ZYMO RESEARCH

The Beauty of Science is to Make Things Simple

INSTRUCTION MANUAL

EZ DNA Methylation RRBS™ Library Prep Kit

Catalog Nos. D5460S

Highlights

- **Simple workflow:** Prepare Reduced Representation Bisulfite Sequencing (RRBS) libraries in as little as 2 hours of hands-on time
- **Low input:** The only RRBS kit that produces NGS libraries from **≥10 ng** of genomic DNA
- **Accurate and reproducible:** Unbiased methylation calling and reproducible CpG coverage

Contents

ZYMO RESEARCH CORP.

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Satisfaction of all Zymo Research products is guaranteed. If you should be dissatisfied with this product, please call 1-888-882-9682.

Notes:

[1]Please refer to Appendix item 7 for the information of how to use this component. This component is not required for mammalian samples or samples with low methylated cytosines in non-CpG context.

[2,3]DNA Wash Buffer and M-Wash Buffer in D5460S come as concentrates and require the addition of ethanol.

For **Assistance**, please contact Zymo Research Technical Support at 1-888-882-9682 or e-mail tech@zymoresearch.com.

Product Contents

EZ DNA Methylation RRBS™ Library Prep Kit (Kit Size)	**D5460S** (12 preps)	**Storage Temp**
MspI (20 U/µl)	20 µl	-20 °C
10× RRBS Buffer	300 µl	-20 °C
rATP (10 mM)	15 µl	-20 °C
RRBS Adapters (10 µM)	15 µl	-20 °C
T4 DNA Ligase (400 U/µl)	15 µl	-20 °C
5-Methylcytosine dNTP Mix (10 mM)	85 µl	-20 °C
Taq DNA Polymerase (2 U/µl)	9 µl	-20 °C
LibraryAmp Master Mix (2×)	250 µl × 2	-20 °C
Index Primer Sets - 12 Sets (10 µM)	30 µl	-20 °C
***E. coli* Non-Methylated Genomic DNA**[1]	5 µg/20 µl	-20 °C
Lightning Conversion Reagent	1.5 ml × 2	RT
M-Binding Buffer	20 ml	RT
M-Wash Buffer[2]	6 ml	RT
L-Desulphonation Buffer	10 ml	RT
DNA Elution Buffer	1 ml	RT
Zymo-Spin™ IC Columns	40	RT
Collection Tubes	40	RT
DNA Binding Buffer	25 ml	RT
DNA Wash Buffer[3]	6 ml	RT
DNase/RNase-free Water	4 ml	RT

Note - Integrity of kit components is guaranteed for up to 6 months from date of purchase. Reagents are routinely tested on a lot-to-lot basis to ensure they provide maximal performance and reliability.

Note: Kit components supplied in two (2) boxes: Box 1 contains all components that can be stored at room temperature (RT) and Box 2 contains components that must be stored at -20 °C.

