ZYMO RESEARCH

The Beauty of Science is to Make Things Simple

INSTRUCTION MANUAL

RRHP™ 5-hmC Library Prep Kit

Catalog Nos. **D5450** & **D5451**

Highlights

- Innovative library preparation for strand-specific mapping of 5-hmC in DNA.
- Streamlined workflow accommodates low (≥ 100 ng) DNA inputs.
- Libraries are ideal for Next-Gen sequencing or array.

Contents

ZYMO RESEARCH CORP.

Phone: (949) 679-1190 ▪ Toll Free: (888) 882-9682 ▪ Fax: (949) 266-9452 ▪ info@zymoresearch.com ▪ www.zymoresearch.com

Satisfaction of all Zymo Research products is guaranteed. If you should be dissatisfied with this product please call 1-888-882-9682.

Product Contents

RRHP™ 5-hmC Library Prep Kit (Kit Size)	**D5450** (12 Rxns.)	**D5451** (25 Rxns.)	**Storage Temperature**
MspI (20 U/µl)	40 µl	2 x 40 µl	-20 °C
10X RRHP Reaction Buffer	200 µl	2 x 200 µl	-20 °C
10X UDPG (1 mM)	600 µl	600 µl	-20 °C
5-hmC Glucosyltransferase (2 U/µl)	50 µl	100 µl	-20 °C
RRHP Adapter 1 (20 µM)	15 µl	15 µl	-20 °C
RRHP Adapter 2 (20 µM)	15 µl	15 µl	-20 °C
T4 DNA Ligase (400 U/µl)	15 µl	2 x 15 µl	-20 °C
Index Primer Sets (6 sets, 10 µM each)	30 µl	30 µl	-20 °C
rATP (10 mM)	15 µl	15 µl	-20 °C
***Taq* DNA Polymerase (2 U/ µl)**	15 µl	15 µl	-20 °C
2X Quest*Taq*™ PreMix	500 µl	2 x 500 µl	-20 °C
dNTPs (10 mM; 2.5 mM/ea)	100 µl	100 µl	-20 °C
Zymo-Spin™ IC Columns	50	100	Room Temp.
Collection Tubes	50	100	Room Temp.
DNA Wash Buffer	6 ml	24 ml	Room Temp.
ADB Buffer	50 ml	50 ml	Room Temp.
DNA Binding Buffer	25 ml	50 ml	Room Temp.
DNA Elution Buffer	4 ml	4 ml	Room Temp.
DNase/RNase-Free Water	4 ml	4 ml	Room Temp.

Note - Integrity of kit components is guaranteed for up to six months from date of purchase. Reagents are routinely tested on a lot-to-lot basis to ensure they provide maximal performance and reliability.

Specifications

- **Sample Sources** – Input ≥ 100 ng intact genomic DNA. DNA can be in water, TE, or low salt buffer and should be free of enzymatic inhibitors.
- **Sequencing** – This system is designed for preparation of libraries compatible with Illumina's TruSeq chemistries for HiSeq™ and MiSeq™ platforms.
- **Equipment Required** – Microcentrifuge and a heat block, water bath, or thermal cycler for heat steps. A thermal cycler is required for PCR and electrophoresis equipment for DNA selection.

