ResearchGate

See discussions, stats, and author profiles for this publication at:
https://www.researchgate.net/publication/279807517

Cover Picture: One-Pot Chemoenzymatic Cascade for Labeling of the Epigenetic Marker 5-Hydroxymethylcytosine (ChemBioChem 13/2015)

ARTICLE *in* CHEMBIOCHEM · JULY 2015

Impact Factor: 3.09 · DOI: 10.1002/cbic.201500329 · Source: PubMed

READS

46

7 AUTHORS, INCLUDING:

Yifat Berkov

Tel Aviv University

39 PUBLICATIONS **373** CITATIONS

SEE PROFILE

Yifat Berkov

Tel Aviv University

11 PUBLICATIONS **61** CITATIONS

SEE PROFILE

Available from: Yuval Ebenstein
Retrieved on: 26 January 2016

One-Pot Chemoenzymatic Cascade for Labelling of the Epigenetic Marker 5-Hydroxymethylcytosine.

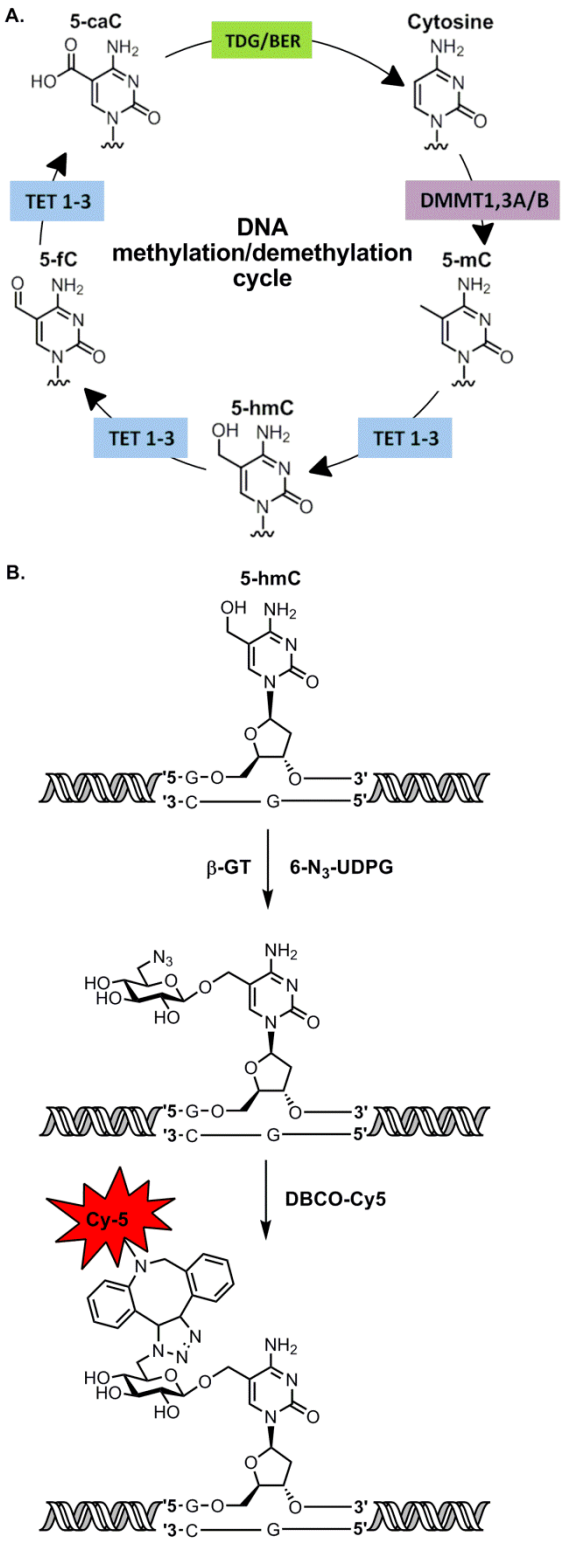
Gil Nifker, Michal Levy-Sakin, Yifat Berkov-Zrihen, Tamar Shahal, Tslil Gabrieli, Micha Fridman* & Yuval Ebenstein*

Abstract: The DNA modification 5-hydroxymethylcytosine (5-hmC) is an epigenetic mark important for regulation of gene expression during development and in tumorigenesis. 5-hmC is selectively glycosylated by the β-glucosyltransferase (β-GT) from the T4 bacteriophage; introduction of an azide on the attached sugar provides a chemical handle for isolation or fluorescent tagging of 5-hmC residues via click chemistry. This approach has not been broadly adopted due to the challenging synthesis and limited commercial availability of the glycosylation substrate, 6-deoxy-6-azido-α-D-glucopyranoside (6-N_3-UDPG). We report on the enzyme-assisted synthesis of this precursor using the uridylyltransferase from *Pasteurella multocida* (PmGlmU). We were able to directly label 5-hmC in genomic DNA via an enzymatic cascade involving successive action of PmGlmU and β-GT. This route provides a facile and cost-effective one-pot chemoenzymatic methodology for 5-hmC analysis.