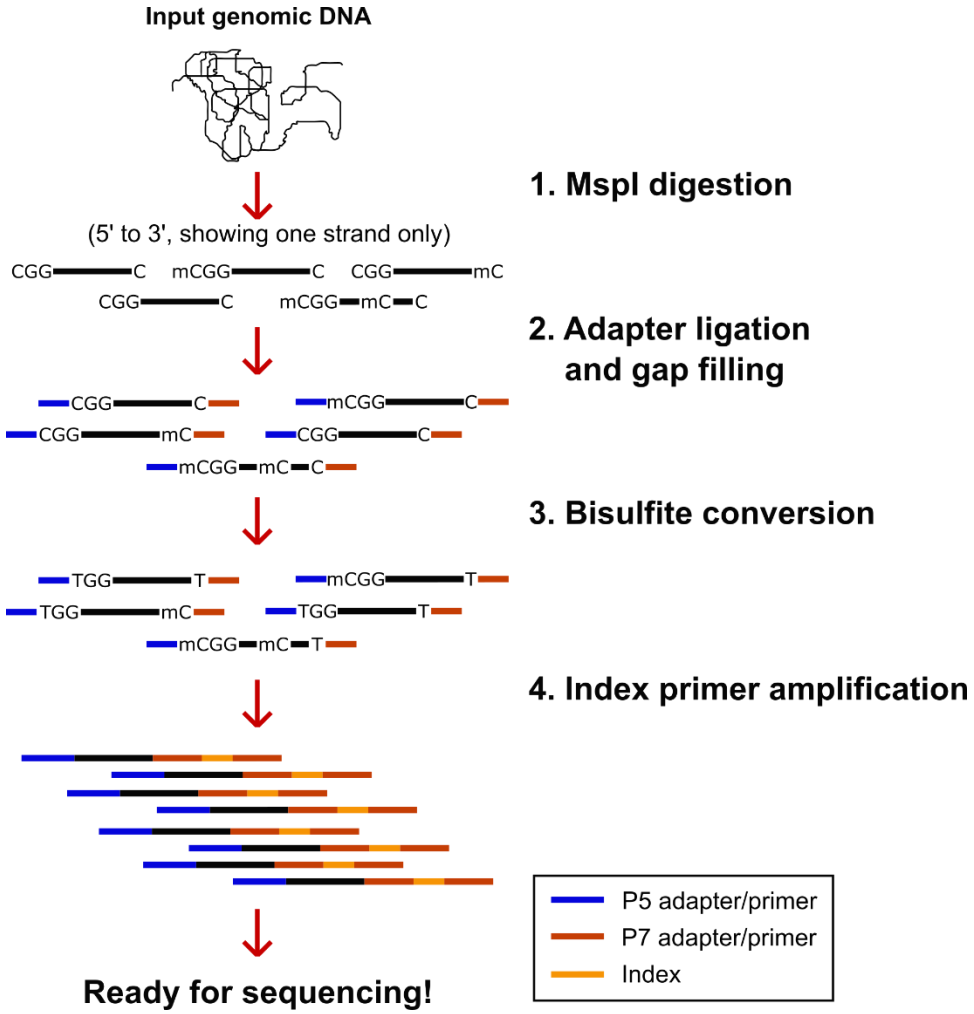
Specifications

- **Sample Sources** – The protocol is designed for 10 – 500 ng genomic DNA input. DNA should be free of enzymatic inhibitors and can be suspended in water, TE, or a low salt buffer. DNA with low 260/280 or 260/230 ratios should be purified prior to processing using the **Genomic DNA Clean & Concentrator™ (Cat. No. D4010)**. This platform is not recommended for samples from species with low CpG density.
- **Sequencing Platform Compatibility** – Libraries are compatible with all Illumina's sequencing platforms except HiSeq X Series.
- **Equipment Required** – Microcentrifuge, thermocycler

Note - ™ Trademarks of Zymo Research Corporation. This product is for research use only and should only be used by trained professionals. It is not intended for use in diagnostic procedures. Some reagents included with this kit are irritants. Wear protective gloves and eye protection. Follow the safety guidelines and rules enacted by your research institution or facility.

Product Description

RRBS (Reduced Representation Bisulfite Sequencing) combines restriction enzyme digestion with bisulfite sequencing to enrich for a CpG-dense fraction of the genome and profile DNA methylation at single-nucleotide resolution. DNA methylation occurs predominantly in CpG contexts, and these CpG dinucleotides are more abundant in select regions of the genome. By enriching for CpG-dense regions and sequencing only the fragments pertaining to those regions, the RRBS platform allows for the capture of a significant amount of methylation data while reducing the amount of sequencing, leading to a substantially decreased cost. This combination makes RRBS the perfect platform for pilot studies. Libraries prepared by **EZ DNA Methylation RRBS™ Library Prep Kit** cover ≥75% CpG islands, >70% gene promoters, ≥75% gene bodies, and 2.5-4 million unique CpG sites at 5-10x coverage when applying to human samples.



Epigenetic services are available at Zymo Research. Please inquire at: services@zymoresearch.com.