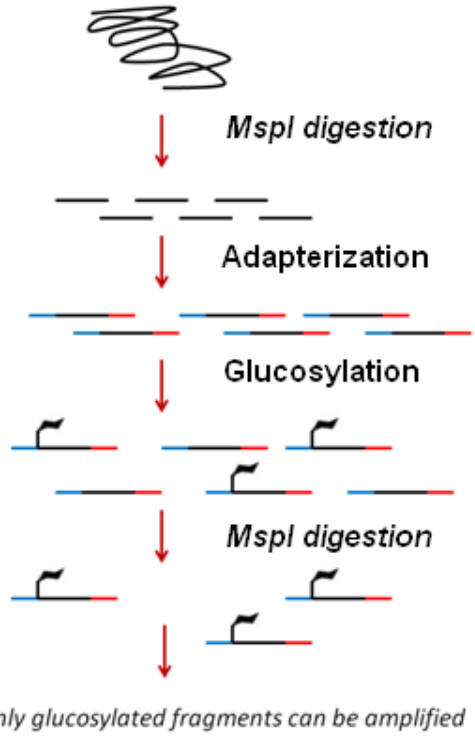
Product Description

While the importance of DNA methylation in epigenetic regulation is well established, the biological role of hydroxymethylation remains elusive. The "sixth base", 5-hydroxymethylcytosine (5-hmC), has been detected in the DNA of embryonic stem cells and other cell types: Neuronal tissue DNA contains the highest levels of 5-hmC, while many carcinomas demonstrate the lowest levels. Recent work suggests that 5-hmC may function in gene regulation and may be involved as an intermediate in active demethylation of 5-methylcytosine (5-mC).

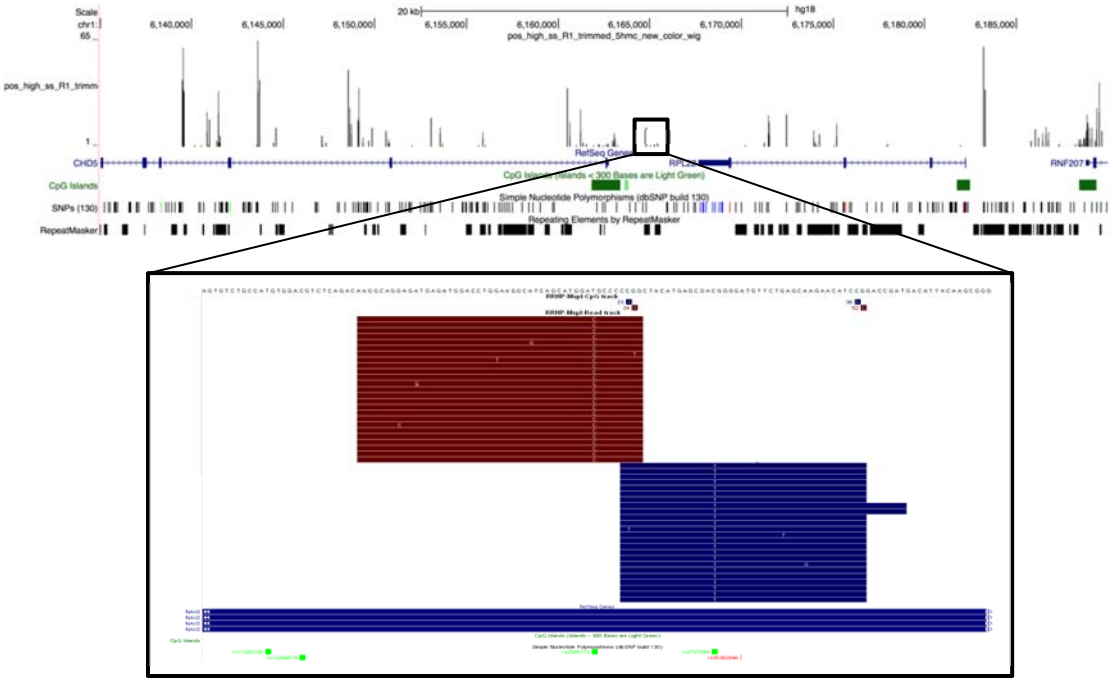
The **RRHP™ 5-hmC Library Prep Kit** is the first comprehensive solution for quantitative analysis of genome-wide 5-hmC positions at single base resolution. The *Reduced Representation Hydroxymethylcytosine Profiling* (RRHP) system is based on blocking MspI digestion by glucosylating 5-hmC within MspI recognition sites. Shown in the schematic below, fragments lacking glucosylated-5-hmC at the adapter-ligation junction will be cleaved and not amplified by PCR. Positive selection relies on fragments containing 5-hmC being successfully amplified and analyzed.

