ZYMO RESEARCH

Epigenetics Made Simple™

Zymo-Seq™ Trio WGBS Library Kit

High quality libraries from precious, fragmented samples

Highlights

- **Co-Detection of Genetic and Epigenetic Information:** Seamlessly analyze both genomic data and DNA methylation in a single experiment, with the validated open-source bioinformatics tools and comprehensive step-by-step guides.
- **True-End Fragment Analysis:** Designed for optimal performance with small or degraded DNA fragments, including cell-free DNA (cfDNA) and FFPE-derived DNA, to capture the fragment's true end.
- **Accurate Methylation Detection:** Achieve precise methylation calling with a direct ligation-based protocol that preserves native termini, ensuring accuracy in each DNA fragment analysis.

Catalog Numbers:
D5462, D5463

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

tech@zymoresearch.com www.zymoresearch.com Toll Free: (888) 882-9682

Table of Contents

Product Contents

Zymo-Seq™ Trio WGBS Library Kit	D5462 (24 prep)	D5463 (96 prep)	Storage Temperature
Lightning Conversion Reagent[1]	3 x 1.5 mL	15 mL	Room Temp.
M-Binding Buffer	20 mL	80 mL	Room Temp.
M-Wash Buffer (concentrate)[2]	6 mL	24 mL	Room Temp.
L-Desulphonation Buffer	10 mL	40 mL	Room Temp.
DNA Elution Buffer	4 mL	16 mL	Room Temp.
Zymo-Spin™ IC Columns	25	2 x 50	Room Temp.
Collection Tubes	25	2 x 50	Room Temp.
DNA Wash Buffer (concentrate)[3]	6 mL	24 mL	Room Temp.
Select-a-Size MagBeads	10 mL	2 x 10 mL	4°C
E coli Non-Methylated Genomic DNA	5 µg/20 µL	5 µg/20 µL	−20°C
Adapter Ligation Buffer 1	48 µL	2 x 96 µL	−20°C
Adapter Ligation Buffer 2[4]	48 µL	2 x 96 µL	−20°C
Adapter Ligation Buffer 3[5]	48 µL	2 x 96 µL	−20°C
Adapter Ligation Master Mix[6]	625 µL	2 x 1.25 mL	−20°C
2X Index PCR Premix	600 µL	2 x 1.2 mL	−20°C
Zymo-Seq™ UDI Primer Set (1-12)[7]	20 µL/Index	-	−20°C
Zymo-Seq™ UDI Primer Plate (1-96)[8]	-	10 µL/Index	−20°C
Instruction Manual	1	1	-

[1] The **Lightning Conversion Reagent** is in a ready-to-use format. The reagent should be stored tightly capped at room temperature with minimum exposure to light.
[2,3] The **M-Wash Buffer** and **DNA Wash Buffer** are supplied as concentrates. See **Buffer Preparation** on pg. 5 for directed amounts of ethanol to be added to each upon first use. Cap bottle tightly after each use to prevent ethanol evaporation.
[4,5,6] The **Adapter Ligation Buffer 2**, **Adapter Ligation Buffer 3**, and **Adapter Ligation Master Mix** reagents are sensitive and should undergo no more than 4 freeze-thaw cycles. Make additional aliquots of each buffer as necessary.
[7,8] The provided **Zymo-Seq™ UDI Primer Set** (Indexes 1-12) (D3008) or **Zymo-Seq™ UDI Primer Plate** (Indexes 1-96) (D3096) contains 12 pre-mixed unique dual-index barcode primers in 1.5 mL tubes or 96 pre-mixed unique dual-index barcode primers in a 96-well plate format respectively. See **Appendix D** for primer specifications, index sequences, and multiplexing considerations.

Specifications

- **Sample Input Material:** Purified cell-free DNA (cfDNA), Sonicated FFPE DNA (average size of 300-600 bp), Sheared genomic DNA (average size of 300-600 bp).
- **Minimum Input:** 5 ng
- **Recommended Maximum Input:** 10 ng. Higher inputs can be used to create libraries; however, the diversity of the library may decline as input increases.
- **Input Quality:** For optimal results, use minimum input of DNA with no RNA or genomic DNA contamination. cfDNA can be concentrated using the **DNA Clean & Concentrator™** (D4013) prior to processing. cfDNA can be suspended in water, DNA Elution Buffer, or TE buffer.
- **Equipment Required:** Thermal cycler(s) with temperature adjustable lids, microcentrifuge, magnetic stand.
- **Total Processing Time:** ~6 hours
- **Hands-On Time:** ~2 hours
- **Bisulfite Conversion Efficiency:** >99.5% of non-methylated cytosine residues are converted to uracil; >99.5% protection of methylated cytosines.
- **Library Storage:** Libraries eluted in **DNA Elution Buffer** (provided) may be stored at ≤ 4°C overnight or ≤ −20°C for long-term storage.
- **Sequencing Platform Compatibility:** Libraries are compatible with all Illumina sequencing platforms. Recommended: HiSeq®, NextSeq®, NovaSeq®.
- **Barcode Sequences:** Available for download here (USA only), or by visiting the Documents section of the D3008 and D3096 product pages at www.zymoresearch.com.

