ZYMO RESEARCH

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INSTRUCTION MANUAL

Pico Methyl-Seq™ Library Prep Kit

Catalog Nos. **D5455** & **D5456** (For Illumina-based Sequencing)

Highlights

- Post-bisulfite library preparation for Whole Genome Bisulfite Sequencing (WGBS).
- Accommodates ultra-low DNA input and compatible with FFPE samples.
- Simple, ligation- and gel-free workflow can be completed in a few hours.

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:**

M-Wash Buffer in D5455 comes with ethanol.

DNA Wash Buffer in D5455 and M-Wash Buffer and DNA Wash Buffer in D5456 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

Pico Methyl-Seq™ Library Prep Kit (Kit size)	**D5455** (10 preps)	**D5456** (25 preps)	**Storage Temperature**
Lightning Conversion Reagent	1 tube	3 tubes	Room Temp.
M-Binding Buffer	7 ml	20 ml	Room Temp.
M-Wash Buffer*	6 ml	6 ml (conc.)	Room Temp.
L-Desulphonation Buffer	2 ml	10 ml	Room Temp.
DNA Elution Buffer	1 ml	2 ml	Room Temp.
PrepAmp Polymerase (13 U/µL)	6 µl	15 ul	-20 °C
PrepAmp Buffer (5X)	30 µl	75 µl	-20 °C
PrepAmp Primer (40 µM)	15 µl	30 µl	-20 °C
PrepAmp Pre-Mix	50 µl	120 µl	-20 °C
DNase/RNase-Free Water	1 ml	1 ml	Room Temp.
Zymo-Spin™ IC Columns	50	100	Room Temp.
Collection Tubes	50	100	Room Temp.
DNA Binding Buffer	10 ml	25 ml	Room Temp.
DNA Wash Buffer	6 ml (conc.)	6 ml (conc.)	Room Temp.
LibraryAmp Master Mix (2X)	250 µl	625 µl	-20 °C
LibraryAmp Primers (10 µM)	15 µl	30 µl	-20 °C
Index Primer Sets - 6 Sets (10 µM)	30 µl	30 µl	-20 °C

Note - Integrity of kit components is guaranteed for up to one (1) year 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 and the Box 2 contains components that must be stored at -20 °C.

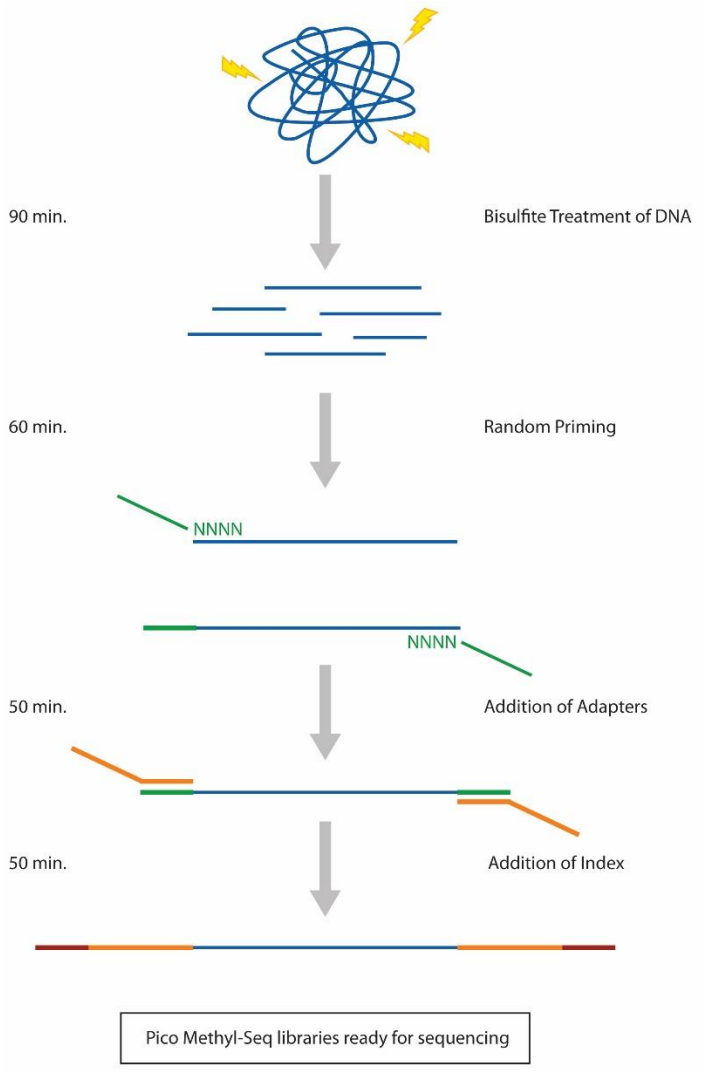
Specifications

- **Sample Sources** – The protocol is designed for 10 pg – 100 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).
- **Sequencing Platform Compatibility** – This system is compatible with Illumina's TruSeq chemistries for the HiSeq™ and MiSeq™ sequencing platforms.
- **Equipment Required** – Microcentrifuge, thermo-cycler

