ZYMO RESEARCH

THE Epigenetics COMPANY™

Zymo-Seq WGBS Library Kit

Bisulfite library preparation in one tube.

Highlights

- Enzymatic reactions consolidated in a single tube for Whole Genome Bisulfite (WGBS) library preparation in less than 4 hours
- Reduced library preparation bias for accurate methylation calling
- Reproducible genome coverage from any species (>90% of CpG sites detected in mammalian samples)

Catalog Number:
D5465

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

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Product Contents

Zymo-Seq WGBS Library Kit	D5465 (24 prep)	Storage Temperature
Lightning Conversion Reagent[1]	3 tubes	Room Temp.
M-Binding Buffer	20 ml	Room Temp.
M-Wash Buffer[2]	6 ml (conc.)	Room Temp.
L-Desulphonation Buffer	10 ml	Room Temp.
DNA Elution Buffer	1 ml	Room Temp.
DNase/RNase-Free Water	4 ml	Room Temp.
Zymo-Spin™ IC Columns	25	Room Temp.
Collection Tubes	25	Room Temp.
E. coli Non-Methylated Genomic DNA	5 µg/20 µl	-20 °C
WGBS Priming Reagent	175 µl	-20 °C
WGBS Synthesis Buffer	225 µl	-20 °C
WGBS Enzyme	30 µl	-20 °C
WGBS Library PCR Mix	525 µl	-20 °C
UDI Tag Primer – 12 Sets	15 µl each	-20 °C
Instruction Manual	1	-

[1] 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.

[2] Add 24 ml of 100% ethanol to the 6 ml M-Wash Buffer concentrate.

Specifications

- **DNA Input -** For optimal results, use 100 ng of high quality, intact genomic DNA as input. Protocol is compatible with inputs between 10 ng to 100 ng. DNA should be free of enzymatic inhibitors and RNA contamination. DNA can be resuspended in water, TE, or a low salt buffer.

- **Processing Time** ~ 4 hours

- **Sequencing Platform Compatibility -** Libraries are compatible with all Illumina sequencing platforms (MiSeq, MiniSeq, HiSeq, NextSeq, NovaSeq)

- **Required Equipment -** Microcentrifuge, thermal cycler with heated lid, magnetic separator, multichannel pipette (suggested)

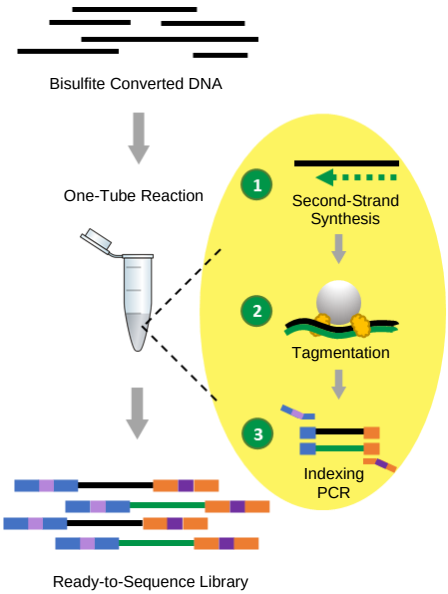
- **Required Materials Not Provided –** Illumina DNA Prep Kit (Illumina, Cat. No. 20018704 or 20018705)

Product Description

Whole Genome Bisulfite Sequencing (WGBS) allows for the detection of methylated cytosines at single-base resolution throughout the entire genome. Methylation levels can be quantified for all cytosines regardless if they are in a CpG, CHG, or CHH context. Conventional methods for generating WGBS libraries require several enzymatic reactions and purification steps. **Zymo-Seq WGBS Library Kit** simplifies these procedures into a single-tube, so ready-to-sequence libraries can be generated in as little as 4 hours.

To prepare Zymo-Seq WGBS libraries, intact genomic DNA is first bisulfite converted. Then, the following library preparation procedures are completed in a single tube: (1) second-strand synthesis, (2) adapterization via tagmentation, and (3) library amplification and indexing. After purification, libraries are ready for sequencing on Illumina instruments. The process has been optimized to ensure libraries offer high genomic coverage and accurate methylation calling.

Zymo-Seq WGBS Library Kit Workflow



Protocol

Section 1: Bisulfite Conversion

Buffer Preparation:

✓ Add 24 ml of 100% ethanol to the 6 ml **M-Wash Buffer** concentrate.

