurons did not show DNA hypomethylation or any notable phenotype, the CNS deletion resulted in postnatal lethality of high contribution chimeras or in selective loss of mutant cells in viable low contribution chimeras[25]. Finally, Bmi-1 has been shown to be required for maintenance of HSC and neural stem cell (NSC) self-renewal[57,65,66].

Epigenetic Marks in Pluripotent Cells

Embryonic stem (ES) cells or more generally pluripotent cells represent a developmental ground state that is maintained by a complex autoregulatory network of transcription factors that include Oct4, Sox2, and Nanog[40,76]. This ground state is of great interest because pluripotent cells have a very unique epigenetic signature and are largely unaffected by the loss of DNA methylation and certain histone modifications[16,37,46,54,61,80]. Although DNA methylation deficient ES cells cannot readily differentiate, they do maintain pluripotency and contribute to germline competent chimeras upon restoration of Dnmts[36,37]. PRC2 deficient ES cells show global loss of H3K27me3 and are more prone to differentiation[11,16,26,60,67]. However, while it had been previously suggested that PRC2 is essential to repress developmental genes and execute differentiation programs[11,26,60,67], recent work from Magnuson and colleagues suggests that PRC2 is not required to maintain pluripotency[16]. Low and high passage Eed -/- ES cells generated early embryonic chimeras with contribution to all germlayers, However, no Eed -/- MEFs could be derived and contribution was rare in late gestation (beyond E12.5 dpc) embryos. Since Eed is required for proper PRC2 assembly, this suggests an essential role in differentiation, but not pluripotency. While the loss of epigenetic marks does not diminish pluripotency, it does nonetheless play a role in regulating gene expression within pluripotent cells. The transcription factor Elf5 plays a central role in the regulation of trophectoderm development and is highly methylated and repressed in undifferentiated ES cells[59]. Murine ES cells cannot normally differentiate into trophectoderm, however, Dnmt1 mutant ES cells that have lost the ability to maintain Elf5 methylation gain the ability to differentiate effectively into trophectoderm[59]. Using murine ES cells deficient for all three methyltransferases[54] Fouse *et al.* demonstrated upregulation of over 350 genes in the absence of DNA methylation in the mutant ES cells. The authors found minimal overlap between these upregulated genes and polycomb and/or Oct4, Nanog target genes, suggesting an independent class of genes regulated by methylation[27]. During initial ES cell differentiation, Oct4 and Nanog need to be repressed. Although DNA methylation is not essential for silencing Oct4, it is required for stably maintaining the repressed state[24].

Epigenetic Regulation of Complex Mammalian Genomes

Promoters

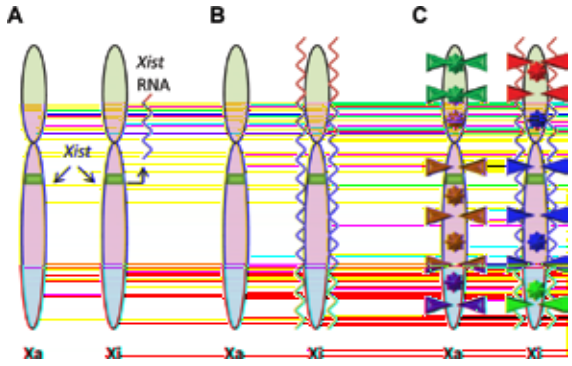
Mammalian RNA polymerase II promoters can be separated into low (LCP), intermediate (ICP), and high CpG promoters (HCP)[56,74,82]. Nearly all HCPs are enriched for H3K4me3 in ES cells and devoid of DNA methylation[55,56]. Around 22% of these also enrich for H3K27me3 and exhibit a so-called bivalent chromatin structure. Bivalent domains are enriched for key developmental transcription factors and tend to be either not or poorly expressed[6]. Many of these resolve into a univalent state upon differentiation, though some remain bivalent in certain cell types. These remaining bivalent domains correlate well with gene expression profiles from the adult tissues where they should ultimately become activated[56]. LCPs rarely contain H3K4me3 (only 6.5%) and frequently show high levels of DNA methylation[55,82]. Using high throughput bisulfite sequencing, we compared the DNA methylation patterns of ~1 million CpGs across 17 different samples[55]. Several hundred HCPs had lost H3K4me3 and gained DNA methylation upon differentiation. Importantly, these changes were solely due to the extended culture and not recapitulated *in vivo*[55]. In fact, most of the DNA methylation changes associated with differentiation were observed at distal putative regulatory regions 1 to >100 kb away from known promoters.

Distal Regulatory Regions

Gene expression in large genomes is dependent upon long-range control of gene transcription[83]. Distal regulatory regions, including enhancers, silencers and boundary elements, are subject to dynamic epigenetic changes during cellular differentiation[55]. The comparison of murine ES cells and ES-derived neural progenitor cells revealed nearly 20,000 distal sites that lost the active marks H3K4me1 and me2 upon differentiation and a similar number that gained H3K4 methylation. In all cases, the change in histone methylation was accompanied by changes in DNA methylation[55]. Although the majority of these distal sites overlap with highly conserved regions, their functional relevance needs to be further investigated.

Imprinting

McGrath, Solter, Surani and colleagues showed in the early 1980s that the maternal and paternal genomes are not equivalent and contained allele specific imprinting marks[3,53,78]. Insulin Growth Factor 2 (*Igf2*) and its receptor (*Igf2r*) were the first imprinted genes to be discovered[77]. A hallmark of imprinted genes is the monoallelic expression and parent of origin specific DNA methylation patterns[39]. DNA methylation has been shown to be required to maintain monoallelic expression[15]. Using conditional and reversible deletion of Dnmt1, Jaenisch and colleagues have generated “imprint-free” ES cells and mice[36]. The fact that most imprints are lost upon transient removal of Dnmt1 suggest that alternative mechanisms are either unstable or insufficient to propagate monoallelic expression[36]; while chimeric mice from this system are viable, they are prone to develop tumors. Loss of imprinting (LOI) of *Igf2* has been shown to increase the frequency of intestinal tumors and is frequently found in the normal mucosa of patients with colorectal cancer[72].



A) The non-coding RNA *Xist* is transcribed from the X inactivation center of the inactive X chromosome, Xi. B) *Xist* binds throughout the length of Xi. C) The silenced Xi displays suppressive histone modifications (red triangles) and DNA methylation at intragenic and promoter loci (red stars). The active X chromosome (Xa) displays activating histone modifications (green triangles) and gene body methylation (green stars).

Mammalian X-Chromosome Inactivation

To achieve comparable X-linked gene expression levels in female (XX) cells similar to male (XY) cells (dosage compensation), they have to silence one of their two X-chromosomes[1] (Figure 2). X-inactivation occurs shortly after implantation or after differentiation of female ES cells. The up-regulation of a long non-coding RNA, *Xist*, and the subsequent coating of the inactive X-chromosome is believed to be sufficient for the initiation of X-inactivation[1]. The spreading of *Xist* leads to chromosome wide transcriptional silencing and late replication of the inactive X chromosome (Xi). The silencing of Xi is further accompanied by histone modifications (H3K9me3 on the inactive and H3K4me3 on the active X) as well as DNA methylation. In a hierarchical model, the order of events would be as follows: *Xist* coating of paternal or maternal Xi, late replication timing, histone hypoacetylation, gain of DNA methylation[1]. Interestingly, the active X (Xa) displays more than two times as much allelic DNA methylation than the Xi. Most of this methylation is found within the gene bodies[33]. The fact that all of these genes are biallelically methylated prior to differentiation suggests a mechanism that leads to promoter hypomethylation and gene body hypermethylation[33]. DNA methylation, histone hypoacetylation, and *Xist* act synergistically to maintain X-inactivation[18]. While *Xist* seems largely dispensable for the maintenance of X-inactivation, DNA methylation and histone deacetylation are essential. Loss of DNA methylation leads to measurable reactivation of the Xi[18]. Additional evidence for the role of DNA methylation in regulation of X-inactivation comes from the human ICF (immunodeficiency, centromeric instability and facial anomaly) syndrome caused by a germline mutation in the DNMT3B gene[45]. In contrast to somatic cells, female ES cells maintain two active X-chromosomes. Interestingly, its has been shown that murine female ES cells show global DNA hypomethylation, which might be a result of two active X-chromosomes as well as lower level of Dnmt3a expression[85].

Imprinted X-Inactivation

X-inactivation is imprinted during early development in placental mammals[1]. The paternal X-chromosome is preferentially inactivated during the first lineage differentiation that gives rise to the extra-embryonic tissues, whereas X-inactivation in the embryo proper is random. Interestingly, in nuclear transfer experiments X-inactivation in extra-embryonic tissues specifically targets the silenced chromosome of the somatic donor, but remains random in the embryo proper[23]. When female ES cells with two active X chromosomes were used as donors, the X-inactivation was random in all tissues[23]. This work suggests that the imprinting marks set during gametogenesis are equivalent to those established during somatic X-inactivation.

Non-coding RNAs and the Mammalian Epigenome

Several large non-coding RNAs that regulate epigenetic modifications in *cis* (*XIST and AIR*) or in *trans* (HOTAIR) have been previously identified and studied[12,13,71]. A recent genome-wide analysis of K4-K36 domains (H3K4me3 marking the promoter and H3K36me3 marking the transcribed region) revealed a large number of conserved non-protein coding transcripts[29]. Using published ChIP data, Guttman *et al.* identified 118 non-coding RNA promoters that overlapped with Oct4 and Nanog binding sites in ES cells. This new data suggests a possible role of large non-coding RNAs, in addition to small RNAs, in the regulation of the complex pluripotency network[40]. Recently, two groups have shown an additional level of connectivity between non-coding RNAs and epigenetic modifications. The imprinted *Air* transcript is located in the second intron of *Igf2r* and regulates its expression in the embryo in *cis*[49]. In the placenta, it has at least two additional targets *Slc22a3* and *Slc22a2*. Recent work by Nagano *et al.* suggests that *Air* accumulates at the *Slc22a3* promoter and directly recruits the H3K9 histone methyltransferase G9A[58]. Loss or truncation of *Air* results in loss of imprinting and biallelic expression of the target genes. *Air* mediated repression of *Igf2r* in the placenta, however, appears independent of G9A[58]. The second report has shown the involvement of a non-coding RNA (*RepA*) in initiation and spreading of X-chromosome inactivation. Loss of *RepA* results in the failure to induce full-length *Xist* and recruitment of PRC2 to induce H3K27 trimethylation on the inactive X-chromosome[84]. Together these examples point to a general mechanism whereby RNAs can guide chromatin-modifying complexes to their specific sites of action.

Sequencing the Human Epigenome(s)

After sequencing dozens of genomes, from fly to human, researchers are now tackling the next frontier: The Human Epigenome. What started as several ambitious projects mostly in Europe is now a major focus of the NIH Roadmap. The initial Human Epigenome Project pilot study focused on DNA methylation and reported single nucleotide resolution data for 253 loci at the human MHC[68]. The scale-up was completed 2 years later and has provided methylation data for 40,000 CpG dinucleotides (~2,500 loci) across many different samples[22]. Histone modifications can readily be read on a genome-wide scale using chromatin immunoprecipitation followed by high throughput sequencing (ChIP-Seq)[2,56]. Currently two technologies are available that allow the final scale-up to achieve genome-wide DNA methylation analysis[21,55]. Eventually, shotgun bisulfite sequencing will be affordable across many genomes and this approach has already been successfully implemented in plants[17,47]. Due to the larger size (3,000 Mb vs. 130 Mb) and the uneven distribution of CpG dinucleotides across mammalian genomes, we have developed a reduced representation approach that allows a reproducible sampling of a smaller, but highly enriched genomic fraction covering ~90% of CpG islands, imprinted regions, highly conserved non coding elements (HCNEs), distal regulatory regions, and repetitive regions[55]. An alternative approach to nucleotide resolution analyis is termed MeDIP-Seq, which is a combination of methylated DNA immunoprecipitation (MeDIP) and next-generation sequencing[4,21]. Based on this MeDIP-Seq study, it is expected that up to two mammalian-sized methylomes of ~100 bp resolution can be generated from a single run of an Illumina Genome Analyzer II.

Conclusions

Aided by recent advances in high throughput sequencing technologies researchers have already begun to generate extensive maps of histone modifications and DNA methylation across many mammalian cell types. The coordinated effort of the NIH Roadmap will further assist this endeavor. Changes to the epigenetic state play a central role in many diseases. While cancer is one of the best studied examples, many other pathologies, including diabetes, neurological

disorders, and aging may have epigenetic contributions. Analysis of disease state epigenomes will be the second phase of the NIH Roadmap and, when compared with normal reference maps, this data should provide a valuable foundation from which to approach the mechanisms of chromatin mediated genome regulation, DNA methylation and other epigenetic events in mammals. Furthermore, it should provide a general framework for understanding aberrant epigenetic regulation in human development and disease.

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REVIEW

Epigenetics & Cancer

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Epigenetics Controls Reading of Genetic Code

Epigentics refers to high level information residing above the genetic code. While each cell in the body is equipped with the same genetic manual, epigenetic instructions tell cells how to make a difference. These instructions determine the access to pages with genetic information by directing the way the DNA is packaged into chromatin. DNA organized in loose chromatin is readily available for gene expression. Conversely, DNA tightly packed into dense chromatin has the letters of genetic code effectively buried and unavailable for reading and transcription. Distinct epigenetic marks decide which sets of genes may be expressed and which genes are kept silent. Once established, epigenetic patterns are stable and are transferred into subsequent generations of somatic cells. Permanent inactivation of one X chromosome in females and parental imprinting of specific genes on autosomes are the best examples of stable epigenetic silencing exerted on one allele. Transcription factors present in the cell nucleus regulate expression of only the other non-silenced allele.

Histones and methylation of DNA are key molecular players with well documented roles in epigenetic molding of chromatin. Histones are small proteins that form spools for wrapping of DNA into nucleosomes. Nucleosomes are the smallest structural unit of chromatin and consist of 8 histone core molecules (doublets of histone 2A, 2B, 3, and 4) with two loops of DNA (147 bp) coiled around them. N-terminal parts of histone proteins protrude from nucleosome cores. Amino acids in histone tails can be modified by numerous enzymes bringing acetylation, methylation, phosphorylation, ubiquitination and other substitutions, creating a complex 'bar' code[1,2]. Based on this histone code, activating or repressive complexes are attracted to DNA and shape chromatin into relaxed or tightly packed structures. Acetylation and phosphorylation of histones are usually activation marks associated with open chromatin structure. Methylation could also be an activation mark, such as trimethylation of lysine 4 in histone[3]. Remarkably, several amino acids further on the same histone molecule methylation of lysine 9 is a mark associated with silencing of euchromatin or densely packed pericentromeric heterochromatin[3]. Methylation of lysine 27 mediated by the Polycomb repressive complex[4,5] is an important silencing mechanism of key differentiation factors in embryonic stem cells[6] (Figure 1).

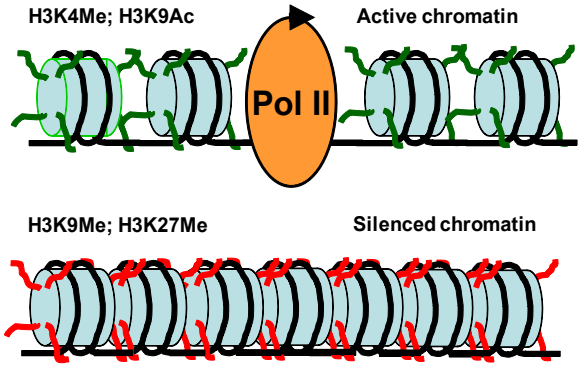
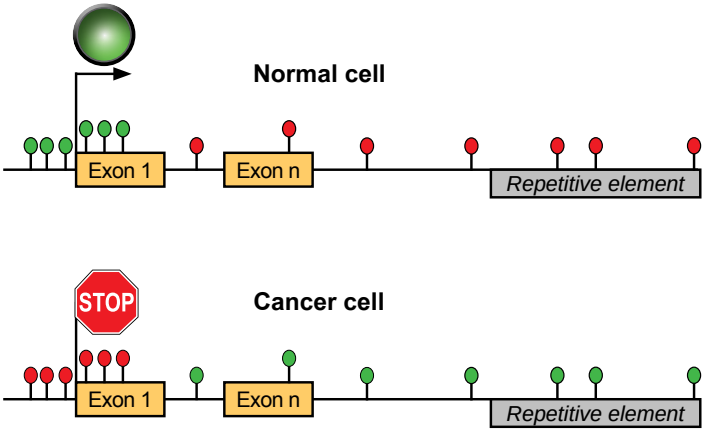


Figure 1. Modification of histone tails and chromatin organization. Methylation of lysine 4 (H3K4Me) and acetylation of lysine 9 (H3K9Ac) on histone 3 are hallmarks of active chromatin. Pol II shows RNA polymerase starting gene transcription. Methylation of lysine 9 or lysine 27 are found on silenced repressive chromatin that is not transcribed.

In contrast to a dazzling variety of histone modifications[7,8] DNA methylation is just a simple addition of a single methyl (CH_3-) group to cytosine at position 5. In mammals, it can almost exclusively happen only on cytosines preceding guanine (CpG) in the DNA sequence. The majority of CpG sites in human DNA are methylated[9]. Methyl groups flag approximately 5-6% of cytosines in healthy cells[10]. CpG methylation is a silencing epigenetic mark and may have developed as a defense against expression of parasitic DNA elements[11]. Moreover, 5-methylcytosine is prone to spontaneous deamination and point mutation to thymine[12]. Consequently, CpG dinucleotides are depleted almost fivefold in the human genome[13]. Although 5-methylcytosine represents only 1% bases in the human genome, its potential mutagenic hazard is well illustrated by the fact that CpG dinucleotide is involved in one third of point mutations causing human genetic disorders[14] and a similar proportion of single nucleotide polymorphisms detected in gene exons[15].

Despite the mutational threat it poses, CpG methylation is essential for life. DNA methylation is established and maintained by specific DNA methyltransferases (DNMT1, 3a and 3b). In mammals, DNA methylation is required for proper embryogenesis and development[16], for sustaining chromosomal stability[17], telomere length[18], and predetermined gene expression states[19] (see review, page 5). The methylation pattern is faithfully passed into dividing somatic cells in a similar fashion as the genetic code[20]. It is 'photocopied' on newly made DNA strands by DNA methyltransferase 1 (DNMT1) that works together with the protein UHRF1 as an all-in-one scanner and copier[21,22]. UHRF1 detects CpG sites methylated only on a single DNA strand and flips the existing 5-methylcytosine out of the DNA helix. DNMT1 then adds a new methyl group on CpG on the complementary DNA strand[23-25].

There is an important exception to a general rule that CpG sites are both sparse and methylated. About half of human genes have their transcription start sites flanked with islands containing tens or hundreds of CpG sites crammed in hundreds to thousands bases-long stretches of DNA. Cytosines in these CpG islands are typically unmethylated which makes them less susceptible to mutational pressure and may explain their survival throughout evolution. By keeping CpG islands methylation-free, normal cells maintain passageways for gene expression open, clean and clear of obstructions[9,19] (Figure 2).



CpG islands are protected from methylation in normal cells. CpG sites away from transcription start sites and in repetitive elements are typically methylated. The situation gets reversed in cancer resulting in focal hypermethylation and global hypomethylation. CpG islands flanking start sites of some genes may become methylated. Intragenic CpG sites and repeats are become unmethylated. Green lollipops show unmethylated, red lollipops methylated CpG sites.