Product Description

The **Zymo-Seq™ Trio WGBS Library Kit** provides an optimized and reliable workflow for the preparation of methyl-seq libraries from cell-free DNA (cfDNA), FFPE DNA, and genomic DNA. The process is completed in three basic steps: (1) bisulfite conversion using **EZ DNA Methylation-Lightning™** chemistry, (2) direct adapter ligation with innovative splinted adapters, and (3) index PCR amplification. This streamlined workflow has been optimized for use with short, damaged DNA fragments, making whole genome bisulfite sequencing (WGBS) library preparation with difficult samples an efficient process that can be completed in as little as 6 hours.

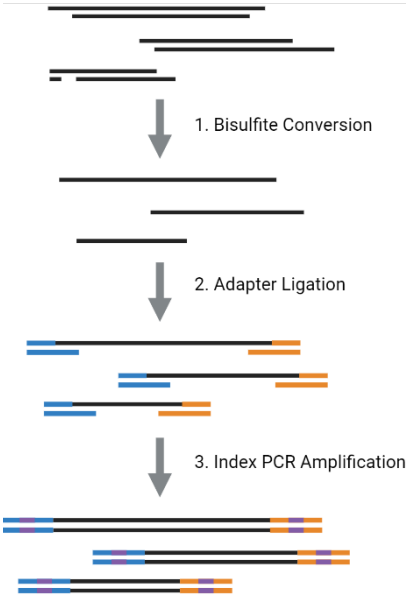

Figure 1. Overview of the Zymo-Seq™ Trio WGBS Library Kit protocol. The simple three-step protocol allows users to effortlessly prepare WGBS libraries from small, damaged DNA with no compromise on quality.

The **EZ DNA Methylation-Lightning™** bisulfite conversion is gentle on already short or damaged DNA fragments, resulting in less degradation of the sample compared to other bisulfite conversion chemistries and methods. The bisulfite conversion is completed rapidly while maintaining the integrity of precious samples.

After bisulfite conversion, DNA remains in a single-stranded conformation, proving more difficult for conventional library preparation methods. This obstacle is circumvented by utilizing unique splinted adapter ligation technology to capture and directly ligate the Illumina-compatible adapters to each end of the bisulfite converted DNA rather than performing more laborious second strand synthesis, end repair, and dA tailing steps. These processes incorporate artificial nucleotides to blunt damaged ends or miss them altogether. The direct adapter ligation eliminates this bias by accurately preserving the methylation status of each fragment terminus. This results in faster library preparation as well as more precise methylation calling across the entire DNA fragment.

The splinted adapter ligation technology is also capable of thoroughly capturing small DNA fragments, allowing for library construction from nicked and very short DNA fragments that would otherwise not be viable when using other methods. Libraries can be prepared from a much greater percentage of DNA input rather than only the DNA fragments that are of convenient size for traditional library preparation.

Once the adapters have been ligated to the DNA, the final step is the amplification and indexing via PCR. The **Zymo-Seq™ UDI Primers** facilitate effortless multiplexing of numerous libraries. After a final clean-up, the DNA WGBS libraries are ready for sequencing on any Illumina instrument.

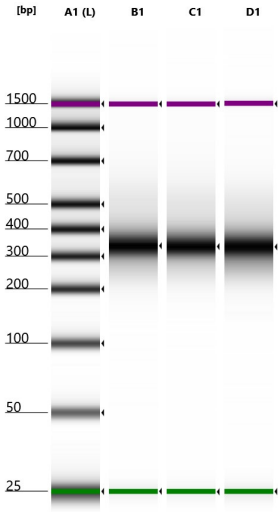

Figure 2. Zymo-Seq™ Trio WGBS Libraries prepared from multiple cfDNA samples. Agilent 4200 TapeStation® HS D1000 of libraries prepared using cfDNA extracted from both healthy and cancerous plasma donors[1]. A1 is a molecular weight marker. B1 is a library prepared with cfDNA from a 59-year-old healthy plasma donor. C1 is a library prepared with cfDNA from a 66-year-old lung cancer NSCLC stage IV plasma donor. D1 is a library prepared with cfDNA from a 69-year-old lung cancer adenocarcinoma stage IV plasma donor. All libraries were prepared using 5 ng purified cfDNA and amplified at 9 index PCR cycles.