This kit includes all of the components necessary for library preparation: DNA is digested with MspI and then ligated to the RRHP Adapters. The DNA is then treated with β-GT, digested again with MspI, and then size-selected from an agarose gel. Lastly, these fragments can be amplified with the Index Primer Set to generate the final RRHP Library. The kit also includes all of the materials required for high efficiency purification of DNA following enzymatic processing steps.

Libraries generated with the RRHP™ 5-hmC Library Prep Kit are ideal for Next-Gen sequencing using TruSeq™ chemistries. Libraries can also be used for microarray-based detection and analysis.



Genome-wide 5-hmC analysis is available as a service from Zymo Research. Please inquire at: **services@zymoresearch.com**



Single-Site Display of 5-hmC. Human brain DNA was prepared using the **RRHP™ 5-hmC Library Prep Kit** and then sequenced with an Illumina HiSeq™ 2000 with 50 bp single-end reads. Sequence was aligned to the human reference genome. Tracks (bottom figure) display relative quantitation of 5-hmC strand distribution (blue = sense, red = antisense) as well as SNP positions within the fragments at a single locus.

Experimental Considerations

1. Input DNA and Digestion – Input DNA should be free of enzymatic inhibitors and relatively intact (≥ 10 kb) to ensure optimal digestion by MspI. Size and quality should be assessed using gel electrophoresis or other highly sensitive method (e.g., Agilent Bionalyzer™) before proceeding with sample preparation. It is recommended to use ≥ 100 ng of input DNA (neuronal origin) or ≥ 500 ng (non-neuronal). The protocol can accommodate up to 1 µg DNA for each reaction. DNA inputs exceeding 1 µg may result in incomplete digestion of the DNA.

2. Downstream Applications – The adapters and primers included in this workflow are designed for sequencing with Illumina's TruSeq™ chemistries. Completed libraries are also suitable for cloning, colony screening, and Sanger sequencing. Additionally, libraries generated using this workflow are suitable for hybridization in tiling arrays and other array systems.

Buffer Preparation

✓ *Before starting:* Add 24 ml 100% ethanol (26 ml 95% ethanol) to the 6 ml **DNA Wash Buffer** concentrate. Add 96 ml 100% ethanol (104 ml 95% ethanol) to the 24 ml **DNA Wash Buffer** concentrate.

Protocol

Section I: Digestion of Genomic DNA

1. Add the following to a PCR tube:

Genomic DNA (≤ 1 µg)	X µl
10X RRHP Reaction Buffer	5 µl
MspI (20 U/µl)	1.5 µl
H_2O	Y µl
	50 µl

Note: *To ensure complete digestion, we recommend the input DNA does not exceed 1 µg per reaction.*

2. Mix, spin briefly, and then incubate the reaction at 37 °C for 8 hours.

3. Transfer the digestion to 300 µl **DNA Binding Buffer** in a **Zymo-Spin™ IC Column** in a **Collection Tube**. Spin at ≥ 10,000 x *g* for 30 seconds.

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

5. Transfer the **Zymo-Spin™ IC Column** to a 1.5 ml microcentrifuge tube. Add 15 µl **DNA Elution Buffer** directly to the column matrix and wait for 1 minute. Spin at ≥ 10,000 x *g* for 1 minute to elute the digested DNA.

Section II: Adapter Ligation, Extension, and Library Glucosylation

1. Add the following to a PCR tube:

Digested DNA	15 µl
10X RRHP Reaction Buffer	2 µl
T4 DNA Ligase (400 U/µl)	1 µl
RRHP Adapter 1 (20 µM)	0.5 µl
RRHP Adapter 2 (20 µM)	0.5 µl
rATP (10 mM)	0.5 µl
H_2O	0.5 µl
	20 µl

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

Notes:

For best results, use a volume of DNA ≤ 15 µl.

