ZYMO RESEARCH

THE Epigenetics COMPANY™

Zymo-Seq RRBS Library Kit

DNA Methylation Profiling Across Diverse Species

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

Catalog Numbers:
D5460 & D5461

Scan with your smart-phone camera to view the online protocol/video.

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Product Contents

Zymo-Seq RRBS Library Kit	D5460 (24 preps)	D5461 (48 preps)	Storage Temperature
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
Zymo-Seq™ UDI Primer Set (Indexes 1-12)[1]	20 µL / Index	20 µL / Index	-80 °C
E. coli Non-Methylated Genomic DNA[2]	5 µg/20 µL	5 µg/20 µL	-20 °C
Lightning Conversion Reagent[3]	1.5 mL × 3	1.5 mL × 5	Room Temp.
M-Binding Buffer	20 mL	20 mL × 2	Room Temp.
M-Wash Buffer (concentrate)	6 mL	6 mL	Room Temp.
L-Desulphonatoin Buffer	10 mL	10 mL	Room Temp.
DNA Elution Buffer	1 mL × 2	4 mL	Room Temp.
Zymo-Spin™ IC Columns	50 × 2	50 × 3	Room Temp.
Collection Tubes	50 × 2	50 × 3	Room Temp.
DNA Binding Buffer	25 mL	50 mL	Room Temp.
DNA Wash Buffer (concentrate)	6 mL	6 mL × 2	Room Temp.
DNase/RNase-Free Water	4 mL	4 mL	Room Temp.
Instruction Manual	1	1	-

[1] The Zymo-Seq™ UDI Primer Set (Indexes 1-12) (Cat. No. D3008) contains 12 pre-mixed unique dual-index barcode primers in 1.5 mL tubes. Refer to **Appendix A** for specifications and barcode sequences.
[2] Refer to **Appendix D, "Evaluating Bisulfite Conversion Efficiency"** for the use of this component. This component is not required for mammalian samples or those with low methylated cytosines in non-CpG context.
[3] 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.

Specifications

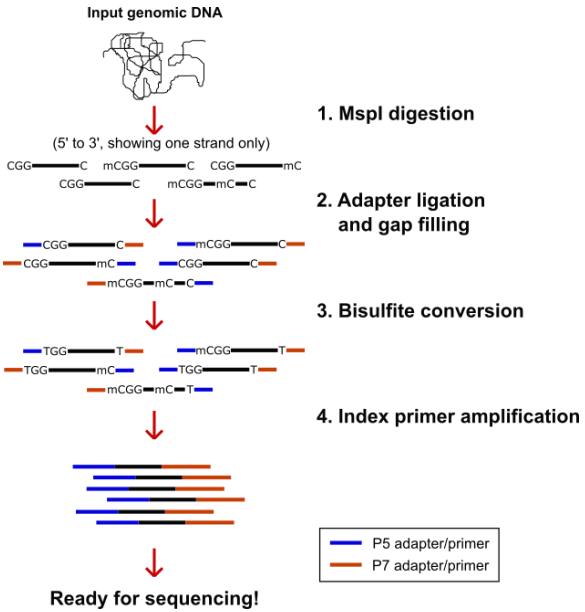
- **Sample Input Material –** Genomic DNA suspended in water, TE, or a low salt buffer.
- **Input Amount –** 10 – 500 ng.
- **Input Quality –** DNA should be free of enzymatic inhibitors. DNA with A260/A280 or A260/A230 ratios < 1.8 should be purified prior to processing using the **Genomic DNA Clean & 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[1].
- **Equipment Required –** Microcentrifuge, thermal cycler with a heated lid.

[1] Illumina® originally limits the applications on HiSeq X exclusively for whole-genome libraries. Please confirm with the sequencing service provider for acceptability and additional details if expecting to sequence RRBS libraries on HiSeq X Series sequencers.

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.

Zymo-Seq RRBS Library Kit Workflow[1]



[1] 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.

Protocol

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).