Epigenetics and Genetics Are Accomplices in Cancer Development

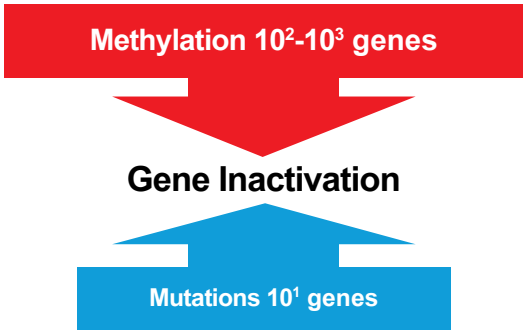
Cancer is caused by failure of checks and balances that control cell numbers in response to the needs of the whole organism. Inappropriate function of genes that promote or inhibit cell growth or survival can be caused by errors introduced into the genetic code itself or by faulty epigenetic mechanisms deciding which genes can and cannot be expressed. Epigenetic lesions and genetic mutations are acquired during the life of an individual and accumulate with aging. Both types of events, either individually or in cooperation, can result in the loss of control over cell growth and development of cancer[26].

DNA methylation patterns undergo complex changes in cancer. The total amount of methylated cytosine is usually decreased resulting in global hypomethylation. Decreased cytosine methylation typically affects satellite DNA, repetitive sequences, and CpG sites located in gene bodies (introns and inner exons). Mice with crippled function of DNMT1 have widespread genomic hypomethylation, activated endogenous retroviral elements, and develop aggressive T lymphomas[27,28]. The cause of reduced amount of methylcytosine observed in human tumors has not been determined. Despite global hypomethylation, high activity of DNA methyltransferases has been detected in multiple human tumor types. This increase may be related to higher proliferation rate of malignant cells[29].

Besides global hypomethylation, most cancers also show focal hypermethylation in distinct subsets of promoter-associated CpG islands (Figure 2). Affected genes are permanently silenced, since methylation marks are propagated through mitosis and are maintained in the malignant clone. Aberrant *de novo* hypermethylation occurring in transformed cells serves as an alternative mechanism for inactivation of tumor suppressor genes. Hundreds to thousands of genes can be epigenetically silenced by CpG island hypermethylation in human cancer[26,30,31], suggesting a general disturbance of epigenetic memory. Methylation affects individual cancer patients with varying extent. While some patients have minimal changes, others show concordant hypermethylation of multiple genes. This phenomenon was first described as CpG island methylator phenotype (CIMP) in colorectal cancer and confirmed in many other types of cancer and leukemia[29]. Epigenetic DNA methylation changes in cancer appear to be considerably more frequent events than genetic mutations. Mass sequencing of more than 20,000 transcripts in breast and colorectal cancers revealed about 80 harmless and less than 15 potentially oncogenic mutations per tumor[32] (Figure 3).

Most mutations and epigenetic alterations occurring in cancer cells are probably harmless. Many genes methylated in tumors are not expressed in relevant normal tissues and their silencing by methylation is inconsequential. However, disabling of genes that are critically important to controlling cell proliferation contributes to the development of a malignant phenotype in the same manner as inactivating mutations of tumor suppressor genes[33]. Epigenetic silencing by DNA methylation of cyclin-dependent kinase inhibitors[34-37], DNA repair genes[38], apoptosis mediators[39,40], nuclear receptors[41-43], transcription factors[44], cell adhesion molecules[45], and many other genes has been reported in multiple cancer types[46-49]. Perhaps the most convincing evidence for CpG island methylation causing the same harmful effect as inactivating mutations of tumor suppressor genes in cancer is documented in studies describing *RB1*[50], *p16* (*CDKN2A*)[51], *VHL*[52], and *MLH1*[53] genes. Each of these genes can be inactivated by either DNA methylation or a mutation with equal consequences for cancer development.

A complex code created by covalent modifications of amino acids in histone tails is also heavily involved in chromatin disturbances in cancer[54-56]. Some genes mutated in cancer recruit histone modifying enzymes and thus alter gene expression. For example, the *PML-RAR*



Hundreds to thousand of genes are hypermethylated in cancer cells while only tens of genes are affected by mutations. Both epigenetic silencing and inactivating mutations or deletions result in gene inactivation.

gene translocation in acute promyelocytic leukemia recruits histone deacetylases that change the chromatin structure from active to silenced and contribute to leukemic transformation[57]. Mutations also directly target histone-modifying enzymes. For example, histone acetylase CBP is mutated, and histone 3 lysine 4 activating methyltransferase MLL gets rearranged in leukemia[58,59]. EZH2, a histone 3 lysine 27 trimethyltransferase is frequently overexpressed in cancer and likely plays an important role in tumorigenesis[60].

Stem Cell Epigenome and Cancer

Temporary silencing of developmental genes in stem cells is an important mechanism for keeping their chromatin in a plastic state. Silencing is brought by EZH2, a member of the Polycomb repressive complex 2 causing methylation of lysine 27 on histone 3. Embryonic stem cells display the silencing mark together with an activating methylation mark on lysine 4. Genes with this bivalent chromatin structure are poised for expression driving differentiation into a specific lineage just after removal of the silencing histone mark[61]. Interestingly, developmental genes silenced by EZH2 in embryonic stem cells appear to be more prone to DNA methylation in cancer[61-65]. The exact relationship between histone 3 lysine 27 methylation and DNA methylation is not clear. It has been proposed that EZH2 and other proteins of the Polycomb repressive complex recruit DNA methyltransferases to specific promoters[66]. Recent studies in prostate cancer suggest that EZH2-mediated histone methylation and DNA methylation are not concurrent mechanisms of gene silencing. They may occur side by side[67] or in sequence, where relatively plastic Polycomb-mediated repression is replaced by DNA hypermethylation that seals reprogramming of the cancer epigenome[68]. Alterations of epigenomes in cancer stem cells may promote their self-renewal and make them less responsive to differentiation signals than their stem cell counterparts in normal tissues. Tight heritable silencing of crucial gene sets would hold cells in a stem cell state; make them vulnerable to mutations and further epigenetic changes in cancer progression[26].

Epigenome Mapping

With the impact of epigenetics in health and disease firmly established, national and international initiatives have been established to coordinate the scientific community's efforts. The National Institutes of Health recently announced a Roadmap Epigenomics Program to define and map epigenetic modifications genome wide. An international Alliance for the Human Epigenome and Disease (AHEAD) has been formed by the American Association for Cancer Research Epigenome Task Force and the European Union Network of Excellence Scientific Advisory Board. Inspired by the great success of the human genome sequencing, the goals of the AHEAD project are to generate high-resolution reference epigenome maps. First, a defined subset of robust epigenetic markers will be analyzed in a limited number of human tissues at different stages. To support epigenetic data, a bioinformatics infrastructure will be established[69].

Rapidly developing technologies greatly transformed our capacity to map epigenetic marks in a large scale. Most recently, ultra-high-throughput technologies using massive parallel sequencing brought a new exciting tool for epigenomic analyses. These techniques can simultaneously read millions of short DNA fragments. Genome wide maps of specific histone marks captured by chromatin immunoprecipitation were generated using this deep sequencing technology[70,71].

DNA methylated regions in the genome can be detected on high density microarrays after isolation by restriction digests with methylation-sensitive enzymes[72,73], methylcytosine-specific antibodies[74] or methyl binding proteins[75]. Replacing microarrays with deep sequencing of isolated methylated DNA further broadens the spectrum of analyzed sequences and also provides more accurate quantitative information than microarray detection.

The gold standard of DNA methylation analysis is sequencing of DNA chemically modified by bisulfite treatment that converts unmethylated cytosines to uracils while leaving methylated cytosines intact[76] (see "Perfecting Bisulfite Treatment for DNA Methylation Detection" on page 17). Massive sequencing of the whole bisulfite converted genome will provide DNA methylation maps with a single nucleotide resolution. This task was successfully accomplished in *Arabidopsis* with a relatively small 120 megabase genome[77] and in a fraction of mammalian genome enriched for CpG islands[78].

Epigenetic Therapy of Cancer

Mapping and characterizing epigenomic changes will transform our understanding of pathology and enhance our ability to diagnose and treat cancer. Epigenetic alterations are easier to reverse than mutations affecting the genetic code. Two inhibitors of DNA methyltransferases, azacytidine and deoxyazacytidine, have already been approved by the Food and Drug Administration as effective drugs for treatment of patients with myelodysplastic syndromes. An inhibitor of histone deacetylases, vorinostat (suberoylanilide hydroxamic acid), is approved for the treatment of cutaneous T-cell lymphoma. Other epigenetic drugs targeting histone modifying enzymes or DNA methylation are in clinical trials or development[79]. Detailed understanding of chromatin dysregulation will undoubtedly be translated into new and more effective ways of cancer treatment.

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REVIEW

Epigenetic Regulation of Gene Expression in Insects & Plants

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Abstract

Epigenetic gene regulatory mechanisms are important in a variety of phenomena throughout the eukaryotes including the silencing of transposons, sex chromosome dosage compensation, imprinting, and the inappropriate gene expression that occurs in cancer cells. In addition, recent findings suggest that epigenetic mechanisms play an unforeseen and expanding role in the normal regulation of genes during development. This review will focus on advances made in our understanding of epigenetic gene regulation in insect and plants.

Introduction

The understanding of epigenetic gene regulation in insects and plants has been driven by discoveries in two model organisms, the common fruit fly *Drosophila melanogaster* and the small experimental plant *Arabidopsis thaliana*. The regulatory systems show many striking commonalities between organisms in these two different kingdoms of life, uncovering basic properties of epigenetic gene regulation that are conserved over billions of years and therefore likely found in the vast majority of eukaryotes. There are also interesting differences between plant and insect systems, and even important differences between different insect or plant species, suggesting that the evolution of epigenetic systems is a driving force behind the diversity of eukaryotic organisms.

Epigenetic Gene Regulation In Insects

Drosophila was one of the first well studied model genetic organisms and early genetic studies in *Drosophila* have shed light not only on epigenetic mechanisms within insects, but also revealed highly conserved paradigms for the activation or silencing of genes controlled by chromatin structure within other eukaryotes. Chromatin is the physiological template for gene transcription and is composed of DNA, histones, and other non-histone proteins. The basic repeating unit of chromatin is the nucleosome octamer, which is composed of the core histones H3, H4, H2A, and H2B, wrapped around 147 base pairs of DNA[1]. The histone N-terminal tails, which protrude out from the core octamer, have been the topic of recent intense study. Histone tails are subject to a host of post-translational modifications including methylation, acetylation, phosphorylation, ubiquitination, and ADP-ribosylation. The "histone code" hypothesis[2-4] posits that histone modifications direct the binding of specific proteins that mediate chromatin function, and thus gene regulation. Genomes are organized into two different general types of chromatin: heterochromatin and euchromatin. Heterochromatin is mostly located near centromeres and telomeres and corresponds to repeat rich areas of the genome. Heterochromatin is usually repressive to gene transcription, and is composed of regular arrays of nucleosomes with particular configurations of histone modifications including methylation of lysine 9 of histone H3 (H3K9), methylation of lysine 20 of histone H4 (H4K20), and low levels of acetylation of a number of sites on both histone H3 and H4. Euchromatin on the other hand generally constitutes most of the gene coding portions of genomes, and is more generally permissive to gene transcription. Euchromatin is composed of less regularly spaced nucleosomes and the histones are more frequently acetylated on H3 and H4 as well as methylated on lysine 4 of H3 (H3K4).

Mechanisms for the inheritance of constitutive heterochromatin discovered through the genetics of position effect variegation: Position effect variegation (PEV) is a phenomenon in which a normally active gene that is present at the boundary between euchromatin and heterochromatin shows a variable or mosaic pattern of gene expression due to the silencing or activation of gene expression in different cells. Screens for mutations that alter PEV in *Drosophila* have uncovered important factors involved in the processes of gene silencing or gene activation. One of the most important was Suppressor of position variegation effect[3-9], *Su(var)3-9*[5], which encodes a SET domain-containing histone methyltransferase enzyme[6]. *Su(var)3-9* is specific for methylating H3K9 and is the main factor maintaining this mark in heterochromatin. The function of *Su(var)3-9* is also conserved in other organisms. For example, in the yeast *Schizosaccharomyces pombe*, the *Su(var)3-9* homolog CLR4 is involved in silencing of the mating type region and centromeric heterochromatin[7], and loss of the mammalian Suv39h histone methyltransferases impairs heterochromatin and genome stability[8]. After the discovery of the function of the *Su(var)3-9* SET domain, many other SET domains have been shown to methylate histones at other positions including H3K4, H3K27, H3K36, and H4K20 and to be critical in epigenetic gene regulation[9]. Methylation of lysine residues of histone tails can either be associated with transcriptional activation or transcriptional repression. In addition, lysines can accept three methyl groups, and can therefore be monomethylated, dimethylated, or trimethylated (hereafter denoted as m, m^2, and m^3), and abundant evidence shows functional differences between these methylation states.

A second factor affecting PEV was *Su(var)2-5*, HETEROCHROMATIN PROTEIN1 (HP1), a conserved protein and a major constituent of heterochromatin[10]. HP1 contains a chromodomain which specifically binds to methylated H3K9 [the site created by *Su(var)3-9*], and this binding is essential for heterochromatin

formation *in vivo*[7,11-13]. HP1 serves as a platform for the recruitment of other proteins. For instance, in the yeast *S. pombe* the HP1 homolog SWI6 is known to recruit both silencing factors and antisilencing factors to effect an equilibrium between various chromatin modifications that confers the steady state level of gene activity[14].

The Polycomb and Trithorax systems: Drosophila genetics also uncovered two very important and conserved systems for the long term silencing or activation of gene expression, the Polycomb and Trithorax systems. A group of proteins called the Polycomb group proteins (PcG) were initially discovered by mutations in genes required to prevent the inappropriate expression of a group transcription factors important for early development called the homeotic (Hox) genes[15]. Two groups of PcG proteins form two protein complexes called PRC1 and PRC2. The key component of the PRC2 complex is Enhancer of *zeste* [E(*z*)], which encodes a SET domain histone H3 methyltransferase protein specific for trimethylation of H3K27 (H3K27m3). The presence of H3K27m3 generally causes gene repression. A key component of PRC1 is the protein Polycomb, which contains a chromodomain that specifically binds to H3K27m3[16-18]. Chromatin immunoprecipitation studies show that PRC1 and PRC2 are stably localized to the Hox genes, and both are required for Hox gene silencing. Although it is not entirely clear how the Polycomb complexes cause gene silencing, it is known that the PRC1 complex contains the component dRING, which causes the ubiquitylation of histone H2A at lysine 119, and this modification is required for gene silencing. Furthermore, two Jumonji domain proteins, UTX and JMJD3, act as specific histone demethylases for H3K27, and likely work in opposition to the E(z) histone methyltransferase to strike the right balance of H3K27 methylation levels[15].

The Trithorax group (TrxG) of proteins were also discovered through *Drosophila* genetics, as suppressors of the PcG mutant phenotypes[10]. Trithorax proteins are localized to the same loci as the PcG proteins, and act in opposition to PcG by stimulating transcription. The key TrxG protein is Trithorax, which encodes a SET domain containing histone methyltransferase protein specific for H3K4 trimethylation, a mark of active chromatin. Both PcG and TrxG group proteins are recruited to specific DNA sequences called Polycomb Response Elements (PREs). While precisely how they are recruited is a major unanswered question in the field, it is most likely that a variety of different DNA binding transcription factors are involved[15]. In mammals it even less clear since no identifiable PREs have been found.

The paradigm of Hox gene regulation suggested a model in which silencing established during early development is then maintained by PcG proteins throughout the remainder of development. However, later work has shown exceptions to this general rule, and suggests a more dynamic interaction between Polycomb silencing, Trithorax activation, and the activity of specific transcription factors, the interplay of which determines the final state of gene activity[15]. In this way the Polycomb and Trithorax systems provide a layer of epigenetic regulation within which canonical sequence specific DNA binding transcription factors act. Additionally, it is now clear that besides the Hox genes, hundreds of other *Drosophila* genes, as well as thousands of mammalian genes, are under control of the Polycomb and Trithorax systems, highlighting them as important and widely used epigenetic mechanisms[15].

The role of chromatin and epigenetic modification during transcription: The role of *Drosophila* Trithorax in gene regulation turns out to be much more widespread than just the set of PcG associated genes. Indeed, it appears that all actively transcribing genes contain H3K4m3 at their promoters[15,19]. The yeast (*Saccharomyces cerevisiae*) homolog of Trithorax is SET1, which is a key component of the COMPASS complex, and is responsible for all H3K4 methylation in yeast. SET1 is recruited to transcribed genes by interacting with the phosphorylated form of the carboxy-terminal domain of RNA polymerase II, a site at which many other factors are also binding during the process of transcription. COMPASS then appears to travel with Pol II and is responsible for H3K4 methylation that is associated with transcriptional elongation[19]. Interestingly, H3K4 methylation is in part dependent on a second histone modification, monoubiquitination of histone H2B. The Trithorax homolog in mammals is the MIXED LINEAGE LEUKEMIA (MLL) gene, which is frequently mutated in particular types of leukemia. Like in other organisms MLLs are associated with RNA polymerase II and co-localize to Pol II binding sites[19].

Other histone modifications are also important in the process of transcription[20]. As just one example, H3K36 methylation is associated with the transcribed bodies of genes, and is deposited along with transcriptional elongation by RNA polymerase II. H3K36 functions to create a more repressive chromatin environment to suppress transcription from cryptic initiation sites within the body of the genes that might otherwise interfere with the primary transcript from the main Pol II promotor[20]. H3K36 methylation appears to function by creating a binding site for the chromodomain of Eaf3, a subunit of the RpdS histone deacetylases complex. The RpdS complex then hypoacetylates the nucleosomes within the body of genes, a condition generally associated with transcriptional suppression.

Role of nucleosome remodeling in gene regulation: Nucleosomes are not evenly spaced along DNA, and often the removal or shifting of nucleosomes from promoters is necessary for transcription factors to gain access to the DNA. A comprehensive way of studying nucleosome positioning is to use high throughput sequencing of DNAs that are associated with mononucleosomes that have been released from chromatin by digestion with micrococcal nuclease. One such recent study showed that, like in other eukaryotes, most active *Drosophila* genes usually contain a small region of the promoter that is generally depleted of nucleosomes[21]. In addition, a special variant of H2A called H2A.Z is enriched at the 5´ ends of genes. Interestingly, *Drosophila* genes also showed a nucleosome free region at the 3´ end of active genes, which might possibly play a role in the termination of transcription[21].

Drosophila genetics again helped in our understanding of proteins that help shape these nucleosomal patterns. One class of TrxG mutations identified genes known as ATP-dependent chromatin remodeling proteins, which use the energy of ATP hydrolysis to alter nucleosome positioning. One in particular is the TrxG protein Brahma, a *Drosophila* homolog of the well-characterized yeast protein SWI2/SNF2. *In vitro*, Brahma can disrupt regular arrays of nucleosomes on DNA, and *in vivo* Brahma localizes with RNA polymerase II, suggesting a general role in gene activation[10]. In addition, Brahma containing complexes are also known in some cases to cause repression of gene expression, a function that is also likely carried out by its nucleosome remodeling capacity.

The role of small RNAs in epigenetic gene regulation: In the yeast *S. pombe*, small interfering RNAs (siRNAs) are critical for the establishment and maintenance of gene silencing and repressive H3K9 methylation[22]. This phenomenon also appears to be conserved in *Drosophila* because mutation of three RNAi components, PIWI, AUBERGINE or SPINDLE-E causes reduction of H3K9 methylation and delocalization of the heterochromatin factor HP1[22]. Additional insight into this type of regulation came from studies the small RNAs that associate with the PIWI proteins, the Piwi-interacting RNAs or piRNAs[23]. piRNAs seem to be especially important in targeting transposons throughout the genome, and appear to act primarily by slicing up and destroying transposon RNAs. Interestingly, in mammals a similar system appears also to target transposons, and mammalian piRNAs were recently shown to play an important role in the establishment of DNA methylation during male germ cell development[24]. While these pathways are only beginning to be

Royal jelly is a honey bee secretion that is used in the nutrition of the larvae. It is secreted from the hypopharyngeal glands in the heads of young workers and used with other nutrients, to feed all of the larvae in the colony, including those destined to become workers. If a queen is needed, a larva is chosen and will receive large quantities of royal jelly exclusively as its food source for the first four days of its growth, and this rapid, early feeding triggers the development of queen morphology, including the fully developed ovaries needed to lay eggs.

deciphered, it is clear that small RNAs are playing vital roles in the targeting of epigenetic marks to the genome.