[1] cfDNA samples were extracted from 5 mL plasma using the ***Quick*-cfDNA™ Serum & Plasma Kit** (D4076) and concentrated using the **DNA Clean & Concentrator-5™** (D4013).

Protocol

Buffer Preparation

✓ Preparation of the **Select-a-Size MagBeads**:

1. Add 300 uL of the **Select-a-Size MagBead Concentrate** to the 10 mL **Select-a-Size MagBead Buffer** bottle.
2. Resuspend by pipetting up and down and/or vortexing. Store at 4°C-8°C.

✓ Preparation of the **M-Wash Buffer** concentrate:

3. Add the following volumes of ethanol to the **M-Wash Buffer** concentrate:

M-Wash Buffer	If Using 100% Ethanol	If Using 95% Ethanol
6 mL M-Wash Buffer (D5001-4)	Add 24 mL	Add 26 mL
24 mL M-Wash Buffer (D5002-4)	Add 96 mL	Add 104 mL

4. Initial and mark date of ethanol addition to the bottle.

✓ Preparation of the **DNA Wash Buffer** concentrate:

1. Add the following volumes of ethanol to the **DNA Wash Buffer** concentrate:

DNA Wash Buffer	If Using 100% Ethanol	If Using 95% Ethanol
6 mL DNA Wash Buffer (D4003-2-6)	Add 24 mL	Add 26 mL
24 mL DNA Wash Buffer (D4003-2-24)	Add 96 mL	Add 104 mL

2. Initial and mark date of ethanol addition to the bottle.

Before Starting:

- ✓ Refer to **Appendix C: *In Situ* Bisulfite Conversion Controls** for considerations regarding the provided ***E. coli* Non-Methylated Genomic DNA** in library preparation and analysis.
- ✓ Components that are stored at −20°C should be thawed and kept on ice unless otherwise stated. Return to −20°C storage after use.
- ✓ Mix each component well before use by pipetting up and down, flicking, inverting, or gently vortexing. Centrifuge briefly to collect all contents potentially caught on the sides or caps of the tubes before using.
- ✓ Avoid multiple freeze-thaws of the **Zymo-Seq™ UDI Primers**. Make additional aliquots as necessary.
- ✓ The **Adapter Ligation Buffer 2**, **Adapter Ligation Buffer 3**, and **Adapter Ligation Master Mix** are very sensitive to freeze-thaw and should only be thawed 4 times maximum. During the first thaw, make additional aliquots as necessary to maintain library quality.
- ✓ Before using the **Select-a-Size MagBeads**, allow them to equilibrate to room temperature for 30 minutes.

Section 1: Bisulfite Conversion

Before Starting:

✓ Ensure that the indicated volume of ethanol has been added to the **M-Wash Buffer** (see pg. 5 **Buffer Preparation**).

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

Component	Volume
Input DNA (5 ng – 10 ng)	X µL
E. coli Non-Methylated Genomic DNA (optional)[2]	Y µL
DNase/RNase-Free Water	Up to 20 µL
Total Volume	**20 µL**

2. Add 130 µL of **Lightning Conversion Reagent** to each sample and mix well by pipetting.

3. Centrifuge briefly to ensure there are no droplets in the cap or on the sides of the tube. Place the 0.2 mL PCR tube(s) in a thermal cycler (lid temp 105°C) and perform the following steps:

Temperature	Time
98°C	8 minutes
54°C	60 minutes
4°C	≤ 20 hours (optional)

4. Place a **Zymo-Spin™ IC Column** into a provided **Collection Tube** and add 600 µL of **M-Binding Buffer**.

5. Load the sample (from Step 3) into the **Zymo-Spin™ IC Column** containing the **M-Binding Buffer**. Close the cap and mix by inverting the column several times.

[1] DNA inputs >20 µL must be processed using multiple conversion reactions. Replicate reactions can be cleaned using the same column for each by repeating Steps 4-6 up to 5 times.

[2] Using the ***E. coli* Non-Methylated Genomic DNA** (D5016) as a spike-in is highly recommended for determining bisulfite conversion efficiency. If using directly from the tube, the *E. coli* DNA can be spiked in at 2-5% wt of the input DNA (e.g., 100-250 pg into 5 ng of DNA). If fragmented to 200-300 bp, the *E. coli* DNA can be spiked in at 0.5-1% wt of the input DNA (e.g., 25-50 pg into 5 ng of DNA). See **Appendix C** for additional information.

6. Centrifuge at full speed (≥ 10,000 x g) for 30 seconds. Discard the flow-through[1].

7. Add 100 µL of **M-Wash Buffer** to the column. Centrifuge at full speed (≥ 10,000 x g) for 30 seconds.

8. Add 200 µL of **L-Desulphonation Buffer** to the column and let stand at room temperature (20-30°C) for 15-20 minutes[2]. After the incubation, centrifuge at full speed (≥ 10,000 x g) for 30 seconds. Discard the flow-through.