Notes:

Components that are stored at -20 °C are better to be thawed and kept on ice.

[1]A master mix of the reagents is recommended when processing multiple samples in parallel.

[2]Refer to Appendix item 7 to ensure the input samples are appropriate for library preparation and bioinformatic analysis.

Buffer Preparation: *Before starting…*

✓ Add 24 ml 100% ethanol (26 ml 95% ethanol) to the 6 ml **DNA Wash Buffer** concentrate (D4003-2-6).
✓ Add 24 ml 100% ethanol (26 ml 95% ethanol) to the 6 ml **M-Wash Buffer** concentrate (D5001-4).

Protocol

Section 1: MspI Digestion
Section 2: Adapter Ligation
Section 3: Bisulfite Conversion
Section 4: Index Primer Amplification
Section 5: Library Validation and Quantification

Section 1: MspI Digestion

1. Mix the following components in a 0.2 ml PCR tube[1]:

Components	Volume
Genomic DNA in TE buffer/H_2O (10-500 ng recommended)[2]	X µl
10× RRBS Buffer	4 µl
MspI (20 U/µl)	0.5 µl
DNase/RNase-free Water	(35.5 – X) µl
Total	**40 µl**

2. Incubate the mixture in a thermocycler according to the following:

Temperature °C	Time	Notes
37	4 h	
4	Forever	4°C storage step is optional

Section 2: Adapter Ligation

1. Add the following components to the product from Section 1 for adapter ligation:

Components	Volume
10× RRBS Buffer	1 µl
rATP (10 mM)	0.5 µl
RRBS Adapters (10 µM)	0.5 µl
MspI (20 U/µl)	1 µl
T4 DNA Ligase (400 U/µl)	1 µl
DNase/RNase-free Water	6 µl
Product from **Section 1**	40 µl
Total	**50 µl**

2. Mix well. Incubate the ligation mixture in a thermocycler[1] according to the following:

Step	Temperature °C	Time	Notes
1	21	3 h	
2	37	1 h	
3	20	1 h	
4	Repeat Steps 2-3		
5	4	Forever	4°C storage step is optional

3. Add the following gap filling components to the ligation product:

Components	Volume
Taq DNA Polymerase (2 U/µl)	0.5 µl
5-methylcytosine dNTP Mix (10 mM)	1.5 µl
Ligation product from **step 1 and 2**	50 µl
Total	**52 µl**

4. Mix well. Incubate the mixture in a thermocycler at 74°C for 30 minutes.

5. Purify the product with the **DNA Clean-up & Concentrator™ (DCC™)** as follows and elute in 20 µl **DNA Elution Buffer**.

5.1 In a 1.5 ml tube, add a 7:1 volume ratio of **DNA Binding Buffer** to the product from **step 4**. (i.e. add 364 µl DNA Binding Buffer to 52 µl product), mix well, and transfer to a **Zymo-Spin™ IC column** in a **Collection Tube[2]**. Spin at ≥ 10,000 x *g* for 30 seconds.

5.2 Add 200 µl **DNA Wash Buffer** to the column. Spin at ≥ 10,000 x *g* for 30 seconds. Discard the flow-through. Repeat this wash step[3].

5.3 Transfer the **Zymo-Spin™ IC column** to a 1.5 ml tube. Add 20 µl **DNA Elution Buffer** directly to the column matrix and let stand for 1 minute at room temperature[4]. Spin at ≥ 10,000 x *g* for 30 seconds to elute.

Section 3: Bisulfite Conversion

1. Mix the following components in a 0.2 ml PCR tube:

Components	Volume
Lightning Conversion Reagent	130 µl
Product from **Section 2**	20 µl
Total[5]	**150 µl**

2. Incubate the mixture in a thermocycler[5] according to the following:

Notes:

[1]The thermocycler program takes 7 hours. We recommend setting up the reaction and leave for overnight to complete step 2 in Section 2 and continue the workflow the following day for your convenience.

Notes:

[2]Collection tubes can hold up to 800 µl liquid.

