

Cancer Research Application Note No. 3

Rapid and Reliable Methylation Detection in Archival Tissue Samples using High-Resolution Melting Analysis

Marija Balic, Martin Pichler, Ellen Heitzer,
Jasmin Strutz, Nadia Dandachi

Division of Oncology
Department of Internal Medicine
Medical University of Graz
Auenbruggerplatz 15, 8036, Graz, Austria



Marija
Balic

Martin
Pichler

Ellen
Heitzer

Jasmin
Strutz

Nadia
Dandachi

Reproduced from J Mol Diagn 2009, 11:102-108 with permission from the American Society for Investigative Pathology.

Original Publication: "High quality assessment of DNA methylation in archival tissues from patients with colorectal cancer using quantitative high-resolution melting analysis" in the J Mol Diagn 2009, 11(2).

1 Introduction

Promoter hypermethylation is a frequent mechanism for the repression of gene transcription in cancer and is regarded as one of the hallmarks of cancer. Analysis of DNA methylation is a promising tool for early cancer detection, risk assessment and response to therapy (1, 2).

The most popular approaches for DNA methylation detection rely on the treatment of genomic DNA with sodium bisulfite (3), which converts cytosine into uracil while 5-methyl cytosine remains unmodified. This modification results in a sequence difference allowing for identification of methyl cytosines in a subsequent PCR amplification (4).

The most precise methylation profiling can be achieved by bisulfite sequencing, which allows identification of single methyl cytosines. Several simpler PCR-based methods have also been developed which are especially important for small scale research labs. One of these newer and easier methods is high-resolution melting analysis (HRM), based on the "melting" properties of DNA in solution (5). The principle of this method is that bisulfite-treated DNA templates with different contents of methyl cytosine can be distinguished by melting analysis based on differences in melting temperatures (6). HRM is a relatively simple and cost-effective method

since it does not require expensive probes and reference gene assays for normalization. With HRM all CpGs within the amplicon are analyzed, enabling the assay to distinguish heterogeneous from homogeneous methylation by the shape of the melting curve. This factor can be of importance because methylation patterns at promoter CpG islands are typically not homogeneous (7, 8).

Archival tissues are an important source for testing important biomarkers. The majority of methods have been tested for their performance on formalin-fixed paraffin-embedded (FFPE) tissues. The aim of the present study was to establish and evaluate HRM assays for detection of promoter

methylation on archival FFPE tissues from individuals with colorectal cancer. As a proof of principle, we demonstrated the applicability of HRM for detection of promoter methylation using assays for O6-methylguanine-DNA methyltransferase (MGMT), adenomatous polyposis coli (APC), glutathione S-transferase P1 (GSTP1) and phosphatase and tension homolog deleted on chromosome 10 (PTEN) promoters in methylated DNA dilution matrix. In a second step, HRM assays for MGMT and APC were tested on DNA isolated from fresh and FFPE human cancer cell lines. These established MGMT and APC HRM assays were analyzed using archival FFPE colorectal tumor specimens.

2 Materials and Methods

Controls and research samples

CpGenome Universal Methylated DNA (Chemicon/Millipore, Billerica, MA) and DNA from peripheral blood mononuclear cells (PBMNCs) were used as methylated and unmethylated controls. Methylation standards (50%, 25%, 10%, 5%, 1% and 0.1%) were generated by diluting methylated control DNA in the unmethylated PBMNC DNA. Human cancer cell lines (MCF-7, MDA-MB-231, SKBR3, T47D, MDA-MB-453, DU145, LNCAP, and PC3) were included for evaluation experiments and obtained either directly from cultures or after formalin-fixation and paraffin-embedding as adapted from Kerstens H et al (9). After optimization of the HRM assay, we analyzed archival tissue samples from individuals with colorectal carcinoma.

DNA extraction and bisulfite modification

Healthy volunteers' PBMNC DNA and DNA from cultured cancer cell lines was isolated using the QIAamp® DNA Blood Midi Kit (Qiagen, Hilden, Germany) according to the supplier's recommendation. For DNA isolation from archival research tissues, 10 µm thick sections from a FFPE block containing tumor tissue were used. DNA was isolated using the High Pure PCR Template Preparation Kit (Roche) with following modifications: (a) incubation of tissue specimen with 200 µl lysis buffer for 30 minutes at +98°C, (b) proteinase K treatment for 1 hour at +65°C, (c) incubation of lysed specimens for 10 minutes at +98°C, (d) centrifugation at room temperature for 20 minutes at 20,000 x g, (e) after transferring the lysate to a new tube, centrifugation at room temperature for an additional 15 minutes at 20,000 x g, and finally (f) transferring lysate to a new tube, adding 200 µl isopropanol and precipitating at -20°C for at least two hours.