The purity of a genomic DNA sample is critical. For best results, ensure that the sample is free of excess salts, alcohol, or other organic contaminants.

Yields may be increased if the **DNA Elution Buffer** is heated before being added to the column. Also, sequential elutions with small volumes (2 x 7.5 µl) can be used for complete elution of DNA from the column.

2. Mix, spin briefly, and then incubate at 16 °C for 2 hours.

   Note: *If desired, adapter ligation can be carried out overnight at 16 °C without diminishing the quality of the library.*

3. Following the incubation, add the following to the tube:

***Taq* DNA Polymerase (2 U/µl)**	0.5 µl
dNTPs (10 mM; 2.5 mM/ea)	1.5 µl
	22 µl total volume

4. Mix briefly, spin, and then incubate at 72 °C for 30 minutes in a thermal cycler with a heated lid. Hold at 4 °C.

5. Following the incubation, add the following to the tube:

10X RRHP Reaction Buffer	3 µl
10X UDPG (1 mM)	5 µl
β-Glucosyltransferase (2 U/µl)	3 µl
H_2O	17 µl
	50 µl total volume

6. Incubate the reaction at 37 °C for 2 hours.

7. Transfer the reaction to 500 µl **DNA Binding Buffer** in a **Zymo-Spin™ IC Column** in a **Collection Tube**. Mix thoroughly, and then spin at ≥ 10,000 x *g* for 30 seconds.

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

9. Transfer the **Zymo-Spin™ IC Column** to a 1.5-ml microcentrifuge tube. Apply 15 µl **DNA Elution Buffer** directly to the column matrix and wait for 1 minute. Spin at ≥ 10,000 x *g* for 1 minute to elute the glucosylated DNA.

Note:

The glucosylation reaction can proceed overnight, or be stored at -20 °C prior to DNA purification.

Section III: Final Digestion

1. Add the following to a PCR tube:

Glucosylated DNA	15 µl
10X RRHP Reaction Buffer	3 µl
MspI (20 U/µl)	1.5 µl
H_2O	10.5 µl
	30 µl

2. Mix, spin briefly, and then incubate at 37 °C for 3 hours.

3. Transfer the reaction to 65 °C for 15 minutes for complete enzyme inactivation. Add loading dye (1X final concentration) and then store on ice until the next step.

Section IV: Size Selection

1. Proceed with size selection*. For the best resolution, use a 2% (w/v) agarose/TAE gel w/EtBr. Gels should be electrophoresed at 110 V such that the range from 100-500 bp can be confidently isolated in an excised gel slice.

2. Add each excised gel slice to 3 mass volumes **ADB Buffer** (1:3) in a 1.5 ml microcentrifuge tube. Incubate at 50 °C for at least 30 minutes. Vortex the mixture periodically to ensure complete solubilization of the gel matrix.

3. Transfer to a **Zymo-Spin™ IC Column** in a **Collection Tube**. If the total volume exceeds 800 µl, load the column twice. Spin at ≥ 10,000 x *g* for 30 seconds.

4. Discard the flow-through. Add 300 µl **DNA Wash Buffer** to the column. Spin at ≥ 10,000 x *g* for 30 seconds. Repeat this wash step.

5. Transfer the **Zymo-Spin™ IC Column** to a 1.5-ml microcentrifuge tube. Add 15 µl **DNA Elution Buffer** directly to the column matrix and wait for 1 minute. Spin at ≥ 10,000 x *g* for 1 minute to elute the size-selected DNA library.

Notes:

*Alternative size selection methods, such as the Caliper LabChip® XT, Sage Science Pippin Prep™, or Agencourt AMPure® Beads can also be used.

Gels should be poured with wells that can accommodate up to 40 µl of sample. If possible, empty wells should be left between samples to avoid cross-contamination.

For optimal gel recovery, determine the mass of excised gel slice and use 3 mass volumes of **ADB** for gel solubilization. (e.g., dissolve a 200 mg agarose slice with 600 µl **ADB**)

Section V: Library Enrichment with Limited Amplification

The kit supplies six (6) **Index Primer Sets** for barcoding different samples for multiplexed sequencing. The index information is provided in the table below. Make sure to use a primer set only once per single lane.