9. Add 200 µL of **M-Wash Buffer** to the column. Centrifuge at full speed (≥ 10,000 x g) for 30 seconds. Repeat this wash step for two washes total.

10. Place the column into a 1.5 mL microcentrifuge tube and add 19 µL[3] of **DNA Elution Buffer** directly to the column matrix. Let incubate for 1-5 minutes[4], and then centrifuge at full speed (≥ 10,000 x g) for 30 seconds to elute the DNA.

This is a safe stopping point. Bisulfite-converted DNA can be safely stored at ≤ −20°C for up to one month.

[1] The capacity of the **Collection Tube** with the column inserted is 800 µL. Empty the **Collection Tube** as necessary to prevent contamination of the column matrix by the flow-through.
[2] Incubation with **L-Desulphonation Buffer** for longer than 20 minutes may result in degradation and lower yield of converted DNA.
[3] Sequential elutions of smaller volumes ≥ 6 µL (e.g., 9.5 µL x 2 for 19 µL total) can help ensure complete elution of all DNA from the column.
[4] Longer incubations of the **DNA Elution Buffer** on the column for up to 5 minutes can ensure greater elution efficiency.

Section 2: Adapter Ligation

Before Starting:

- ✓ Thaw the **Adapter Ligation Buffer 1**, **Adapter Ligation Buffer 2**, and **Adapter Ligation Buffer 3** on ice.
- ✓ Thaw the **Adapter Ligation Master Mix** to room temperature. Once thawed, vortex for at least 30 seconds and invert to mix well.
- ✓ The **Adapter Ligation Buffer 2**, **Adapter Ligation Buffer 3**, and **Adapter Ligation Master Mix** should only be thawed 4 times maximum. Make additional aliquots as necessary upon first thaw.

1. Preheat a thermal cycler to 98°C (lid temp 105°C) and another thermal cycler to 37°C (lid temp 45°C).
   Note: If only a single thermal cycler is available, set to 98°C (lid temp 105°C) initially and change the temperature to 37°C (lid temp 45°C) during the 2-minute return to ice incubation (Step 6). Leave the lid open to help cool.

2. Combine the following on ice in a 0.2 mL PCR tube:

Component	Volume
Bisulfite-converted DNA	18 µL
Adapter Ligation Buffer 1	2 µL
Total Volume	**20 µL**

3. Mix entire reaction thoroughly by pipetting or gently vortexing then centrifuge briefly to ensure there are no droplets in the cap or on the sides of the tube.

4. Incubate the tube on ice for 2 minutes.

5. Heat shock by immediately placing the tube at 98°C (lid temp 105°C) for 3 minutes.

6. Immediately return the tube to ice and incubate for at least 2 minutes to fully denature the DNA[1].

7. Thoroughly mix the **Adapter Ligation Master Mix** tube by vortexing for at least 30 seconds and inverting several times[2].

[1] If using only one thermal cycler, set the temperature to 37°C (lid temp 45°C) during this incubation so that the temperature is ready by Step 10. Leave the lid open to help cool it faster.
[2] The **Adapter Ligation Master Mix** is very viscous. Mix well after thawing and right before use.

8. Add the following on ice in the order defined below to the tube:

Component	Volume
Denatured DNA	20 µL
Adapter Ligation Buffer 2	2 µL
Adapter Ligation Buffer 3	2 µL
Adapter Ligation Master Mix	26 µL
Total Volume	**50 µL**

9. Mix entire reaction thoroughly by pipetting up and down 20-25 times, vortexing, and inverting to ensure complete homogenization[1]. Centrifuge very briefly to ensure there are no droplets in the cap or on the sides of the tube.

10. Incubate the tube at 37°C (lid temp 45°C)[2] for 1 hour in a thermal cycler.

11. After the 1-hour adapter ligation reaction, add 60 µL of **DNA Elution Buffer** to the sample to bring the volume up to 110 µL and mix well by pipetting.

12. Follow the clean-up protocol in **Appendix A** on pg. 13. Allow the **Select-a-Size MagBeads** to equilibrate to room temperature for 30 minutes prior to use. For elution, resuspend the beads in 15 µL of **DNA Elution Buffer** and aspirate all 15 µL eluate after separation from the beads into a new tube.

This is a safe stopping point. The purified adapter-ligated DNA can be safely stored at ≤ −20°C for up to one month.

[1] After addition of the **Adapter Ligation Master Mix**, the reaction will become very viscous. It is possible to mix by pipetting up and down, although additional vortexing and inversion is recommended for complete homogenization.

[2] If using only one thermal cycler, ensure that the temperature has reached 37°C (lid temp 45°C) before starting the incubation. If it is still cooling down, leave the samples on ice until the thermal cycler is ready.