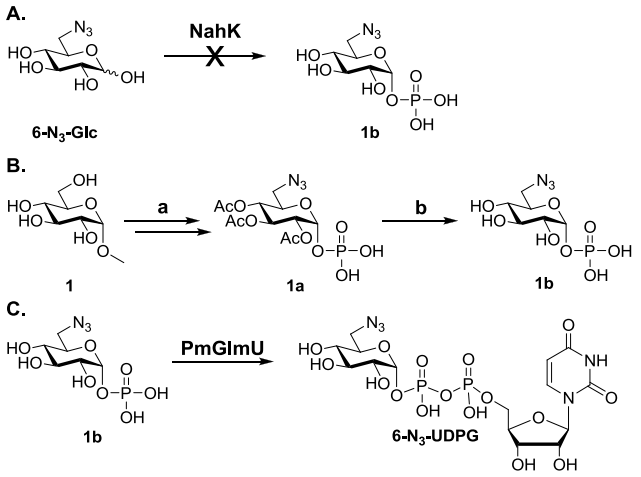
Since its discovery in mammalian DNA in 2009, the epigenetic modification 5-hydroxymethylcytosine (5-hmC) has attracted increasing attention from the genomics community. [1–4] 5-hmC is the initial oxidized form of 5-methylcytosine (5-mC) a cytosine methylated by DNA methyltransferase (DMMT) enzymes. 5-hmC is generated by the action of the Ten-eleven translocation (TET) family of oxygenases that specifically bind 5-methyl C in CpG dinucleotides and catalyze the oxidation of the methyl group to 5-hmC. TET enzymes can then further oxidize this modification to 5-formylcytosine (5-fC) and 5-carboxycytosine (5-caC.)[2,5–7] These subsequent oxidation steps, along with the activity of Thymine-DNA glycosylase (TDG) and base excision repair mechanism (BER) compose an active methylation / demethylation cycle of the CpG sites, releasing gene repression mediated by DNA methylation (Scheme 1A).[8] Although little is known about the exact distribution of 5-hmC in various tissues, it has been shown that 5-hmC patterns are stable for specific cell states and that alterations in these patterns are observed during development and cell differentiation.[2,7,9–12] Another established observation is that 5-hmC levels are lower than normal in a broad range of cancers, potentially providing a new diagnostic and prognostic marker for cancer by simple global quantification of genomic 5-hmC levels. [2,13,14]

[*] G. Nifker, Dr. M. Levy-Sakin, Dr. Y. Berkov-Zrihen, Dr. T. Shahal, T. Gabrieli, Dr. M. Fridman, Dr. Y. Ebenstein
School of Chemistrey,Tel-Aviv University
Tel Aviv 69978 (Israel)
E-mail: mfridman@post.tau.ac.il
uv@post.tau.ac.il

Supporting information for this article is given via a link at the end of the document.



Scheme 1. A. DNA methylation/demethylation cycle. B. Labelling process of 5-hmC in genomic DNA: β-GT mediated glycosylation and copper-free click labelling with DBCO-Cy5.



Scheme 2. Reagents and conditions: A. NahK (0.4 mg/mL), ATP, Tris pH 8 (100 mM), $MgCl_2$ (5 mM), DTT (2 mM), 37ºC, 16 hours. B. a) Compound **1a** was prepared according to a previously reported protocol[15]; b) H_2O: MeOH:Et_3N (2:2:1); 96% isolated yield; C. PmGlmU (0.4 mg/mL), UTP, Tris pH 8 (100 mM), $MgCl_2$ (10 mM), DTT (3 mM), 37 ºC, 16 hours; 19% isolated yield.