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

1. Assemble the following components in a PCR tube:

Size-selected DNA library	15 µl
Index Primer Set (10 µM/ea)†	1 µl
2X Quest*Taq*™ Master Mix	25 µl
H_2O	9 µl
	50 µl

†Selection of the primer set depends upon multiplexing strategy. For more information on indexing, refer to Appendix A.

Notes:

The thermal profile is based on the use of Quest*Taq*™ Master Mix from Zymo Research. Initial denaturation and extension steps can vary depending on the polymerase used.

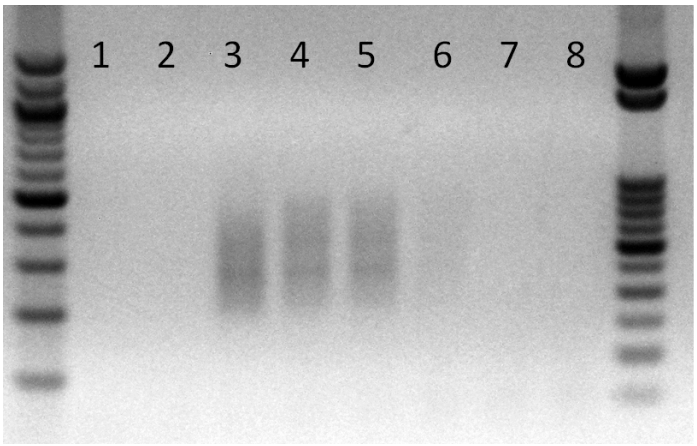
*Precise size distribution will depend upon the genomic DNA sample. A prerequisite for library construction should include *in silico* digestion with MspI to anticipate library characteristics.

Samples with low 5hmC abundance may require additional cycling.

2. Cycle with the following thermal profile:

95 °C	3 min	
95 °C	30 sec	} 10 cycles
59 °C	30 sec	
72 °C	30 sec	
72 °C	1 min	
4 °C	hold	

3. After the first 10 cycles, sample 5 µl of the reaction mix for analysis by agarose gel electrophoresis. Cycling is complete upon visualization of a faint product between 150-500 bp*. If no visible product appears in the gel, add an additional 2 cycles until a product does appear.



1. No DNA in adapterization
2. 500 ng Adapterized DNA – βGT, /MspI
3. 500 ng Adapterized DNA + βGT, /HpaII
4. 500 ng Adapterized DNA + βGT, /MspI
5. 500 ng Adapterized DNA + βGT, /MspI
6. 100 ng Adapterized DNA + βGT, /MspI
7. 100 ng Adapterized DNA – βGT, /MspI
8. NTC

Library Enrichment by Limited Amplification. Human brain DNA was fragmented with MspI then ligated together with RRHP adapters to create sequencing libraries. When glucosylated (libraries 3-6), the library is resistant to subsequent MspI or HpaII digestion and can be amplified. Non-glucosylated samples (libraries 2 and 7) are susceptible to digestion and cannot be amplified for library enrichment. Digestion with HpaII, an isoschizomer of MspI with methylation sensitivity, allows for inclusion of methylated fragments in the final library display.

4. After cycling is complete, add 5 volumes of **DNA Binding Buffer** to the reaction (5:1) and transfer to a **Zymo-Spin™ IC Column** in a **Collection Tube**. Spin at ≥ 10,000 x *g* for 30 seconds.

5. Discard the flow-through from the **Collection Tube** and apply 200 µl **DNA Wash Buffer** to the column. Spin at ≥ 10,000 x *g* for 30 seconds. Repeat this wash step.

6. Transfer the **Zymo-Spin™ IC Column** to a 1.5-ml microcentrifuge tube. Add 15 µl **DNA Elution Buffer** directly to the column matrix and wait for 1 minute. Spin at ≥ 10,000 x *g* for 1 minute to elute the completed DNA library.