Section 3: Index PCR Amplification

Before Starting:

✓ If utilizing the **Zymo-Seq™ UDI Primer Plate**, wait for the wells to thaw completely before use. Spin down in a plate centrifuge. Pierce the foil with a 10 µL pipette tip, then throw away the tip and use a clean pipette tip to aspirate the primers.

1. Combine the following on ice to a 0.2 mL PCR tube containing the purified adapter-ligated DNA[1]:

Component	Volume
Adapter-Ligated DNA	15 µL
Zymo-Seq™ UDI Index Primers	10 µL
2X Index PCR Premix	25 µL
Total Volume	**50 µL**

2. Mix entire reaction thoroughly by pipetting or gently vortexing then briefly centrifuge.

3. Perform the following steps in a thermal cycler (lid temp 105°C):

Step	Temperature	Time	Recommended Number of Cycles
1	98°C	3 minutes	
2	98°C	20 seconds	10 ng DNA = 8-9 cycles 5 ng DNA = 9-10 cycles
3	65°C	30 seconds	
4	72°C	30 seconds	
Repeat Steps 2-4 for X cycles			
5	72°C	1 minute	
6	4°C	Hold	

This is a safe stopping point. Amplified DNA samples can be safely stored overnight at 4°C. Otherwise, continue directly to Step 4 on the next page.

[1] See **Appendix D** for **Zymo-Seq™ UDI Primer** information and multiplexing guidelines.

4. Follow the clean-up protocol in **Appendix A** on pg.13. Allow the **Select-a-Size MagBeads** to equilibrate to room temperature for 30 minutes prior to use. For elution, resuspend the beads in 20 µL of **DNA Elution Buffer** and aspirate all 20 µL eluate after separation from the beads.

The eluate is the final library. Libraries can be safely stored for months at ≤ −20°C

Appendices

Appendix A: Select-a-Size MagBead Clean-Up Protocol

Before Starting:

- ✓ Ensure that the indicated volume of ethanol has been added to the **DNA Wash Buffer** (see pg. 5 **Buffer Preparation**).
- ✓ Allow the **Select-a-Size MagBeads** to equilibrate to room temperature for 30 minutes prior to use.
- ✓ Resuspend the magnetic particles immediately before use by vortexing the **Select-a-Size MagBeads** until homogenous.

1. Add 60 µL of the **Select-a-Size MagBeads** to the tube. Mix thoroughly by pipetting until homogenous and incubate for 5−10 minutes at room temperature.

2. Place the tube on a magnetic stand for 3 minutes, or until the supernatant is clear.

3. Carefully remove the supernatant without disturbing the magnetized bead pellet[1].

4. Without removing from the magnetic stand, add 200 µL of **DNA Wash Buffer** to the tube, incubate for at least 30 seconds, and then remove the supernatant completely without disturbing the magnetized bead pellet. Repeat this wash step for two washes total.

5. Remove the tube from the magnetic stand and centrifuge very briefly. Then return the tube to the magnetic stand, wait for the beads to pellet, and remove any residual **DNA Wash Buffer** with a 10 µL pipette tip.

6. Leave the tube on the magnetic stand and keep the cap open for 2−3 minutes to allow the beads to air dry[2,3].

[1] Avoid aspirating any beads when removing the supernatant.
[2] Do not over dry the beads as this may negatively impact recovery. Beads should remain a glossy brown color.
[3] When performing the clean-up in **Section 2: Adapter Ligation**, the beads are more likely to disperse around the tube and are more susceptible to drying faster than normal.

7. Cap and remove the tube from the magnetic stand. Add the indicated volume of **DNA Elution Buffer** and fully resuspend the beads by pipetting up and down[1]. Incubate for 5 minutes at room temperature.

    a. If performing clean-up in **Section 2: Adapter Ligation**, add 15 µL of **DNA Elution Buffer** to fully resuspend the beads.

    b. If performing clean-up in **Section 3: Index PCR Amplification**, add 20 µL of **DNA Elution Buffer** to fully resuspend the beads.

8. Place the tube back on the magnetic stand for 2 minutes or until the supernatant is clear.

9. Transfer the indicated volume of eluate to a new tube. Discard the beads.

    a. If performing clean-up in **Section 2: Adapter Ligation**, aspirate all 15 µL eluate and transfer to a 0.2 mL PCR tube.

    b. If performing clean-up in **Section 3: Index PCR Amplification**, aspirate all 20 µL eluate and transfer to a new 1.5 mL microcentrifuge tube.

This is a safe stopping point. If moving on to Section 3: Index PCR Amplification, the purified adapter-ligated DNA can be safely stored at ≤ −20°C for up to a month. If this is the final clean-up, DNA libraries can be safely stored at ≤ −20°C for months.