DNA methylation in the Honeybee: Cytosine DNA methylation is a conserved gene silencing mechanism that functions to suppress transposable elements as well as to regulate processes such as X chromosome inactivation and genomic imprinting. Surprisingly, while methylation systems are widely conserved throughout eukaryotes, evidence for the existence of methylation in *Drosophila* is controversial. *Drosophila* may have a very small amount of methylation, but has clearly lost most of the methylation machinery found in other eukaryotes[25]. However, this loss seems specific to certain groups of insects, because the Honeybee, *Apis mellifera*, was recently shown to have all of the key DNA methyltransferase enzymes needed for methylation at CpG sites, and CpG methylation has been detected at endogenous honeybee genome sequences[26]. While the function of methylation in honeybee is far from clear, a fascinating recent finding suggests that methylation plays a profound role in development. Adult female honeybees develop either as sterile workers, or if fed royal jelly, as fertile queens. Remarkably, silencing of one of the honeybee DNA methyltransferases, Dnmt3, causes an effect similar to feeding with royal jelly, suggesting that an inhibitor of DNA methylation in royal jelly may be responsible for its transformative effects[27]. In the future, genome wide profiling of DNA methylation in honeybee should shed further light this phenomenon, as well as help us understand the other roles that DNA methylation may play in insects.

Epigenetic Gene Regulation In Plants

Arabidopsis has become the premier model plant for studying a variety of processes, and has become an important system for studying epigenetic regulatory systems that are widely conserved in other eukaryotes. Like insects and other eukaryotes, it has a full complement of histone modification enzymes, nucleosome remodeling complexes, and a DNA methylation system that in many ways resembles that of mammals. The most powerful aspect of *Arabidopsis* is the excellent forward and reverse genetics available. Saturation mutant screens can be used to identify genes involved in any process, and loss of function mutations for nearly any gene can be obtained from community stock centers.

Polycomb regulation of gene expression: Like many other eukaryotes, plants use the conserved Polycomb system to regulate gene expression. *Arabidopsis* encodes the key components of the PRC2 complex, but interestingly is completely lacking in PRC1 proteins. Instead, *Arabidopsis* appears to have recruited its HP1 homolog, called LHP1, to bind to and silence H3K27m3 associated chromatin[28,29]. The most well studied example of an *Arabidopsis* gene regulated by the Polycomb system is *FLC*. *FLC* encodes a transcription factor that inhibits flowering. During early plant development, *FLC* is highly expressed and prevents flowering, thereby prolonging vegetative development. However, *FLC* expression can be silenced by exposure of the plants to long periods of cold, which in nature indicates the passing of winter, and this leads to flowering in favorable conditions in the spring. After the initial cold stimulus, *FLC* repression is maintained for long periods of time by the Polycomb system, allowing for flowering to occur at the normal time. At the end of the plant life cycle, sometime around the time of meiosis, *FLC* is reactivated again, allowing for resetting and the continuation of this cycle in the next sexual generation.

DNA methylation: Arabidopsis has become one of the best organisms for genetic studies of cytosine DNA methylation because of its facile forward and reverse genetics and its small and well-annotated genome. *Arabidopsis* DNA methylation systems have much in common with mammalian systems. However, unlike in mammals where DNA methylation mutants are inviable, *Arabidopsis* can tolerate mutations that virtually eliminate methylation, allowing for detailed genetic analysis. DNA methylation in *Arabidopsis* is found in three different sequence contexts, CG, CHG (where H = A, T, or C) and CHH or asymmetric. Furthermore, DNA methyltransferase activities are generally classified as either maintenance or *de novo*. The methylation of hemimethylated symmetrical sequences (CG and CHG) following DNA replication is maintenance methylation. This results in stable patterns of methylation that are maintained throughout development or, in many cases, between generations[30-32]. Methylation that occurs at previously unmethylated sites is called *de novo* methylation. For symmetric sites, *de novo* methylation need only occur once, after which maintenance activity is sufficient. However, for asymmetric cytosines, which cannot be recognized as hemimethylated sites following DNA replication, methylation is "maintained" by the persistent activity of *de novo* methyltransferases[33].

CG methylation (sometimes referred to as CpG methylation) is the most common type found in mammalian genomes and is also prevalent in *Arabidopsis*. It is maintained by the maintenance DNA methyltransferase called MET1, which is a homolog of mammalian Dnmt1 that performs the same function[34,35]. Dnmt1 acts on newly synthesized DNA at replication forks, and shows a preference for hemimethylated CG sites. Mouse Dnmt1 mutants show early embryonic lethality, but *Arabidopsis met1* mutants which eliminate CG methylation are viable, yet display a number of specific developmental abnormalities. Remarkably, these abnormalities can be segregated away from *met1*, and they map to discrete loci such as the *SUPERMAN* and *FWA* genes important in development[36,37]. These DNA methylation defects, or "epialleles", including hypermethylation of SUPERMAN and hypomethylation of *FWA* are heritable and can be used for forward genetic screens and other classical genetic experiments[33]. Recently, an important MET1/Dnmt1 accessory factor has been discovered called VIM1/ORTH in plants and UHRF1 in mammals[38]. These factors bind directly to hemimethylated DNA and aid in the recruitment of the DNA methyltransferase to replication foci to faithfully replicate DNA methylation.

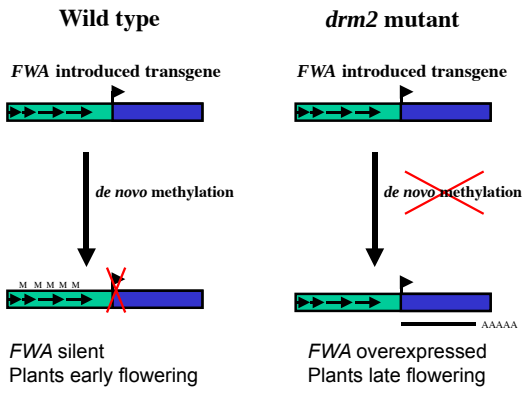
CHG DNA methylation is mostly found in plant genomes, and is controlled by the plant specific DNA methyltransferase CHROMOMETHYLASE3 (CMT3)[39]. Mutant screens were used to uncover the *cmt3* mutants, by looking for mutations that suppress the phenotypes associated with hypermethylation and silencing of the *SUPERMAN* locus[40]. Additional *cmt3* alleles were found from a similar but independent screen using the methylated and silent PAI loci[41].

These screens also uncovered a second gene required for CHG methylation called *KRYPTONITE* (*KYP*), which encodes a histone methyltransferase protein that is specific for H3K9m2[42,43]. The key to understanding the mechanism by which CMT3 and KYP cooperate to maintain CHG methylation came from determining the function of particular domains in these proteins. CMT3 contains a chromodomain that can bind to methylated histone H3 tails[44], which is the product KYP. KYP on the other hand contains a domain, the SRA, which can bind directly to DNA methylated at CHG sites[45], the product of CMT3. This suggests a model in which CHG DNA methylation is maintained by a positive feed forward loop between the activities of CMT3 and KYP.

CHH DNA methylation is inherited in a manner very similar to that of *de novo* DNA methylation. In *Arabidopsis*, both *de novo* DNA methylation and the maintenance of CHH methylation is mainly controlled by the DRM2 genes, which are orthologs of the *Dnmt3* genes which perform the same functions in mammals[46]. The function of DRM2 was discovered through genetics experiments using the above-mentioned *FWA* gene. FWA can adopt two very stable epigenetic states, either DNA methylated and silent, or unmethylated and active. Once established, these states are remarkably stable over many plant generations; an excellent example of the ability of DNA methylation to be meiotically heritable. The methylation of the *FWA* gene is confined to two tandem direct repeats near the *FWA* promoter[37] (Figure 1). When a new transgenic copy of the *FWA* gene is introduced into plants by *Agrobacterium*-mediated transformation, these repeats are reliably *de novo* methylated, and the new transgene is then transcriptionally silent. However, when *FWA* transgenes are introduced into plants with a *drm2* mutation, the *de novo* DNA methylation of *FWA* is blocked, the transgene remains active, and the plants develop a late flowering phenotype due to the ectopic expression of the *FWA* protein[46] (Figure 1). Thus, *FWA* transformation serves as a convenient assay to screen for mutants that block the establishment of DNA methylation.

The DRM2 DNA methyltransferase appears to be guided by small interfering RNAs (siRNAs), because mutations in a number of RNA silencing genes were found to mimic *drm2* mutants in the *FWA* transformation assay. These include the genes *ARGONAUTE4*, *DICER-LIKE 3* and *RNA DEPENDENT RNA POLYMERASE2*[32]. This, in many ways, resembles heterochromatin establishment in *S. pombe* where these same types RNA silencing factors are critical for the establishment of H3K9 histone methylation and gene silencing[22]. The finding of RNA silencing factors being involved in *de novo* DNA methylation is consistent with much earlier observations of so-called RNA directed DNA methylation (RdDM), first observed when cytoplasmically replicating RNA viroids were seen to cause *de novo* methylation of homologous genomic DNA sequences[47]. When RdDM is directed to promoter sequences, for instance as driven by inverted repeat expressing transgenes, it can cause transcriptional gene silencing[48-50]. These results suggest that siRNAs are at the heart of *de novo* DNA methylation processes, which can help explain the sequence specificity of gene silencing, likely involving the pairing of siRNAs with either DNA or nascent RNA transcripts. However, many questions remain concerning the precise mechanisms involved. Interestingly, recent studies suggest that siRNAs transfected into mammalian cells can also cause RNA directed gene silencing, and that antisense RNAs can trigger transcriptional gene silencing and DNA methylation, suggesting that certain aspects of this pathway may be conserved in mammals[51,52]. In addition, plant RNA directed DNA methylation resembles the phenomenon of piRNA directed DNA methylation that occurs in mammalian male germ cells[24].

DNA demethylation also plays an important role in shaping DNA methylation patterns throughout the genome. For instance, the *FWA* gene is normally methylated throughout the plant life cycle, but *FWA* is an imprinted gene that becomes specifically demethylated during



: Scheme showing that the drm2 mutant blocks de novo methylation normally seen in plants transformed with FWA. : *FWA* transformed plants with either an early or late flowering phenotype.

female gametophyte development[53]. DNA demethylation is required for expression of FWA and is actively carried out by the DME DNA glycosylase[54]. DNA glycosylases are normally involved in DNA repair, but DME encodes an enzyme that can specifically remove methylated cytosine. Another DNA glycosylase, ROS1, shows more general effects on DNA demethylation on transgenes and at various locations throughout the genome[55]. The molecular mechanisms for DNA demethylation in mammals have been very elusive, but it seems possible that similar mechanisms could be at play[56].

Epigenomics: Arabidopsis has been one of the leading systems in the emerging area of epigenomics research, in large part due to the well-developed genomic resources in this model plant. The *Arabidopsis* genome is roughly 130 megabases (about the size of *Drosophila*), and is one of the most well annotated to date. Relative to most animal genomes the *Arabidopsis* genome is also relatively compact, with small introns, relatively little repetitive DNA, and an average spacing between genes of only around 5 kilobases. This has made whole genome tiling array experiments, as well as whole genome sequencing approaches, much more practical.

The first whole genome profiling of DNA methylation of any organisms came from *Arabidopsis* researchers using Affymetrix or Nimblegen whole genome tiling arrays to detect DNA that had been immunoprecipitated with a methyl-DNA specific antibody[57,58]. As expected, these studies showed that the majority of transposons and repeat elements in the genome were methylated, and that this methylation was highly correlated with endogenous siRNAs. Unexpectedly, over 1/3 of the protein coding genes were also methylated, even though it had been previously thought that *Arabidopsis* genes were mostly devoid of methylation. This methylation was peculiar in that it was restricted to the coding portions of genes that were generally highly expressed, was anticorrelated with siRNAs, and was strictly in a CG sequence context. The function of this methylation is presently unclear, though it has been proposed to function to suppress the initiation of cryptic transcripts within genes, similar to the known role of H3K36 histone methylation in yeast[59].

More recently, by coupling the technique of genomic bisulfite sequencing with ultra high throughput DNA sequencing methods using the Illumina Genome Analyzer, researchers have refined *Arabidopsis* methylation patterns further. Sodium bisulfite converts C to U, but methyl C is protected from conversion. Thus after PCR amplification and sequencing of bisulfite converted DNAs, Cs indicate methylation while Ts indicate unmethylation. Deep sequencing of bisulfite converted DNA was used to establish single nucleotide resolution DNA methylation maps of *Arabidopsis*, revealing a number of addition interesting properties[60,61]. For instance, precise and quantitative sequence specificity for methylation conditioned by the three different methylation systems could be readily determined. It also allowed the study of previously inaccessible repetitive elements of the genome such as telomeres and rDNA. These methods can be used in virtually any genome, and once sequencing throughput is increased, it should become practical to utilize these techniques to analyze entire mammalian genomes routinely.

High-resolution mapping techniques are also being utilized to study histone modifications. As one example, whole genome tiling array experiments showed that at least 4,400 *Arabidopsis* genes (17% of all genes) are associated with H3K27m3[62], including a wide variety of transcription factors and developmentally important loci. This suggests that the type of Polycomb mediated long-term epigenetic regulation exemplified by the *FLC* locus plays a major role in a wide variety of developmental and physiological processes. Clearly, high resolution profiling of other histone modifications and chromatin proteins in *Arabidopsis* will shed additional light on mechanisms of epigenetic inheritance.

Conclusions and Future Directions

Epigenetics has evolved from an initially rather peculiar set of poorly understood phenomena to one of the hottest fields of biology. The explosion of epigenetics research came about in part because of the realization that the same epigenetic mechanisms that regulate phenomena such as PEV are involved in the regulation of many genes during development. The process of transcription itself involves a complex set of chromatin interactions and the main function of transcription factors is often to modify the underlying chromatin structure to permit or restrict transcription. A revolution taking place in biology is the widespread use of genomic techniques to study basic cellular processes, and the field of epigenetics is benefiting from this tremendously. The use of tiling microarrays and high throughput sequencing to discover the positions of chromatin proteins and particular histone modifications is becoming commonplace, and as these techniques are further improved they will soon become part of the basic toolkit of most laboratories. Ultimately, we need to understand the complex interplay of the various epigenetic systems and how they converge to effect proper gene control. We also know relatively little about how epigenetic regulatory complexes are targeted. For instance, it is not clear how Polycomb and Trithorax complexes find their target genes, or how DNA methylation is directed to repeats and other sequences. So while we now have a basic understanding about many epigenetic regulatory systems, there is also much to be learned. The future of exciting epigenetics research is bright indeed.

Acknowledgement

The author thanks Steve Jacobsen for assistance and critical review of the manuscript.

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Arabidopsis is a genus in the family *Brassicaceae*. They are small flowering plants related to cabbage and mustard. This genus is of great interest since it contains thale cress (*Arabidopsis thaliana*), one of the model organisms used for studying plant biology and the first plant to have its entire genome sequenced. Changes in thale cress are easily observed, making it a very useful model.

Perfecting Bisulfite Treatment for DNA Methylation Detection

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Zymo Research Corporation

Abstract

Sodium bisulfite can deaminate or convert cytosine in DNA into uracil, but does not affect 5-methylcytosine. Bisulfite treatment of DNA is a pre-requisite for DNA methylation analysis for many epigenetics-based studies involving methylation profiling and the quantification of methylation status. However, analytical procedures involving bisulfite-treated DNA are often subject to variability due to DNA degradation, incomplete conversion, and low yields of DNA. We have systematically investigated the procedure of bisulfite treatment of DNA paying particular attention to the chemistries involved in the process and to conversion rates in an effort to limit variability between samples and to improve upon conventional methods. We found conventional bisulfite DNA conversion chemistries could be improved without the levels of DNA degradation typically resulting from incubation of reaction mixtures at high temperature and nonphysiological pH. Essential to this process was prohibiting the over-conversion of 5-methylcytosine into uracil that can occur in some situations and reaction conditions. We found the bisulfite conversion process could be simplified and the variability between treatments kept to a minimum by coupling heat denaturation with the bisulfite conversion process and by using in-column desulphonation to clean and purify the converted DNA. This new method was found to yield an average of > 80% recovery of input DNA with > 99% C to U conversion. The method has been specifically designed to accommodate (in addition to purified DNA) biological fluids, cells, or tissue directly as the input material. This makes its application for FFPE and LCM-derived samples particularly well suited.

Introduction

DNA methylation is a naturally occurring event in both prokaryotic and eukaryotic organisms. In prokaryotes, DNA methylation provides a way to protect host DNA from digestion by restriction endonucleases that are designed to eliminate foreign DNA, and in higher eukaryotes DNA methylation functions in the regulation/control of gene expression[1]. It has been demonstrated that aberrant DNA methylation is a widespread phenomenon in cancer and may be among the earliest changes to occur during oncogenesis[2]. DNA methylation has also been shown to play a central role in gene imprinting, embryonic development, X-chromosome gene silencing, and cell cycle regulation. In many plants and animals, DNA methylation consists of the addition of a methyl group to the fifth carbon position of the cytosine pyrimidine ring via a methyltransferase[3]. The majority of DNA methylation in mammals occurs in 5´...CpG...3´dinucleotides, but other methylation patterns do exist. In fact, about 80 percent of all 5´...CpG...3´ dinucleotides in mammalian genomes are found to be methylated, whereas the majority of the twenty percent that remain non-methylated are within promoters or in the first exons of genes.

The ability to detect and quantify DNA methylation efficiently and accurately has become essential for the study of cancer, gene expression, genetic diseases, as well as many other important aspects of biology. To date, a number of methods have been developed to detect and quantify DNA methylation including: high-performance capillary electrophoresis[4] and methylation-sensitive arbitrarily primed PCR[5]. However, the most common technique used today remains the bisulfite conversion method[6] (Figure 1). This technique involves treating methylated DNA with bisulfite, which converts non-methylated cytosines into uracil. Methylated cytosines remain unchanged during the treatment. Once converted, the methylation profile of the DNA can be determined by PCR amplification followed by DNA sequencing (Figure 2).

Among the most challenging aspects of traditional bisulfite conversion protocols is the loss of DNA due to harsh reaction conditions and laborious manipulations. In addition, conversion efficiencies can vary dramatically using traditional protocols. Incomplete conversion frequently hinders reliable interpretation of the DNA methylation status under investigation. In this project we investigated the bisulfite conversion process systematically to improve the reaction efficiency, recovery, quality, and reliability for processing small amounts of input DNA, cells and difficult to process FFPE samples.

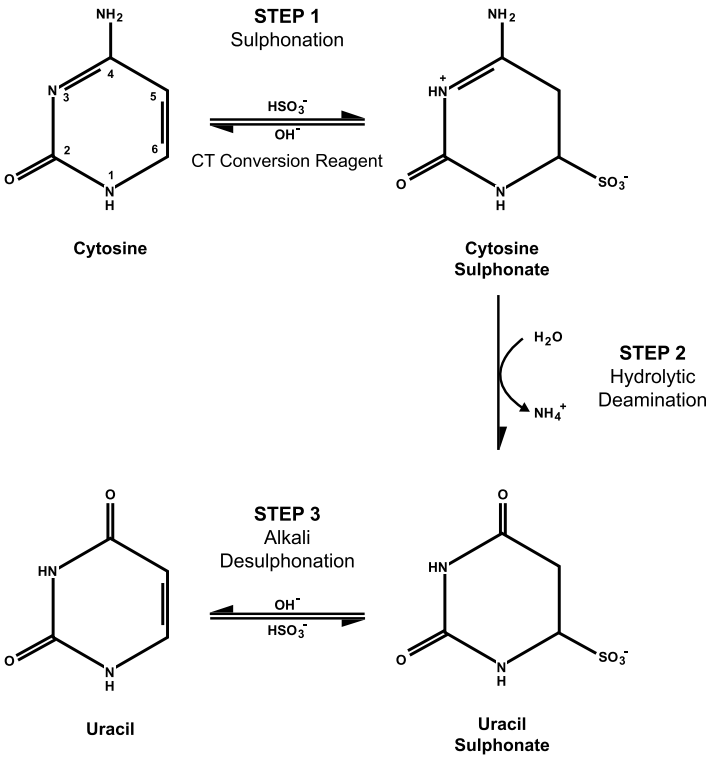


Figure 1. Deamination reaction of sodium bisulfite with cytosine to Sodium bisulfite treatment is currently the "gold standard" in distinguishing between cytosine and 5-methylcytosine in complex genomes. In this process, unmethylated cytosine undergoes conversion to uracil whereas the unreactive 5-methylcytosine remains unchanged.

