ZYMO RESEARCH

The Beauty of Science is to Make Things Simple

INSTRUCTION MANUAL

Zymo-Seq RRBS™ Library Kit

Catalog Nos. **D5460** & **D5461**

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

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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]Refer to Appendix Item 7 for information regarding the use of this component. This component is not required for mammalian samples or samples with low methylated cytosines in non-CpG context.

[2]The **Lightning Conversion Reagent** is in a ready-to-use liquid format. The reagent should be stored tightly capped at room temperature with minimum exposure to light.

[3,4]DNA Wash Buffer and M-Wash Buffer are provided as concentrates and require the addition of ethanol prior to use.

Product Contents

Zymo-Seq RRBS™ Library Kit (Kit Size)	**D5460** (24 preps)	**D5461** (48 preps)	**Storage Temp**
MspI (20 U/µl)	40 µl	40 µl × 2	-20 °C
10× RRBS Buffer	300 µl	300 µl	-20 °C
rATP (10 mM)	15 µl	15 µl × 2	-20 °C
RRBS Adapters (10 µM)	15 µl	15 µl× 2	-20 °C
T4 DNA Ligase (400 U/µl)	30 µl	30 µl × 2	-20 °C
5-Methylcytosine dNTP Mix (10 mM)	85 µl	85 µl	-20 °C
Taq DNA Polymerase (2 U/µl)	15 µl	15 µl × 2	-20 °C
LibraryAmp Master Mix (2×)	625 µl	625 µl × 2	-20 °C
Index Primer Sets - 12 Sets (10 µM)	30 µl	30 µl × 2	-20 °C
***E. coli* Non-Methylated Genomic DNA**[1]	5 µg/20 µl	5 µg/20 µl	-20 °C
Lightning Conversion Reagent[2]	1.5 ml × 3	1.5 ml × 5	RT
M-Binding Buffer	20 ml	20 ml × 2	RT
M-Wash Buffer[3]	6 ml	6 ml	RT
L-Desulphonation Buffer	10 ml	10 ml	RT
DNA Elution Buffer	1 ml × 2	4 ml	RT
Zymo-Spin™ IC Columns	50 × 2	50 × 3	RT
Collection Tubes	50 × 2	50 × 3	RT
DNA Binding Buffer	25 ml	50 ml	RT
DNA Wash Buffer[4]	6 ml	6 ml × 2	RT
DNase/RNase-free Water	4 ml	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.