[3]A dry spin can be included after the wash steps to ensure complete removal of wash buffer.

[4]Yields can often be enhanced if the **DNA Elution Buffer** is heated before application to the column or doing two sequential elutions of 10 µl.

[5]Some thermocyclers may not allow a reaction volume larger than 100 ul. Simply set the maximum allowed if encountering such a case.

Temperature °C	Time	Notes
98	8 min	
54	1 h	
4	≤ 20 h	4°C storage step is optional

3. Add 600 µl **M-Binding Buffer** to a **Zymo-Spin™ IC Column** in a **Collection Tube**. Add the bisulfite-converted sample to the column and invert several times to mix. Spin at ≥ 10,000 x *g* for 30 seconds.

4. Discard the flow-through from the **Collection Tube** and add 100 µl **M-Wash Buffer** to the column. Spin at ≥ 10,000 x *g* for 30 seconds.

5. Add 200 µl **L-Desulphonation Buffer** to the column and let stand at room temperature (20°C-30°C) for 15-20 minutes[1]. After the incubation, spin at ≥ 10,000 x *g* for 30 seconds.

6. Add 200 µl **M-Wash Buffer** to the column and spin at ≥ 10,000 x *g* for 30 seconds. Discard the flow-through. Repeat this wash step.

7. Transfer the **Zymo-Spin™ IC column** to a 1.5 ml tube. Add 24 µl **DNA Elution Buffer** directly to the column matrix and let stand for 1 minute. Spin at ≥ 10,000 x *g* for 30 seconds to elute the bisulfite-converted DNA.

Notes:

[1]Incubation with L-Desulphonation Buffer for more than 20 minutes may result in degradation of input DNA and lower yields of conversed DNA.

Section 4: Index Primer Amplification

1. Mix the following components in a 0.2 ml PCR tube:

Components	Volume
LibraryAmp Master Mix (2×)	25 µl
Index Primer Set (10 µM)[2]	1 µl
Product from **Section 4**	24 µl
Total	**50 µl**

Notes:

[2]Do not use the same index primer set for multiple samples if those samples will be multiplexed together on the same sequencing lane. Refer to the Appendix for detailed information about the index primer sets.

2. Incubate the mixture in a thermocycler to amplify the libraries according to the following, with recommended amplification cycle numbers based on input gDNA amount.

Step	Temperature °C	Time
1	94	30 s
2	94	30 s
3	55	30 s
4	68	1 min
5	Repeat Steps 2-4 for **X** times	
6	68	5 min
7	4	Forever

Input gDNA (ng)	Empirical X[1]
500	7-9
300	9-11
100	11-13
30	13-15
10	15-17

3. Purify the PCR product with the **DCC™** (refer to **Section 2.5**) and elute in 15 µl **DNA Elution Buffer**.[2]

Section 5: Library Validation and Quantification

1. The size distribution of the libraries from **Section 4** can be visualized using gel electrophoresis, Agilent 2200 TapeStation, or any equivalent instrument. No index primer dimers should appear at ~ 120 bp.[3]

