ZYMO RESEARCH

The Beauty of Science is to Make Things Simple

INSTRUCTION MANUAL

Quest 5-hmC™ DNA Enrichment Kit

Catalog Nos. **D5420** & **D5421**

Highlights

- Clean & uniform enrichment of 5-hmC DNA by J-Binding Protein (JBP-1).
- Simple three-step workflow.
- Enriched DNA is ideal for PCR, qPCR, Next-Gen sequencing, arrays, and more.

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.

Product Contents

Quest 5-hmC™ DNA Enrichment Kit (Kit Size)	D5420 (25 Rxns.)	D5421 (50 Rxns.)	Storage Temperature
JBP Binding Buffer	50 ml	100 ml	Room Temp.
5-hmC DNA Elution Buffer	1.5 ml	1.5 ml	Room Temp.
JBP Capture MagBeads	250 µl	500 µl	4 ℃
5-hmC Control DNA	25 µl	25 µl	-20 ℃
Control Primers (20 µM each)	25 µl	25 µl	-20 ℃
5-hmC Glucosyltransferase (2 U/µl)	50 µl	100 µl	-20 ℃
5X 5-hmC GT Reaction Buffer (contains UDPG)	600 µl	600 µl	-20 ℃
Magnetic Rods	4	4	Room Temp.

Note - Integrity of kit components is guaranteed for up to six (6) months from date of purchase.

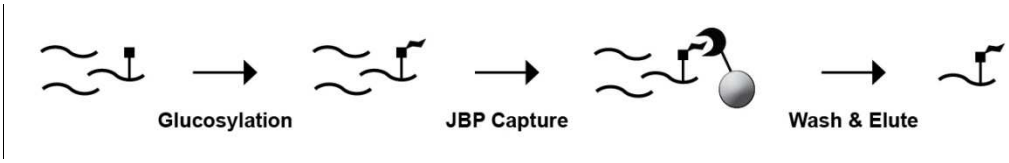
Specifications

- **Sample Sources** – This protocol is developed for processing fragmented genomic DNA as the input material. DNA should be free of enzymatic inhibitors and can be in water, TE, or low salt buffer.
- **Input DNA** – 5-4,000 ng DNA
- **Purity** – Enriched 5-hmC DNA is of suitable purity for a wide variety of downstream applications including PCR, qPCR, Next-Gen sequencing, whole genome amplification, arrays, and more.
- **5-hmC Glucosyltransferase** – One unit (1 U) of recombinant **5-hmC Glucosyltransferase (5-hmC GT)** is sufficient to glycosylate 1 µg of 5-hmC containing DNA in 1 hour at 37 ℃.
- **Equipment Required** – Microcentrifuge, heat block, incubator, and a rotator/rocker are required. A magnetic stand is recommended, but this kit includes magnetic rods if such a stand is unavailable. A thermal cycler will be necessary to perform PCR.

Product Description

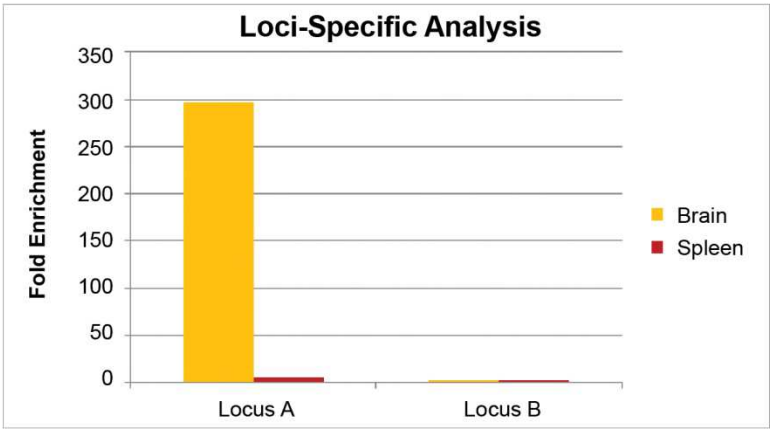
While the importance of DNA methylation in epigenetic regulation is well established, the biological role of hydroxymethylation remains elusive. The "sixth base", 5-hydroxymethylcytosine (5-hmC), has been detected in the DNA of embryonic stem cells and other cell types. Brain tissue DNA contains the highest levels of 5-hmC. 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 **Quest 5-hmC™ DNA Enrichment Kit** features J-base binding protein (JBP) for the specific enrichment of 5-hmC containing DNA. The consolidated workflow makes the procedure reliable for robust analysis of multiple samples. Simply glucosylate the input DNA, add **JBP Capture MagBeads**, and then wash and elute the enriched 5-hmC DNA.