One microgram of genomic DNA was subjected to bisulfite conversion using the EpiTect® Bisulfite kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Bisulfite converted DNA was eluted in 40 µl volume and was subsequently used for HRM and MethyLight analysis.

MethyLight assay

The MethyLight assays for MGMT and APC have been described previously (10–12). Briefly, PCR was carried out on the LightCycler® 480 Instrument in a 20 µl volume containing: 1x LightCycler® 480 Probes Master, 500 nmol/l of each primer, 200 nmol/l of probe, and 50 ng bisulfite treated DNA. Each reaction was performed in triplicate. The cycling conditions were as follows: monocolour hydrolysis probes detection format, 1 cycle of +95°C for 10 minutes, 45 cycles of +95°C for 10 seconds, +60°C for 30 seconds, and +72° for 1 second. COL2A1 was used to normalize for the amount of input DNA.

High-resolution melting analysis

PCR amplification and HRM were performed on the LightCycler® 480 Instrument (Roche) as adapted from the published protocol by Wojdacz and Dobrovic (5). Primers were designed as described by Wojdacz and Dobrovic. They included no more than 1 to 2 CpG sites and were placed at or adjacent to the 5' end. Amplicons were designed to be no greater than 200 bp considering the fact that FFPE DNA is highly fragmented and larger amplicons result in lower melting resolution. An overview of the HRM primers used is listed in Table 1.

Gene Name	Primer Sequence 5'–3'	GenBank Accession Number	Amplicon Location	Amplicon Size (bp)	CpGs/Amplicon
APC	Fw AAGTAGTTGTGTAATTCGTTGGAT Rv CACCTCCATTCTATCTCCAATA	NT_034772	14488467-14488615	149	11
MGMT	Fw GCGTTTCGGATATGTTGGGATAGT Rv AACGACCCAAACACTCACCAAA	X61657	1023-1132	110	15
GSTP1	Fw GTGAAGCGGGTGTGTAAGTTT Rv TAAACAAACAACAAAAAACC	X08058	862-981	120	13
PTEN	Fw TCGGTTGGGTTTTGGGTAGAGG Rv CGCAAACCTACTAAACATACCCAATAT	AF406618	1938-2093	156	11

Table 1: HRM Primers and amplicon information

PCR was carried out in a final volume of 20 µl containing: 1x LightCycler® 480 High Resolution Melting Master, 200 nmol/l of each primer for MGMT, PTEN and GSTP1 and 500 nmol/l for APC, 50 ng bisulfite treated DNA template, with 3 mmol/l final MgCl₂ for MGMT and PTEN, and 4 mmol/l final MgCl₂ for APC and GSTP1. Each reaction was performed in triplicate. The cycling conditions were as follows: SYBR Green 1/ LightCycler® 480 High Resolution Melting Dye detection format; 1 cycle of +95°C for 10 minutes, 50 cycles of +95°C for 10 seconds, a touch down of +64°C to +58°C for 10 seconds (1°C/cycle), and +72°C for 20 seconds; followed by an HRM step of +95°C for 1 minute, +40°C for 1 minute, +74° for 5 seconds and continuous acquisition to +90°C at 25 acquisitions per 1°C. Each plate included multiple water blanks. PBMNC DNA from healthy individuals was used as a negative control.

A standard curve with known methylation ratios was included in each assay and the resulting equation was used to deduce the methylation ratio of each research tumor sample. The resulting relative signal values (%) reflect the proportion of non-methylated alleles and methylated alleles.

HRM data were analyzed using the Gene Scanning and TM Calling software modules (Roche). Melting curves were normalized by calculation of two normalization regions before and after the major fluorescence decrease representing the melting of the PCR product. This algorithm allows the direct comparison of the samples that have different starting fluorescence levels. Output plots are in the form of normalized temperature-shifted melting curves, showing the decrease in fluorescence against increasing temperature.

3 Results and Discussion

The sensitivity of the HRM assays was tested in standard dilution series. With all HRM assays (APC, MGMT, GSPT1, and PTEN), we were able to reproducibly detect 1% methylated DNA in the background of unmethylated DNA (Figure 1A-D). Importantly, our data were reproducible in three distinct replicates even in the mix with the low amount of methylated DNA. For certain applications, such as detection of rare events, high sensitivity of the assay is important.