Product Description

Conventional methods for generating libraries for Whole Genome Bisulfite Sequencing (WGBS) typically involve fragmenting genomic DNA, end repairing, adapterization, bisulfite conversion, followed by limited amplification. The drawback to the conventional workflow is the requirement for large starting amounts of DNA and fragmentation of adapterized libraries during bisulfite conversion. This can result in the loss of sample throughout the library preparation and an under-representation of methylation sites in the DNA.

The Pico Methyl-Seq™ Library Prep Kit provides a streamlined workflow for making WGBS libraries. Input DNA is randomly fragmented during the initial bisulfite treatment step followed by three rounds of amplification with uniquely designed primers. The procedure can accommodate as little as 10 pg input DNA (including that derived from FFPE samples), making it ideal for methylation analysis of precious, limited, and target-enriched samples.



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WGBS, as well as other epigenetic services, are available from Zymo Research. Please inquire at:

services@zymoresearch.com

Buffer Preparation: *Before starting…*

- ✓ **D5455:** Add 26 ml 95% ethanol to the 6 ml **DNA Wash Buffer** concentrate.
- ✓ **D5456:** Add 26 ml 95% ethanol to the 6 ml **M-Wash Buffer** concentrate and 26 ml 95% ethanol to the 6 ml **DNA Wash Buffer** concentrate.

Protocol

Section 1: Bisulfite Conversion of Genomic DNA
Section 2: Amplification with PrepAmp Primer
Section 3: Purification with the DNA Clean & Concentrator™ (DCC™)
Section 4: Amplification with LibraryAmp Primers
Section 5: Amplification with Index Primer
Section 6: Validation and Quantification of Library

Notes:

Bisulfite conversion step in **Section I** features the Zymo EZ DNA Methylation – Lightning kit.

Section 1: Bisulfite Conversion of Genomic DNA

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

Component	Volume
Lightning Conversion Reagent	130 µl
Input DNA (10 pg - 100 ng)	20 µl (DNA + H_2O)
Total	**150 µl**

2. Incubate the mixture in a thermo-cycler according to the following:

Temperature °C	Time	Notes
98	8 min	
54	1 hr	
4	≤ 20 hours	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.

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

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

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

Yields can often be enhanced if the **DNA Elution Buffer** is heated before application to the column.

7. Add 8 µl 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.

Section 2: Amplification with the PrepAmp Primer

1. *Priming Reaction*: Mix the following components for the priming reaction on ice:

Priming Components	Volume
PrepAmp Buffer (5X)	2 µl
PrepAmp Primer (40 µM)	1 µl
Bisulfite-converted DNA	7 µl
Total	**10 µl**

2. *PrepAmp Mix*: Combine the following components for the PrepAmp mix in a separate tube on ice:

PrepAmp mix components	Volume
PrepAmp Buffer (5X)	1 µl
PrepAmp Pre-mix	3.75 µl
PrepAmp Polymerase (13 U/µL)	0.3 µl
Total	**5.05 µl**

Note:

For genomic inputs <100 pg, It is recommended to dilute the 40 µM primer to 20 µM and use 1 µl of this dilution to avoid primer dimer formation that can interfere with sequencing

Notes:

The time between the additions of PrepAmp Polymerase is ~ 24 minutes.

3. Incubate the Priming Reaction tubes in a thermo-cycler with the following program for a total of two (2) cycles with the lid temperature set at 25°C:

Important: Make sure that the lid temperature is set at 25°C. Thermo-cycler capabilities vary. Refer to Note 4 in Appendix, pg. 8, for alternative ramping rates. *Note: During Step 3 of **Cycle 1**, add 5.05 µl PrepAmp mix. During Step 3 of **Cycle 2**, add only 0.3 µl PrepAmp Polymerase.*

Step	Temperature (°C)	Time
1	98	2 min
2	8	1 min **Hold**
3 (hands-on)	**Cycle 1**: Pulse-spin tubes and add 5.05 µl PrepAmp **mix** **Cycle 2**: Pulse-spin tubes and add 0.3 µl PrepAmp **Polymerase**	
4	8	4 min
5	16	1 min (Ramp rate: 0.1°C s^{-1})
6	22	1 min (Ramp rate: 0.1°C s^{-1})
7	28	1 min (Ramp rate: 0.1°C s^{-1})
8	36	1 min (Ramp rate: 0.1°C s^{-1})
9	36.5	1 min (Ramp rate: 0.1°C s^{-1})
10	37	8 min
11	Go to Step 1 and repeat once	
12	4	Forever

Section 3: Purification with the DNA Clean-up & Concentrator™ (DCC™)

Collection tubes can hold up to 850 µl.