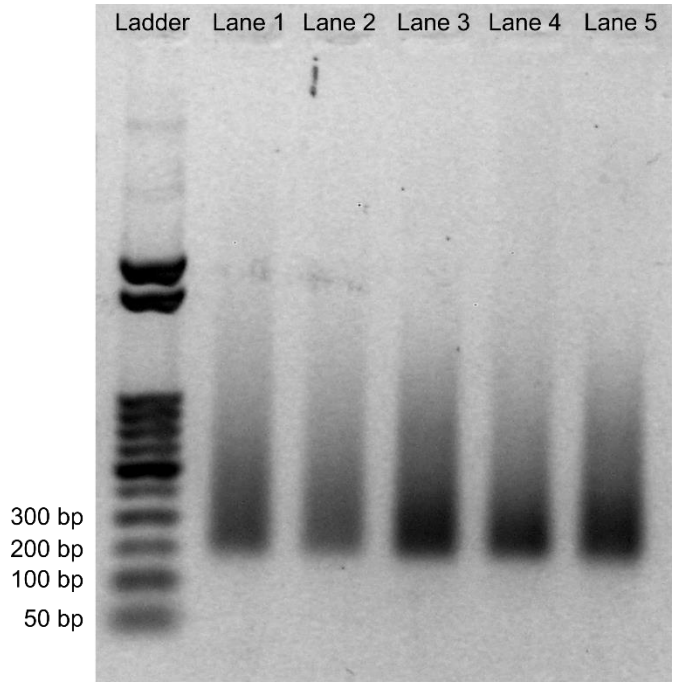


Figure 1. A 2% agarose gel (1×TAE buffer) of libraries prepared using the EZ DNA Methylation RRBS™ Library Prep Kit. The libraries in lane 1 through 5 were generated from 500, 300, 100, 30, and 10 ng of rat genomic DNA, respectively. Library sizes range from 150-500 bp.

Notes:

[1]This table should only serve as a starting point. The cycle number can vary depending on the quality of DNA and should be optimized prior to working with precious samples.

[2]If using agarose gel to validate the libraries, either go to Section 5.1 **prior to purification,** or elute to a higher volume to ensure enough libraries for all characterizations and quantification.

[3]Please refer to Appendix item 2 if primer dimers show up.

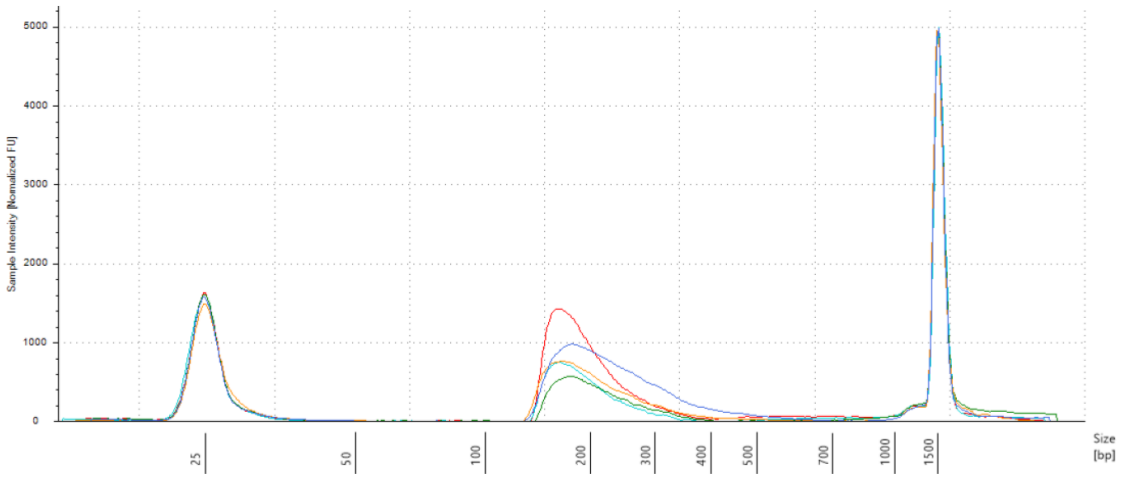


Figure 2. Five RRBS libraries were analyzed using a D1000 ScreenTape on the Agilent 2200 TapeStation. The libraries were generated from 500, 300, 100, 30, and 10 ng of rat genomic DNA, shown in blue, orange, green, red, and cyan traces, respectively. Library sizes range from 150 to 500 bp mainly. Standards were at 25 and 1500 bp, respectively.

2. KAPA Library Quantification Kits for Illumina® platforms are recommended for library quantification prior to sequencing. The libraries are now ready for sequencing.

Appendix: Additional Considerations for Library Preparation, Sequencing, and Bioinformatic Analysis

1. The kit provides 12 index primer sets allowing up to 12-plex libraries on one single lane. Please use the color balance strategy from Illumina's Index Adapters Pooling Guide to select a compatible group of index primer sets for multiplexing.