In contrast, when low level methylation is detected in tumor tissues, the biological significance is still not established. This remains to be studied, independent of the method used for detection of methylation. However, the ability of HRM to reproducibly detect low amounts of methylated DNA in the background of unmethylated DNA, regardless of the DNA origin, will facilitate the evaluation of the biological significance of low levels of methylated DNA.

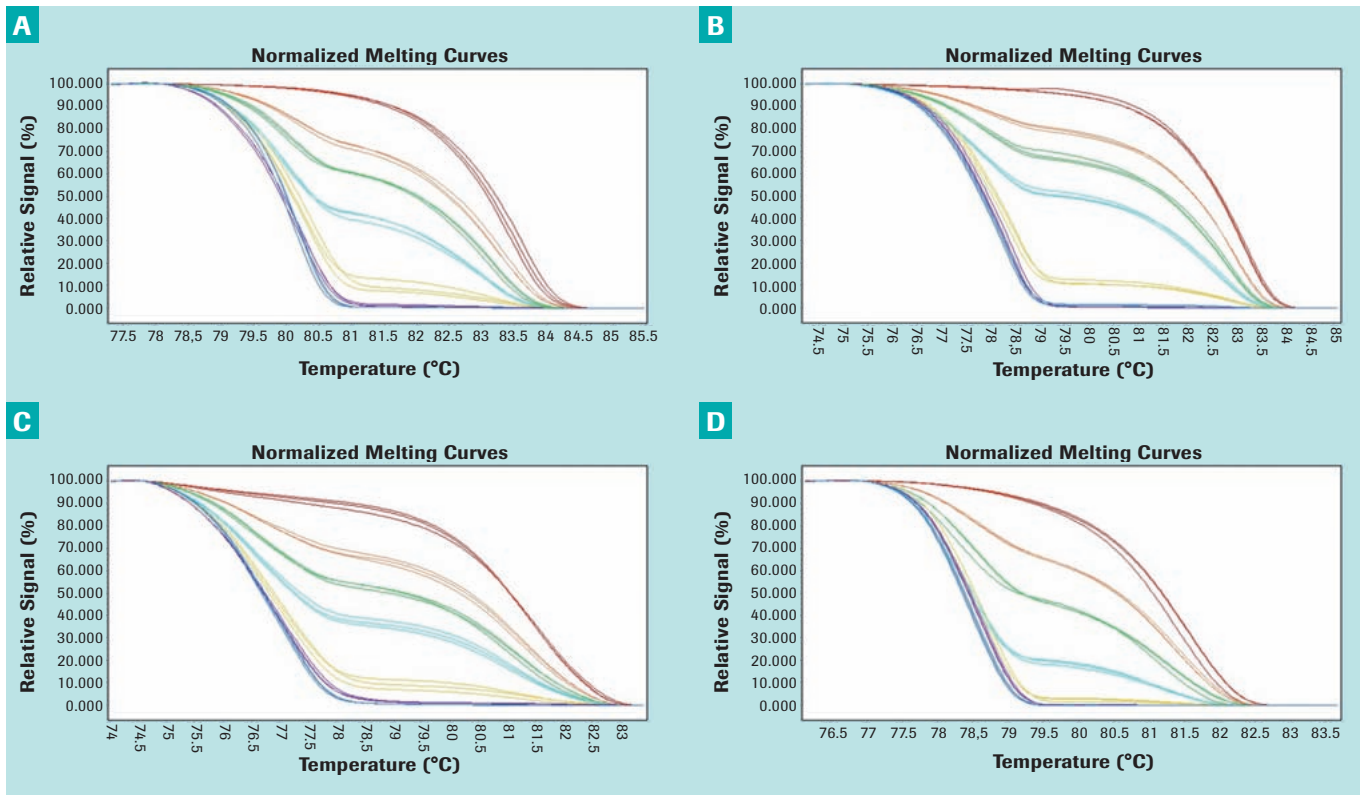


Figure 1: Sensitivity of HRM assays for MGMT (A), APC (B), GSTP1 (C) and PTEN (D) methylation. Assays were run at annealing temperatures ranging from +64°C to +58°C using a touchdown protocol. Data were analyzed using the “Gene Scanning” software module. Methylation standards are displayed as triplicates. Standards 100% red lines, 50% orange lines, 25% green lines, 10% cyan lines, 1% yellow lines, 0.1% violet lines and 0% blue lines.