1. In a 1.5 ml tube, add a 7:1 ratio of DNA Binding Buffer to the product from the PrepAmp Polymerase Reaction (i.e. add 175 µl DNA Binding Buffer to 25 µl product), mix well, and transfer to a Zymo-Spin™ IC column in a Collection Tube. Spin at ≥ 10,000 x *g* for 30 seconds.

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

2. Add 200 µl DNA Wash Buffer to the column. Spin at ≥ 10,000 x *g* for 30 seconds. Repeat this wash step.

Sequential elutions of small volumes (2 X 6 µl) can ensure complete elution of all DNA from the column.

3. Transfer the Zymo-Spin™ IC column to a 1.5-ml microcentrifuge tube. Add 12 µl DNA Elution Buffer directly to the column matrix and let stand for 1 minute at room temperature. Spin at ≥ 10,000 x *g* for 30 seconds to elute.

Section 4: Amplification

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

Component	Volume
LibraryAmp Master Mix (2X)	12.5 µl
LibraryAmp Primers (10 µM)	1 µl
Purified product from **Section 3**	11.5 µl
Total	**25 µl**

2. Incubate the mixture in a thermo-cycler with the following program for a total of six (6) amplification cycles:

Temperature °C	Time	
94	30 s	
94	30 s	←
45	30 s	
55	30 s	Repeat 5 times*
68	1 min	
68	5 min	
4	Forever	

* For 100 ng of genomic DNA input. For other quantities of starting input, refer to Appendix, pg. 8.

3. Purify the PCR product with the DCC™ (refer to **Section 3**) and elute in 12.5 µl DNA Elution Buffer.

Section 5: Amplification with the Index Primer

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

Component	Volume
LibraryAmp Master Mix (2X)	12.5 µl
Index Primer (10 µM)	0.5 µl
Purified product from **Section 4**	12.0 µl
Total	**25 µl**

2. Incubate the mixture in a thermo cycler with the following program for a total of ten (10) amplification cycles:

Temperature ºC	Time	
94	30 s	
94	30 s	←
58	30 s	Repeat 9 times
68	1 min	
68	5 min	
4	Forever	

3. Purify the PCR product with the DCC™ (refer to **Section 3**) and elute in 12 µl DNA Elution Buffer.

Note:

It is recommended to validate and quantify the library using an Agilent 2200 TapeStation or Advanced Analytical Fragment Analyzer.

Section 6: Validation and Quantification of the Library

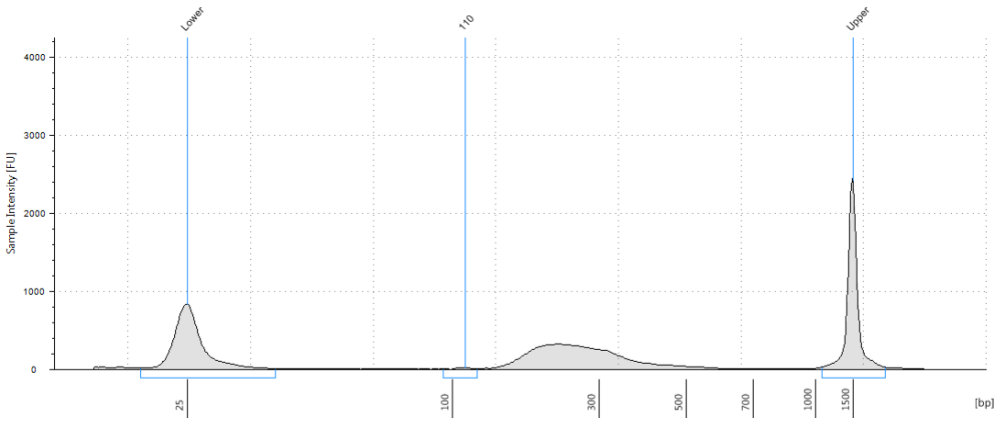

Figure 1. Agilent 2200 TapeStation D1K electropherogram of a Pico Methyl-Seq™ library prepared using 100 pg of human liver DNA. Fragment sizes range from 150 bp – 500 bp.