Results

The newly developed EZ DNA Methylation-Direct™ Kit was tested on the basis of DNA recovery and conversion efficiency as well as its application to a broad-range of sample sources including FFPE-derived samples.

DNA recovery following bisulfite treatment: As a test for DNA recovery with the EZ DNA Methylation-Direct™ Kit, the percentage of DNA eluted from the column following bisulfite conversion was determined for a range (125-1000 ng) of DNA inputs (Figure 3).

Pre-existing kit technologies (i.e., the EZ DNA Methylation™ and EZ DNA Methylation-Gold™ Kits) were included in the test for comparison purposes. The amounts of DNA recovered (post-conversion) with each of the technologies was quantified by UV-spectrophotometric analysis using a value of 40 µg/ml for A_{260} = 1.0 (see "Tips & FAQs for Methylation Analysis", page 41), and averages of replicates were compared to the initial amount of input DNA to determine recovery percentage. The results indicate the EZ DNA Methylation-Direct™ Kit promoted an average recovery of 88% for all DNA input levels tested. In comparison, the EZ DNA Methylation-Gold™ Kit demonstrated a slight decline in recovery as a function of the amount of input DNA, decreasing 13% from 1000 ng to 125 ng. Similarly, the trend in recovery with the EZ DNA Methylation™ Kit decreased 18%. The results show that the recovery of input DNA was highest (~88%) with the EZ DNA Methylation-Direct™ Kit compared to the other bisulfite conversion technologies for the input levels of DNA tested.

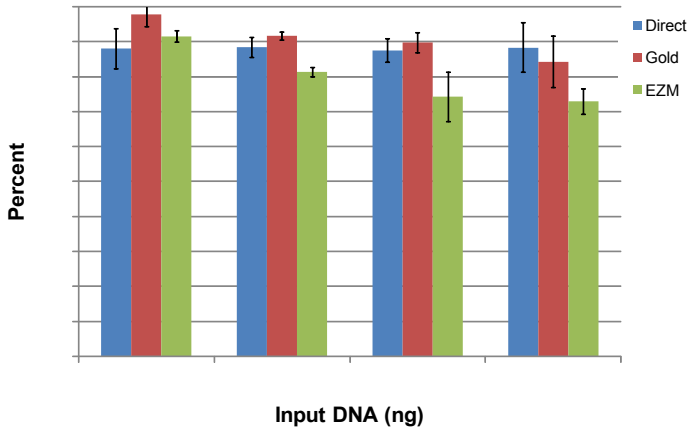
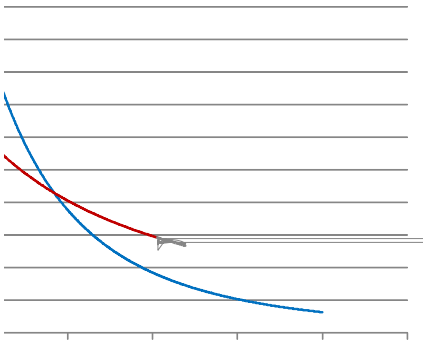


Figure 3. High efficiency recovery of input DNA. A range from 125-1,000 ng of genomic DNA was processed using the EZ DNA Methylation-Direct™ (Direct), EZ DNA Methylation-Gold™ (Gold), or EZ DNA Methylation™ (EZM) Kits. Data are given as the average percent recoveries ± SD for measurements performed in quadruplicate.

Conversion reaction kinetics: As a test for the conversion efficiency of DNA with the EZ DNA Methylation-Direct™ Kit, reaction kinetics for bisulfite conversion with the kit or a conventional procedure were determined and compared (Figure 4). A plasmid (~4 kb) methylated *in vitro* at all 5´…CpG…3´ dinucleotides using the CpG Methylase from Zymo Research (Cat. Nos. E2010, E2011) was used for test purposes. For both EZ DNA Methylation-Direct™ Kit and conventional procedures, the linearized, methylated plasmid DNA was bisulfite converted, purified, amplified, cloned, and transformed into *E. coli*. DNA from selected transformants were sequenced to determine which cytosines were methylated (see Figure 2). Conversion errors were tabulated for each group of sequences and graphed as a percentage of either unconverted, nonmethylated cytosines (incomplete conversion) or converted, methylated cytosines (over-conversion). At t = 3 hours, it was determined that DNA converted with the EZ DNA Methylation-Direct™ Kit demonstrated incomplete conversion levels of only 0.2% with 0.3% over-conversion. In comparison, conventional conversion demonstrated incomplete conversion levels of 0.9% with 1.2% over-conversion.



Bisulfite conversion efficiency was evaluated over time, comparing the EZ DNA Methylation-Direct™ Kit with a conventional conversion procedure. Conversion errors were tabulated for each group of sequences and graphed as a percentage of either unconverted nonmethylated cytosines (Incomplete Conversion) or converted methylated cytosines (Over Conversion).

Lower limits for cellular input: To test the lower-limits of sample input required for reproducible DNA conversion and recovery (sensitivity) with the EZ DNA Methylation-Direct™ Kit, primary fibroblasts were serially diluted (1:10) to achieve from 0 to 1,000 cells in Digestion Buffer, then proteinase K-treated, bisulfite-treated, and recovered according to the product instructions. Eluted, bisulfite converted DNA was amplified by PCR and the products were separated in a 2% (w/v) agarose/EtBr/TAE gel (Figure 5). The expected amplicon was visible in increasing amounts only in those lanes with an input of ≥ 10 cells. This demonstrates that 10 cells, or ~50 pg DNA, is sufficient to attain adequate conversion and recovery with the EZ DNA Methylation-Direct™ Kit.

Processability of FFPE-tissues: The compatibility of the EZ DNA Methylation-Direct™ Kit with archived samples was tested with formalin-fixed, paraffin-embedded (FFPE) tissues. Prior to processing with the kit, FFPE mouse, liver, and kidney tissues were de-paraffinized by canonical methods using xylene. Approximately 0.5 mg of dewaxed tissue was processed for each sample and compared to its non-FFPE counterpart (frozen) of the same tissue type. PCR was performed (post-conversion) using 1 µl (10% v/v) of the eluted DNAs and the products were visualized in a 2% (w/v) agarose/EtBr/TAE gel (Figure 6). Amplicons were observed for all sample types indicating the EZ DNA Methylation-Direct™ Kit is suitable for both non- and FFPE derived tissue specimens.

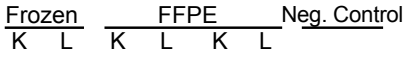


Figure 6. Bisulfite Conversion of FFPE Tissues. Formalin-fixed, paraffin-embedded (FFPE) and frozen mouse kidney (K) and liver (L) tissues were processed using the EZ DNA Methylation-Direct Kit™. PCR was performed and products were visualized in a 2% (w/v) agarose/EtBr/TAE gel.

Sample compatibility assessment: Finally, to test the diversity of samples that can be effectively processed with the EZ DNA Methylation-Direct™ Kit, several different sample types were used as input material. These samples were of two groups: purified DNA (plasmid, genomic) or tissues (blood, kidney). Samples were processed with the "Proteinase-K & Bisulfite Conversion Steps" of the EZ DNA Methylation-Direct™ Kit protocol or with just the "Bisulfite Conversion Step." The plasmid control, human, and mouse genomic DNAs were processed with both methods. Human and mouse blood and mouse kidney samples were processed using the "Proteinase-K & Bisulfite Conversion Steps." All sample DNAs were converted with the technology featured in the EZ DNA Methylation-Direct™ Kit. Eluted, bisulfite converted DNA for each of the samples was amplified by PCR and the products (ranging in size from 150 to 470 bp) were visualized in a 2% (w/v) agarose/EtBr/TAE gel (Figure 7). The experiment demonstrates that a wide-variety of samples can be effectively processed using the EZ DNA Methylation-Direct™ Kit.

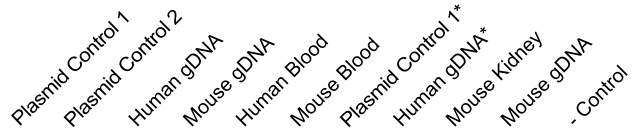


Figure 7. "Direct" Bisulfite DNA Conversion from Diverse Sample Sources. Several different sample types were processed using the EZ DNA Methylation-Direct™ Kit. Two groups of samples are represented, those that were processed using the "Proteinase-K & Bisulfite Conversion Steps" and those that used only the bisulfite conversion procedures (*). PCR was performed and products were visualized in a 2% (w/v) agarose/EtBr/TAE gel.

Blood and tissue samples require the "Proteinase-K & Bisulfite Conversion Steps" of the protocol, but purified DNA only requires the "Bisulfite Conversion Step".

Discussion

Traditional bisulfite conversion of DNA involving "homebrews" is cumbersome and has been marred with inconsistencies stemming from low recovery, incomplete or over conversion, and low sensitivity. Here we have demonstrated the use of the EZ DNA Methylation-Direct™ Kit for consistent, high recovery of bisulfite treated DNA from a range of sample inputs. The design of the kit and its reagents were developed for data consistency and to mitigate the loss of DNA typical of "homebrew" reactions and second-tier products available from most commercial sources. Also, unlike other products currently available, the technologies featured in the EZ DNA Methylation-Direct™ Kit ensure consistent recovery of input DNA even from as few as ten (10) cells or as little as ~50 pg DNA. This is facilitated through the fine tuning of bisulfite conversion chemistries that enable the reaction to proceed to near completion (i.e., 99.8% conversion of non-methylated cytosine) while maintaining the integrity of methylated cytosine during the process. Also, key to the high recovery of converted DNA is the state-of-the-art column/plate design featured in the EZ DNA Methylation-Direct™ Kit line of products. The *Fast-Spin* columns and plates have been designed to ensure rapid desulphonation as well as high recovery of converted DNA. Elution of buffers from the column/plate matrices is complete, negating buffer carryover. Thus, eluted, bisulfite converted DNA is pure and ready for analysis. Further, the columns allow DNA to be eluted in ultra-small volumes (≥ 6 µl) for highly concentrated DNA if required. Finally, an optimized Proteinase K digestion step is included in the protocol that facilitates the efficient sampling of even the most problematic samples including FFPE and LCM derived tissues. The ability to process samples directly from cells, tissues, or previously purified DNA gives unprecedented ease of use in dealing with such diverse sample types. Together, these factors combine to establish the EZ DNA Methylation-Direct™ Kit as the pinnacle of bisulfite conversion technology available today in providing unmatched consistency, accuracy, sensitivity, and ease of use.

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FEATURED TECHNOLOGIES

Introduction

Zymo Research offers a compendium of products for DNA methylation detection and other aspects of epigenetics-based research.

DNA Methylation Detection

The EZ DNA Methylation™ family of bisulfite conversion kits (pages 23-28) provides complete C to U conversion in DNA for rapid and precise methylation detection by a host of downstream procedures (*see figure on opposite page*). The products have been specifically developed for use with purified DNA or cells, tissue (including FFPE and LCM), blood, and biological fluids with lower range inputs of approximately 10 cells or 50 pg DNA. Recovery of bisulfite-converted DNA following in column desulfonation and cleanup is typically >90% (see Figure 3 page 18).

Validated methylated and non-methylated DNA standards (pages 29-30) are provided for use as controls with the EZ DNA Methylation™ line and any other commercially available or "homebrew" bisulfite conversion procedures. Also, a bisulfite converted DNA standard is provided for downstream analyses including PCR and MSP requiring a validated template that has been completely methylated then converted with bisulfite.

For robust amplification of bisulfite-treated DNA a unique hot start Zymo*Taq*™ DNA polymerase (page 37) is available that reduces non-specific PCR product formation from "difficult" to amplify templates.

For DNA methylation detection for the first time user, we offer the EZ DNA Methylation-Startup™ Kit (page 28) that consolidates bisulfite conversion and amplification of DNA into a single product.

Additional Tools for Epigenetics Research

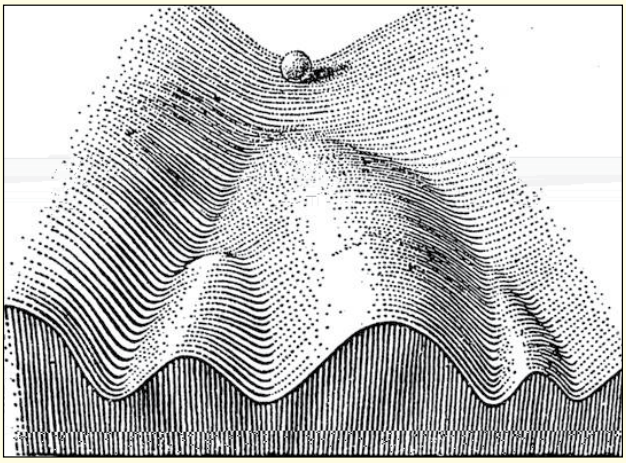
For bisulfite-independent analysis of DNA methylation, a specific anti-5-methylcytosine monoclonal antibody is available individually (page 32) or as part of a Methylated-DNA IP Kit (page 31). The latter is designed for MeDIP enrichment of methylated DNA for genome-wide methylation analysis by array, qPCR, ultra-deep sequencing, etc. Also, the ChIP DNA Clean & Concentrator™ (page 34) provides a quick, two (2) minute method for purification of high quality DNA from any step in a standard ChIP procedure irrespective of the antibody(ies) used by the researcher.

A highly efficient CpG Methylase (M.SssI) (page 33) is offered for complete methylation of cytosines in the dinucleotide context 5′…CpG…3′. It has been specifically engineered to ensure rapid, complete, and reproducible methylation of DNA for subsequent analysis.

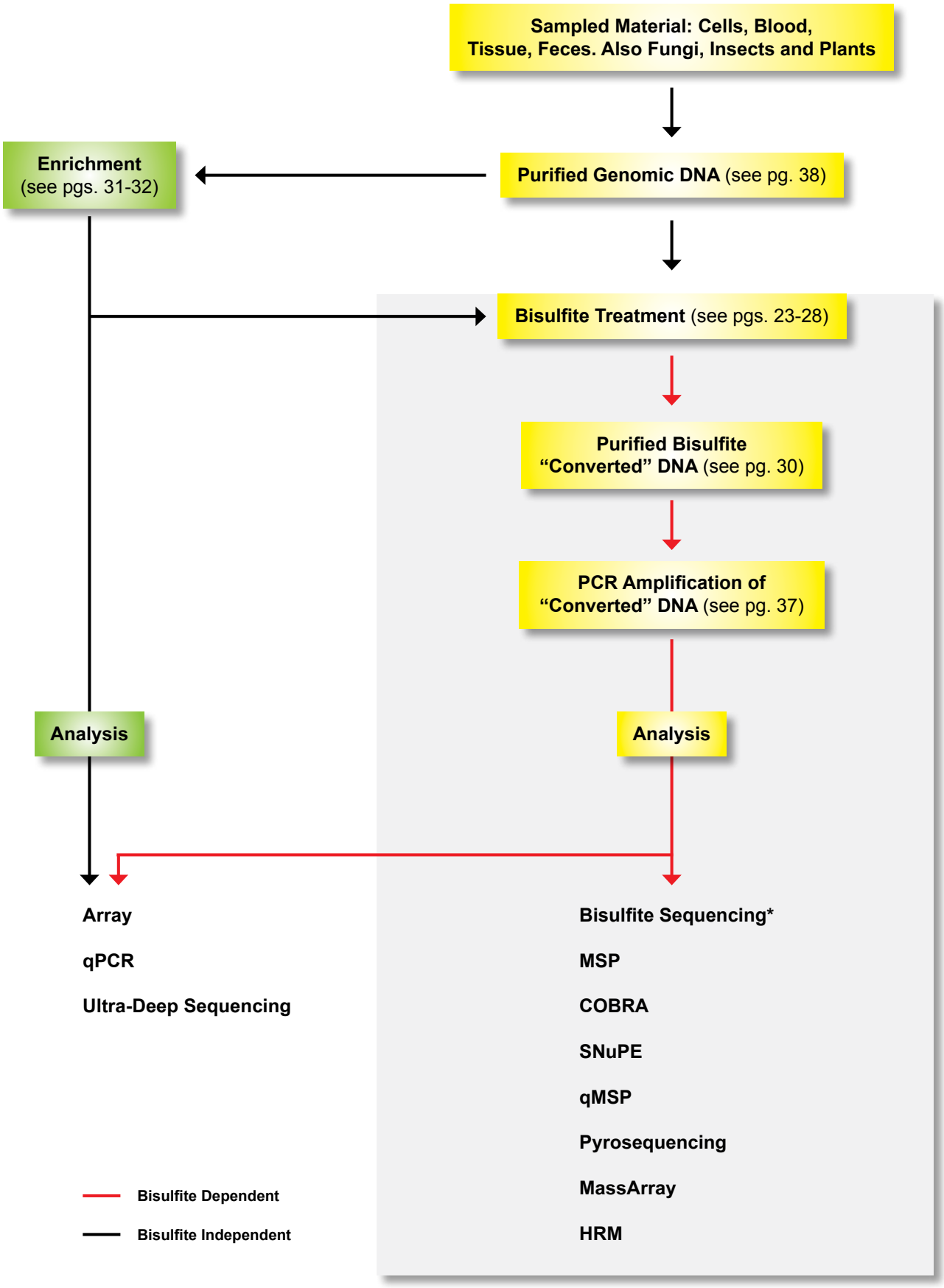
Several aspects of epigenetics are strongly linked to non-coding RNAs, especially small RNAs that can direct cytosine methylation and histone modifications that are implicated in gene expression regulation in complex organisms. The ZR RNA MicroPrep™ and ZR RNA MiniPrep™ (page 36) provide quick methods for the isolation of high quality total RNA from small amounts of cells and tissue. Both kits isolate large and small RNAs that can include those non-coding regulatory species that affect gene expression. For high resolution extraction and purification of small RNAs (e.g., miRNA) from polyacrylamide gel slices, the ZR small-RNA™ PAGE Recovery Kit (page 35) is available.

Finally, other products useful to the epigenetics researcher are presented (see pages 39-40). These include products for rapid DNA recovery and concentration from both liquid sources and agarose gel slices, a single step procedure for PCR inhibitor removal from DNA, a rapid plasmid miniprep for purification of transfection quality DNA directly from cultured *E. coli*, and a kit that enables researchers to generate their own ultra-competent cells for 20 second transformations without heat shock.

. In this view of embryonic regulation, cell fate determination is based on selection between pre-existing, intrinsically robust fates. The dynamics of this developmental selection is depicted as a "landscape" with hills and valleys. The phenotypic state of a cell at any time in its development is indicated by the position of a marble on that landscape. The marble will spontaneously roll down the valleys (stable developmental paths) leading to a distinct phenotype. The lowest points in the valleys correspond to the distinct, stable phenotypes within a given set of fates the cell may experience. However, recent research involving nuclear transfer and reprogramming of embryonic stem cells indicate that the marble can be rolled back to the top of the hill and that lateral movement across the landscape (over peaks) may also be possible in effecting the ultimate fate of the cell.