One of the most robust tools for 5-hmC analysis involves the use of β-glucosyltransferase (β-GT) from the T4 bacteriophage as depicted in Scheme 1B. This enzyme specifically transfers a glucose moiety from UDP-α-D-glucopyranoside onto 5-hmC. An elegant way of harnessing this activity for 5-hmC detection was presented by Song et al. A synthetic form of the UDP sugar was prepared by substituting the glucose with a 6-azido-glucose. [15] Treatment of genomic DNA with β-GT and UDP-6-deoxy-6-azido-α-D-glucopyranoside (6-N_3-UDPG) results in attachment of an azido-sugar to every 5-hmC residue. The modified 5-hmC can be selectively tagged utilizing copper-free click chemistry[16], enabling global quantification and whole genome 5-hmC mapping by next generation sequencing.[17,18] We have recently demonstrated that by attaching a fluorescent reporter molecule to the modified 5-hmC, it is possible to quantify 5-hmC in genomic DNA with sensitivity as low as 0.002%.[19] This high sensitivity is a pre-requisite for clinical utility of 5-hmC analysis as it allows quantitation of 5-hmC in samples such as white blood cells and solid tumors that contain extremely low levels of this modification.[14,20] Broad implementation of the β-GT/6-N_3-UDPG-based 5-hmC labelling is limited by lack of commercial availability of 6-N_3-UDPG.[21] Moreover, the previously reported synthesis of 6-N_3-UDPG is challenging, and NDP-sugars are unstable once dissolved in aqueous solution, preventing their long-term storage.[15,22]

Chen et al. previously reported the utilization of N-acetylhexosamine 1-kinase (NahK_ATCC55813) and PmGlmU for the one-pot enzymatic synthesis of UDP-GlcNAc derivatives.[23] As both NahK_ATCC55813 and PmGlmU exhibited relaxed substrate specificity, this method enabled the preparation of a highly diverse set of UDP-glucosamine derivatives. We reasoned that these two enzymes could be utilized for generation of 6-N_3-UDPG. Hence, we used the commercially available sugar 6-deoxy-6-azido-D-glucopyranoside (6-N_3-Glc, scheme 2A) as a substrate for NahK_ATCC55813 and PmGlmU.[23]

Incubation of this sugar with the kinase NahK_ATCC55813 did not yield the desired sugar phosphate, and thus we prepared 1-phospho-6-deoxy-6-azido-α-D-glucopyranoside from methyl-O-α-D-glucopyranoside (compounds **1b** and **1**, respectively, Scheme 2B) with the expectation that this sugar-phosphate would serve as a substrate for PmGlmU. As expected, incubation of compound **1b**, UTP, and PmGlmU yielded the desired 6-N_3-UDPG (Scheme 2C). The reaction was monitored by ESI-MS and was complete after 16 hours when the ratio of the mass signal intensity of UTP (m/z $[M-H]^-$, 483.00) and 6-N_3-UDPG (m/z $[M-H]^-$, 590.07) remained constant at ~1:2. The best conversion was observed when the sugar-phosphate **1b** and UTP were used in a 1:1 ratio. Isolation and purification of the product by DEAE column chromatography followed by C18 reverse-phase preparative HPLC gave the desired 6-N_3-UDPG in 19% isolated yield and over 99% purity as indicated by ^{1}H-NMR, ^{13}C-NMR, ^{31}P-NMR, HR-ESI-MS, and HPLC (see supporting information).

We first compared the labelling efficiency of 5-hmC in genomic DNA using the chemoenzymatically produced 6-N_3-UDPG to that using the commercial product. Genomic DNA extracted from mouse brain was treated with the 6-N_3-UDPG from both sources in the presence of β-GT.