Schematic Overview of The Quest 5-hmC™ DNA Enrichment Kit Workflow

The enriched DNA is suitable for PCR, qPCR, Next-Gen sequencing, whole genome amplification, and arrays. The kit also includes **5-hmC Control DNA** to monitor enrichment efficiency.



Loci-Specific Validation of 5-hmC by qPCR. Human DNA from brain and spleen tissues was randomly fragmented, glucosylated, and enriched using the **Quest 5-hmC™ DNA Enrichment Kit**. The enriched DNA was quantitatively amplified using real-time PCR at Loci A and B. Regions (Loci A and B) shown to be high and low in hydroxymethylation were initially identified from Next-Gen sequencing data.

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

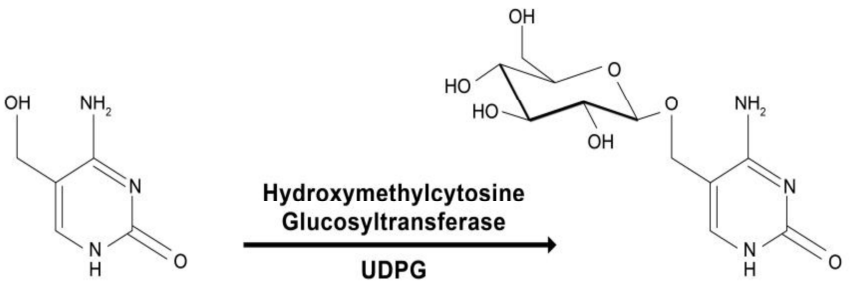
Experimental Considerations

1. DNA Fragmentation and Input – Input DNA should be free of enzymatic inhibitors and in the range of 5-4,000 ng per enrichment reaction. DNA must be fragmented according to your specific requirements. For genome-wide analysis of 5-hmC distribution using Next-Gen sequencing or arrays, any technique for random fragmentation is generally acceptable. For region-specific interrogation of 5-hmC by qPCR, restriction enzyme digestion is recommended.

2. DNA End Modification – If required, various adaptors can be added to the ends of the fragmented DNA using established procedures. End-repair should not interfere with the enrichment.

3. 5-hmC Control DNA – It is recommended that DNA samples be "spiked" with 0.5-1 µl of the supplied **5-hmC Control DNA** *(especially during the preliminary stages of an investigation)* to monitor the enrichment efficiency. The control is comprised of 5-mC and 5-hmC plasmid DNA constructs at a ratio of 9:1, respectively. **Control Primers** are included to selectively amplify the **5-hmC Control DNA** following enrichment. Restriction enzyme digestion of the PCR product is used to assess the efficiency of enrichment. See Appendix A (Page 5) for detailed information regarding this process.

4. Glucosylation – The supplied **5-hmC Glucosyltransferase** (**5-hmC GT**) specifically "tags" 5-hydroxymethylcytosine in DNA with a glucose moiety to yield glucosyl-5-hydroxymethylcytosine, which specifically binds to the **JBP Capture MagBeads**.



5-Hydroxymethylcytosine **Glucosyl-5-Hydroxymethylcytosine**

Glucosyltransferase activity is Mg^{2+}-dependent and requires UDPG as a glucose donor. The supplied **5X 5-hmC GT Reaction Buffer** is supplemented with UDPG and is compatible with NEBuffer #4 (NEB) for enzymatic digestion. So, restriction enzyme digestion and glucosyltransferase reactions can be performed simultaneously in the same mixture. See Appendix B (Page 7) for more specifics regarding this process.

Protocol

Section I: Glucosylation

1. Set up the following in a 1.5 ml microcentrifuge tube.
   (Alternatively, DNA fragmentation/glucosylation can be performed at the same time using the procedure outlined in Appendix B, page 7.)

Input DNA (5-4,000 ng)	X µl
5X 5-hmC GT Reaction Buffer	10 µl
5-hmC Glucosyltransferase	2 µl
ddH_2O	X µl
	50 µl final

   *Note: If the enriched DNA will be used for qPCR, a reaction without **5-hmC Glucosyltransferase** should be included to serve as a negative control.*

2. Mix by pipetting (*or gently tapping*), spin briefly, and then incubate at 37 ℃ for one hour. Proceed with the **Enrichment** below.