Technology Overview: DNA Methylation Analysis



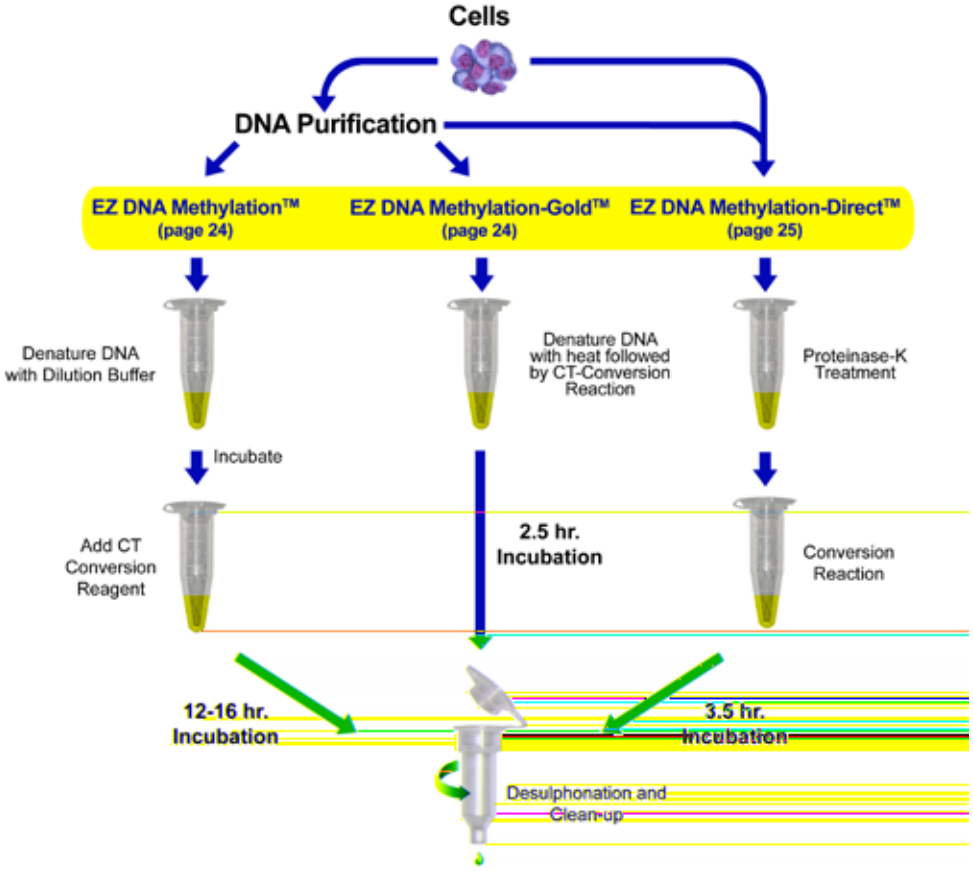
* See glossary on page 43 for specific definitions.

Bisulfite Conversion Kits for Accurate DNA Methylation Analysis

Bisulfite treatment for the deamination of (C)ytosine to (U)racil in DNA has remained the "gold standard" for a number of downstream applications developed over recent years to assess DNA methylation status. Most commonly used methods for locus- and multi-locus specific methylation rely on pretreatment of DNA with bisulfite, since bisulfite conversion and DNA sequencing remain the only means to accurately quantitate methylation at the level of the individual nucleotide.

The EZ DNA Methylation™ family of kits from Zymo Research remains the most popular and cited technologies available for bisulfite conversion and DNA methylation detection today (see page 27 for selected references). They have been validated by countless researchers at both academic institutions and companies alike. The EZ DNA Methylation™ kits featured on pages 24-26, 28 have been specifically engineered for complete conversion of as little as 50 pg DNA in as fast as 3 hours *…reliably!*... with DNA recoveries >80%.

Kits are available in single column and 96-well plate formats.



Bisulfite-Treated DNA Ready for Analysis

A core technology of Zymo Research's (spin column format) bisulfite DNA *Fast-Spin* Developed and manufactured by Zymo Research, its innovative design makes it perfect for rapid in-column desulphonation and high-yield elution of bisulfite-treated DNA. These unique columns allow purification of up to 5 µg of DNA in ≥ 6 µl eluate with no buffer retention or carryover.

EZ DNA Methylation™ Kit

Simple, Validated Procedure for Precise DNA Methylation Detection

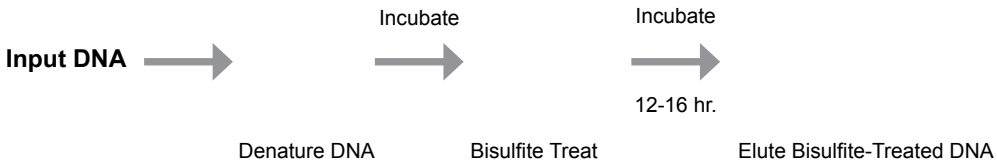
- Desulphonation and recovery of bisulfite-treated DNA with a spin column or 96-well plate
- Recovered DNA is ideal for downstream analyses including PCR, endonuclease digestion, sequencing, microarrays, etc.

The **EZ DNA Methylation™ Kit** features a simplified procedure that streamlines bisulfite treatment of DNA. The kit is based on the three-step reaction that takes place between cytosine and sodium bisulfite where cytosine is converted into uracil. The product's innovative in-column desulphonation technology eliminates otherwise cumbersome precipitations. The kit is designed to reduce template degradation, minimize DNA loss during treatment and cleanup, while ensuring complete conversion of the DNA. Purified, converted DNA is ideal for PCR amplification for downstream analyses including endonuclease digestion, sequencing, microarrays, etc. These kits are validated for use with Illumina's *GoldenGate*® and *Infinium*® Assays.

Input DNA: 500 pg - 2 µg DNA per treatement with 200-500 ng optimal.

Conversion Efficiency: >99%

Recovery: >80%



Product	Format	Cat. No.	Size	Price
EZ DNA Methylation™ Kit	Spin Column	D5001	50 rxns.	$112.00
		D5002	200 rxns.	$390.00
EZ-96 DNA Methylation™ Kit	Shallow-Well	D5003	2x96 rxns.	$310.00
	Deep-Well	D5004	2x96 rxns.	$310.00

EZ DNA Methylation-Gold™ Kit

Complete Bisulfite Conversion of GC-Rich DNA in Less Than 3 Hours

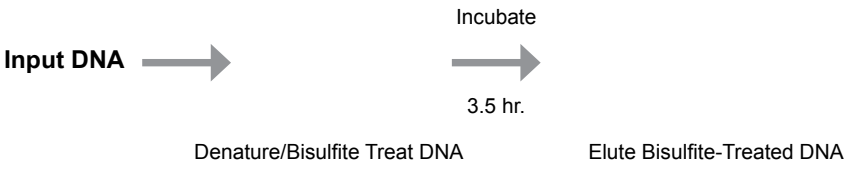
- A coupled heat denaturation/conversion reaction step streamlines the conversion of non-methylated cytosines into uracil.
- DNA precipitations are omitted. Instead, DNA is cleaned and desulphonated in a single step using state-of-the-art spin column technology.

The **EZ DNA Methylation-Gold™ Kit** is a refinement of our popular EZ DNA Methylation™ Kit (above). The EZ DNA Methylation-Gold™ Kit integrates DNA denaturation and bisulfite conversion processes into one-step. This is accomplished using temperature denaturation to replace chemical denaturation in the previous protocol. Also, the kit has been streamlined for high yield recovery of DNA following bisulfite treatment. Recovered DNA is ideal for PCR amplification for downstream analyses including endonuclease digestion, sequencing, microarrays, etc.

Input DNA: 500 pg - 2 µg DNA per treatement with 200-500 ng optimal.

Conversion Efficiency: >99%

Recovery: >75%



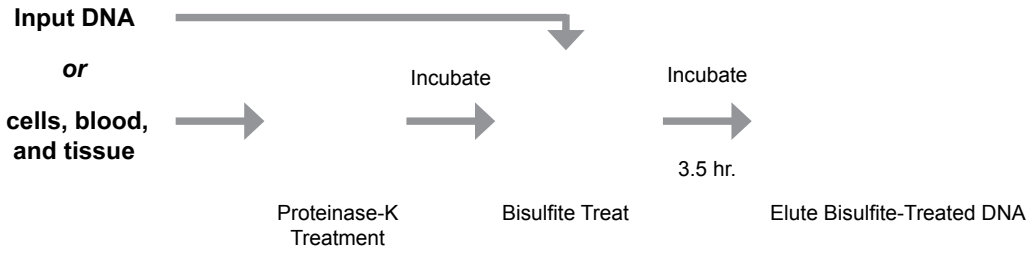
Product	Format	Cat. No.	Size	Price
EZ DNA Methylation-Gold™ Kit	Spin Column	D5005	50 rxns.	$121.00
		D5006	200 rxns.	$410.00
EZ-96 DNA Methylation-Gold™ Kit	Shallow-Well	D5007	2x96 rxns.	$320.00
	Deep-Well	D5008	2x96 rxns.	$320.00

EZ DNA Methylation-Direct™ Kit

DNA Methylation Detection Directly From Cells!

- Complete bisulfite conversion of DNA directly from blood, tissue, or cells.
- Compatible with small sample inputs - as few as 10 cells or 50 pg DNA.
- Well-suited for FFPE and LCM-derived samples.

The **EZ DNA Methylation-Direct™ Kit** is a further refinement of our popular EZ DNA Methylation™ and EZ DNA Methylation-Gold™ Kits. The EZ DNA Methylation-Direct™ Kit features simple and reliable DNA bisulfite conversion directly from blood, tissue, and cells without the prerequisite for DNA purification. The increased sensitivity of this kit makes it possible to amplify bisulfite converted DNA from as few as 10 cells or 50 pg DNA. Like the EZ DNA Methylation-Gold™ Kit, DNA denaturation and bisulfite conversion processes are combined into a single step (see below). All kits streamline the three step process of bisulfite conversion of nonmethylated cytosine in DNA into uracil. In addition the methylation kits share innovative in-column desulphonation technology that eliminates otherwise cumbersome DNA precipitation steps while ensuring researchers consistent results every time. The kits have been designed to minimize template degradation, loss of DNA during treatment and clean-up, and to provide complete conversion of unmethylated cytosines. Recovered DNA is ideal for PCR amplification for downstream analyses including restriction endonuclease digestion, sequencing, microarrays, etc.

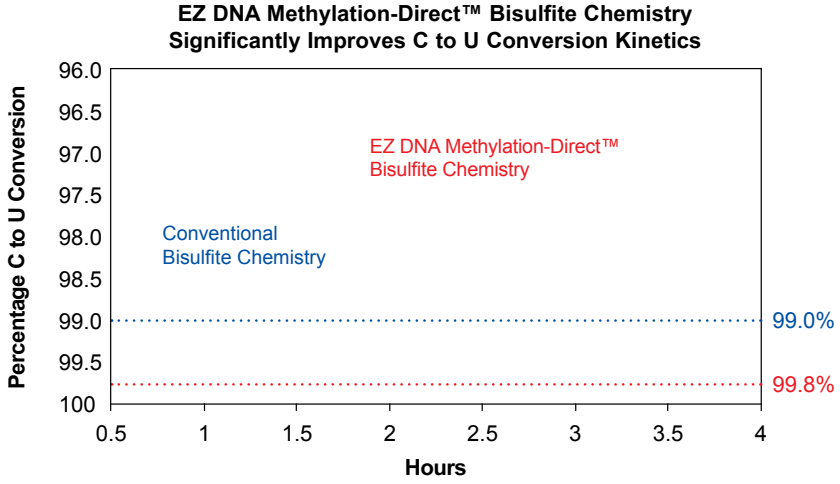


Starting Materials: Compatible with cells from solid tissue, tissue culture, whole blood, buffy coat, biopsies, LCM (Laser Capture Micro-Dissection) and FFPE samples, etc.

Conversion Efficiency: > 99.5% of non-methylated C residues are converted to U; > 99.5% protection of methylated cytosines.

DNA Recovery: > 80% sensitivity of detection (lower limit): 10 cells or 50 pg DNA.

Format: Single Column, 96-Well



Methylated DNA was converted using either conventional or bisulfite chemistries. Recovered DNA was amplified by PCR, then cloned. Sequences from individual clones were analyzed and quantitated. These data show that EZ DNA Methylation-Direct™ bisulfite chemistry improves the rate and extent (> 99.8%) of C to U conversion of DNA as compared to conventional bisulfite chemistry.

Product	Cat. No.	Size	Price
EZ DNA Methylation-Direct™ Kit	D5020	50 rxns.	$161.00
	D5021	200 rxns.	$452.00
EZ-96 DNA Methylation-Direct™ Kit	D5022	2x96 rxns. (Shallow-well Silicon-A™ Plate)	$362.00
	D5023	2x96 rxns. (Deep-well Zymo-Spin I-96™ Plate)	$362.00

EZ DNA Bisulfite DNA Clean-up Kit™

Rapid In-Column Desulphonation and DNA Cleanup From "Homebrew" Reactions

- Consistent, high yield recovery of bisulfite-treated DNA from any "homebrew" or commercial reaction mixture containing bisulfite.
- Simple spin column procedure with small elution volumes (≥ 6 µl) for concentrated DNA.

The **EZ Bisulfite DNA Clean-up Kit™** has been specifically designed for the purification of bisulfite-treated DNA from any "homebrew" or commercial reaction mixture containing bisulfite.

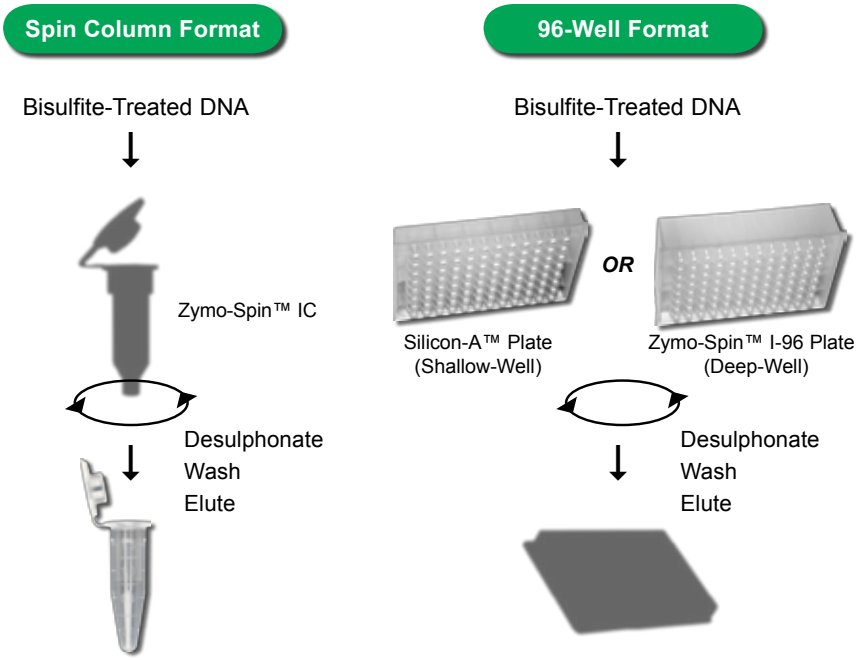
The product features innovative *Fast-Spin*, in-column desulphonation and wash technologies that eliminate DNA loss, buffer carryover and the need for ethanol/isopropanol precipitations. The procedure is easy and DNA clean-up can be completed in just minutes. Bisulfite-treated DNA purified with the EZ Bisulfite DNA Clean-up Kit™ is ideal for PCR amplification for downstream DNA methylation analysis including endonuclease digestion, sequencing, microarrays, etc.

DNA Input: Up to 5 µg DNA (per column) from "homebrew" or commercial reaction mixtures containing bisulfite.

DNA Recovery: Typical yields are between 80-90%.

Elution: DNA can be eluted with ≥ 10 µl buffer or water.

Detergent Tolerance: ≤ 5% Triton X-100, ≤ 5% Tween-20, ≤ 5% Sarkosyl, ≤ 0.1% SDS, and others.



DNA is perfect for...
- PCR
- Endonuclease Digestion
- Arrays
- Sequencing

Product	Cat. No.	Size	Price
EZ Bisulfite DNA Clean-up Kit™	D5025	50 rxns.	$81.00
	D5026	200 rxns.	$274.00
EZ-96 Bisulfite DNA Clean-up Kit™	D5027	2x96 rxns. (Shallow-well Silicon-A™ Plate)	$214.00
	D5028	2x96 rxns. (Deep-well Zymo-Spin I-96™ Plate)	$214.00

Selected Citations Featuring EZ DNA Methylation™ Kit Technology

Cancer

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17. Hanson, JA *et al.* Gene Promoter Methylation in Prostate Tumor-Associated Stromal Cells. *J Nat Cancer Inst* **98:** 255-261 (2006).
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7. Eiges R *et al.* Developmental Study of Fragile X Syndrome Using Human Embryonic Stem Cells Derived From Preimplantation Genetically Diagnosed Embryos. *Cell Stem Cell* **1:** 568-577 (2007).
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10. Zhang, F *et al.* Active tissue-specific DNA demethylation conferred by somatic cell nuclei in stable heterokaryons. *Proc Natl Acad Sci USA* **104:** 4395-4400 (2007).
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2. Weisenberger, DJ *et al.* DNA methylation analysis by digital bisulfite genomic sequencing and digital MethyLight. *Nucleic Acids Res* **36:** 4689-4698 (2008).
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6. Yegnasubramanian, S *et al.* Combination of methylated-DNA precipitation and methylation-sensitive restriction enzymes (COMPARE-MS) for the rapid, sensitive and quantitative detection of DNA methylation. *Nucleic Acids Res* **34:** e19 (2006).

EZ DNA Methylation-Startup™ Kit

- A *complete* system for DNA methylation detection: DNA bisulfite treatment, robust hot start PCR, and a universally methylated human control with primers.
- Designed for the first time user requiring a consolidated product to perform DNA methylation analysis.

Zymo Research's technologies for bisulfite conversion and DNA methylation detection remain the most popular and cited to date. The **EZ DNA Methylation-Startup™ Kit** is designed to include these technologies for the first time user requiring a consolidated product to perform DNA methylation analysis.

The EZ DNA Methylation-Startup™ Kit provides the necessary technologies required for complete bisulfite-conversion of DNA for PCR and methylation analysis. This kit includes bisulfite conversion reagents that allow for direct sampling of blood, cells, and fresh or FFPE tissues without the prerequisite for upstream DNA purification. However, purified DNA can also be bisulfite treated directly. A fully methylated **Universal Methylated Human DNA Standard** is provided together with a special primer set for PCR to control for and assess conversion efficiency. Finally, a unique **Zymo*Taq*™ DNA Polymerase** (see page 37) is included for robust amplification of bisulfite-treated DNA.

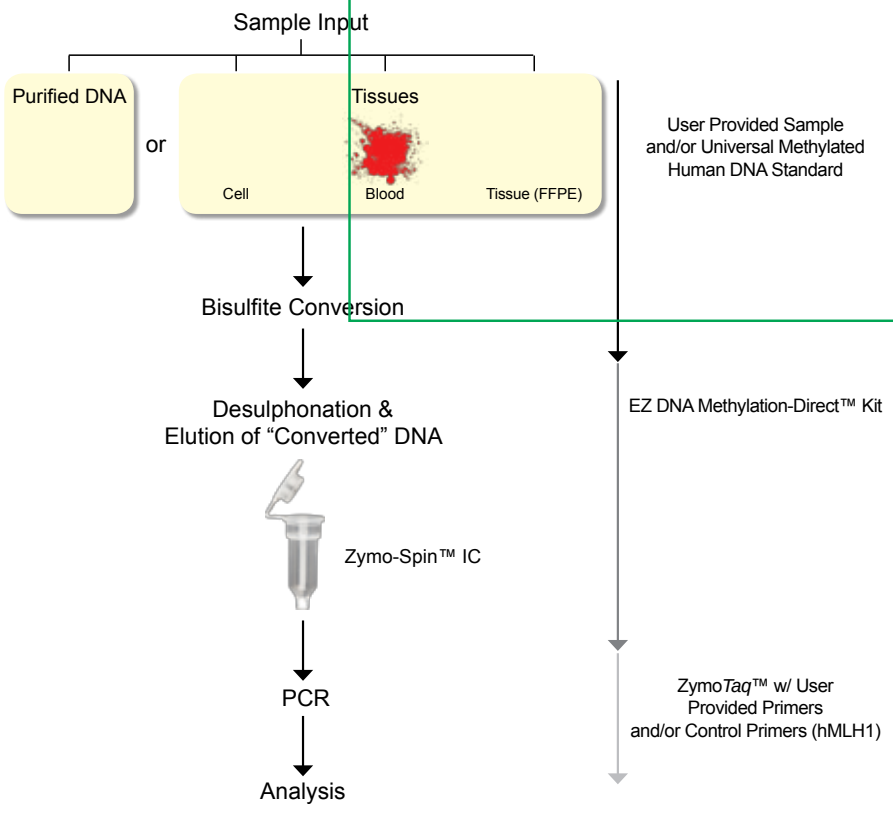
Application: Workflow design allows direct sample input for bisulfite treatment of DNA followed by robust PCR amplification of the "converted" DNA for methylation analysis.