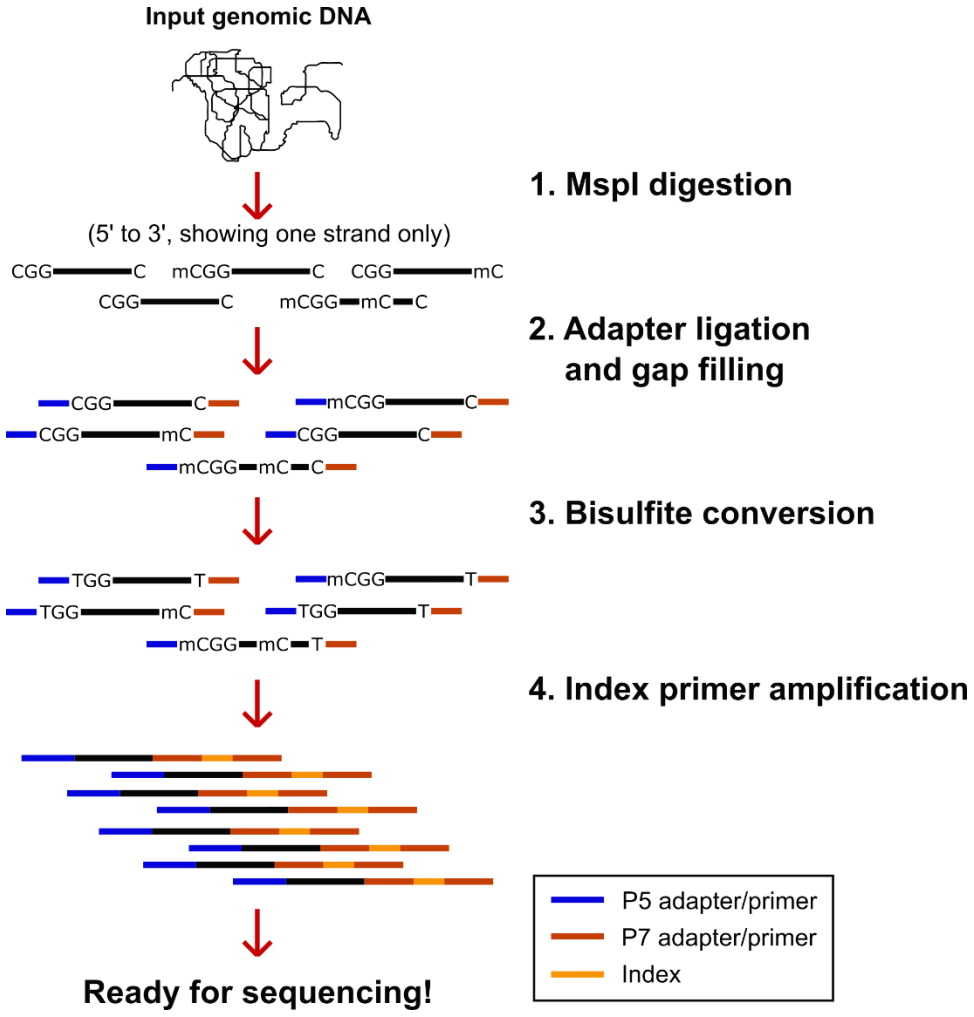
Note: Kit components supplied in two (2) boxes: Box 1 contains all components that can be stored at room temperature (RT, 20-30 °C) and Box 2 contains components that must be stored at -20 °C.

Specifications

- **Input:** 10 – 500 ng of genomic DNA.
- **Sample Sources:** Purified genomic DNA. The 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 protocol is not recommended for samples from species with low CpG density.
- **Sequencing Platform Compatibility:** Libraries are compatible with all Illumina sequencing platforms except HiSeq X Series.
- **Equipment Required**: Microcentrifuge, thermocycler with a heated lid

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 **Zymo-Seq RRBS™ Library Kit** cover ≥75% CpG islands, ≥70% gene promoters, ≥75% gene bodies, and 2.5-4 million unique CpG sites at 5-10x coverage when applied to human samples.



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

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

Notes:

The bisulfite reaction chemically converts unmethylated cytosine bases to uracil. Index primer amplification with standard dNTPs and a uracil-tolerant *Taq* DNA polymerase generates libraries with thymines in place of the originally unmethylated cytosines. To illustrate the sequencing readout clearly, T (thymines) are drawn in place of U (uracils) in the workflow.

Notes:

Components that are stored at -20 °C should 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:

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

Protocol

The protocol consists of 5 sections as follows:
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:

Step	Temperature °C	Time
1	37	4 h
2	4	hold

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 and incubate the mixture in a thermocycler[1] according to the following:

Step	Temperature °C	Time
1	21	3 h
2	37	1 h
3	20	1 h
4	Repeat Steps 2-3	
5	4	hold

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 2**	50 µl
Total	**52 µl**

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

5. 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 the 52 µl product), mix well, and transfer to a **Zymo-Spin™ IC column** in a **Collection Tube**. Centrifuge at ≥ 10,000 x *g* for 30 seconds.

6. Add 200 µl **DNA Wash Buffer** to the column. Centrifuge at ≥ 10,000 x *g* for 30 seconds. Discard the flow-through. Repeat this wash step.[2]

7. 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[3]. Centrifuge 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	**150 µl**

2. Incubate the mixture in a thermocycler[4] according to the following:

Step	Temperature °C	Time
1	98	8 min
2	54	1 h
3	4	hold for **≤ 20 h**

Notes:

[1]The thermocycler program takes 7 hours. We recommend setting up the reaction for overnight ligation and continuing the workflow the following day for convenience.

[2]An optional dry spin can be included after the wash steps to ensure complete removal of wash buffer.