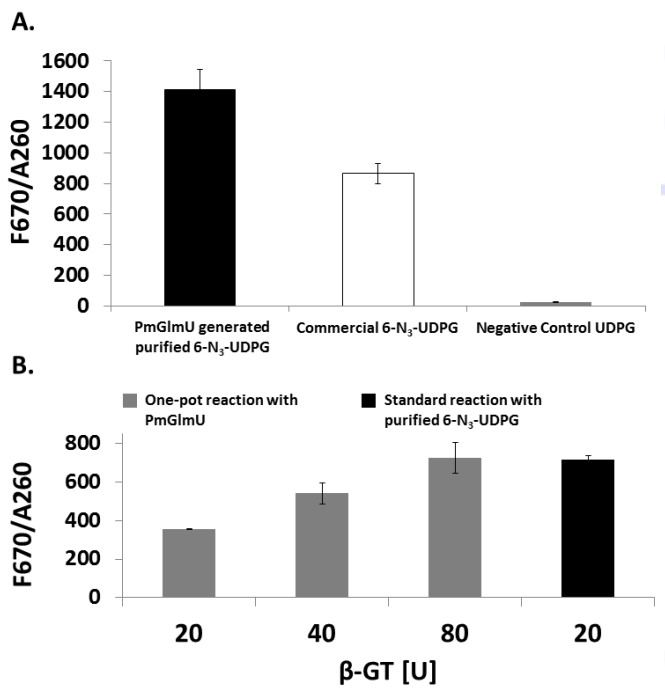


Figure 1. A: 5-hmC labelling of genomic DNA with the PmGlmU generated purified 6-N_3-UDPG compared to labelling with the commercial product in the presence of β-GT. UDP-α-D-glucopyranoside (UDPG) was used as a negative control. B: 5-hmC labelling efficiency in one-pot reaction with PmGlmU and increasing amounts of β-GT vs. standard reaction with purified 6-N_3-UDPG. For both experiments, the efficiency was measured by analysis of the ratio of the 670 nm fluorescence (5-hmC) to 260 nm absorbance (DNA content). Experiments were performed in duplicates and the results were obtained from two independent experiments.

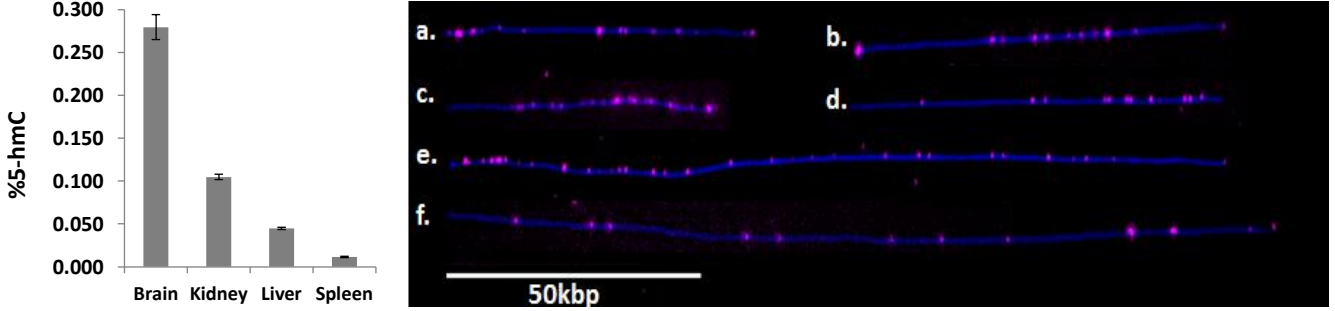


Figure 2. A: Global quantification of 5-hmC levels in mouse tissues (calculated from the calibration scale Figure s_2 in supporting information): brain 0.28±0.01%; kidney 0.105±0.003%; liver 0.045±0.001%; spleen 0.011±0.001%. Results were obtained from double measurements of the same DNA, error bars on graphs refers to their standard deviation. B: Single-molecule detection of 5-hmC. DNA extracted from human peripheral blood mononuclear cells was stained with YOYO-1(blue) and 5-hmC was labelled with Cy5 (magenta). Molecules a-f contain 0.024%, 0.015%, 0.038%, 0.015%, 0.020%, 0.008% 5-hmC in percent of total nucleotides, respectively. The overall genomic 5-hmC content was 0.0027% (2.7 residues per 100 kbp).

The samples were then fluorescently labelled with Cy5-cycloalkyne via copper-free click chemistry as illustrated in Scheme 1B. Fluorescence intensity at 670 nm from the conjugated Cy5 was used to assess the relative degree of labelling. The normalized fluorescence intensities indicated that for the given labelling conditions, the chemoenzymatically generated 6-N_3-UDPG provided 63% higher labelling efficiency (Figure 1A). We also observed elevated labelling efficiency when synthetic 5-hmC DNA standards were used in the reaction (Figure s_1, supporting information).

In order to operate a one-pot labeling scheme we checked whether β-GT could be added directly to the PmGlmU reaction mixture in the DNA labelling process. Mouse kidney genomic DNA was introduced into the crude mixture with varying amounts of β-GT, and the degree of labelling was monitored by fluorescence and compared to a similar reaction performed with the purified 6-N_3-UDPG. Figure 1B shows the results of the one-pot 5-hmC labelling enzymatic cascade with increasing amounts of added β-GT. Upon addition of 80 units of β-GT (4x the standard amount), the labelling efficiency using the crude mixture reached that attained with the purified 6-N_3-UDPG. Thus, direct one-pot 5-hmC labelling of genomic DNA was achieved, mitigating the need to purify the chemoenzymatically generated 6-N_3-UDPG.