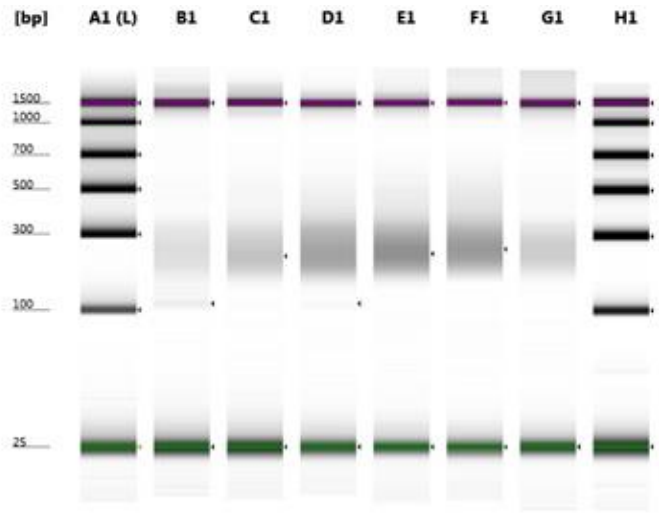

Figure 2. Agilent 2200 TapeStation D1K gel of libraries prepared (from B1-G1) using 10 pg, 20 pg, 100 pg, 1 ng, 10 ng, and 100 ng, respectively.

Appendix: Notes for General Consideration

1. The library procedure is designed for a starting input of 100 ng gDNA, but can be scaled-up or -down as needed (see table below).

Genomic DNA	Total number of cycles in **Section 4** (pg. 6)
10 pg to 1 ng	10
1 ng to 50 ng	8
50 ng to 100 ng	6
≥ 100 ng	May need to be diluted after **Section 4** before proceeding to the final amplification.

2. A higher DNA Binding Buffer to sample ratio (>3:1) may be used when utilizing low DNA inputs to maximize recovery during purification steps.

3. The PrepAmp Polymerase in **Section 2** is not heat stable and will be denatured at high temperatures. Make sure the lid is set at 25°C throughout the thermo-cycling reaction.

4. The thermo-cycler program in **Section 2** can be adjusted to fit thermo-cycler capabilities as long as the temperature ramp from 8°C to 37°C spans 8 minutes.

5. Primer dimers at 126 bp may appear as a result of a high PrepAmp Primer concentration relative to the amount of input DNA. The PrepAmp Primer (40 µM) can be diluted to 20 µM to alleviate this issue (**Section 2**).

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

7. Each library should be sequenced to obtain at least 800 million reads for 15-20X coverage of sites. This number is based on the size of the human genome. Sequencing can be scaled-up or -down according to genome size. Recommended: Treat Pico Methyl-Seq™ libraries as 50 bp single-end reads when mapping back to the reference genome. Sequentially trimming of the first four bases may increase mapping efficiency.

8. Index sequences*

Index	Illumina Index	Sequence
A	2	CGATGT
B	4	TGACCA
C	5	ACAGTG
D	6	GCCAAT
E	7	CAGATC
F	12	CTTGTA

*The index sequences correspond to Illumina Index sequences for multiplexing and are copyrighted to Illumina, Inc.

Ordering Information

Product Description	Cat. No.	Kit Size
Pico Methyl-Seq™ Library Prep Kit	D5455	10 preps
Pico Methyl-Seq™ Library Prep Kit	D5456	25 preps

For Individual Sale	Cat. No.	Quantity
EZ DNA Methylation-Lightning™ Kit	D5030 D5031	50 rxns. 200 rxns.
Lightning Conversion Reagent	D5030-1 D5032-1	10 rxns. 96 rxns.
M-Binding Buffer	D5005-3 D5006-3 D5040-3	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	D4003 D4004	50 preps. 200 preps.
DNA Binding Buffer	D4003-1-L D4004-1-L	50 ml 100 ml
DNA Wash Buffer (concentrate)	D4003-2-6 D4003-2-24	6 ml 24 ml
DNA Elution Buffer	D3004-4-1 D3004-4-4	1 ml 4 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

Related Products for 5-mC Analysis:

Product Name	Size	Cat. No.
***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
EZ Bisulfite DNA Clean-up Kit™	50 Preps. 200 Preps. 2 x 96 Preps. 2 x 96 Preps.	D5025 D5026 D5027 D5028
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
Quest 5-hmC™ Detection Kit	25 Preps. 50 Preps.	D5410 D5411
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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