[1] When performing the clean-up in **Section 2: Adapter Ligation**, the resuspended beads behave differently and will be more challenging to remove from the sides of the tube. Briefly centrifuge tubes if necessary. Take care to ensure the beads are still fully resuspended.

Appendix B: Library Quantification and Characterization

Libraries can be quantified using a preferred method (i.e., NanoDrop, Qubit®, TapeStation®, etc.). However, quantitative PCR is the recommended method for accurately determining library concentration prior to loading on to the Illumina sequencers.

Libraries should be visualized by using an automated electrophoresis instrument (i.e., Agilent TapeStation®, Agilent Bioanalyzer®, etc.) to determine that the correct library size is present. We recommend running on High Sensitivity tapes/chips for optimal library characterization. If adapter dimers are present, they will form an approximately 130-180 bp band. Yields will vary depending on the total quantity and quality of sample input DNA.

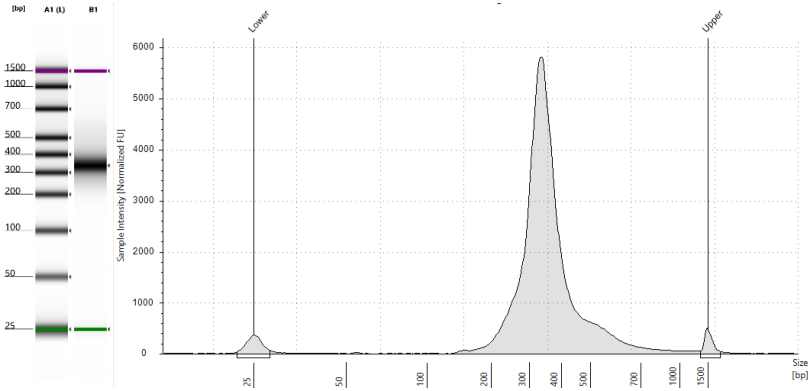

Figure 3. Characterization of a typical Zymo-Seq™ Trio WGBS library. Agilent 4200 TapeStation® HS D1000 gel (left) and electropherogram (right) of a library prepared with the Zymo-Seq™ Trio WGBS Library Kit from 5 ng healthy male donor cfDNA and indexed using 9 PCR cycles. The kit typically produces libraries with two to three visible peaks. These will correspond to an adapter dimer peak at approximately 130-180 bp which may not be present, a mono-nucleosome peak at approximately 290-350 bp, and a di-nucleosome peak at approximately 480-550 bp. **A1** is a molecular weight marker and **B1** is the final cfDNA WGBS library.

Appendix C: *In Situ* Bisulfite Conversion Controls

The provided ***E. coli* Non-Methylated Genomic DNA** (D5016) can be used *in situ* to determine the bisulfite conversion efficiency. The *E. coli* DNA can be spiked in at different percentages of input cfDNA depending on if it is intact or not. Due to the splinted adapters, the likelihood of each adapter being ligated to either end of a DNA fragment is inversely correlated to fragment size (i.e., the smaller the DNA fragment, the more likely both adapters are to ligate; the larger the DNA fragment, the less likely both adapters are to ligate).

The *E. coli* DNA can be used as-is, however prior fragmentation of the DNA will allow for better coverage. Therefore, we recommend the following amounts of spike-in *E. coli* DNA depending on the situation:

- **Intact *E. coli* DNA:** The *E. coli* DNA comes as ready-to-use intact genomic DNA at high molecular weight. Bisulfite conversion will fragment the DNA somewhat; however, it will still be much larger than the cfDNA input without additional fragmentation. To use directly, spike in at 2-5% wt of input cfDNA (e.g., spiking in 100-250 pg of intact *E. coli* genomic DNA into 5 ng of cfDNA sample). The amount can be adjusted to allow for more coverage.

- **Fragmented *E. coli* DNA (200-300 bp):** For better coverage, the *E. coli* DNA can be fragmented to approximately 200-300 bp in average size prior to use. When fragmented to a smaller size, the spike in amount can be reduced to 0.5-1% wt of input cfDNA (e.g., spiking in 25-50 pg of fragmented *E. coli* genomic DNA into 5 ng of cfDNA sample). The amount can be adjusted to allow for more coverage. Fragmenting the *E. coli* DNA beforehand is recommended and will allow for better coverage, although it is not required for accurate bisulfite conversion efficiency determination.

The bisulfite conversion efficiency can be determined by the percentage of unmethylated cytosines in the aligned *E. coli* reads. The reference genome of *E. coli* strain K-12 substrain MG1655 can be used for alignment and analysis. It can be accessed at the following web address: https://www.ncbi.nlm.nih.gov/genome/167?genome_assembly_id=16152

Appendix D: Unique Dual Index Primer Sets

Indexes in the **Zymo-Seq™ UDI Primer Set (Indexes 1-12)** are dispensed in 1.5 mL tubes (D3008), and the **Zymo-Seq™ UDI Primer Plate (Indexes 1-96)** are dispensed in a single-use foil-sealed 96-well plates (D3096). Indexes 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 pages at www.zymoresearch.com.