[3]Yields can often be enhanced when performing two sequential elution of 10 µl.

[4]Some thermocyclers may not allow a reaction volume larger than 100 µl. Simply set the maximum allowed and put the reaction in if encountering such a case.

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, close the cap, and invert ~ 8 times to mix. Centrifuge 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. Centrifuge 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, centrifuge at ≥ 10,000 x *g* for 30 seconds.

6. Add 200 µl **M-Wash Buffer** to the column and centrifuge 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. Centrifuge 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 converted DNA.

Section 4: Index Primer Amplification

1. Mix the following components in a 0.2 ml PCR tube[2]:

Components	Volume
LibraryAmp Master Mix (2×)	25 µl
Index Primer Set (10 µM)	1 µl
Product from **Section 3**	24 µl
Total	**50 µl**

[2]Each index primer set contains both forward and reverse primers. Do not use the same index primer set for multiple samples if those samples will be multiplexed in the same sequencing lane. Refer to Appendix Item 1 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 shown in the table below.

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	hold

Input gDNA (ng)	Empirical X[3]
500	7-9
300	9-11
100	11-13
30	13-15
10	15-17

[3]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.

3. Purify the PCR product following steps 5 to 7 from **Section 2** and elute in 15 µl **DNA Elution Buffer**. *If using agarose gel to validate the size distribution of the libraries, either go to step 1 in Section 5 **prior to PCR clean-up** or **elute in a higher volume** to ensure the volumes of libraries are large enough for both validation and quantification.*

Section 5: Library Validation and Quantification

1. The size distribution of the libraries from **Section 4** can be visualized using gel electrophoresis (Figure 1A), Agilent 2200 TapeStation (Figure 1B), or any equivalent instrument. No primer dimer bands should appear at ~ 100 – 120 bp.[1]