Inter-assay variability of the HRM assay was evaluated by comparing methylation results, obtained from the same set of bisulfite converted methylation standards tested, in four independent assays on four different days. Resulting data showed small and reproducible run-to-run HRM variations. Further, we tested if bisulfite treatments performed on different days influenced the HRM results. Therefore, we compared methylation standards prepared from four separate bisulfite treatments analyzed in the same HRM assays. Data indicated good reproducibility in standards of different bisulfite treatments. Figure 2 shows low bisulfite-to-bisulfite variation indicated by the low standard deviations obtained for both APC and MGMT HRM assays.

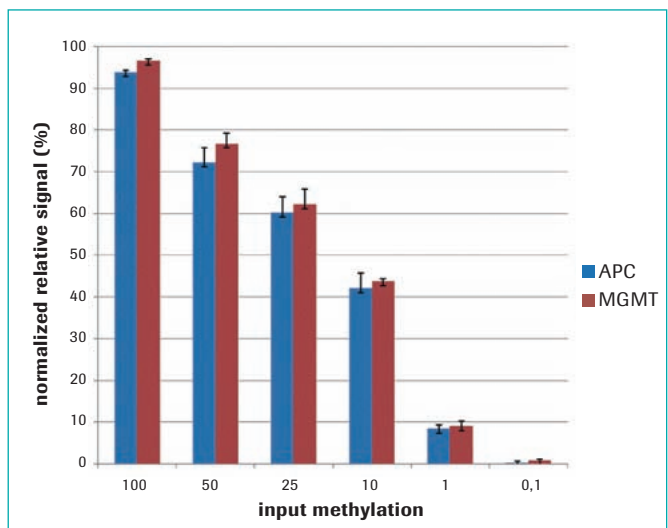


Figure 2: Influence of bisulfite treatment on methylation results. Bisulfite treatment was performed on four different days and bisulfite treated methylation standards were subjected to the same HRM assay. Bars represent the mean of four independent experiments and the corresponding standard deviations.

In the next step, we evaluated the impact of formalin-fixation and paraffin-embedding on detection of promoter hypermethylation. To test the reproducibility of the HRM assays on FFPE DNA, we performed six independent experiments on fresh and FFPE cell line DNA. We were able to reliably detect promoter hypermethylation of APC and MGMT. Figure 3 (A–C) shows similar reproducibility and concordance of the APC HRM for fresh and FFPE cell line DNA. In five of eight cell lines analyzed, we detected comparable APC methylation levels in both fresh and FFPE

DNA. Figure 3A and B depict representative melting curves of one HRM assay. Figure 3C demonstrates the mean methylation values and corresponding standard deviations obtained from six independent experiments on fresh and FFPE cell line DNA. Similar results were obtained for MGMT HRM assay (Figure 4A, B and C). All results were compared and confirmed with MethyLight assay. Figure 5 shows the concordance of detected APC methylation between HRM and MethyLight.