We next evaluated 6-N_3-UDPG that was produced by PmGlmU for global quantification of 5-hmC in genomic DNA extracted from various mouse tissues.

The chemoenzymatically prepared 6-N_3-UDPG enabled simple, fluorescence based quantification of the tissue-specific 5-hmC levels as shown in Figure 2A. The quantification is in agreement with our previous observations (Figure s_2, supporting information).[19]

Finally, the labelling procedure was used to detect, for the first time, the 5-hmC patterns on individual DNA molecules extracted from human peripheral blood mononuclear cells.[24–26] These cells are known to have extremely low levels of 5-hmC and are difficult to characterize using other 5-hmC analysis methods. [20] Figure 2B shows several such DNA molecules with magenta-colored fluorescent spots indicating the pattern of Cy5-tagged 5-hmC residues along the DNA. This level of sensitivity allowed quantification of the percentage of 5-hmC on a DNA strand by simply counting the number of spots on a measured length of a DNA molecule in the image (see supporting information for details). The percentage of 5-hmC relative to the total number of nucleotides was 0.0027%. This is a level of sensitivity below the detection limit of most previously published assays.[3]

To conclude, the chemoenzymatic generation of 6-N_3-UDPG from a simple sugar precursor was accomplished. The method enables a more robust generation of the 5-hmC labelling reagent 6-N_3-UDPG, thereby avoiding the limitation of prolonged storage of NDP sugar, which has limited stability. The reported enzymatic coupling offers an alternative to the chemical synthesis of 6-N_3-UDPG and facilitates a simplified isolation of the pure desired product. The labelling capacity of the crude enzymatic coupling mixture enabled us to directly label 5-hmC in genomic DNA via a one-pot enzymatic cascade involving successive action of PmGlmU and β-GT. This approach is a facile and cost-effective methodology for 5-hmC analysis and its applicability to low 5-hmC containing samples such as white blood cells opens new opportunities for the utility of 5-hmC as a routine biomarker.

Acknowledgements

MF acknowledges funding from a grant from the Israel Science Foundation [Grant no. 58/10]. YE acknowledges funding from the i-Core program of the Israel Science Foundation [Grant no. 1902/12], the Marie Curie Career Integration Grant, and the European Research Councils starter grant [Grant no. 337830]. MLS would like to thank Tel Aviv University Center for Nanoscience and Nanotechnology and the Marian Gertner Institute for Medical Nanosystems for financial support.

Keywords 5-Hydroxymethylcytosine • Sugar nucleotides • Chemoenzymatic synthesis • Single molecule

[1] S. Kriaucionis, N. Heintz, *Science* **2009**, *324*, 929–930.

[2] M. Tahiliani, K. P. Koh, Y. Shen, W. A. Pastor, Y. Brudno, S. Agarwal, L. M. Iyer, R. David, L. Aravind, A. Rao, *Science* **2009**, *324*, 930–935.

[3] C. E. Nestor, J.P. Reddington, M. Benson, R.R. Meehan, in Funct. Anal. DNA Chromatin, Vol. 1094, Springer Science+Buisness Media, New York, 2014, pp. 243–258.

[4] E. L. Moen, C. J. Mariani, M. Jeff-eke, E. Litwin, J. N. Nikitas, L. A. Godley, *Immunol. Rev.* **2015**, *263*, 36–49.

[5] Y. Xu, F. Wu, L. Tan, L. Kong, L. Xiong, J. Deng, A. J. Barbera, L. Zheng, H. Zhang, S. Huang, et al., *Mol. Cell* **2011**, *42*, 451–464.

[6] S. Ito, A. C. D'Alessio, O. V. Taranova, K. Hong, L. C. Sowers, Y. Zhang, *Nature* **2010**, *466*, 1129–1133.

[7] T.-P. Gu, F. Guo, H. Yang, H.-P. Wu, G.-F. Xu, W. Liu, Z.-G. Xie, L. Shi, X. He, S. Jin, et al., *Nature* **2011**, *477*, 606–10.

[8] U. Müller, C. Bauer, M. Siegl, A. Rottach, H. Leonhardt, *Nucleic Acids Res.* **2014**, *42*, 8592–604.