Index Primer Set	Standard Illumina Designation	TruSeq™ Single Index Sequence
A	2	CGATGT
B	4	TGACCA
C	5	ACAGTG
D	6	GCCAAT
E	7	CAGATC
F	12	CTTGTA
G	1	ATCACG
H	3	TTAGGC
I	8	ACTTGA
J	9	GATCAG
K	10	TAGCTT
L	11	GGCTAC

*The index sequences correspond to Illumina TruSeq™ Single Indexes index sequences for multiplexing and are copyrighted to Illumina, Inc. Oligonucleotide sequences © 2019 Illumina, Inc. All rights reserved.

2. The kit is designed to reduce the chance of primer dimers after index primer amplification at the recommended numbers of PCR cycle. However, if a primer dimer peak shows up at ~ 120 bp on the size distribution, a bead cleanup for size selection is recommended to remove the dimers for better sequencing quality. An example product for such bead cleanup is D4084/D4085 Select-a-Size DNA Clean & Concentrator MagBead Kit.

3. Libraries prepared with this kit are non-directional. As such, the original-top, original-bottom, and the complementary strands for each will be represented.

4. Each library should be sequenced to obtain at least 30 million reads for an approximately 10× average coverage of CpG sites (numbers based on libraries from human or mouse gDNA and may vary from sample to sample). We recommend 50 bp paired-end sequencing with Illumina's TruSeq™ technology.

5. RRBS libraries have first few base bias due to MspI digestion and an unbalanced genomic composition with high AT content due to bisulfite conversion. Therefore, follow the sequencer's instruction to spike in a PhiX control at a recommended percentage of the total libraries to improve cross-talk and phasing calculation and balance the sequence diversity.

6. To trim the adapter sequences and remove the filled-in nucleotides introduced during library preparation, we highly recommend applying a publicly accessible wrapper script called **Trim Galore!**[1] to the raw reads. Apply the parameters '—non_directional', '—rrbs',

Notes:

[1]**Trim Galore!** and its up-to-date documentations can be accessed at https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/.

and ‘paired-end’ when executing the script to ensure correct analysis.

```
    Example: trim_galore ––non_directional --rrbs ––paired <read1.fq.gz>
<read2.fq.gz>
```

7. To calculate bisulfite conversion rate, we suggest using the percentage of unmethylated cytosines in non-CpG contexts for mammalian samples; if the samples may be significantly methylated on cytosines in non-CpG contexts, the provided unmethylated *E.coli* genomic DNA (D5016) can be spiked into the input samples (2-5 wt%, i.e., 2-5 ng of *E.coli* genomic DNA to 100 ng of input genomic DNA) before starting the library preparation and calculate the percentage of unmethylated cytosines in non-CpG contexts on the aligned *E.coli* reads. The reference genome of *E.coli* MG1655 can be used for the alignment.

Ordering Information

Product Description	Cat. No.	Kit Size
EZ-Methylation RRBS™ Library Prep	D5460 D5461	24 preps. 48 preps.

For Individual Sale	Cat. No.	Quantity
EZ DNA Methylation-Lightning™ Kit	D5030T D5030 D5031	10 rxns. 50 rxns. 200 rxns.
Lightning Conversion Reagent	D5030-1 D5032-1	1 tube 1 bottle
M-Binding Buffer	D5001-3 D5002-3 D5005-3 D5006-3 D5040-3	20 ml 80 ml 30 ml 125 ml 250 ml
M-Wash Buffer (concentrate)	D5001-4 D5002-4 D5007-4 D5040-4	6 ml 24 ml 36 ml 72 ml
L-Desulphonation Buffer	D5030-5 D5031-5 D5046-5	10 ml 40 ml 80 ml
DNA Clean & Concentrator™-5 Kit (supplied with capped spin columns)	D4013 D4014	50 preps. 200 preps.
DNA Binding Buffer	D4003-1-25 D4003-1-L D4004-1-L	25 ml 50 ml 100 ml
DNA Wash Buffer (concentrate)	D4003-2-6 D4003-2-24 D4003-2-48	6 ml 24 ml 48 ml
DNA Elution Buffer	D3004-4-1 D3004-4-4 D3004-4-10 D3004-4-16 D3004-4-50	1 ml 4 ml 10 ml 16 ml 50 ml
DNase/RNase-free Water	W1001-1 W1001-4 W1001-6 W1001-10 W1001-30	1 ml 4 ml 6 ml 10 ml 30 ml
Zymo-Spin™ IC Columns (capped)	C1004-50 C1004-250	50 columns 250 columns
Collection Tubes	C1001-50 C1001-500 C1001-1000	50 tubes 500 tubes 1000 tubes
***E.coli* Non-Methylated Genomic DNA**	D5016	5 µg/20 µl