The following procedure should be performed at room temperature (15-30°C) unless specified.

1. Mix the following components in a 0.2 ml PCR tube (not provided).

Component	Volume
Input Genomic DNA (10-100ng)	X µl
E. Coli Non-Methylated Genomic DNA (optional)[1]	Y µl
DNase/RNase-Free Water	Up to 20 µl
Total	20 µl

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

3. Centrifuge briefly to ensure that there are no droplets in the caps or sides of tube. Place the sample in a thermal cycler with a heated lid[2] and perform the following steps:

Step	Temperature	Time
1	98°C	8 min
2	54°C	60 min
3	4°C	Hold ≤ 20 hr (*optional)*

4. Place a **Zymo-Spin™ IC Column** into the provided **Collection Tube**. Add 600 µl of **M-Binding Buffer** to each column.

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.

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[1] Using the ***E. coli* Non-methylated Genomic DNA** (Cat. D5016) as a spike-in is highly recommended for determining bisulfite conversion rate. The *E. coli* DNA can be spiked in at 0.5-1% wt of the input genomic DNA (ex: 0.5-1 ng into 100 ng of DNA input). See **Appendix A (pg. 13)** for additional information.

[2] If the thermocycler cannot program up to 150 µl, set it to the highest volume possible (typically, 100 µl). This will not affect bisulfite conversion performance.

6. Centrifuge ≥10,000 x *g* for 30 seconds. Discard the flow-through.

7. Add 100 µl of **M-Wash Buffer** to the column. Centrifuge ≥10,000 x *g* for 30 seconds.

8. Add 200 µl of **L-Desulphonation Buffer** to the column and let stand at room temperature for 15-20 minutes[1]. After the incubation, centrifuge ≥10,000 x *g* for 30 seconds.

9. Add 200 µl of **M-Wash Buffer** to the column. Centrifuge ≥10,000 x *g* for 30 seconds. Repeat this wash step.

10. Place column into a 1.5 ml microcentrifuge tube and add 15 µl of **DNase/RNase-Free Water** directly to the column matrix and let stand for 1 minute. Centrifuge for 30 seconds ≥10,000 x *g* to elute the Bisulfite Converted DNA.

Safe stopping point: The sample can be safely stored at -20°C overnight. Thaw samples on ice before proceeding to the next step.

[1] Incubation with L-Desulphonation Buffer for longer than 20 minutes may result in degradation and lower yield of converted DNA.

Section 2: Library Preparation

2.1 Second Strand Synthesis

Before Starting:

- ✓ Thaw all -20°C reagents on ice and spin down briefly before use.
- ✓ Create the following priming and synthesis reaction program on the thermal cycler for a total reaction volume of 30 µl and with the **heated lid off or set below 37°C.**

Step	Temperature	Time	
1	98°C	2 min	Priming Reaction
2	8°C	1 min	
3	8°C	Hold	
4	8°C	4 min	Synthesis Reaction
5	12°C	1 min	
6	16°C	1 min	
7	20°C	1 min	
8	24°C	1 min	
9	28°C	1 min	
10	32°C	1 min	
11	37°C	8 min	
12	4°C	Hold	

- ✓ **Reagent preparation:** Freshly prepare the **synthesis reaction master mix**[1] in a new microcentrifuge tube using the table below. Mix well by pipetting and store on ice for use in step 4.

Component	Volume per reaction
WGBS Synthesis Buffer	8.4 µl
WGBS Enzyme[2]	1.2 µl
Total	9.6 µl

1. Transfer 15 µl of Bisulfite Converted DNA into a new PCR tube.

2. Add 7 µl of **WGBS Priming Reagent** (Red Cap) to each tube and mix thoroughly by pipetting. Briefly spin down.

3. Place the tube in the thermal cycler and run Steps 1-3 (Priming Reaction) of the program.

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[1] The volume specified already accounts for pipetting error; do not scale up the reaction. The **synthesis reaction master mix** should be prepared fresh and used immediately.

[2] The **WGBS enzyme** is unstable at high temperatures. Store the enzyme and synthesis reaction master mix on ice during use.

4. After reaching 8°C hold, immediately remove the tube from the thermal cycler and spin down briefly[1]. Transfer 8 µl of the prepared **synthesis reaction master mix** to the tube and mix by pipetting. Briefly spin down.

5. Return the tube to the thermal cycler and run Steps 4-12 (Synthesis Reaction) of the program.

Safe stopping point: The sample can be safely stored at -20°C overnight. Thaw samples on ice before proceeding to the next step.