Primer Sequences:

Forward Primer Sequence (i5):
5′-AATGATACGGCGACCACCGAGATCTACAC**NNNNNNNN**ACACTC
TTTCCCTACACGACGCTCTTCCGATCT-3′

Reverse Primer Sequence (i7):
5′-CAAGCAGAAGACGGCATACGAGAT**NNNNNNNN**GTGACTGGAG
TTCAGACGTGTGCTCTTCCGATCT-3′

UDI Primer Plate (D3096) Setup:

To use UDI primers, pool ≥ 2 libraries in numerical order (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 E: Considerations for Sequencing and Data Analysis

***Preparation for Clustering*:**

Accurate determination of the final library concentration is critical to achieve optimal clustering and sequencing results. For this, we recommend using quantitative PCR (e.g., KAPA® Library Quantification Kit).

Bisulfite conversion reduces the complexity of the library's nucleotide content. Complexity can be increased by loading PhiX or multiplexing with a high-diversity library. Optimal PhiX loading will vary based on the sequencer and sequencer software; please contact Illumina technical support for recommendations.

***Sequencing Parameters*:**

Libraries generated with this workflow are suitable for any read length but increased read lengths will require greater amounts of adapter trimming for the shorter library fragments. For most applications, 100 base paired-end (PE) reads are enough to generate substantial amounts of high-quality data for genome-wide coverage. The sequencing depth will be dependent on the genome size, genome coverage, and site coverage required. Sites with more than 10X coverage have a higher reliability in 5mC calling, but certain sites may have less coverage due to gene copy number, variability in library preparation, or clustering efficiency during sequencing. Using 100 bp PE sequencing, we recommend at least 500 million reads for human DNA WGBS, and at least 200 million reads for mouse DNA WGBS.

***Adapter Trimming*:**

Libraries should be trimmed to remove any adapter sequence. No other trimming is required. Use the following sequences to trim the adapters:

Read 1: AGATCGGAAGAGCACACGTCTGAACTCCAGTCA
Read 2: AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

***Alignment Parameters*:**

Libraries prepared with this kit are directional. As such, the original-top and original-bottom strands will be represented. We recommend aligning to reference genome hg38 for human DNA WGBS, and reference genome mm10 for mouse DNA WGBS.

Appendix F: Considerations Before Starting and Performing Bisulfite Conversion

If the formalin-fixed, paraffin-embedded (FFPE) derived DNA input is sufficient (≥20 ng), we recommend performing sonication to shear the FFPE DNA to an average size of 300-600 bp for best results. This should yield enough sheared DNA for the 5 ng minimum input required for the kit (concentration of the DNA may need to be performed for best results). Perform **Section 1: Bisulfite Conversion** as normal, then refer to the **FFPE DNA Protocol** below for required adjustments to **Sections 2 and 3**.

If sonication is not possible or the FFPE DNA input is insufficient for sonication, the DNA may still be processed directly by performing the bisulfite conversion as instructed in the **EZ DNA Methylation-Gold Kit** (D5005, D5006) instead of **Section 1: Bisulfite Conversion**:

1. The following reagents will need to be purchased separately to perform the **EZ DNA Methylation-Gold Kit** protocol. For users who plan to process all 24 or 96 preps with this protocol, please refer to the following table for ordering information:

Product Name	24 Preps	96 Preps
CT Conversion Reagent	3 x D5001-1	2 x D5001-1-50
M-Dilution Buffer	1 x D5005-2	1 x D5006-2
M-Dissolving Buffer	1 x D5005-6	1 x D5005-6
M-Desulphonation Buffer	1 x D5001-5	2 x D5001-5

2. Perform the bisulfite conversion as instructed in the **EZ DNA Methylation-Gold Kit** manual. Use ≤50 ng of FFPE DNA input, with higher input yielding best results. Elute the bisulfite-converted DNA in 18 µL of the provided **DNA Elution Buffer**.

FFPE DNA Protocol

After the bisulfite conversion process by either **Section 1: Bisulfite Conversion** or with the **EZ DNA Methylation- Gold** protocol, continue the **Zymo-Seq Trio WGBS Library Kit** protocol as instructed with the following adjustments:

1. In **Section 2, Step 11**: After the 1-hour adapter ligation reaction, add 85 µL of **DNA Elution Buffer** to the sample to bring the volume up to 135 µL and mix well by pipetting.