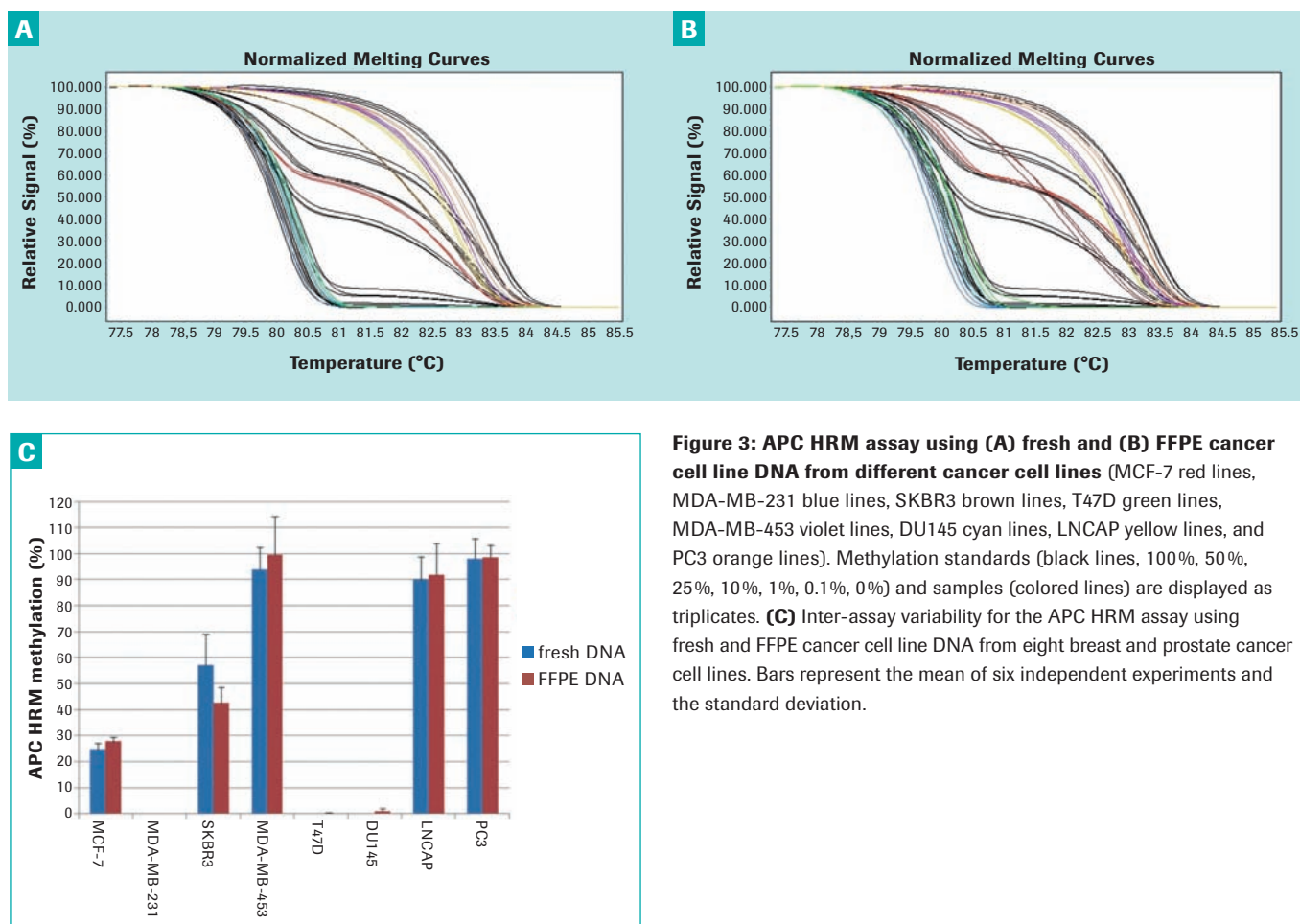


Figure 3: APC HRM assay using (A) fresh and (B) FFPE cancer cell line DNA from different cancer cell lines (MCF-7 red lines, MDA-MB-231 blue lines, SKBR3 brown lines, T47D green lines, MDA-MB-453 violet lines, DU145 cyan lines, LNCAP yellow lines, and PC3 orange lines). Methylation standards (black lines, 100%, 50%, 25%, 10%, 1%, 0.1%, 0%) and samples (colored lines) are displayed as triplicates. (C) Inter-assay variability for the APC HRM assay using fresh and FFPE cancer cell line DNA from eight breast and prostate cancer cell lines. Bars represent the mean of six independent experiments and the standard deviation.

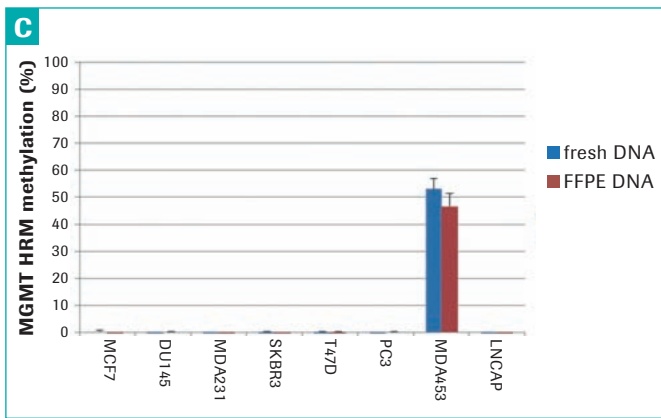
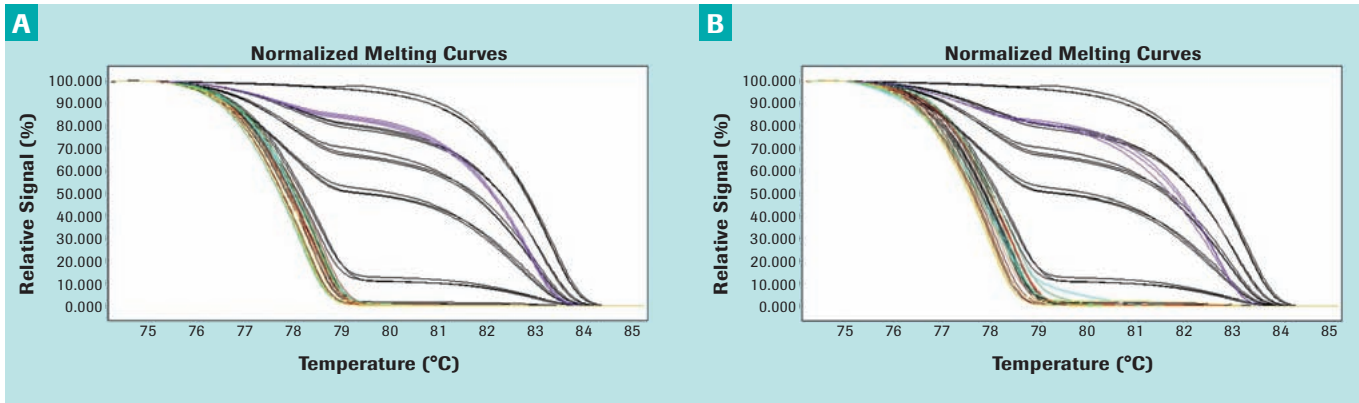