[9] D. Globisch, M. Münzel, M. Müller, S. Michalakis, M. Wagner, S. Koch, T. Brückl, M. Biel, T. Carell, *PLoS One* **2010**, *5*, 1–9.

[10] M. Münzel, D. Globisch, T. Brückl, M. Wagner, V. Welzmiller, S. Michalakis, M. Müller, M. Biel, T. Carell, *Angew. Chem., Int. Ed.* **2010**, *49*, 5375–5377.

[11] W. Li, M. Liu, *J. Nucleic Acids* **2011**, *2011*, 870726.

[12] G. Ficz, M. R. Branco, S. Seisenberger, F. Santos, F. Krueger, T. A Hore, C. J. Marques, S. Andrews, W. Reik, *Nature* **2011**, *473*, 398–402.

[13] L. I. Kroeze, B. A. van der Reijden, J. H. Jansen, *Biochim. Biophys. Acta* **2015**, *1855*, 144–154.

[14] L. G. Pellakuru, A. Toubaji, P. Argani, *Oncotarget* **2011**, *2*, 627–637.

[15] C.-X. Song, K. E. Szulwach, Y. Fu, Q. Dai, C. Yi, X. Li, Y. Li, C.-H. Chen, W. Zhang, X. Jian, et al., *Nat. Biotechnol.* **2011**, *29*, 68–72.

[16] J.C. Jewett, C.R. Bertozzi, *Chem. Soc. Rev.* **2010**, *39*, 1272–1279.

[17] K. E. Szulwach, X. Li, Y. Li, C.-X. Song, H. Wu, Q. Dai, H. Irier, A. K. Upadhyay, M. Gearing, A. I. Levey, et al., *Nat. Neurosci.* **2011**, *14*, 1607–1616.

[18] J. P. Thomson, J. M. Hunter, H. Lempiäinen, A. Müller, R. Terranova, J. G. Moggs, R. R. Meehan, *Nucleic Acids Res.* **2013**, *41*, 5639–5654.

[19] T. Shahal, N. Gilat, Y. Michaeli, O. Redy-keisar, D. Shabat, Y. Ebenstein, *Anal. Chem.* **2014**, *86*, 8231–8237.

[20] C. E. Nestor, R. Ottaviano, J. Reddington, D. Sproul, D. Reinhardt, D. Dunican, E. Katz, J. M. Dixon, D. J. Harrison, R. R. Meehan, *Genome Res.* **2012**, *22*, 467–477.

[21] A. B. Robertson, J. A. Dahl, R. Ougland, A. Klungland, *Nat. Protoc.* **2012**, *7*, 340–50.

[22] G. K. Wagner, T. Pesnot, R. a Field, *Nat. Prod. Rep.* **2009**, *26*, 1172–94.

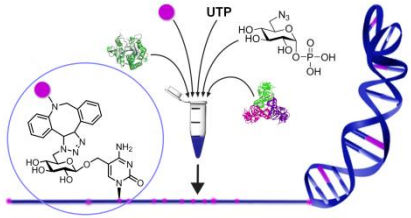
[23] Y. Chen, V. Thon, Y. Li, H. Yu, L. Ding, K. Lau, J. Qu, L. Hie, X. Chen, *Chem. Commun.* **2011**, *47*, 10815.

[24] S. Zirkin, S. Fishman, H. Sharim, Y. Michaeli, J. Don, Y. Ebenstein, *J. Am. Chem. Soc.* **2014**, *136*, 7771–7776.

[25] Y. Michaeli, T. Shahal, D. Torchinsky, A. Grunwald, R. Hoch, Y. Ebenstein, *Chem. Commun.* **2013**, *49*, 8599–601.

[26] M. Levy-Sakin, A. Grunwald, S. Kim, N. R. Gassman, A. Gottfried, J. Antelman, Y. Kim, S. O. Ho, R. Samuel, X. Michalet, et al., *ACS Nano* **2014**, *8*, 14–26.

A one-pot chemoenzymatic protocol for labelling and analysis of the epigenetic marker 5-hydroxymethylcytosine is reported. The reported route provides a facile methodology for the mapping of this epigenetic modification on genomic DNA.



Gil Nifker, Michal Levy-Sakin, Yifat Berkov-Zrihen, Tamar Shahal, Tslil Gabrieli, Micha Fridman & Yuval Ebenstein**

Page No. – Page No.

Title