Related Products for 5-mC Analysis:

Product Name	Size	Cat. No.
Pico Methyl-Seq Library Prep Kit	10 preps. 25 preps.	D5455 D5456
***OneStep* qMethyl™ Kit**	1 x 96	D5310
***OneStep* qMethyl™-Lite**	1 x 96	D5311
Zymo*Taq*™ DNA Polymerase	50 rxns. 200 rxns.	E2001 E2002
Zymo*Taq*™ PreMix	50 rxns. 200 rxns.	E2003 E2004
EZ DNA Methylation™ Kit	50 rxns. 200 rxns. 2 x 96 rxns. 2 x 96 rxns.	D5001 D5002 D5003 D5004
EZ DNA Methylation-Gold™ Kit	50 rxns. 200 rxns. 2 x 96 rxns. 2 x 96 rxns.	D5005 D5006 D5007 D5008
EZ DNA Methylation-Direct™ Kit	50 rxns. 200 rxns. 2 x 96 rxns. 2 x 96 rxns.	D5020 D5021 D5022 D5023
EZ DNA Methylation-Startup™ Kit	50 rxns.	D5024
Universal Methylated DNA Standard	1 Set	D5010
Universal Methylated Human DNA Standard	1 Set	D5011
Universal Methylated Mouse DNA Standard	1 Set	D5012
Human HCT116 DKO Methylation Standards	1 Set	D5014
Human HCT116 DKO Non-methylated DNA Standard	5 µg	D5014-1
Human HCT116 DKO Methylated DNA Standard	5 µg	D5014-2
Bisulfite Converted Universal Methylated Human DNA Standard	1 set	D5015
***E. coli* Non-methylated Genomic DNA**	5 µg	D5016
Methylated-DNA IP Kit	10 rxns.	D5101
ChIP DNA Clean & Concentrator™	50 preps.	D5205
Anti-5-Methylcytosine Monoclonal Antibody (clone 10G4)	50 µg 200 µg	A3001-50 A3001-200
CpG Methylase (M.SssI)	200 Units 400 Units	E2010 E2011

Additional Products for Epigenetics Research:

Product Name	Size	Cat. No.
RRHP™ 5-hmC Library Prep Kit	12 Preps. 25 Preps.	D5450 D5451
Human Matched DNA Set	1 Set	D5018
Mouse 5-hmC & 5-mC DNA Set	1 Set	D5019
5-Methylcytosine & 5-Hydroxymethylcytosine DNA Standard Set	1 Set	D5405
DNA Degradase™	500 Units 2,000 Units	E2016 E2017
DNA Degradase Plus™	250 Units 1,000 Units	E2020 E2021
5-hmC Glucosyltransferase	100 Units 200 Units	E2026 E2027
5-Hydroxymethyl dCTP [100 mM]	10 µmol	D1045
5-Hydroxymethylcytosine dNTP Mix [10 mM]	2.5 µmol	D1040
5-Methyl dCTP [10 mM]	1 µmol	D1035
5-Methylcytosine dNTP Mix [10 mM]	2.5 µmol	D1030
Zymo-Spin™ ChIP Kit	10 Preps. 25 Preps.	D5209 D5210

Need assistance with bioinformatics analysis?
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