Figure 4: MGMT HRM assay using (A) fresh and (B) FFPE cancer cell line DNA from different cancer cell lines (MCF-7 red lines, MDA-MB-231 blue lines, SKBR3 brown lines, T47D green lines, MDA-MB-453 violet lines, DU145 cyan lines, LNCAP yellow lines, and PC3 orange lines). Methylation standards (black lines, 100%, 50%, 25%, 10%, 1%, 0.1%, 0%) and samples (colored lines) are displayed as triplicates. (C) Inter-assay variability for the MGMT HRM assay using fresh and FFPE cancer cell line DNA from eight breast and prostate cancer cell lines. Bars represent the mean of six independent experiments and the standard deviation.

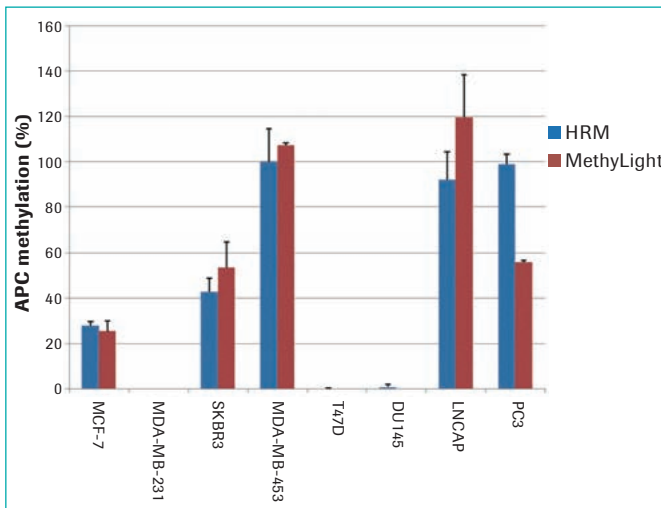


Figure 5: Comparison of HRM assay with MethyLight for APC methylation detection using different cancer cell lines. Bars represent the mean of six independent experiments and the standard deviation.

Interestingly, the SKBR3 cell line showed a different APC melting profile compared to the other tested cancer cell lines (Figure 6) indicating heterogeneous methylation. As shown in

Figure 6A and B, SKBR3 revealed a shift of the melting peak. Heterogeneous methylation of SKBR3 was confirmed by direct bisulfite sequencing (Figure 6C).