Components: The EZ DNA Methylation-Startup™ Kit is a three component kit: The EZ DNA Methylation-Direct™ Kit (D5020), the Universal Methylated Human Standard (D5011), and 2X Zymo*Taq*™ PreMix (E2003).

DNA Recovery: Typical yields of bisulfite-converted DNA are between 80-90%.

Elution: DNA can be eluted with ≥ 10 µl buffer or water.

Workflow of the EZ DNA Methylation-Startup™ Kit



Validated Methylated and Non-Methylated DNA Standards

Zymo Research offers a comprehensive set of DNA standards, including methylated, non-methylated, and bisulfite-treated DNA standards for use as controls with the EZ DNA Methylation™ line of products or other commercially available bisulfite conversion kits.

The **Universal Methylated DNA Standards** contain DNA from either bacterial (plasmid), human, or mouse sources that have been enzymatically methylated at all cytosines in the dinucleotide sequence 5´...CpG...3´. The **Human Methylated & Non-methylated DNA Set** contains non-methylated genomic DNA isolated from a cell line having low levels of naturally occurring methylation in combination with an enzymatically methylated version of the same non-amplified genomic DNA. The ***E. coli* Non-methylated Genomic DNA** is ideal for use as a completely non-methylated control. The **Bisulfite Converted Universal Methylated Human DNA Standard** is validated to have had all non-methylated cytosines in 5´...CpG...3´ context methylated and then converted to uracils following bisulfite treatment.

All of the DNA standards from Zymo Research have been optimized for use in PCR with their respective primer set but can also be used with a primer set of your choice.

Universal Methylated DNA Standard
Universal Methylated Human DNA Standard
Universal Methylated Mouse DNA Standard

The **Universal Methylated DNA Standard**, **Universal Methylated Human DNA Standard**, and **Universal Methylated Mouse DNA Standard** are designed for use as controls to assess the efficiency of bisulfite-mediated conversion of DNA in combination with the EZ DNA Methylation™, EZ DNA Methylation-Gold™, or EZ DNA Methylation-Direct™ kits. The primer sets included with the standards have been designed and validated to amplify the DNA only after bisulfite conversion.

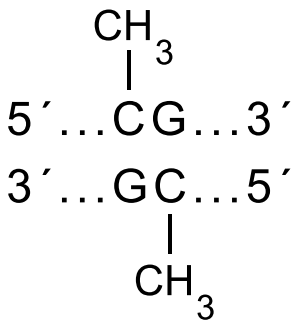
The control DNAs have been enzymatically modified *in vitro* with CpG Methylase (see page 33 and figure below), resulting in methylation at all cytosines in the dinucleotide sequence 5´...CpG...3´. Each primer set has been specifically designed to amplify a fragment of the supplied DNA following bisulfite treatment. The methylated cytosines remain unconverted following bisulfite treatment, whereas non-methylated cytosines are converted into uracils and detected as thymines following PCR.

Assess Bisulfite Conversion Efficiency with Fully Methylated DNA Standards & Primer Sets

Available As:

- Linearized Plasmid
- Human Genomic DNA
- Mouse Genomic DNA

Compatible with Zymo Research's Full Line of Bisulfite Conversion Products



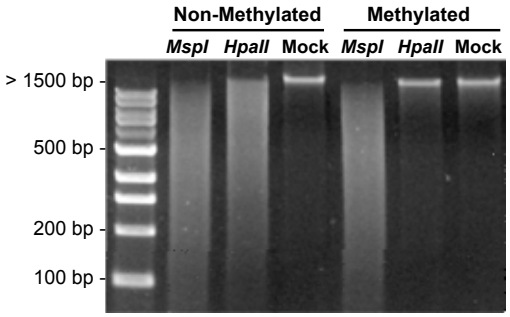
CpG Methylase methylates all cytosine residues in double-stranded, CpG context.

Product	Cat. No.	Size	Price
Universal Methylated DNA Standard	D5010	1 set	$122.00
Universal Methylated Human DNA Standard	D5011	1 set	$182.00
Universal Methylated Mouse DNA Standard	D5012	1 set	$182.00

Human Methylated & Non-methylated DNA Set

The **Human Methylated & Non-methylated DNA Set** consists of two control DNAs (a methylated human DNA standard and a non-methylated human DNA standard) together with a set of specifically designed primers that can be used in conjunction with the EZ DNA Methylation™, EZ DNA Methylation-Gold™, and EZ DNA Methylation-Direct™ kits from Zymo Research to assess the efficiency of bisulfite-mediated conversion of DNA.

The non-methylated human DNA is purified from the HCT116 DKO (double knock-out) cell line, which contains genetic knockouts of both DNA methyltransferases DNMT1 (-/-) and DNMT3b (-/-). The DNA derived from HCT116 DKO cells has a low level of DNA methylation (< 5%) and therefore can be used as a negative control for DNA methylation analysis (see below). The methylated human DNA standard is purified HCT116 DKO DNA that has been enzymatically methylated at all cytosine positions comprising CG dinucleotides by CpG Methylase (EC 2.1.1.37) (see also page 33) and can be used as a positive control for DNA methylation analysis.



An assay for complete methylation by M.SssI methytransferase. Non-methylated and methylated DNA from HCT116 DKO cells was digested with restriction enzymes MspI and HpaII. MspI digests both non-methylated and methylated DNA. HpaII is sensitive to CpG methylation.

Product	Cat. No.	Size	Price
Human Methylated & Non-methylated DNA Set (DNA w/ primers)	D5014	1 set	$382.00
Human HCT116 DKO Non-Methylated DNA (DNA only)	D5014-1	5 µg	$212.00
Human HCT116 DKO Methylated DNA (DNA only)	D5014-2	5 µg	$212.00

Bisulfite Converted Universal Methylated Human DNA Standard

The **Bisulfite Converted Universal Methylated Human DNA Standard** is designed for use as a control for bisulfite-mediated conversion of DNA and especially the downstream analyses including PCR, MSP, and other amplification based assays. This DNA is identical to our Universal Methylated Human DNA Standard, but has been bisulfite converted using Zymo Research's advanced conversion technologies. The primer set included with the standard has been designed and validated to amplify the bisulfite converted DNA.

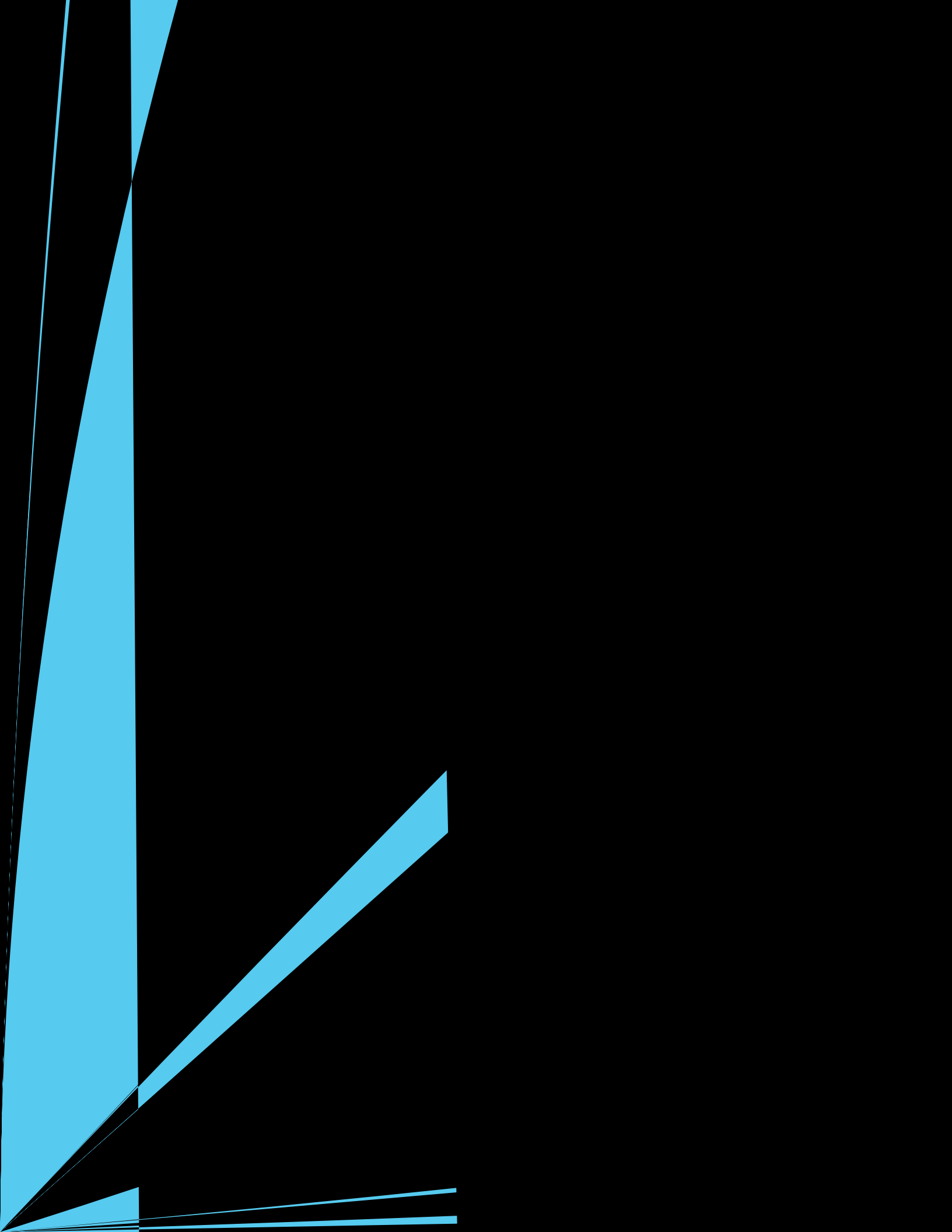
Product	Cat. No.	Size	Price
Bisulfite Converted Universal Methylated Human DNA Standard	D5015	1 set	$122.00

E. coli Non-Methylated Genomic DNA

This non-methylated genomic DNA is from a Dam$^-$ and Dcm$^-$ strain (ER2925) of *E. coli*. It is useful for DNA methylation analyses requiring DNA with absolutely no methylation.

ER2925 Genotype: *ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 R(zgb210::Tn10)TetS endA1 rpsL136 dam13::Tn9 xylA-5 mtl-1 thi-1 mcrB1 hsdR2*.

Product	Cat. No.	Size	Price
***E. coli* Non-Methylated Genomic DNA**	D5016	5 µg	$98.00



Specific Anti-5-Methylcytosine Antibody for Methylation Analysis

Anti-5-Methylcytosine Monoclonal Antibody (Clone 10G4)

- A highly specific anti-5-methylcytosine monoclonal antibody for defined, reproducible results.
- Ideal for use in the immunoprecipitation or immunodetection of methylated DNA

The ability to detect and quantify DNA methylation (i.e., 5-methylcytosine) efficiently and accurately has become essential for the study of cancer, gene expression, genetic diseases, as well as many other important aspects of epigenetics.

The mouse **Anti-5-Methylcytosine Monoclonal Antibody (Clone 10G4)** has been developed to facilitate differentiation between methylated and non-methylated cytosines in DNA. Specificity of this clone is to 5-methylcytosines in single-stranded DNA with no detectable cross-reactivity to non-methylated cytosines. The antibody has proven to be a valuable tool in the characterization of DNA methylation and has been successfully used for immunopreciptiation-based assays such as Methylated DNA Immunoprecipitation (MeDIP) (see opposite page).

Product Type: Monoclonal Antibody

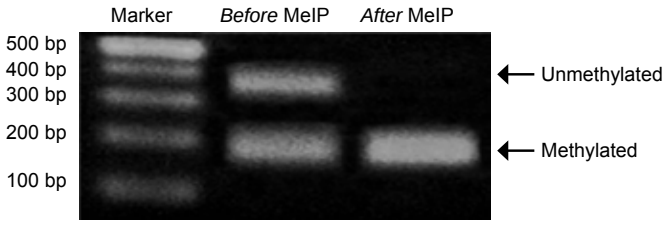
Format: Purified

Clone: 10G4

Source: Mouse

Isotype: IgG1

Specificity: 5-Methylcytosine in single-stranded DNA from any source including human, rat, mouse, plants, etc.



Methylated DNA is efficiently enriched using the 5-Methylcytosine DNA was immunoprecipitated using the mouse Anti-5-Methylcytosine 10G4 antibody from a mixed methylated/non-methylated DNA population. Methylated DNA can be cut with NcoI whereas non-methylated DNA is resistant to NcoI digestion. The DNA (post-IP) was subsequently amplified by PCR and digested with NcoI. Products were then separated in a 2.0% (w/v) agarose/TAE/EtBr gel. The image above demonstrates specific enrichment of methylated versus non-methylated DNA by the Anti-5-Methylcytosine 10G4 antibody.

Application		Recommended Dilution
ELISA	Yes	1:500 to 1:1,000
Flow Cytometry	N/D**	N/A*
Immunoblotting	Yes	N/A*
Immunofluorescence	Yes	N/A*
Immunohistology	N/D**	N/A*
Immunoprecipitation (IP) of Methylated DNA	Yes	2-4 µg per IP
Western Blotting	N/D**	N/A*

*N/A = Data Not Available. **N/D = Not Determined

Space filling model of mouse IgG. The two heavy chains are shown in blue and the two light chains are shown in red.

Product	Description	Cat. No.	Size	Price
Anti-5-Methylcytosine	Mouse monoclonal antibody developed to facilitate the differentiation between methylated and non-methylated cytosines in DNA.	A3001-50	50 µg/50 µl	$152.00
		A3001-200	200 µg/200 µl	$442.00

Methylase for Complete, *In Vitro* DNA Methylation

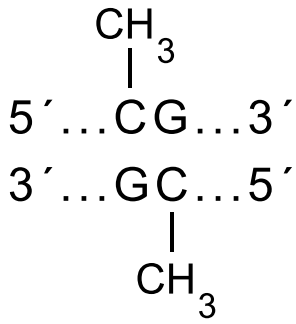
CpG Methylase (M.SssI)

- For complete, *in vitro* methylation of DNA for methylation analysis.
- Methylation of chromatin DNA for DNA accessibility studies.
- Inhibition of endonucleases with overlapping CpG sequence recognition.
- [^{3}H]-labeling of DNA.

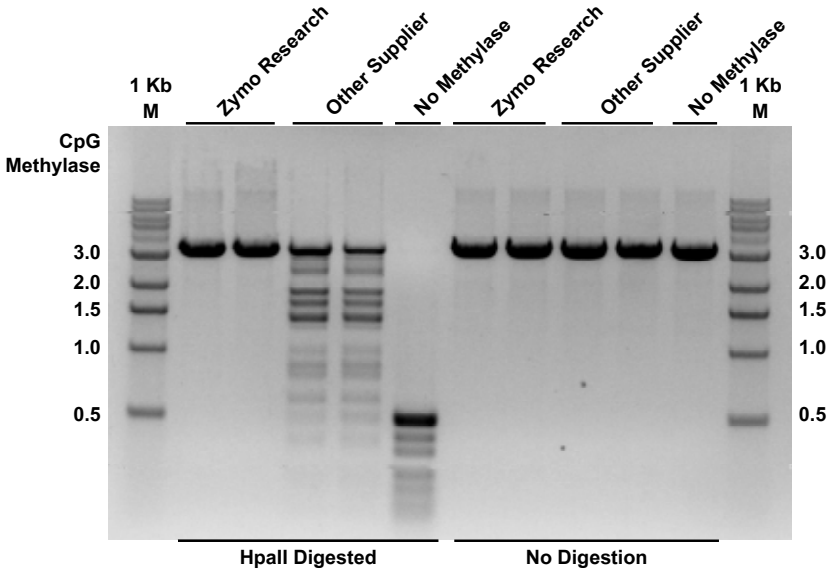
The **CpG Methylase** (EC 2.1.1.37) from Zymo Research completely methylates all cytosines (C^5) in double-stranded, non-methylated and hemi-methylated DNA having the dinucleotide sequence 5′…CpG…3′. This recombinant Methylase is isolated from an *E. coli* strain that expresses the methyltransferase gene from *Spiroplasma* sp. strain MQ1. Reaction conditions have been optimized to maximize the processivity of the enzyme to ensure rapid, complete, and reproducible methylation of DNA for accurate DNA methylation analysis.

Enzyme Concentration: 4 units/µl.

Unit Definition: One unit is defined as the amount of enzyme required to protect 1 µg of λ DNA against cleavage by BstUI restriction endonuclease in a total reaction volume of 20 µl for 1 hour at 37°C.



CpG Methylase methylates all cytosine residues in double-stranded, CpG context.



The CpG Methylase from Zymo Research catalyzes complete methylation

Methylase activities of CpG Methylase from Zymo Research versus that of another supplier were tested for complete methylation of equivalent amounts of a linearized plasmid DNA using reaction conditions recommended by the supplier. Completion of CpG methylation was assessed by resistance to digestion with a methylation-specific endonuclease (HpaII) and subsequently analyzed in an agarose gel. As shown in the figure above, the CpG Methylase from Zymo Research completely methylated the CpG sites in the DNA whereas that of the other supplier did not. Samples were assayed in duplicate.

Product	Description	Cat. No.	Size	Price
CpG Methylase	Methylates all cytosine residues (C^5) in double-stranded, non-methylated and hemi-methylated DNA having dinucleotide sequence 5′…CpG…3′.	E2010	200 Units	$147.00
		E2011	400 Units	$243.00

Quick ChIP DNA Cleanup

ChIP DNA Clean & Concentrator™

- Two (2) minute DNA clean up from any step in a standard ChIP protocol.
- DNA is ideal for PCR, arrays, DNA quantification, Southern blot analysis, sequencing, and other molecular applications.

The **Chromatin Immunoprecipitation (ChIP) DNA Clean & Concentrator™** provides a hassle-free method for the rapid purification and concentration of high quality DNA from any step in a standard ChIP protocol. This includes samples that have undergone reverse cross-linking, Proteinase K or RNase A digestion, mechanical or nuclease-mediated DNA shearing, and samples eluted from chromatin-antibody-bead complexes. Additionally, this product may also be used to purify DNA from PCR and other enzymatic reactions. The specially formulated ChIP DNA Binding Buffer promotes DNA adsorption to the column in the presence of detergents, antibodies, and proteinases that are often used for ChIP. Simply add the ChIP DNA Binding Buffer to your sample and transfer the mixture to the supplied Zymo-Spin™ Column. This kit may be applied to any routine ChIP procedure to determine DNA concentration of samples that have undergone reverse cross-linking following DNA shearing. It can also be used for the removal of TES, 0.1M $NaHCO_3$ and 1% SDS from DNA eluted from chromatin-antibody-bead complexes and can be used to purify DNA from buffers containing up to 1% SDS or 5% NP-40, Tween-20, Triton X-100 or Sarkosyl.

DNA Size Limits: From 50 bp to ~ 23 kb.