Appendix A: Additional Considerations for Library Preparation and Sequencing

Negative Controls

For initial experiments, it is recommended to also prepare a library to serve as a negative control that is *not* treated with β-GT. The presence of any product from the limited amplification of this negative control library will indicate: (a) incomplete MspI restriction in *Section III,* or (b) non-specific contamination occurred during the library preparation process. A non-templated PCR should also be performed during this final step.

PCR Amplification

The **2X Quest*Taq*™ Master Mix** included with this kit is optimized for non-biased amplification of glucosylated DNA. Use of other polymerases may introduce biased amplification.

Alternative Digestion Scheme for Methylation Analysis

This system allows for the alternative use of HpaII, an isoschizomer of MspI, for generation of a final library that includes methylated positions at the adapter-fragment junction, which would normally be excluded from a library generated with MspI.

This allows for a dual-display of 5-mC ***and*** 5-hmC sites from the same sample: At the final digestion step, the library can be split in two for digestion with either MspI or HpaII. These libraries can be compared directly in the same sequencing run using differently indexed primers. Sites appearing only in the HpaII sequencing reads can be regarded as exclusively methylated. When the same site is profiled in the MspI sequencing reads, having the same amount of read depth, it can be regarded as predominantly hydroxymethylated. Ratios between the two can be used to estimate the relative abundances of 5-mC and 5-hmC at individual sites.

Multiplexing

The **Index Primer Sets** included with this kit are indexed to allow multiplexing for up to six (6) samples on a single MiSeq™ or HiSeq™ lane. The index included in each primer can be found in the table on page 6.

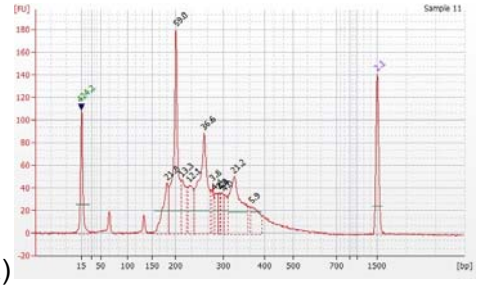
Typical Library Characteristics

Many key indicators of library quality can be observed in agarose gels including:

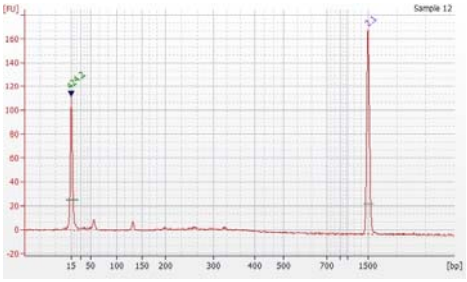
- Intensity
- Size distribution and uniformity (as predicted from *in silico* digestion)
- Presence of primer dimerization

In general, finalized libraries should be analyzed using sensitive methods (e.g., Bioanalyzer™) to observe size distributions and accurately determine concentrations.

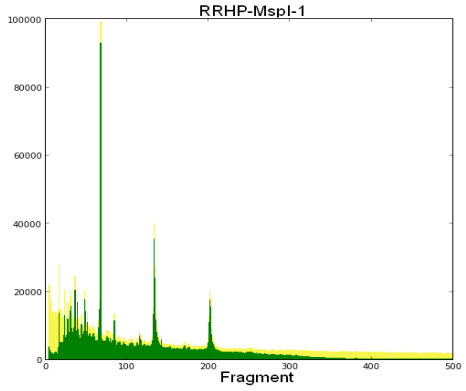
(a)



(b)



(c)



Library Distribution Closely Correlates with *in silico* MspI Digestion. Human brain DNA was processed according to the RRHP protocol and examined using a Bioanalyzer™ and a DNA 1000 Chip. (a) The glucosylated sample shows robust amplification due to protection from final MspI digestion. Peaks indicating the most abundant library fragments correlate closely with the sizes predicted *in silico*, but are shifted ca. +120 bp due to adapterization. (b) The non-glucosylated control sample shows no amplification following final MspI digestion. (c) Predicted (yellow) and mapped (green) library fragments are overlaid to demonstrate the correlation of sequencing results with predicted coverage.