Before Starting:

- ✓ Refer to Appendix D, "Evaluating Bisulfite Conversion Efficiency" to ensure if the provided *E. coli* Non-Methylated Genomic DNA or other spike-in is needed for library preparation and subsequent bioinformatic analysis.
- ✓ Components that are stored at -20 °C or -80 °C should be thawed and kept on ice. Centrifuge briefly to collect contents that are potentially caught at the tube lids or the inner sidewalls.
- ✓ Avoid multiple freeze-thaws of the Zymo-Seq™ UDI Primer Sets. Make aliquots as necessary.
- ✓ Ensure the thermal cycler's lid temperature is set to 100-105 °C for each program.
- ✓ For Section 1, Step 1 and Section 2, Step 3 and 5, a master mix of the reagents is recommended when processing multiple samples in parallel.
- ✓ When mixing the components, pipet up and down or flick the tube to ensure sufficient mixing. Centrifuge briefly to collect the contents.
- ✓ All centrifugation steps should be performed at ≥ 10,000 x *g*.

Section 1: MspI Digestion

1. Mix the following components in a 0.2 mL PCR tube for each sample.

Components	Volume
Genomic DNA in TE buffer/H_2O	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 tube in a thermal cycler according to the following program (reaction volume: 40 µL).

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

Section 2: Adapter Ligation

3. Add the following components to the tube from Section 1, Step 2.

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, Step 2	40 µL
Total	**50 µL**

4. Mix well and incubate the tube in a thermal cycler according to the following program[1] (reaction volume: 50 µL).

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

5. Add the following components to the tube from Section 2, Step 4.

Components	Volume
Taq DNA Polymerase (2 U/µL)	0.5 µL
5-methylcytosine dNTP Mix (10 mM)	1.5 µL
Product from Section 2, Step 4	50 µL
Total	**52 µL**

6. Incubate the tube in a thermal cycler at 74 °C for 30 minutes.

7. In a 1.5 mL tube, add 364 µL of **DNA Binding Buffer** to the product from Section 2, Step 6. Mix well and transfer the mixture to a **Zymo-Spin™ IC Column** in a **Collection Tube**. Centrifuge for 30 seconds.

[1] This thermal cycler program takes 7 hours. We recommend setting up the reaction for overnight ligation and continuing the workflow on the following day for convenience.

8. Add 200 µL of **DNA Wash Buffer** to the column. Centrifuge for 30 seconds. Discard the flow-through. Repeat this wash step.[1]

9. Transfer the **Zymo-Spin™ IC Column** to a clean 1.5 mL tube. Add 20 µL of **DNA Elution Buffer** directly to the column matrix and let stand for 1 minute at room temperature[2]. Centrifuge for 30 seconds to elute.

[1] An optional dry spin can be included after the wash steps to ensure complete removal of the wash buffer.
[2] Yields can often be enhanced when performing two sequential elution of 10 µL.

Section 3: Bisulfite Conversion

10. Mix the following components in a 0.2 mL PCR tube.

Components	Volume
Lightning Conversion Reagent	130 µL
Product from Section 2, Step 9	20 µL
Total	**150 µL**

11. Incubate the tube in a thermal cycler according to the following program (reaction volume: 150 µL[1]).

Step	Temperature	Time
1	98℃	8 min
2	54℃	1 h
3	4℃	Hold for ≤ 20 h

12. Add 600 µL of **M-Binding Buffer** to a **Zymo-Spin™ IC Column** in a **Collection Tube**. Add the bisulfite converted sample from Section 3, Step 11 to the column, close the cap, and invert ~ 8 times to mix. Centrifuge for 30 seconds.

13. Discard the flow-through from the **Collection Tube** and add 100 µL of **M-Wash Buffer** to the column. Centrifuge for 30 seconds.

14. Add 200 µL of **L-Desulphonation Buffer** to the column and let stand at room temperature (20°C-30°C) for 15-20 minutes.[2] After the incubation, centrifuge for 30 seconds.

15. Add 200 µL of **M-Wash Buffer** to the column and centrifuge for 30 seconds. Discard the flow-through. Repeat this wash step.

