

## 5-Hydroxymethylcytosine (5-hmC) Specific Enrichment

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**[Abstract]** 5-Hydroxymethylcytosine (5-hmC) is a newly discovered DNA modification in mammalian genomes. This protocol is to be a highly efficient and selective chemical approach to label and capture 5-hmC, taking advantage of a bacteriophage enzyme that adds a glucose moiety to 5-hmC specifically, which could in turn be used for high-throughput mapping via next-generation sequencing.

### Materials and Reagents

1. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S3014)
2. Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, catalog number: E9884)
3. Trizma base (Tris) (Sigma-Aldrich, catalog number: T1503)
4. HEPES (Genscript, catalog number: C01621)
5. Magnesium chloride (MgCl<sub>2</sub>) (Sigma-Aldrich, catalog number: M8266)
6. Dimethyl sulfoxide (DMSO) (Fisher Chemical, catalog number: D128)
7. Tween 20 (Sigma-Aldrich, catalog number: P9416)
8. DBCO-S-S-PEG3-Biotin conjugate (Click Chemistry Tools, catalog number: A112P3)
9. 1, 4-Dithiothreitol, ultrapure (DTT) (Roche Applied Science, catalog number: 3117006001)
10. Wizard Genomic DNA Purification Kit (Promega Corporation, catalog number: A1120)
11. 10 kDa Amicon Ultra-0.5 ml centrifugal filters (EMD Millipore, catalog number: UFC501008)
12. QIAquick Nucleotide Removal Kit (QIAGEN, catalog number: 28304)
13. Dynabeads MyOne Streptavidin C1 (Life Technologies, Invitrogen™, catalog number: 650-01)
14. Qiagen MinElute PCR Purification Kit (QIAGEN, catalog number: 28004)
15. UltraPure Agarose (Life Technologies, Invitrogen™, catalog number: 16500500)
16. β-glucosyltransferase (β-GT) (New England Biolabs)
17. DNA marker
18. 10x β-GT reaction buffer (see Recipes)

19. 2x binding and washing (B&W) buffer (see Recipes)
20. TE (see Recipes)

### **Equipment**

1. Sonication device (Covaris)
2. Desktop centrifuges
3. Water bath (Thermo Fisher Scientific)
4. Gel running apparatus (Bio-Rad Laboratories)
5. Nanodrop 1000 (Thermo Fisher Scientific)
6. Labquake tube shaker (Barnstead/ThermoLyne)
7. Magnetic separation stand (Promega Corporation, catalog number: Z5342)
8. Qubit 2.0 Fluorometer (Life Technologies, Invitrogen™)
9. Hydroxymethyl collector (Active Motif, catalog number: 55013)
10. Micro Bio-Spin 6 column (Bio-Rad Laboratories, catalog number: 732-6222)

### **Procedure**

1. Fragment purified genomic DNA into short fragments depending on different genome-wide sequencing platforms (usually sonicated to 300 bp). Follow the manual from Covaris to set up the fragmentation program for 300 bp. DNA buffer for sonication can be TE, EB (10 mM Tris, pH 8.5) or water.
2. Verify the size distribution of the fragmented genomic DNA on 2% agarose gel using 100 bp DNA marker. Measure the DNA concentration after fragmentation using Nanodrop or by fluorometric quantification of dsDNA, ideal concentration should be higher than 600 ng / $\mu$ l.
3. Concentrate the DNA if necessary by 10 kDa Amicon Ultra-0.5 ml centrifugal filters or ethanol precipitation. Measure DNA concentration again after concentration using Nanodrop.
4. Use appropriate starting DNA amounts and set up the reaction according to the following conditions (for brain tissue).
5. Mix by pipetting and incubate in a 37 °C water bath for 1 h.