Section II: Enrichment

1. Add 500 µl **JBP Binding Buffer** and 10 µl **JBP Capture MagBeads** *directly* to the glucosylation reaction above.

2. Incubate the reaction with constant end-over-end rotation at room temperature for 60 minutes.

3. Briefly spin down the reaction at 300-500 x *g* in a microcentrifuge. Place the tube in a magnetic stand[1] and allow 1-3 minutes for the bead pellet to form. Gently aspirate the supernatant and discard.

4. Add 400 µl of **JBP Binding Buffer** and re-suspend the beads. Pipet this suspension into a new microcentrifuge tube; and place in a magnetic stand[1]. Allow 1-3 minutes for the bead pellet to form and then gently aspirate the supernatant. Repeat this step two more times with 500 µl of **JBP Binding Buffer** using the same tube.

5. Remove any residual binding buffer and then add 20 µl **5-hmC DNA Elution Buffer**. Re-suspend the beads by pipetting (*or gently tapping*) and then briefly spin down the contents at 1,000 x *g*. Transfer the tube to a magnetic stand[1] and allow 1-3 minutes for the bead pellet to form. Collect the supernatant.

   ►The supernatant contains enriched 5-hmC DNA suitable for your downstream applications. The 5-hmC enriched DNA can be used immediately or stored at -20℃ for short term or -80℃ for long term storage.

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

Note:

[1]If a magnetic stand is unavailable, use the supplied **Magnetic Rods**.

Appendix A: 5-hmC Control DNA

The **5-hmC Control DNA** contains 5-mC and 5-hmC plasmid DNA constructs at ratio of 9:1, respectively. The 5-mC DNA has C positions in CpG context enzymatically methylated, while the 5-hmC DNA has C positions in CpG context enzymatically hydroxymethylated. Both DNAs have nearly identical sequences with the exception of a BamHI restriction site in the 5-mC template and a KpnI restriction site in the 5-hmC template (see Page 6). Enrichment can be evaluated (*see below*) by amplifying a 400 bp product from any sample "spiked" with **5-hmC Control DNA** using the supplied **Control Primers** followed by a digestion with KpnI. Successful enrichment is based on cleavage of the 400 bp amplicon to 200 bp fragments (*see gel image below*).

Evaluation of Enrichment using 5-hmC Control DNA

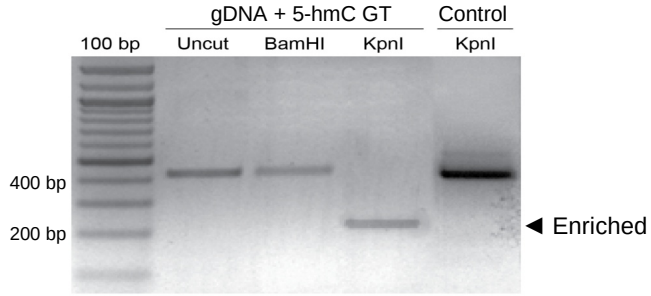
Following enrichment from any sample "spiked" with **5-hmC Control DNA**, use 1 µl for PCR using the **Control Primers** as indicated below:

1	µl	Enriched DNA
1	µl	20 µM **Control Primers**
10	µl	Quest*Taq*™ PreMix
8	µl	ddH_2O
20	µl	total reaction volume

When using the Quest*Taq*™ PreMix (not included), we recommend the following thermal cycling parameters:

95 ℃	3 min	
95 ℃	30 sec	28 cycles
59 ℃	30 sec	
72 ℃	30 sec	
72 ℃	1 min	
4 ℃	hold	

Following amplification, use 5 µl of the PCR directly for KpnI digestion. Allow the digestion to proceed at 37 ℃ for at least 45 minutes. Products can be visualized in a 1.0% agarose gel with standard ethidium staining.



Monitoring 5-hmC Enrichment Using 5-hmC Control DNA. Enriched human brain DNA "spiked" with **5-hmC Control DNA** was used for PCR using the **Control Primers.** PCR procducts were then digested with KpnI and BamHI endonucleases. Only DNA treated with **5-hmC GT** demonstrated enrichment by cleavage with KpnI. The **5-hmC Control DNA** without **5-hmC GT** treatment did not cleave showing no enrichment.

Note:

Quest*Taq*™ PreMix is for *non-biased* PCR amplification of C, 5-mC, and 5-hmC DNA and is available from Zymo Research (Cat. Nos. E2050 & E2051).

Quest*Taq*™ qPCR PreMix is available for real-time applications (Cat. Nos. E2052 & E2053).