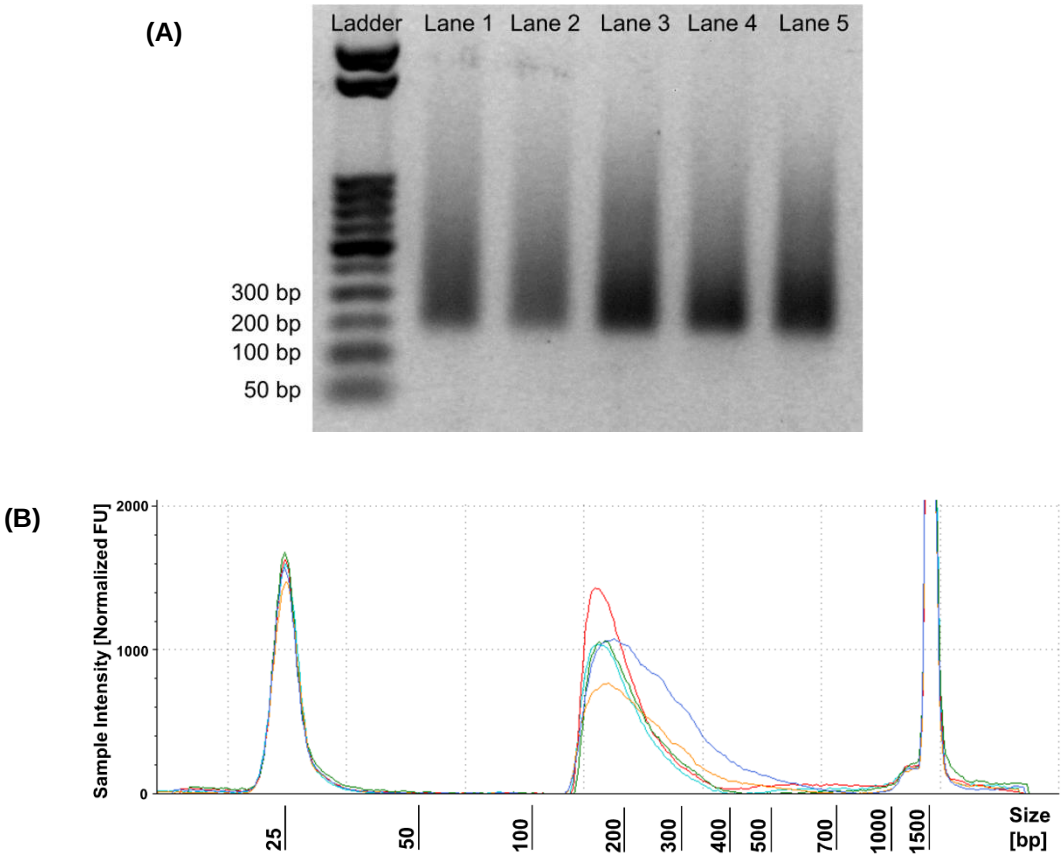


Figure 1. Libraries prepared from 500, 300, 100, 30, and 10 ng of rat gDNA characterized on **(A)** a 2% agarose gel (lane 1 to 5, respectively) or **(B)** a D1000 ScreenTape on the Agilent 2200 TapeStation (traces in blue, orange, green, red, and cyan, respectively; standards at 25 and 1500 bp).

2. KAPA Library Quantification Kits[2] or similar for Illumina® platforms are recommended for library quantification[3] prior to sequencing. The libraries are ready for sequencing after KAPA quantification.

Notes:

[1]Refer to Appendix Item 2 if primer dimers are observed.

[2]Please refer to www.kapabiosystems.com for more information.

[3]It is a good practice to quantify the libraries on a Nanodrop or a Qubit with the settings for dsDNA to obtain an estimate of the library amount **prior to** KAPA quantification.

Notes:

[1]If additional index primer sets or dual index primer sets are needed, please contact Technical Support at 1-888-882-9682 or email tech@zymoresearch.com.

[2]Please search "Index Adapters Pooling Guide + Illumina" in a web browser search engine to obtain the most recent version of the document.

[3]The index sequences correspond to Illumina TruSeq™ Single Indexes index sequences for multiplexing and are copyrighted to Illumina, Inc. Oligonucleotide sequences

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

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

1. The kit provides 12 index primer sets allowing up to 12 libraries pooled in one sequencing lane.[1] Please use the color balance strategy from Illumina's Index Adapters Pooling Guide[2] to select compatible index primer sets for multiplexing.

Index Primer Set	Standard Illumina Designation	TruSeq™ Single Index Sequence[3]
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

2. The kit is designed to reduce the formation of primer dimers after index primer amplification at the recommended numbers of PCR cycle. However, if primer dimers show up at ~ 100 – 120 bp on the size distribution profile, a bead clean-up is recommended to remove the dimers for better sequencing quality. An example product from Zymo Research is Select-a-Size DNA Clean & Concentrator™ MagBead Kit (Cat. No. D4084).

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 recommend applying a publicly accessible wrapper script called **Trim Galore!**[4] to the raw reads. Apply the parameters '—non_directional', '—rrbs', and 'paired-end' when executing the script to ensure correct analysis. For example:

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

7. To calculate the bisulfite conversion rate, we suggest using the percentage of the 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* (Escherichia coli) genomic DNA (D5016)[1] can be spiked into the input samples (0.5-5 wt%, i.e., 0.5-5 ng of the unmethylated *E.coli* genomic DNA to 100 ng of input genomic DNA) before starting the library preparation, and calculate the percentage of the unmethylated cytosines in non-CpG contexts on the aligned *E.coli* reads. The reference genome of *E.coli* strain K-12 substrain MG1655[2] can be used for alignment and analysis.

Notes:

[1]Use a different spike-in of your choice to avoid cross mapping if your sample is homologous to *E.coli* or shares common sequences with *E.coli*.

[2]The *E.coli* reference genome can be accessed at NCBI genome database (https://www.ncbi.nlm.nih.gov/genome/167?genome_assembly_id=161521).

Ordering Information

Product Description	Kit Size	Cat. No.
Zymo-Seq RRBS™ Library Kit	24 preps. 48 preps.	D5460 D5461

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

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 7D21)	50 µg 200 µg	A3002-50 A3002-200
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

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