16. Transfer the **Zymo-Spin™ IC Column** to a 1.5 mL tube. Add 21 µL of **DNA Elution Buffer** directly to the column matrix and let stand for 1 minute. Centrifuge for 30 seconds to elute the bisulfite-converted DNA.

[1] If the thermal cycler does not allow a reaction volume ≥ 100 µL, simply set the maximum volume allowed.
[2] Incubate with the L-Desulphonation Buffer for more than 20 minutes may result in degradation of the DNA sample and lower the yield of the converted DNA.

Section 4: Index Primer Amplification

17. Mix the following components in a 0.2 mL PCR tube.

Components	Volume
LibraryAmp Master Mix (2×)	25 µL
UDI Primer Set (2.5 µM)[1]	4 µL
Product from Section 3, Step 16	21 µL
Total	**50 µL**

18. Incubate the tube in a thermal cycler according to the following program (reaction volume: 50 µL). Adjust the total cycles based on the input gDNA amount as listed on the right side of the table[2].

Step	Temperature	Time	
1	94℃	30 sec	
2	94℃	30 sec	500 ng = 8 cycles
3	55℃	30 sec	300 ng = 10 cycles
4	68℃	1 min	100 ng = 12 cycles
5	--- Go to Step 2 ---		30 ng = 14 cycles
6	68℃	5 min	10 ng = 16 cycles
7	4℃	hold	

19. In a 1.5 mL tube, add 350 µL of **DNA Binding Buffer** to the product from Section 4, Step 18. Mix well and transfer the mixture to a **Zymo-Spin™ IC Column** in a **Collection Tube**. Centrifuge for 30 seconds.

20. Add 200 µL of **DNA Wash Buffer** to the column. Centrifuge for 30 seconds. Discard the flow-through. Repeat this wash step.[3]

21. Transfer the **Zymo-Spin™ IC Column** to a clean 1.5 mL tube. Add 15 µL of **DNA Elution Buffer** directly to the column matrix and let stand for 1 minute at room temperature[4]. Centrifuge for 30 seconds to elute.

The eluate is your final RRBS library. Please store at ≤ 4°C overnight or ≤ -20°C for long-term storage.

[1] Do not use the same index primer set for multiple samples if they will be multiplexed in the same sequencing lane. See **Appendix A** for index primer sequences and barcodes.
[2] The listed PCR cycle numbers for various input gDNA amount should only serve as a starting point. The PCR cycle number can vary depending on the quality of the input and should be optimized prior to working with precious samples.
[3] An optional dry spin can be included after the wash steps to ensure complete removal of the wash buffer.
[4] If using agarose gel to validate the size distribution of the libraries, please elute in a higher volume at Section 4, Step 21 to ensure enough volumes for both agarose gel-based validation and subsequent quantification.

Appendices

Appendix A: Unique Dual Index (UDI) Primer Sets

The **Zymo-Seq UDI Primer Set (Indexes 1-12) (Cat. No. D3008)** are included in the **Zymo-Seq RRBS Library Kit (Cat. No. D5460/D5461)**. To achieve a higher multiplexibility, please order the **Zymo-Seq UDI Primer Plate (Indexes 1-96) (Cat. No. D3096)** separately. Indexes in the **Zymo-Seq UDI Primer Set (Indexes 1-12)** and the **Zymo-Seq UDI Primer Plate (Indexes 1-96)** come as pre-mixes and the forward and reverse primers are provided at 5 µM total concentration (2.5 µM each).

The complete index sample sheet is available for download here (USA Only), or by visiting the Documents section of the D3008 and D3096 product page at www.zymoresearch.com. The primer sequences are as below (**NNNNNNNN** correspond to the "Bases in Adapter" columns in the index sample sheet).