| Component                       | Volume       | Final concentration   |
|---------------------------------|--------------|-----------------------|
| Water                           | _ $\mu$ l    |                       |
| 10x $\beta$ -GT reaction buffer | 2 $\mu$ l    | 1x                    |
| Up to 10 $\mu$ g genomic DNA    | _ $\mu$ l    | Up to 500 ng/ $\mu$ l |
| UDP-6-N3-Glc (3 mM)             | 0.67 $\mu$ l | 100 $\mu$ M           |
| $\beta$ -GT (40 $\mu$ M)        | 1 $\mu$ l    | 2 $\mu$ M             |
| Total volume                    | 20 $\mu$ l   |                       |

6. Mix by pipetting and incubate in a 37 °C water bath for 1 h.
7. Clean up the reaction with QIAquick Nucleotide Removal Kit, using 10  $\mu$ g DNA per column. Elute with 30  $\mu$ l water per column and combine.
8. Dissolve 10 mg DBCO-S-S-PEG3-Biotin conjugate in 384  $\mu$ l DMSO to make 30 mM stock solution and store at -80 °C. Dilute stock solution with DMSO to make 4.5 mM working solution and store at -20 °C. Stock solution is stable at -80 °C for years. Working solution is stable at -20 °C for 3 months.
9. Add DBCO-S-S-PEG3-Biotin conjugate working solution in the eluted DNA solution from step 6) to a final concentration of 150  $\mu$ M (*i.e.* 1  $\mu$ l of working solution per 30  $\mu$ l DNA solution).
10. Mix by pipetting and incubate in a 37 °C water bath for 1 h.
11. Clean up the reaction with QIAquick Nucleotide Removal Kit, using 10  $\mu$ g DNA per column. Elute with at least 30  $\mu$ l water per column and combine. The ideal combined elution volume is 100  $\mu$ l, maximum volume is 150  $\mu$ l.
12. Measure DNA concentration using Nanodrop, calculate the DNA recovery.
13. Wash 50  $\mu$ l of Dynabeads MyOne Streptavidin C1 three times with 100  $\mu$ l of 1x B&W buffer following its manual. Separate the beads with a magnetic stand and resuspend the beads in 100  $\mu$ l of 2x B&W buffer.
14. Save 10 ng DNA from step 11, add the rest (100 to 150  $\mu$ l) to the resuspended beads from the previous step.
15. Incubate for 15 min at room temperature with gentle rotation on a Labquake tube shaker.
16. Separate the beads with a magnetic stand and wash the beads three times with 200  $\mu$ l of 1x B&W buffer.
17. Elute the DNA by incubating the beads in 50  $\mu$ l freshly prepared 100 mM DTT. For 2 h at room temperature with gentle rotation on a Labquake tube shaker.
18. Separate the beads with a magnetic stand. Aspirate the eluent and load to a Micro Bio-Spin 6 column following its instruction to remove the DTT.
19. Purify the eluted DNA from previous step by Qiagen MinElute PCR Purification Kit and elute DNA in 10  $\mu$ l EB buffer. Quantify DNA using Qubit Fluorometer. The DNA is ready for downstream genome-wide sequencing library preparation.

## **Recipes**

1. 10x  $\beta$ -GT reaction buffer  
500 mM HEPES (pH 7.9)  
250 mM MgCl<sub>2</sub>
2. 2x binding and washing (B&W) buffer  
10 mM Tris (pH 7.5)  
1 mM EDTA  
2 M NaCl  
0.02% Tween 20
3. TE  
10 mM (Tris pH 8.0)  
1 mM EDTA

## **Acknowledgments**

The protocol described here is adapted from one reported previously (Song *et al.*, 2011).

## **References**

1. Song, C. X., Szulwach, K. E., Fu, Y., Dai, Q., Yi, C., Li, X., Li, Y., Chen, C. H., Zhang, W., Jian, X., Wang, J., Zhang, L., Looney, T. J., Zhang, B., Godley, L. A., Hicks, L. M., Lahn, B. T., Jin, P. and He, C. (2011). [Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine](#). *Nat Biotechnol* 29(1): 68-72.