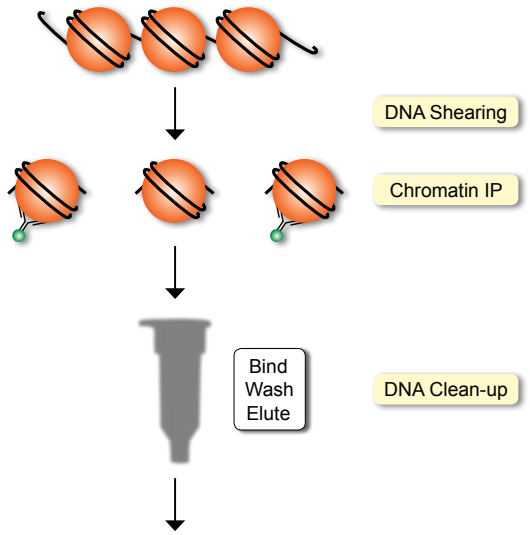
DNA Recovery: For DNA 50 bp to 10 kb the recovery is 70-90%. For DNA 11 kb to 23 kb the recovery is 50-70%.

DNA Binding Capacity: Up to 5 µg total DNA per spin column.

Detergent Tolerance: ≤ 5% Triton X-100, ≤ 5% Tween-20, ≤ 5% Sarkosyl, ≤ 1% SDS, and others.

Format: Spin Column

Overview of ChIP DNA Clean & Concentrator™ Procedure



GAL 7 ***GAL10***

Galactose Induction **- + - +**

Saccharomyces cerevisiae liquid cultures were incubated at 30°C for 45 min. in YEP medium with or without 2% galactose to induce galactose (*GAL*) genes. Following cross-linking, cell lysis, and DNA shearing, ChIP was performed using an antibody specific for RNA polymerase II. Reverse cross-linking was followed by Proteinase K digestion and DNA purification using the ChIP DNA Clean and Concentrator™. PCR was performed using primers specific to the *GAL* regions and the products were subsequently analyzed by agarose gel electrophoresis.

Product	Description	Cat. No.	Size	Price*	
ChIP DNA Clean & Concentrator™	Hassle-free method for the rapid purification and concentration of high quality DNA from any step in a standard ChIP protocol.	D5201	50 uncapped rxns.	~~$82.00~~	$41.00
		D5205	50 capped rxns.	~~$86.00~~	$43.00

*Use code: **PN1950** to receive 50% off online order. Offer good for U.S. customers only.

Reliable, High-Resolution Recovery of Small RNA from Polyacrylamide Gels

ZR small-RNA™ PAGE Recovery Kit

- Quick (*45 minute*) recovery of small RNA fragments from polyacrylamide gels.
- *Fast-Spin* column technology allows RNA to be eluted into minimal volumes (≥ 6 µl).
- Eluted RNA is ultra clean and ready for subsequent analysis and molecular manipulation.

The **ZR small-RNA™ PAGE Recovery Kit** provides an easy and efficient method for the rapid purification of high quality small RNAs in less than 45 minutes.

The ZR small-RNA™ PAGE Recovery Kit is a refinement of the "crush & soak" method that incorporates a unique buffer system together with *Fast-Spin* column technology for improved recovery and added convenience. The recovered RNA can be concentrated at the elution step in volumes as small as 6 µl and is ideal for any downstream enzymatic reaction or manipulation.

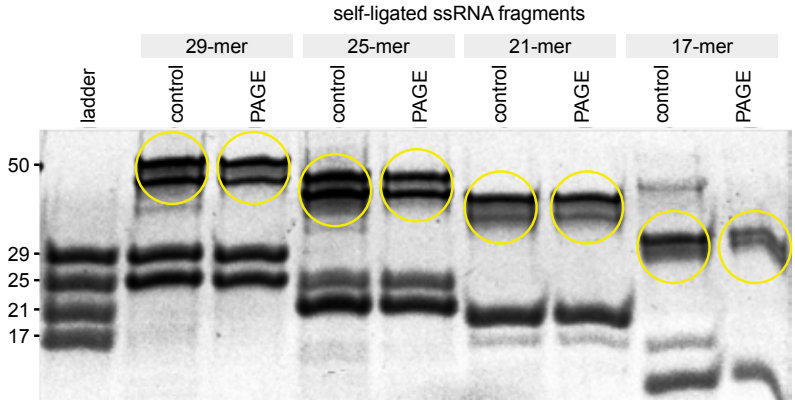
Sample Sources: Single- or double-stranded RNA fragments (17-200 nucleotides) resolved in polyacrylamide gels (tested up to 25% (w/v) polyacrylamide) stained with ethidium bromide or ssRNA-specific dyes (e.g. GelStar®).

Format: Spin column

RNA Purity: High quality total RNA (A_{260}/A_{280} > 1.8, A_{260}/A_{230} > 1.8) is recovered.

RNA Recovery: The recovery rate for fragments 17-28 nucleotides is ≥ 50%. Total binding capacity of the supplied Zymo-Spin™ IC Columns is ≥ 5 µg.

Equipment Needed: Microcentrifuge, 37-65ºC heat source, dry ice or -80ºC freezer.



ladder = ZR small RNA ladder (Cat. # R1090)
control = ssRNA oligo ligation control
PAGE = recovered ssRNA oligo self-ligated

In the image above, the RNA fragments were recovered from a 17.5% (w/v) native polyacrylamide gel using the ZR small-RNA™ PAGE Recovery Kit. All fragments shown were resolved in a native PAGE gel following ligation. T4 polynucleotide kinase and T4 RNA ligase I (New England Biolabs) were used for the phosphorylation and subsequent ligation of the ssRNA samples. Ligated RNAs are circled in yellow. RNA in the gel was visualized with GelStar® Stain (Lonza).

Product	Description	Cat. No.	Size	Price
ZR small-RNA™ PAGE Recovery Kit	Rapid purification of high quality small RNAs.	R1070	20 preps.	$121.00

Small/Total RNA Recovery from Small Volume Samples

ZR RNA MicroPrep™ and ZR RNA MiniPrep™

- Quick (*~10 minute*) RNA isolation from a wide range of sources using *Fast-Spin* column technology.
- RNA eluted into volumes ≥6 µl is suitable for use in RT-PCR and other RNA-based procedures.
- Omits the use of organic denaturants, β-mercaptoethanol and proteases.
- RNA*later*™ compatible.

The **ZR RNA MicroPrep™** and **ZR RNA MiniPrep™** both provide a quick method for high quality total RNA isolation from small amounts of cells and tissue. The kits isolate both large and small RNA species without the use of phenol or reducing agents. Small RNAs (e.g. tRNAs, microRNAs) can be recovered quantitatively following a simple adjustment within the RNA isolation protocol - no extra steps required! RNA from 10^1 to 10^5 (ZR RNA MicroPrep™) or 10^2 to 10^7 (ZR RNA MiniPrep™) cells can be eluted into volumes as little as 6 µl or 25 µl, respectively, in less than 15 minutes.

Sample Sources: Cells from culture or small amounts of solid tissue.

Sample Size: 10^1 to 10^5 (ZR RNA MicroPrep™) and 10^2 to 10^7 (ZR RNA MiniPrep™) cells in suspension or solid form.

RNA Recovery: RNA can be eluted into small volumes, ≥6 µl (ZR RNA MicroPrep™) or ≥25 µl (ZR RNA MiniPrep™) allowing for a highly concentrated sample. Maximum RNA binding capacity of provided column is ~5 µg (ZR RNA MicroPrep™) or ~25 µg (ZR RNA MiniPrep™).

RNA Purity: High quality total RNA (A_{260}/A_{280} > 1.8, A_{260}/A_{230} > 1.8) is recovered.

Storage: ≤-70ºC. The addition of RNase inhibitors is optional but highly recommended for prolonged storage.

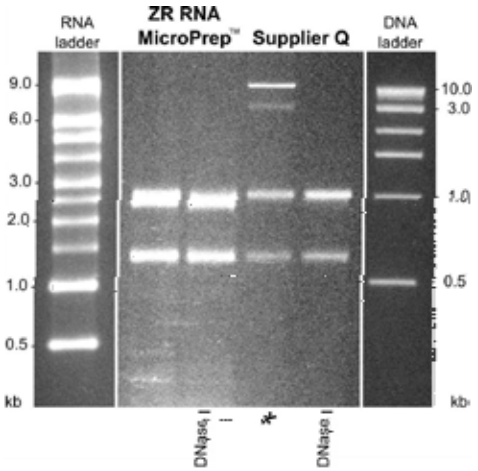
Total RNA isolated using the was resolved in an agarose gel (2-4) and small RNAs from the same sample were also resolved in a native polyacrylamide gel (6-7). Input = 10^5 yeast cells spiked with 1µg ZR small-RNA ladder (Cat. #R1090).

1 ZR 1kb DNA Marker (Cat. #M5003, M5006) [agarose gel]

2-4 2, 4, or 9 µg total RNA (yeast) + ZR small-RNA ladder mix [agarose gel]

5 ZR small-RNA ladder (17-29 bp ssRNA oligos) [PAGE]

6-7 300, 600 ng ZR small-RNA ladder isolated with ZR RNA MicroPrep™ [PAGE]



E. coli

The samples were resolved in 2% (w/v) agarose gel. RNA Millenium Markers (Ambion) and ZR 1kb DNA Marker (Zymo Research) were used.

= genomic (>10 kb) and plasmid (>3 kb) DNA contamination

DNase I = samples treated with DNase I

• 50% Off

Product	Description	Cat. No.	Size	Price*	
ZR RNA MicroPrep™	High quality total RNA isolation from small amounts of cells and tissue. RNA from 10^1 to 10^5 cells can be eluted into volumes as little as 6 µl.	R1060	50 preps.	~~$181.00~~	$90.50
		R1061	200 preps.	~~$581.00~~	$290.50
ZR RNA MiniPrep™	High quality total RNA isolation from small amounts of cells and tissue. RNA from 10^2 to 10^7 cells can be eluted into volumes as little as 25 µl.	R1064	50 preps.	~~$181.00~~	$90.50
		R1065	200 preps.	~~$581.00~~	$290.50

*Use code: **PN1950** to receive 50% off online order. Offer good for U.S. customers only.

Robust Amplificaton of Bisulfite-Treated DNA

Zymo*Taq*™ DNA Polymerase

- Reduces non-specific PCR product formation.
- Hot start DNA polymerase for robust amplification.
- Compatible with real-time and quantitative PCR and suitable for TA-cloning.

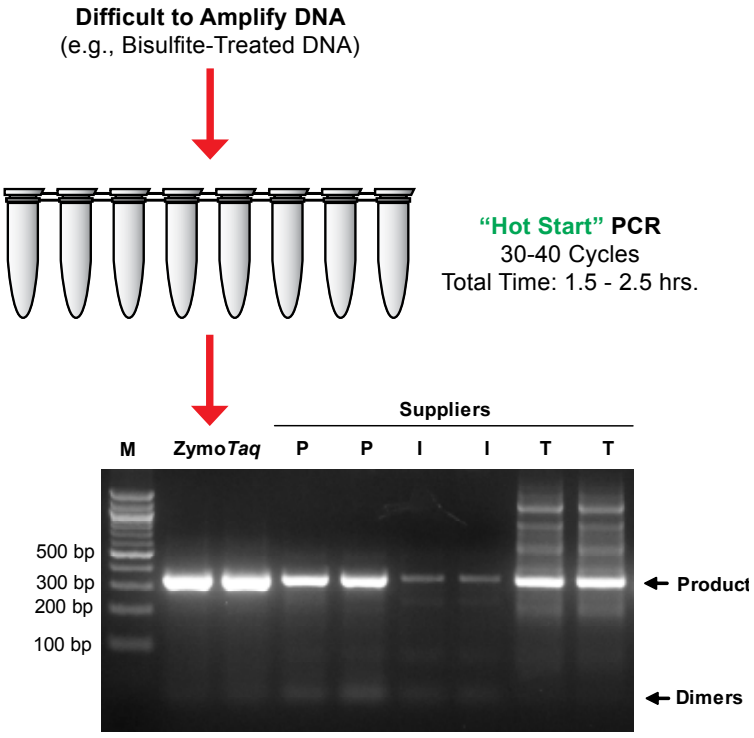
Zymo*Taq*™ DNA Polymerase is a hot start, thermal-stable DNA polymerase that reduces primer dimer and non-specific product formation that can occur when performing PCR with difficult to amplify templates. This unique product is specifically designed for the amplification of bisulfite-treated DNA for methylation detection, real-time and quantitative PCR that are SYBR™ Green and probe based. The product generates specific amplicons with little or no by-product formation. The polymerase has 3´-terminal transferase activity and the addition of "A" overhangs to amplified DNA makes it ideal for use in TA-cloning.

Zymo*Taq*™ DNA Polymerase is simple and easy to use... just heat at 95°C for 10 minutes to activate the polymerase. It is available either as a single buffer premix or as a polymerase system with components provided separately.

Unit Definition: One unit of enzyme is defined as the amount of enzyme required for the incorporation of 10 nM dNTPs into an acid-insoluble form in 30 minutes at 72°C.

Enzyme Concentration: Reaction conditions at 1X (50 µl total volume) will contain 2 U of Zymo*Taq*™ DNA polymerase.

Storage: Store at -20°C for up to 12 months. Avoid repeated freeze/thawing of reagents. Prolonged storage is at -80°C.



Methylated DNA was immunoprecipitated using the Methylated-DNA IP Kit. DNA (post-IP) was used in a PCR assay comparing Zymo Research's hot start Zymo*Taq*™ polymerase vs. that of three other suppliers. Expected amplicon size is 350 bp. PCR products (in duplicate) were separated in a 2.0% (w/v) agarose TAE/EtBr gel. The use of Zymo*Taq*™ generated specific, robust products with minimal non-specific banding as compared to the others.

Product	Description	Cat. No.	Size	Price
Zymo*Taq*™ PreMix	Single tube, 2X premix of hot start polymerase for easy PCR setup.	E2003	50 rxns.	$62.00
		E2004	200 rxns.	$198.00
Zymo*Taq*™ DNA Polymerase	Set of hot start polymerase, 2X reaction buffer,and dNTPs (each provided individually).	E2001	50 rxns.	$62.00
		E2002	200 rxns.	$198.00

Isolation of Genomic DNA Ideal for Methylation Detection

Genomic DNA Isolation Kits

Providing the Best Quality Genomic DNA for Bisulfite Conversion & PCR

- High quality total DNA from a wide range of samples (blood, tissue, feces, soil, insects, fungi, plants, etc.) in as little as ten minutes.
- Innovative buffer chemistries effectively remove PCR inhibitors during the purification process.

Zymo Research offers a range of genomic DNA isolation kits that are suitable for extracting high molecular weight DNA from a wide variety of sample types. Kits are tailor-made for specific applications and feature chemical, Proteinase K, and/or mechanical lysis technologies depending on the starting material (see table below).

DNA Isolation Method	Applications
Chemical Extraction	*Soft tissue samples* from humans, mice, etc., including: whole blood, plasma, serum, cells, buffy coat, buccal cells, biological liquids, crude homogenates, etc.
Proteinase K & Chemical Extraction	*Solid tissue samples* from humans, mice, etc., including: tailsnips, earpunches, hair*, feathers*, and FFPE* samples, as well as all of the above.
Mechanical (Bead Bashing) Homogenization & Chemical Extraction	*Tough tissues and organisms* including: insects, arthropods, fungi, gram (+/-) bacteria, and microorganisms in soil, sludge, feces, or water, as well as most of the above.

Chemical Extraction

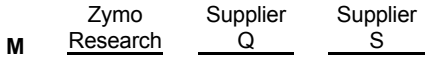
Product (format)	Size	Cat No.	Price
ZR Serum DNA Kit™ (silica bead)	scaleable	D3013	$232.00
ZR Genomic DNA I Kit™ (silica bead)	100 preps. 400 preps.	D3004 D3005	$72.00 $270.00
ZR Genomic DNA II Kit™ (spin column)	50 preps. 200 preps.	D3024 D3025	$81.00 $263.00
ZR-96 Genomic DNA Kit™ (96-well)	2x96 preps. 4x96 preps. 10x96 preps.	D3010 D3011 D3012	$162.00 $310.00 $648.00

Proteinase K & Chemical Extraction

Product (format)	Size	Cat No.	Price
Pinpoint™ Slide DNA Isolation System (spin column)	50 preps.	D3001	$225.00
ZR Genomic DNA™-Tissue MiniPrep (spin column)	50 preps. 200 preps.	D3050 D3051	$103.00 $360.00
ZR-96 Genomic DNA™-Tissue MiniPrep (96-well)	2x96 preps. 4x96 preps. 10x96 preps.	D3055 D3056 D3057	$395.00 $691.00 $1,047.00

Mechanical (Bead Bashing) Homogenization & Chemical Extraction

Product (format)	Size	Cat No.	Price
ZR Soil Microbe DNA Kit™ (spin column)	50 preps.	D6001	$182.00
ZR-96 Soil Microbe DNA Kit™ (96-well)	2x96 preps.	D6002	$559.00
ZR Fungal/Bacterial DNA Kit™ (spin column)	50 preps.	D6005	$132.00
ZR-96 Fungal/Bacterial DNA Kit ™ (96-well)	2x96 preps.	D6006	$482.00
ZR Fecal DNA Kit™ (spin column)	50 preps.	D6010	$182.00
ZR-96 Fecal DNA Kit™ (96-well)	2x96 preps.	D6011	$559.00
ZR Insect & Tissue DNA Kit-5™ (spin column)	50 preps.	D6015	$132.00
ZR Insect & Tissue DNA Kit-25™ (spin column)	50 preps.	D6016	$132.00
ZR-96 Insect & Tissue DNA Kit™ (96-well)	2x96 preps.	D6017	$482.00
ZR Plant/Seed DNA Kit™ (spin column)	50 preps.	D6020	$182.00
ZR-96 Plant/Seed DNA Kit™ (96-well)	2x96 preps.	D6021	$559.00



High yield/quality DNA is successfully isolated from frozen porcine whole blood using the . Equivalent amounts (100 µl) of blood were processed without Proteinase-K using the ZR Genomic DNA II Kit™ in half the time as compared to the kits from suppliers Q and S. Equal volumes of eluted DNA were then analyzed in duplicate in a 0.8% (w/v) TAE/agarose/ethidium bromide gel. The size marker "M" is a 1 kb ladder (Zymo Research).

DNA isolated from porcine muscle using the . Equivalent amounts (25 mg) of muscle tissue were processed using the ZR Genomic DNA™-Tissue MiniPrep after incubation with Proteinase K at 55°C for the indicated times or overnight (O/N). Equal volumes of eluted DNA were then analyzed in a 0.8% (w/v) TAE/agarose/ethidium bromide gel. The size marker "M" is a 1 kb ladder (Zymo Research).

Other Products Useful for Epigenetics Research

DNA Clean & Concentrator™-5 & -25

6 µl Elution Volume, 2 Minute Procedure, 0 µl Wash Residue Carryover!

- Clean and concentrate up to 5 µg DNA with ≥ 6 µl elution volume in 2 minutes with 0 µl wash residue carryover.

DNA is effectively desalted and concentrated from PCR, endonuclease digestions, DNA modification reactions, isotope/fluorescence labeling reactions, etc. The DCC™s facilitate the removal of DNA polymerases, modifying enzymes, RNA polymerases, ligases, kinases, nucleases, phosphatases, and restriction endonucleases, as well as free dNTPs and their analogs including radiolabled and fluorescent derivatives. Eluted DNA is suitable for PCR, arrays, ligation, sequencing, etc.

Product	Cat. No.	Size	Price
DNA Clean & Concentrator™-5 Kit (5 µg binding capacity, ≥6 µl elution)	D4003 D4004 D4013 D4014	50 uncapped preps. 200 uncapped preps. 50 capped preps. 200 capped preps.	$65.00 $231.00 $65.00 $231.00
DNA Clean & Concentrator™-25 Kit (25 µg binding capacity, ≥25 µl elution)	D4005* D4006* D4033 D4034	50 uncapped preps. 200 uncapped preps. 50 capped preps. 200 capped preps.	$65.00 $231.00 $65.00 $231.00

* Compatible with NuGEN's Ovation™ RNA Amplification System.