Forward Primer Sequence (i5):
5′-AATGATACGGCGACCACCGAGATCTACAC**NNNNNNNN**ACACTCT TTCCCTACACGACGCTCTTCCGATCT-3'

Reverse Primer Sequence (i7):
5′-CAAGCAGAAGACGGCATACGAGAT**NNNNNNNN**GTGACTGGAGTT CAGACGTGTGCTCTTCCGATCT-3'

Pool ≥ 2 libraries with UDIs down a column not across a row.

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDI_01	UDI_09	UDI_17	UDI_25	UDI_33	UDI_41	UDI_49	UDI_57	UDI_65	UDI_73	UDI_81	UDI_89
B	UDI_02	UDI_10	UDI_18	UDI_26	UDI_34	UDI_42	UDI_50	UDI_58	UDI_66	UDI_74	UDI_82	UDI_90
C	UDI_03	UDI_11	UDI_19	UDI_27	UDI_35	UDI_43	UDI_51	UDI_59	UDI_67	UDI_75	UDI_83	UDI_91
D	UDI_04	UDI_12	UDI_20	UDI_28	UDI_36	UDI_44	UDI_52	UDI_60	UDI_68	UDI_76	UDI_84	UDI_92
E	UDI_05	UDI_13	UDI_21	UDI_29	UDI_37	UDI_45	UDI_53	UDI_61	UDI_69	UDI_77	UDI_85	UDI_93
F	UDI_06	UDI_14	UDI_22	UDI_30	UDI_38	UDI_46	UDI_54	UDI_62	UDI_70	UDI_78	UDI_86	UDI_94
G	UDI_07	UDI_15	UDI_23	UDI_31	UDI_39	UDI_47	UDI_55	UDI_63	UDI_71	UDI_79	UDI_87	UDI_95
H	UDI_08	UDI_16	UDI_24	UDI_32	UDI_40	UDI_48	UDI_56	UDI_64	UDI_72	UDI_80	UDI_88	UDI_96

Appendix B: Library Validation and Quantification

Libraries should be visualized by running an agarose gel or using an automated electrophoresis instrument (*e.g.*, Agilent TapeStation®, Bioanalyzer®, etc) to determine that the correct library size is present, and no adapter dimers have formed (Figure 1).

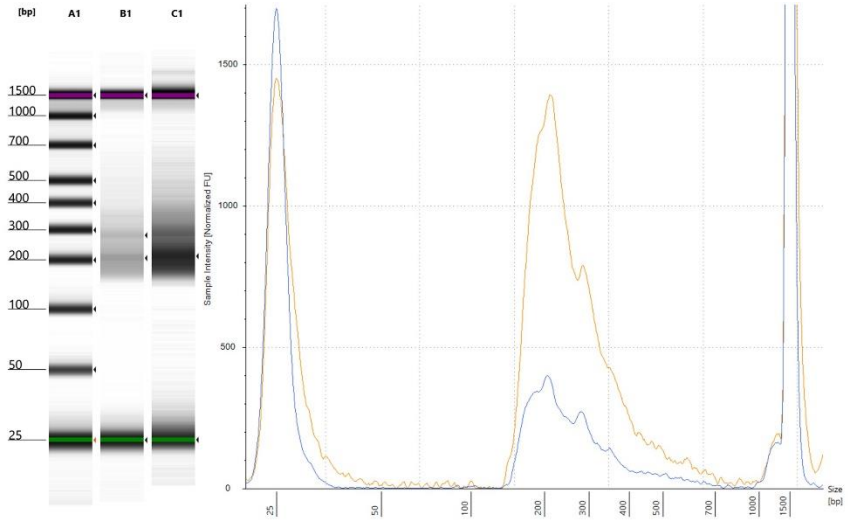


Figure 1. Typical Zymo-Seq RRBS libraries prepared with 500 ng (well B1, blue trace) or 10 ng (well C1, orange trace) of human genomic DNA (Human HCT116 DKO Methylated DNA, Cat. No. D5014-2) characterized on Agilent 2200 TapeStation®. Fragment sizes range from about 150 to 750 bp. The peaks at 25 and 1500 bp in the zoomed-in electropherogram were the interior standards of the D1000 ScreenTape®.