5-mC DNA Sequence (BamHI site (in **bold**), C bases at CpG sites are mC, Priming sites are underlined):

CTGGTCCCGCCACCAAACGTTTCGGCGAGAAGCAGGCCATTATCGCCGGCATGGCGGCCGACGCGCT
GGGCTACGTCTTGCTGGCGTTCGCGACGCGAGGCTGGATGGCCTTCCCCATTATGATTCTTCTCGCTT
CCGGCGGCATCGGGATGCCCGCGTTGCAGGCCATGCTGTCCAGGCAGGTAGATGACGACCATCAGG
GACAGCTTCAA**GGATCC**CTCGCGGCTCTTACCAGCCTAACTTCGATCACTGGACCGCTGATCGTCACG
GCGATTTATGCCGCCTCGGCGAGCACATGGAACGGGTTGGCATGGATTGTAGGCGCCGCCCTATACC
TTGTCTGCCTCCCCGCGTTGCGTCGCGGTGCATGGAGCCGGGCCACCTCGACCTGAATGGAAGCCGG
CGGCACCTCGCTAACGGATTCACCACTCCAAGA

5-hmC DNA Sequence (KpnI site (in **bold**), C bases at CpG sites are hmC, Priming sites are underlined):

CTGGTCCCGCCACCAAACGTTTCGGCGAGAAGCAGGCCATTATCGCCGGCATGGCGGCCGACGCGCT
GGGCTACGTCTTGCTGGCGTTCGCGACGCGAGGCTGGATGGCCTTCCCCATTATGATTCTTCTCGCTT
CCGGCGGCATCGGGATGCCCGCGTTGCAGGCCATGCTGTCCAGGCAGGTAGATGACGACCATCAGG
GACAGCTTCAA**GGTACC**CTCGCGGCTCTTACCAGCCTAACTTCGATCACTGGACCGCTGATCGTCACG
GCGATTTATGCCGCCTCGGCGAGCACATGGAACGGGTTGGCATGGATTGTAGGCGCCGCCCTATACC
TTGTCTGCCTCCCCGCGTTGCGTCGCGGTGCATGGAGCCGGGCCACCTCGACCTGAATGGAAGCCGG
CGGCACCTCGCTAACGGATTCACCACTCCAAGA

Appendix B: Simultaneous DNA Fragmentation/Glucosylation

While random fragmentation of genomic DNA by established methods is ideal for genome-scale analysis, certain applications, such as region-specific 5-hmC qPCR, may require more stringent processing methods. For these types of applications, DNA should be fragmented/glucosylated in the same reaction. The supplied **5X 5-hmC GT Buffer** is compatible with NEBuffer # 4 enabling simultaneous restriction enzyme digestion and glucosylation. An example setup with BfaI endonuclease is shown below.

DNA	X µl
5X 5-hmC GT Reaction Buffer	10 µl
5-hmC Glucosyltransferase	2 µl
BfaI*	1.5 µl
ddH_2O	X µl
	50 µl

Mix by pipetting (*or gently tapping*), spin briefly, and then incubate at 37 ℃ for 4 h ours to ensure complete digestion/glucosylation. Proceed with **Section II: Enrichment** on Page 4.

Notes:

***When choosing a restriction enzyme for** simultaneous DNA fragmentation/ glucosylation*, make sure that there is no recognition site in the region to be analyzed by PCR. In addition, DNA methylation sensitive restriction enzymes should be avoided. The following list provides some recommended restriction enzymes*:

BfaI, DpnII, FatI, AluI, MseI, Csp6I, HpyCH4V, HindIII, and XbaI

Make sure that the chosen enzyme is compatible with NEBuffer #4.

Ordering Information

Product Description	Cat. No.	Kit Size
Quest 5-hmC™ DNA Enrichment Kit	D5420 D5421	25 Rxns. 50 Rxns.

For Individual Sale	Cat. No.	Amount
JBP Binding Buffer	D5420-1-50 D5420-1-100	50 ml 100 ml
5-hmC DNA Elution Buffer	D5420-2	1.5 ml
JBP Capture MagBeads	D5420-3-250 D5420-3-500	250 µl 500 µl
5-hmC Control DNA	D5420-5	25 µl
Control Primers (20 µM each)	D5420-6	25 µl
5-hmC Glucosyltransferase (2 U/µl)	E2026-1 E2027-1	50 µl 100 µl
5X 5-hmC GT Reaction Buffer	E2026-4	600 µl
Magnetic Rods	D5420-4	4 rods

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

Additional Products for Epigenetics Research:

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

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