Appendix B: Considerations for Sequencing and Data Analysis

Preparation for Clustering

Accurate determination of the final library concentration is critical to achieve optimal sequencing results – For this, we recommend using a Bioanalyzer™ or similar.

A final library concentration of 8 - 12 pM is required for optimal clustering using TruSeq™ chemistry. Due to the lack of degeneracy of the first several cycles (at the CCGG junction), clusters can be more difficult to distinguish from one another when higher concentrations of library are used. Complexity can also be gained by loading higher amounts of the PhiX control library.

Parity and Read Lengths

The libraries generated with this workflow are suitable for any of the cycling numbers (50 or 300 cycles) for the HiSeq™ and MiSeq™ systems. Note that these cycling numbers *do not* include the cycles needed for indexing, but rather the number of cycles past the index.

For most applications, 50-base, single-end reads are sufficient to generate substantial amounts of high-quality data for genome-wide coverage. Paired-end reads can also be used, but can require greater amounts of adapter trimming, especially for shorter library fragments.

Sequencing can also be performed with 150-base, paired-end cycling to increase the output per lane. As with any other library, Q-scores can drop as read lengths increase. This scoring should be considered when determining quality-trimming parameters.

Output Requirements

Sufficient genome-wide mapping can be accomplished for most 5-hmC sites with 20 - 25 million reads. In general, more reads allow for profiling of less abundant 5-hmC positions. With default parameters of 50-base, single-end reads and 25 million reads as output, the system can confidently profile positions displaying down to ca. 5% 5-hmC.

Alignment and Trimming

In general, any desired alignment program, such as Bowtie or BWA, can be utilized for performing the alignments *(assuming compatibility with the FASTQ output)*. Default quality trimming parameters can be employed (i.e., using Bowtie 0.12.8, parameters with -best). The 3′ adapter sequences (the P7CG adapter) should be trimmed from the reads. Reads presenting the 5′-CCGG tag can then be mapped onto the genome of interest and counted.

Ordering Information

Product Description	Cat. No.	Kit Size
RRHP™ 5-hmC Library Prep Kit	D5450 D5451	12 Rxns. 25 Rxns.

For Individual Sale	Cat. No.	Amount
5-hmC Glucosyltransferase (2 U/µl)	E2026 E2027	100 U 200 U
2X Quest*Taq*™ PreMix	E2050 E2051	500 µl 2000 µl
dNTPs (10 mM; 2.5 mM/ea)	D1000-1	100 µl
Zymo-Spin™ IC Columns	C1004-50 C1004-250	50 250
Collection Tubes	C1001-50 C1001-500	50 500
DNA Wash Buffer	D4003-2-6 D4003-2-24	6 ml 24 ml
ADB Buffer	D4001-1-50	50 ml
DNA Binding Buffer	D4003-1-L	50 ml
DNA Elution Buffer	D3004-4-4	4 ml
DNase/RNase-Free Water	W1001-4	4 ml

Related Products for 5-hmC Analysis:

Product Name	Size	Cat. No.
Quest 5-hmC™ Detection Kit	25 Preps. 50 Preps.	D5410 D5411
Quest 5-hmC™ Detection Kit-Lite	25 Preps. 50 Preps.	D5415 D5416
Quest*Taq*™ PreMix	50 Rxns. 200 Rxns.	E2050 E2051
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

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Additional Products for Epigenetics Research:

Product Name	Size	Cat. No.
Pico Methyl-Seq™ Library Prep Kit	10 Preps. 25 Preps	D5455 D5456
Zymo-Spin™ ChIP Kit	10 Preps. 25 Preps.	D5209 D5210
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
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

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