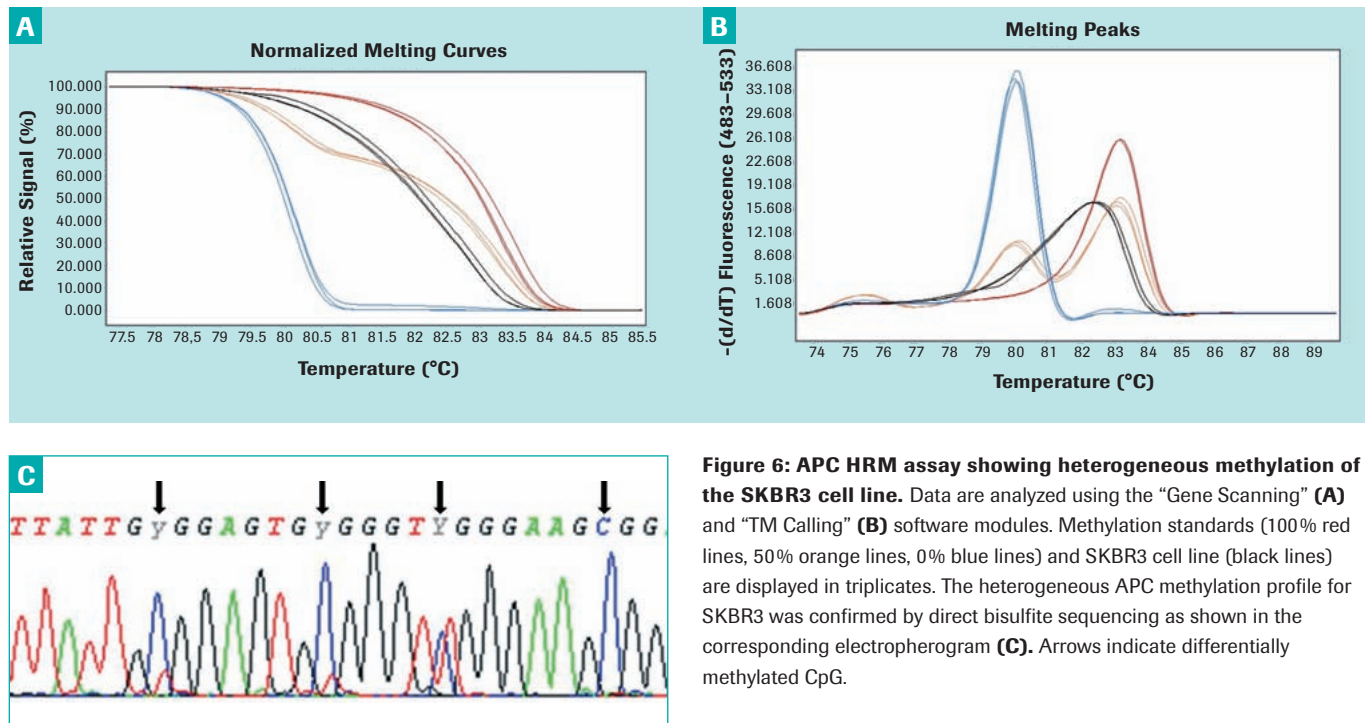


Figure 6: APC HRM assay showing heterogeneous methylation of the SKBR3 cell line. Data are analyzed using the “Gene Scanning” (A) and “TM Calling” (B) software modules. Methylation standards (100% red lines, 50% orange lines, 0% blue lines) and SKBR3 cell line (black lines) are displayed in triplicates. The heterogeneous APC methylation profile for SKBR3 was confirmed by direct bisulfite sequencing as shown in the corresponding electropherogram (C). Arrows indicate differentially methylated CpG.

We then applied HRM for MGMT and APC promoter methylation to study archival FFPE colorectal tumor specimens. Methylation of APC and the MGMT promoter region in colorectal cancer tissue has been known and documented in several independent studies (13–17). We were able to show that normalized melting curves from both FFPE colorectal cancer tissue and methylated DNA dilution matrix demonstrated the same quality. MGMT methylation was identified in 42.4% and APC methylation in 33.3% of colorectal cancer samples. Figure 7 represents results of four representative FFPE samples for the APC (A) and MGMT (B) HRM assay. To underpin the value of HRM based methylation

analysis, we concomitantly performed analysis of all research samples using MethyLight. The overall concordance for MGMT was 91% and for APC 98.5%. MGMT promoter methylation was detected in six cases by HRM, where the methylation was not detected by MethyLight. The APC promoter methylation was detected in one case by HRM, but not with MethyLight. The melting curves of those discrepant cases indicated that there was a low level of methylation detected by HRM (12). Thus, the low number of inconsistent results was most likely due to the lower level of methylation detected by HRM and not by MethyLight.

There are several potential future applications for the HRM based analysis of promoter methylation in FFPE tissues. Identification of the methylation profiles in primary tumors associated with lymph node and distant metastasis would not only elucidate those epigenetic events involved in disease progression but may aid in the definition of a prediction marker panel that can readily be assessed from paraffin embedded tissue specimens (18, 19). Thus, one application may be the risk stratification of individuals based on methylation status of specific markers. For such a potential application the cutoff of 1%, as used in our study, is

sufficient for discrimination. The same assay can be adapted and used to detect low amounts of methylated cells within the tumor, or even to detect low numbers of tumor cells in the background of non-tumor cells in lymph nodes and other organs. Data indicates that lymph node micrometastasis is a critical benchmark in cancer disease and is often the earliest sign of tumor progression (20, 21). Once the protocol has been adapted for such an potential application, as mentioned above, even the lower cut off levels might be used for selection of individuals with low tumor burden.