2. In **Section 2, Step 12**: Follow the clean-up protocol in **Appendix A** on pg. 13 using 50 µL of **Select-a-Size MagBeads**. Allow the **Select-a-Size MagBeads** to equilibrate to room temperature for 30 minutes prior to use. For elution, resuspend the beads in 15 µL of **DNA Elution Buffer** and aspirate all 15 µL eluate into a new tube after separation from the beads.

3. In **Section 3, Step 3**: The recommended number of PCR cycles may be increased to the following depending on the FFPE DNA sample input amount and type used:

Sample Input	Sonicated	Not Sonicated
5 ng	9-10 cycles	14-15 cycles
10 ng	8-9 cycles	13-14 cycles
20 ng	-	12-13 cycles
30 ng	-	11-12 cycles
50 ng	-	9-10 cycles

4. In **Section 3, Step 4**: Follow the clean-up protocol in **Appendix A** on pg. 13 using 40 µL of **Select-a-Size MagBeads**. Allow the **Select-a-Size MagBeads** to equilibrate to room temperature for 30 minutes prior to use. For elution, resuspend the beads in 20 µL of **DNA Elution Buffer** and aspirate all 20 µL eluate after separation from the beads.

Ordering Information

Product Description	Catalog No.	Size
Zymo-Seq™ Trio WGBS Library Kit	D5462 D5463	24 preps. 96 preps.

Individual Kit Components	Catalog No.	Amount
EZ DNA Methylation-Lightning™ Kit	D5030T D5030 D5031	10 rxns 50 rxns 200 rxns
Lightning Conversion Reagent	D5030-1 D5032-1	1.5 mL 15 mL
M-Binding Buffer	D5001-3 D5002-3 D5049-3	20 mL 80 mL 100 mL
M-Wash Buffer (concentrate)	D5001-4 D5002-4 D5007-4	6 mL 24 mL 36 mL
L-Desulphonation Buffer	D5030-5 D5031-5 D5046-5	10 mL 40 mL 80 mL
DNA Elution Buffer	D3004-4-1 D3004-4-4 D3004-4-16	1 mL 4 mL 16 mL
Zymo-Spin IC™ Columns	C1004-50 C1004-250	50 pack 250 pack
Collection Tubes	C1001-50 C1001-500 C1001-1000	50 pack 500 pack 1000 pack
DNA Wash Buffer (concentrate)	D4003-2-6 D4003-2-24 D4003-2-48	6 mL 24 mL 48 mL
Select-a-Size MagBeads	D4084-4-10 D4084-4-50	10 mL 50 mL
***E. coli* Non-Methylated Genomic DNA**	D5016	5 µg/20 µL
Zymo-Seq™ UDI Primer Sets	D3008 D3096	12 indexes 96 indexes

Complete Your Workflow

✓ For extraction of high-quality circulating cell-free DNA from up to 10 mL of serum or plasma, up to 5 mL of saliva, and up to 1 mL of amniotic and cerebrospinal fluid:

***Quick*-cfDNA Serum & Plasma Kit**	
Cat. No. D4076	Recover DNA ≥ 100 bp; 50 preps

✓ For extraction of both circulating cell-free DNA and cell-free RNA including protein-bound, exosomal, microRNA, and other small RNA from serum, plasma, and other biological fluid:

***Quick*-cfDNA/cfRNA Serum & Plasma Kit**	
Cat. No. R1072	Recover DNA ≥ 50 bp and RNA ≥ 17 nt; 50 preps

✓ For an all-in-one RRBS library prep kit perfect for DNA methylation profiling at single-nucleotide resolution in CpG-rich regions of the genome including CpG islands, promoters, and gene bodies:

Zymo-Seq RRBS Library Kit	
Cat. No. D5460	24 preps
Cat. No. D5461	48 preps

✓ For epigenetic NGS analysis solutions, contact our Services Department by phone at (949)-679-1190 Ext. 2, by email at services@zymoresearch.com, or on our website at https://www.zymoresearch.com/pages/services:

Next Generation Sequencing Services	
Targeted Bisulfite Sequencing	Evaluate site-specific DNA methylation
Genome-Wide DNA Methylation	RRBS, Methyl-MiniSeq, and Methyl-MaxiSeq
ChIP-Seq Service	Protein/DNA interactions and histone modifications
Human Epigenetic Age	Quantify epigenetic age with Human DNAge
Mouse Epigenetic Age	Quantify biological age across various tissues

Notes

Notes

100% satisfaction guarantee on all Zymo Research products, or your money back.

Zymo Research is committed to simplifying your research with quality products and services. If you are dissatisfied with this product for any reason, please call 1(888) 882-9682.

Integrity of kit components is guaranteed for up to one year from date of purchase.
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.

ZYMO RESEARCH

The **BEAUTY** *of* **SCIENCE** *is to Make Things* **SIMPLE**®

tech@zymoresearch.com www.zymoresearch.com Toll Free: (888) 882-9682