[1] Ensure that the thermal cycler lid is ≤ 37°C and the samples has cooled to 8°C before adding the synthesis reaction master mix.

2.2 Tagmentation

The following steps 6-18 use Illumina DNA Prep Kit. Users have the option of following the manufacturer's provided protocol specified for DNA tagmentation. However, for clarity and optimal library preparation, it is suggested to follow the instructions described in this instruction manual.

If proceeding with the Illumina DNA Prep protocol, follow the manufacturer's procedure for preparing tagmentation master mix and transfer directly to the reaction from Step 5. Proceed with manufacturer's protocol for post-tagmentation clean-up. For library amplification, proceed to pg. 10, Step 19 of this instruction manual.

6. Allow the **Bead-Linked Transposomes (BLT)**, **Tagmentation Buffer (TB1)**, **Tagment Stop Buffer (TSB)**, and **Tagment Wash Buffer (TWB)**[1] reagents provided in the Illumina DNA Prep kit to equilibrate to room temperature for ~20 minutes.

7. Vortex the **Bead-Linked Transposomes (BLT)** and **Tagmentation Buffer (TB1)** to mix. Ensure that the beads in the BLT are completely resuspended prior to use.

8. Prepare the **tagmentation master mix**[2] using the table below. Vortex gently to ensure that the beads and buffers are thoroughly mixed.

Component	Volume per reaction
BLT	11 µl
TB1	11 µl
Total	22 µl

9. Remove the tube from Step 5 from the thermal cycler, and spin down briefly.

10. Transfer 20 µl of **tagmentation master mix**[3] to the tube. Mix well by pipetting.

11. Place the tube in a thermal cycler with a heated lid[4]. Incubate the samples at 55°C for 15 minutes and hold at 10°C.

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[1] IMPORTANT: After opening, store the Illumina reagents at the appropriate temperature: Store BLT at 4°C, store TB1 at -20°C, and store TSB and TWB at room temperature. If TSB has precipitated, warm the solution to 37°C and vortex until the buffer is clear.

[2] The volume specified already accounts for pipetting error; do not scale up the reaction.

[3] Ensure that the beads in the tagmentation master mix remain evenly resuspended during the aliquot. If not, vortex the master mix in between aliquots.

[4] The heated lid should be set to ≥100°C for all subsequent steps.

12. Remove the tube from step 11 from the thermal cycler and add 10 µl of **Tagment Stop Buffer (TSB)**. Mix well by pipetting.

13. Place the tube in a thermal cycler with a heated lid. Incubate at 37°C for 15 minutes and hold at 10°C.

14. Remove the tube from the thermal cycler and place on a magnetic rack until the beads have fully separated from solution. Remove and discard the supernatant[1].

15. Remove the tube from the magnetic rack and resuspend the beads with 100 µl of **Tagment Wash Buffer (TWB)**. Avoid forming bubbles by gently pipetting.

16. Place the tube on a magnetic rack until the beads have fully separated from solution. Remove and discard the supernatant.

17. Repeat steps 15-16 for one additional wash.

18. Remove the tube from the magnetic rack and resuspend the beads with 100 µl of TWB. Place the tube on a magnetic rack to separate the beads. **Do <u>not</u> remove the supernatant until instructed**. Proceed immediately to Section 2.3 for library amplification.

[1] Avoid drying the beads in between wash steps.

2.3 Library Amplification

19. Prepare the **library amplification master mix**[1] using the table below:

Component	Volume per reaction
WGBS Library PCR Mix	21 µl
DNase/RNase-Free Water	27 µl
Total	48 µl

20. Ensure that the beads from step 18 have fully separated from the supernatant. Remove and discard the supernatant.

21. Remove the tube from the magnetic rack and resuspend the beads using 46 µl of the prepared **library amplification master mix**.

22. Add 4 µl of the appropriate **UDI Tag Primer**[2] to the tube and mix well by pipetting. Spin down briefly to pool any droplets, but do not allow the beads to pellet.

23. Place tube in the thermal cycler and perform the following cycling conditions. Use Table 1 below to adjust the total number of PCR cycles based on the starting genomic DNA input.

PCR Step	Temperature	Time
1	68°C	3 min
2	98°C	3 min
3	98°C	45 sec
4	62°C	30 sec
5	72°C	2 min
6	Repeat PCR Steps 3-5 for X cycles	
7	72°C	1 min
8	4°C	Hold ≤ 18 hr *(optional)*

Table 1. Suggested total number of PCR cycles for various amounts of genomic DNA input.