ZymoClean™ Gel DNA Recovery Kit

Fast Gel DNA Recovery with Minimal Elution Volume

- Quick (*15 minute*) recovery of ultra-pure DNA from TAE or TBE-buffered gels.
- Eluted DNA is well suited for use in DNA ligations, sequencing, labeling reactions, PCR, etc.

The **ZymoClean™ Gel DNA Recovery Kit** provides a simple, rapid method for the purification and concentration of high quality DNA from agarose gel slices. The entire DNA purification/concentration procedure typically takes about 15-20 minutes. The procedure is easy: simply add the specially formulated Agarose Dissolving Buffer (ADB™) to the gel slice containing your DNA sample, let dissolve, and transfer the mixture to the supplied column, then spin, wash, and elute the DNA.

Product	Cat. No.	Size	Price
ZymoClean™ Gel DNA Recovery Kit	D4001 D4002	50 preps. 200 preps.	$69.00 $260.00

OneStep™ PCR Inhibitor Removal Kit

One Step PCR Inhibitor Removal from Impure DNA Preparations

OR

- For high quality DNA (or RNA) that is free of enzymatic inhibitors including polyphenolics, humic/fulvic acids, tannins, melanin, etc.
- Fast, one-step procedure for "cleaning" impure samples prior to PCR, sequencing, RT, or other enzymatic reactions.

The ***OneStep***™ and ***OneStep-96***™ **PCR Inhibitor Removal Kits** contain all the components necessary to efficiently remove contaminants from DNA and RNA preparations that can inhibit downstream enzymatic reactions such as PCR and RT, respectively. The column/plate matrices have been specifically designed for the efficient removal of polyphenolic compounds, humic/fulvic acids, tannins, melanin, etc. Polyphenolic compounds can contaminate DNA and RNA preparations from soil, plants, skin, and fecal samples amongst others. Sample cleanup is as simple as: applying, spinning and recovering a sample from the column or plate.

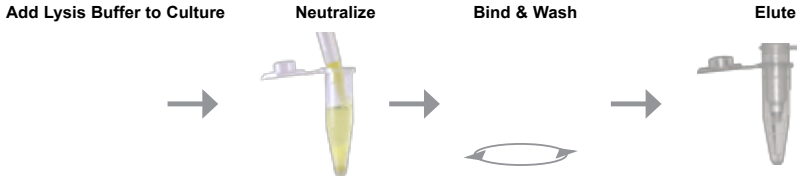
Product	Cat. No.	Size	Price
OneStep™ **PCR Inhibitor Removal Kit**	D6030	50 preps.	$97.00
OneStep-96™ **PCR Inhibitor Removal Kit**	D6035	2x96 preps.	$297.00

Zyppy™ Plasmid Miniprep Kit

Plasmid Purification Directly from E. coli *Cultures*

- Pellet-Free procedure omits conventional cell-pelleting and resuspension steps.
- The fastest, simplest procedure for purifying transfection quality plasmid DNA.

The Zyppy™ Plasmid Miniprep Kit is the fastest and simplest method available to efficiently purify plasmid DNA from *E. coli*. The purified plasmid DNA is of the highest quality and is well suited for use in bacterial transformation, restriction endonuclease digestion, DNA ligation, PCR, transcription, sequencing, transfections, and other sensitive downstream applications.



Product	Cat. No.	Size	Price
Zyppy™ Plasmid Miniprep Kit	D4036 D4019 D4020	50 preps. 100 preps. 400 preps.	$52.00 $95.00 $320.00

ZR DNA Sequencing Clean-up Kit™

Simplest, Most Reliable Method for Dye-Terminator Removal From DNA

- Quick (*2 minute*) Bind, Wash, Elute Procedure.
- Complete elimination of “dye blobs” with high quality Phred scores and long read lengths.

The **ZR DNA Sequencing Clean-up Kit™** provides a simple method for the rapid removal of post-cycle sequencing reaction contaminants (i.e., unincorporated fluorescent dyes, residual salts, dNTPs, primers, and enzymes) from DNA extension products. These contaminants, can often interfere with the quality and signal strength of sequencing data.

Product	Cat. No.	Size	Price
ZR DNA Sequencing Clean-up Kit™	D4050 D4051	50 preps. 200 preps.	$82.00 $241.00
ZR-96 DNA Sequencing Clean-up Kit™	D4052 D4053	2x96 preps. 4x96 preps.	$132.00 $272.00

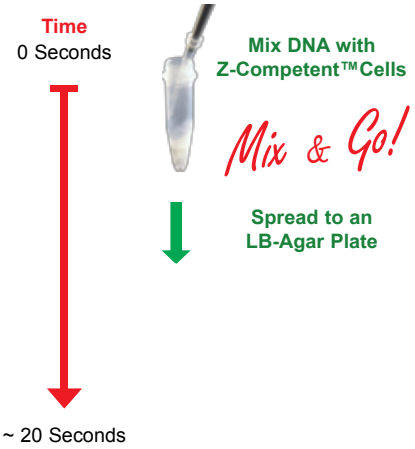
Z-Competent™ *E. coli* Transformation Kit

DNA Transformation in 20 Seconds Without Heat Shock!

Make your own highly competent *E. coli* cells...

- No Heat Shock!
- No Lengthy Incubations!
- No Outgrowth Procedures!
- No Wait!!!

The **Z-Competent™ *E. coli* Transformation Kit** enables the researcher to produce their own “home-made” Z-Competent™ *E. coli* cells from most K12 and B strain derivatives that bypass the requirement of heat-shocking and related procedures. Instead, transformation can be performed by adding DNA to the Z-Competent™ cells and the mixture spread directly to a culture plate - *Mix & Go!*



Product	Cat. No.	Size	Price
Z-Competent™ *E. coli* Transformation Kit	T3001	For up to 20 ml cells	$92.00

Premade Z-Competent™ *E. coli* are also available. Visit www.zymoresearch.com for details.

Tips for Successful Bisulfite Conversion and DNA Methylation Analysis

Visualizing Bisulfite-Treated DNA

Bisulfite-treated DNA can be visualized in agarose/EtBr gels following electrophoresis using a standard UV-light source. However, cooling the gel on ice for 10-15 minutes prior to visualization will greatly enhance the resolution and apparent banding of the DNA.

Visualizing bisulfite-treated DNA in agarose/EtBr gels is best done after In the figures above, bisulfite-treated salmon sperm DNA was desulphonated then purified using the EZ Bisulfite DNA Clean-up Kit™. The DNA, mostly single stranded, was then separated in a 0.8 % (w/v) agarose/TAE/EtBr gel and visualized with a UV-light source immediately following electrophoresis (room temp) and after chilling the gel on ice for 15 minutes. M is a 100 bp DNA ladder (Zymo Research).

Quantifying Bisulfite-Treated DNA

Following bisulfite treatment of genomic DNA, non-methylated cytosine residues are converted into uracil. The recovered DNA is typically A, U, and T-rich. The original base-pairing no longer exists. Instead, it is single stranded with limited non-specific base-pairing at room temperature. The absorption coefficient at 260 nm resembles that of RNA. Use a value of 40 µg/ml for A_{260} = 1.0 when determining the concentration of the recovered bisulfite-treated DNA.

PCR of Bisulfite Converted DNA

PCR Primer Design. Generally, primers of 24 to 32 bases are required for amplification of bisulfite converted DNA. For most eukaryotes, all non-methylated cytosine residues will be converted into uracil during the bisulfite treatment. These Cs should be treated as Ts for primer design purposes. For example, for the sequence 5′-AACCTTACAGGCAC-3′, the corresponding primer after bisulfite treatment should be 5′-AA**TT**TTA**T**AGG**T**A**T**-3′. **MethPrimerDB** (http://medgen.ugent.be/methprimerdb) and **MethPrimer** (http://www.urogene.org/methprimer/index1.html) are useful resources when designing primers for bisulfite PCR.

If the primer contains CpG dinucleotides with uncertain methylation status, then mixed bases with C and T can be used. Usually, there should be no more than three mixed positions per primer and they should be located toward the 5′ end of the primer. It is not recommended to have mixed bases located at the 3′ end of the primer.

PCR Conditions. Usually, 35 to 40 cycles are required for successful PCR amplification of bisulfite converted DNA. Optimal amplicon size is between 150 - 300 bp; however larger amplicons (up to 1 kb) can be generated with optimization of the bisulfite reaction and PCR conditions. We have found that annealing temperatures between 55 - 60°C typically work well. As most non-methylated cytosine residues are converted into uracil, the bisulfite-treated DNA is usually AT-rich and has low GC composition. Thus, it may be necessary to reduce the annealing temperature accordingly.

Non-specific PCR amplification is relatively common with bisulfite treated DNA due to its AT-rich nature. PCR using hot start polymerases (e.g., Zymo*Taq*™ DNA Polymerase, page 37) is strongly recommended for the amplification of bisulfite-treated DNA.

FAQs

Q: Should the input DNA be dissolved in TE, water, or some other buffer prior to treatment with Zymo's bisulfite kits.

A: *Water, TE or modified TE buffers can be used to dissolve DNA and do not interfere with the conversion process.*

Q: At what temperature and for how long can DNA be stored following bisulfite treatment?

A: *The sample should be stored at ≤ -20ºC whenever possible. The quality of the DNA should remain relatively unchanged for up to 3 months.*

Q: Why am I not getting "complete" conversion of DNA using the EZ DNA Methylation-Direct™ Kit?

A: *1) If sampling solid tissue, then it is most likely that too much sample was processed, resulting in incomplete DNA conversion. 2) If sampling FFPE tissue, then it is probable that the DNA was extensively damaged and/or cross-linked resulting in incomplete DNA conversion. 3) If debris is not removed by centrifugation from the Proteinase K digestion, it may interfere with the bisulfite conversion process resulting in incomplete conversion of the DNA.*

Q: Which *Taq* polymerase(s) do you recommend for PCR amplification of converted DNA?

A: *We recommend a "hot start" DNA polymerase (e.g., Zymo*Taq™ *DNA Polymerase, page 37).*

Q: Why are there two different catalog numbers for the EZ-96 DNA Methylation™ product line?

A: *The two different catalog numbers are used to differentiate between the binding plates that are included in the kits. Deep and shallow-well binding plates are available to accommodate most rotors and microplate carriers. Below shows a comparison of the two binding plates.*

Plate Name	Silicon-A™	Zymo-Spin™ I-96
Style	Shallow-Well	Deep-Well
Height of Binding Plate	19 mm	35 mm
Height of Binding/Collection Plate Assembly	43 mm	60 mm
Binding Cap./Minimum Elution Vol.	5 µg/30 µl per well	5 µg/15 µl per well
Catalog Numbers	D5003, D5007, D5022, D5027	D5004, D5008, D5023, D5028

Q: Are your bisulfite kits compatible with technologies from Illumina?

A: *Yes. The EZ DNA Methylation™ Kit technologies from Zymo Research are ideal for Illumina's GoldenGate® and Infinium® Assays.*

Q: What downstream analytical procedures can be used for DNA converted with the EZ DNA Methylation Kits?

A: *DNA converted using any of our EZ DNA Methylation™ kits is ideal for subsequent analysis by canonical sequencing methods, Ms-SNuPE, COBRA, , Bisulfite-PCR, MSP, Bisulfite-Sequencing, mass spectroscopy (e.g., EpiTYPER® from Sequenom), as well as other methods for analysis. (Please see Epigenetics Glossary on page 43 for a detailed explanation of some of these methods).*

EPIGENETICS GLOSSARY

5mC: 5-methylcytosine.

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

Bisulfite conversion: the deamination of non-methylated cytosine bases to uracil by treatment with sodium bisulfite ($NaHSO_3$); 5mC 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 bases within coding sequences of actively transcribed genes found within euchromatin.

ChIP-on-chip: a combination of chromatin immunoprecipitation and DNA hybridization to genomic microarrays.

ChIP-Seq: a method combining chromatin immunoprecipitation and DNA sequencing to analyze specific DNA-protein interactions.

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

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

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.

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

Constitutive heterochromatin: heterochromatin located near centromeres and irreversibly silenced; DNA within constitutive heterochromatin is typically AT-rich; also known as pericentric heterochromatin.

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 *de novo* 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 binds to a DNA sequence, usually the 5th carbon of the cytosine pyrimidine ring in a CpG dinuclotide, 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 5mC class (EC 2.1.1.37) 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 *de novo* methyltransferases DNMT3a and DNMT3b, which function during embryonic development and shortly after birth.

Dosage compensation: equalizing between the two sexes the expression of genes encoded on the X-chromosome.

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 marker: a modifying moiety that carries an epigenetic signal; examples: methylation of DNA, methylation, acetylation, phosphorylation, ubiquitylation, 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 DNA methyltransferase inhibitors (e.g. 5-azacytidine, 5-aza-2'-deoxycytidine) to target silenced tumor suppressor genes in cancer patients.

Epigenetics: genetic traits heritable through cell division and sexual reproduction that are independent of DNA sequence; epigenetic factors include chromatin conformation, DNA methylation, histone modification, and RNAi.

Epigenome: all epigenetic markers 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 sequence (such as CG or CHG) being methylated on one strand only.

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

Histone acetyltransferase (HAT): enzyme that acetylates histones at specific lysine residues; EC 2.3.1.48.

Histone deacetylase (HDAC): enzyme that remove acetyl groups from N(6)-acetyl-lysine residues on a histone; EC 3.5.1.98.

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

Histone code: the hypothesis that the location and species of histone modification, through chromatin remodeling or recruitment of transcription factors, predicts the effect of that modification on gene expression.

Histone methyltransferase: enzyme that adds a methyl group to specific histone residues; histone-lysine N-methyltransferase: EC 2.1.1.43; histone-arginine N-methyltransferase: EC 2.1.1.125.

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

Histone variants: paralogous histones that can replace major 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.

iPS: induced pluripotent stem cells; differentiated cells reprogrammed to pluripotency by ectopic expression of the reprogramming factors Oct4, Sox2, Klf4, and c-Myc.

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 sensitive 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.

Methyl-DIP: Methylated DNA Immunoprecipitation; a technique used to identify methylated DNA by precipitation with an antibody specific for 5mC and hybridization to a genomic microarray.

Ms-SNuPE (Methylation-sensitive single-nucleotide primer extension): 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.

Multipotency: the property of stem cells to differentiate into cells of a specific lineage; example: hematopoietic stem cells.

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 histone 2A, histone 2B, histone 3, and histone 4.

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.

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

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

siRNA (small interfering RNAs): 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 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.

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 females is rendered dormant.

Xist: X inactive specific transcript; the non-coding RNA transcribed from the X-inactivation center (Xic) that binds in *cis* 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.

The image above shows genetically identical mice with differing tail morphologies: one kinky and one straight. A mutation in the axin gene (*axin-fused*) results in mice with kinky tails, and the degree of "kinkiness" varies among genetically identical littermates. For these mice, it was discovered that epigenetic marks were responsible for the resulting phenotype and that these marks could be inherited between sexual generations. Credit: Emma Whitelaw, University of Sydney, Australia.

Product	Size	Cat No. (Format)
Genomic DNA Isolation		
Pinpoint™ Slide DNA Isolation System	50 preps.	D3001
ZR Serum DNA Kit™	scaleable	D3013
ZR Genomic DNA I Kit™	100 preps. 400 preps.	D3004 D3005
ZR Genomic DNA II Kit™	50 preps. 200 preps.	D3024 D3025
ZR-96 Genomic DNA Kit™	2x96 preps. 4x96 preps. 10x96 preps.	D3010 D3011 D3012
ZR Genomic DNA™-Tissue MiniPrep	50 preps. 200 preps.	D3050 D3051
ZR-96 Genomic DNA™-Tissue MiniPrep	2x96 preps. 4x96 preps. 10x96 preps.	D3055 D3056 D3057
ZR Soil Microbe DNA Kit™	50 preps.	D6001
ZR-96 Soil Microbe DNA Kit™	2x96 preps.	D6002
ZR Fungal/Bacterial DNA Kit™	50 preps.	D6005
ZR-96 Fungal/Bacterial DNA Kit™	2x96 preps.	D6006
ZR Fecal DNA Kit™	50 preps.	D6010
ZR-96 Fecal DNA Kit™	2x96 preps.	D6011
ZR Plant/Seed DNA Kit™	50 preps.	D6020
ZR-96 Plant/Seed DNA Kit™	2x96 preps.	D6021
ZR Insect & Tissue DNA Kit-5™	50 preps.	D6015
ZR Insect & Tissue DNA Kit-25™	50 preps.	D6016
ZR-96 Insect & Tissue DNA Kit™	2x96 preps.	D6017
Other Products Useful for Epigenetics Research		
DNA Clean & Concentrator™-5 Kit (5 µg binding capacity, ≥6 µl elution)	50 preps. 200 preps. 50 preps. 200 preps.	D4003 (uncapped) D4004 (uncapped) D4013 (capped) D4014 (capped)
DNA Clean & Concentrator™-25 Kit (25 µg binding capacity, ≥25 µl elution)	50 preps. 200 preps. 50 preps. 200 preps.	D4005 (uncapped) D4006 (uncapped) D4033 (capped) D4034 (capped)
ZymoClean™ Gel DNA Recovery Kit	50 preps. 200 preps.	D4001 D4002
Zyppy™ Plasmid Miniprep Kit	50 preps. 100 preps. 400 preps.	D4036 D4019 D4020
ZR DNA Sequencing Clean-up Kit™	50 preps. 200 preps.	D4050 D4051
ZR-96 DNA Sequencing Clean-up Kit™	2x96 preps. 4x96 preps.	D4052 D4053
***OneStep*™ PCR Inhibitor Removal Kit**	50 preps.	D6030
***OneStep-96*™ PCR Inhibitor Removal Kit**	2x96 preps.	D6035
Z-Competent™ *E. coli* Transformation Kit	For up to 20 ml cells	T3001

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ZR Serum DNA Kit™	scaleable	D3013
ZR Genomic DNA I Kit™	100 preps. 400 preps.	D3004 D3005
ZR Genomic DNA II Kit™	50 preps. 200 preps.	D3024 D3025
ZR-96 Genomic DNA Kit™	2x96 preps. 4x96 preps. 10x96 preps.	D3010 D3011 D3012
ZR Genomic DNA™-Tissue MiniPrep	50 preps. 200 preps.	D3050 D3051
ZR-96 Genomic DNA™-Tissue MiniPrep	2x96 preps. 4x96 preps. 10x96 preps.	D3055 D3056 D3057
ZR Soil Microbe DNA Kit™	50 preps.	D6001
ZR-96 Soil Microbe DNA Kit™	2x96 preps.	D6002
ZR Fungal/Bacterial DNA Kit™	50 preps.	D6005
ZR-96 Fungal/Bacterial DNA Kit™	2x96 preps.	D6006
ZR Fecal DNA Kit™	50 preps.	D6010
ZR-96 Fecal DNA Kit™	2x96 preps.	D6011
ZR Plant/Seed DNA Kit™	50 preps.	D6020
ZR-96 Plant/Seed DNA Kit™	2x96 preps.	D6021
ZR Insect & Tissue DNA Kit-5™	50 preps.	D6015
ZR Insect & Tissue DNA Kit-25™	50 preps.	D6016
ZR-96 Insect & Tissue DNA Kit™	2x96 preps.	D6017
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DNA Clean & Concentrator™-25 Kit (25 µg binding capacity, ≥25 µl elution)	50 preps. 200 preps. 50 preps. 200 preps.	D4005 (uncapped) D4006 (uncapped) D4033 (capped) D4034 (capped)
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ZR DNA Sequencing Clean-up Kit™	50 preps. 200 preps.	D4050 D4051
ZR-96 DNA Sequencing Clean-up Kit™	2x96 preps. 4x96 preps.	D4052 D4053
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***OneStep-96*™ PCR Inhibitor Removal Kit**	2x96 preps.	D6035
Z-Competent™ *E. coli* Transformation Kit	For up to 20 ml cells	T3001

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