The kit is designed to reduce the formation of primer dimers after library amplification at the recommended number of PCR cycles. However, if primer dimers show up at ~ 140 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 the **Select-a-Size DNA Clean & Concentrator™ MagBead Kit** (Cat. No. D4084).

We also recommend quantifying the libraries with qPCR-based methods designed for Illumina® platforms prior to sequencing.[1]

[1] 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** qPCR-based library quantification.

Appendix C: Considerations for Sequencing

Instrument Compatibility

The libraries prepared with this kit are compatible with all Illumina® sequencing platforms except the HiSeq X Series[1].

Sequencing Specifications

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 manual 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.

Each library should be sequenced to obtain at least 30 million reads for an approximately 10× average coverage of the detected CpG sites (numbers based on libraries prepared with human or mouse gDNA and may vary from sample to sample).

We recommend paired-end sequencing with Illumina's TruSeq™ technology at a read length ≥ 50 bp.

[1] Illumina® originally limits the applications on HiSeq X exclusively for whole-genome libraries. Please confirm with the sequencing service provider for acceptability and additional details if expecting to sequence RRBS libraries on HiSeq X Series sequencers.

Appendix D: Bioinformatic Analysis

Library Strand Information

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

Trimming Reads

We recommend trimming adapter sequences and filled-in nucleotides introduced during library preparation from the raw reads. An example using Trim Galore![1] for such trimming is as below:

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

Evaluating Bisulfite Conversion Efficiency

We suggest using the percentage of unmethylated cytosines in all detected cytosines in non-CpG contexts for mammalian samples to evaluate the bisulfite conversion efficiency.

If the samples may be significantly methylated on cytosines in non-CpG contexts, the provided *E. coli* Non-Methylated Genomic DNA (Cat. No. D5016) can be used for evaluating the bisulfite conversion efficiency. This component can be used in two ways.

- As a **spike-in**[2]. Add 0.5-2.0 ng of *E. coli* gDNA to every 100 ng of experimental DNA input and proceed with library preparation as instructed.
- As an **experimental control**. Use the *E. coli* gDNA to prepare libraries in parallel with experimental samples. Use the same amount (ng) of *E. coli* gDNA as what is being used as input for the experimental samples.

Align the sequencing reads to the *E. coli* reference genome[3] and perform methylation calling on the aligned reads. Calculate the percentage of unmethylated cytosines in all detected cytosines. This will provide a good representation of the bisulfite conversion efficiency of the experimental samples.

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[1] Trim Galore! is a publicly available software and accessible at https://github.com/FelixKrueger/TrimGalore.
[2] 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*.
[3] The reference genome of *E.coli* strain K-12 substrain MG1655 can be used for alignment and analysis. It can be accessed at https://www.ncbi.nlm.nih.gov/genome/167?genome_assembly_id=161521.

Ordering Information

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

For Individual Sale	Catalog No.	Amount
Zymo-Seq UDI Primer Set (Index 1-12)	D3008	12 sets
Zymo-Seq UDI Primer Plate (Index 1-96)	D3096	96 sets
EZ DNA Methylation-Lightning™ Kit	D5030 D5031	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	D4003-1-2 D4003-1-L D4004-1-L	25 mL 50 mL 100 mL
DNA Wash Buffer (concentrate)	D4003-3-6 D4003-3-24 D4003-3-48	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

Explore Epigenomics *with NGS*

Discover additional tools to advance your epigenetics research

Single-base DNA Methylation

Pico Methyl-Seq Library Prep Kit (D5455)
Zymo-Seq WGBS Library Kit (D5465)
Zymo-Seq RRBS Library Kit (D5460)

RNA-Seq Libraries

Zymo-Seq RiboFree Total RNA
Library Kit (R3000)

Chromatin Structure

Zymo-Spin ChIP Kit (D5209)

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Technical Assistance
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Notes

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Reagents are routinely tested on a lot-to-lot basis to ensure they provide the highest performance and reliability.

This product is for research use only and should only be used by trained professionals. It is not 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.

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