Genomic DNA Input	Total PCR Cycles
75 – 100 ng	6
25 – 50 ng	8
10 – 25 ng	10

[1] The volume specified already accounts for pipetting error; do not scale up the reaction.

[2] Refer to **Appendix B** (pg. 14) for detailed information about the index primer and multiplexing strategy.

Section 3: Library Purification

Before Starting:

- ✓ Allow the **Sample Purification Beads (SPB)** provided in the Illumina Nextera® kit to equilibrate to room temperature for 30 minutes. Ensure beads are completely resuspended prior to use by inverting and vortexing.

1. Place the tube from step 23 on a magnetic rack and allow the beads to clear from solution. While the beads are on the magnetic rack, transfer 45 µl of the cleared supernatant to a new PCR tube. The BLT beads can be discarded at this point.

2. Transfer 38 µl of **Sample Purification Beads (SPB)** to the supernatant. Mix well by pipetting (~10 times). Incubate at room temperature for 5 minutes.

3. Place the tube on a magnetic rack until the magnetic beads have fully separated from solution.

4. Once the beads have cleared from solution, remove and discard the supernatant[1].

5. While the beads are still on the magnetic rack, add 200 µl of **M-Wash Buffer**[2] to wash the beads. Remove and discard the wash buffer.

6. Repeat step 5 for a total of 2 washes.

7. While the beads are still on the magnetic rack, aspirate out any residual wash buffer.

8. Dry the beads at room temperature for about 3 minutes to ensure that all traces of ethanol have been removed.

9. Remove the tube from the magnetic rack and resuspend the beads with 15 µl of **DNA Elution Buffer** by pipetting up and down until homogenous. Incubate at room temperature for 2 minutes.

10. Place the tube on the magnetic rack for 1-2 minutes to separate the beads from the eluate.

11. Transfer the eluate to a clean microcentrifuge tube. The purified WGBS library is now ready for sequencing.

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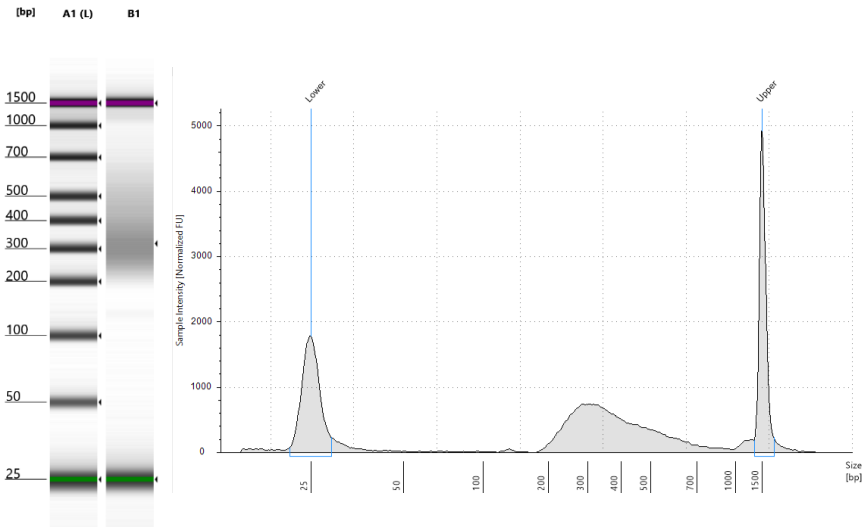
[1] Avoid drying the beads in between wash steps.

[2] Do not resuspend the beads when performing the wash steps.

Section 4: Library Validation and Quantification

Libraries should be visualized (i.e. agarose gel, Agilent TapeStation, Agilent Bionalyzer, etc.) to determine that the correct library size is present, and no adapter dimers have formed. If dimers are present, they will form a ~140 bp band. Libraries ranging from approximately 200-700bp are ideal for sequencing. Below is an example of a completed Zymo-Seq WGBS library visualized on the Agilent Tapestation.

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.



Characterization of completed WGBS libraries. A library was prepared from 100 ng of *Arabidopsis* genomic DNA using the Zymo-Seq WGBS Library Kit and analyzed with Agilent TapeStation 2200. The library preparation produces approximately 200 – 700 bp fragments as shown on the D1000 ScreenTape gel (left) and electropherogram (right). **A1** is a molecular weight marker and **B1** is completed WGBS library.

Appendices

Appendix A: *In Situ* Bisulfite Conversion Controls

Efficient bisulfite conversion (>99%) is important for accurate methylation calling. Bisulfite conversion rate can usually be determined after sequencing by observing the percentage of cytosines in CpH context (H = A, T, C) that were converted to thymines. However, samples, such as plants and pluripotent stem cells, are known to have some level of CpH methylation, which will affect calculation of the bisulfite conversion rate. Therefore, it is best to use an *in situ* control with no CpH methylation to evaluate the conversion efficiency.

The provided *E. coli* Non-methylated Genomic DNA (Cat. D5016) can be used *in situ* to determine the bisulfite conversion rate. The *E. coli* DNA can be spiked in at 0.5-1% of the genomic DNA input before performing the bisulfite conversion. The amount can be adjusted to allow for more coverage or to be in proportion to the genome size of the experimental sample. Bisulfite conversion rate 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.

If your sample is homologous or shares common sequences with *E. coli*, alternative spike-ins should be used. However, there are a few factors to consider:

- **Linearized control:** If using circular DNA (i.e. plasmid or lambda phage DNA), ensure the DNA is linearized for efficient bisulfite conversion.

- **Methylation levels validated:** Ensure that controls produced *in vivo* were grown in a methylation-deficient strain or organism. For example, lambda phage DNA is usually unmethylated, but the phage can be produced in methylation-competent bacterial cells, resulting in low methylation levels of the lambda DNA.

Appendix B: Unique Dual Index Primer Sequences

The kit supplies twelve (12) **UDI Tag Primer Sets**, each with unique i5 and i7 index sequences pooled together.

If multiplexing samples together, use a primer set only once per lane. To ensure color balance during multiplexing, use the Index Tag Primer sequentially (Ex: If pooling 2 samples, use Sets #1-2. If pooling 4 samples, use Sets #1-4.).

UDI Tag Primer Set	i7 Sequence for Sample Sheet	i5 Sequence for NovaSeq, MiSeq, HiSeq 2000/2500	i5 Sequence for MiniSeq, NextSeq, HiSeq 3000/4000
1	GAACTGAGCG	TCGTGGAGCG	CGCTCCACGA
2	AGGTCAGATA	CTACAAGATA	TATCTTGTAG
3	CGTCTCATAT	TATAGTAGCT	AGCTACTATA
4	ATTCCATAAG	TGCCTGGTGG	CCACCAGGCA
5	GACGAGATTA	ACATTATCCT	AGGATAATGT
6	AACATCGCGC	GTCCACTTGT	ACAAGTGGAC
7	CTAGTGCTCT	TGGAACAGTA	TACTGTTCCA
8	GATCAAGGCA	CCTTGTTAAT	ATTAACAAGG
9	GACTGAGTAG	GTTGATAGTG	CACTATCAAC
10	AGTCAGACGA	ACCAGCGACA	TGTCGCTGGT
11	CCGTATGTTC	CATACACTGT	ACAGTGTATG
12	GAGTCATAGG	GTGTGGCGCT	AGCGCCACAC

Appendix C: 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 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 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.

Adapter trimming

Libraries should be trimmed to remove any low-quality bases and to remove any adapter sequence. Use Illumina's Nextera Adapter sequence (CTGTCTCTTATA) for trimming.

Tagmentation creates a 9 bp duplication at the beginning and end of DNA fragments. To ensure that this does not affect accurate methylation calling, the first 15 nucleotides of Read 1 and Read 2 should be removed after adapter and quality trimming has been completed.

Alignment parameters

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

Ordering Information

Product Description	Catalog No.	Size
Zymo-Seq WGBS Library Kit	D5465	24 preps

Individual Kit Components	Catalog No.	Amount
E. coli Non-Methylated Genomic DNA	D5016	5 µg/20 µl
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 D5005-3 D5006-3	20 ml 80 ml 30 ml 125 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 Elution Buffer	D3004-4-1 D3004-4-4 D3004-4-10 D3004-4-16	1 ml 4 ml 10 ml 16 ml
DNase/RNase-free Water	W1001-1 W1001-4 W1001-6 W1001-10	1 ml 4 ml 10 ml 16 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

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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-Seq ATAC Library Kit (D5458)
Zymo-Spin ChIP Kit (D5209)

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Notes

Notes

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