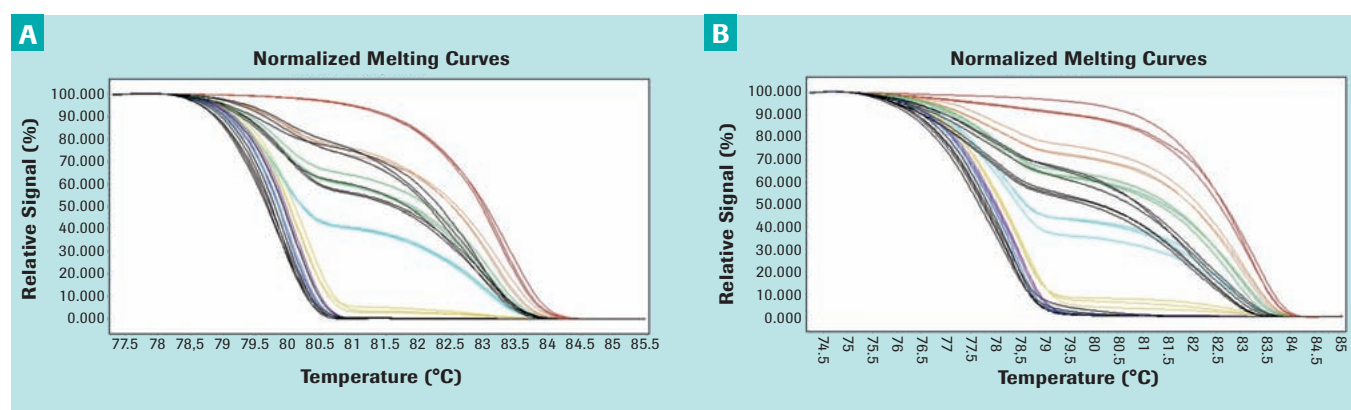


Figure 7: (A) APC and (B) MGMT HRM curves for methylation standards containing varying amounts of methylated DNA (colored lines) and four samples (two methylated and two unmethylated) represented by black lines. Methylation standards and samples are displayed as triplicates. Standards 100% red lines, 50% orange lines, 25% green lines, 10% cyan lines, 1% yellow lines, 0.1% violet lines and 0% blue lines.

4 Conclusion

We demonstrate here the usefulness and applicability of quantitative HRM for promoter methylation for analysis of FFPE tissues. Since FFPE tissue samples are the largest source of material from normal controls and diseased tissues,

their use is of inestimable value for research. Methodical evaluations are of high importance to demonstrate robustness of the assay, facilitating its establishment as a research tool and possibly a future routine test.

Ordering Information

Product	Cat. No.	Pack Size
High Pure PCR Template Preparation Kit	11 796 828 001	Up to 100 purifications
LightCycler® 480 Probes Master (2x concentrated)	04 707 494 001	5 x 1 ml (500 x 20 µl reactions)
	04 887 301 001	10 x 5 ml (5000 x 20 µl reactions)
	04 902 343 001	1 x 50 ml (5000 x 20 µl reactions)
LightCycler® 480 High Resolution Melting Master	04 909 631 001	5 x 1 ml (500 x 20 µl reactions)
LightCycler® 480 Gene Scanning Software	05 103 908 001	1 software package

References

1. Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet.* 2007 Apr;8(4):286-98.
2. Cote RJ, Laird PW, Datar RH. Promoter hypermethylation: a new therapeutic target emerges in urothelial cancer. *J Clin Oncol.* 2005 May 1;23(13):2879-81.
3. Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci U S A.* 1992 Mar 1;89(5):1827-31.
4. Laird PW. The power and the promise of DNA methylation markers. *Nat Rev Cancer.* 2003 Apr;3(4):253-66.
5. Wojdacz TK, Dobrovic A. Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation. *Nucleic Acids Res.* 2007;35(6):e41.
6. Dahl C, Guldborg P. High-resolution melting for accurate assessment of DNA methylation. *Clinical chemistry.* 2007 Nov;53(11):1877-8.
7. Nakagawa H, Chadwick RB, Peltomaki P, Plass C, Nakamura Y, de La Chapelle A. Loss of imprinting of the insulin-like growth factor II gene occurs by biallelic methylation in a core region of H19-associated CTCF-binding sites in colorectal cancer. *Proc Natl Acad Sci U S A.* 2001 Jan 16;98(2):591-6.
8. Song JZ, Stirzaker C, Harrison J, Melki JR, Clark SJ. Hypermethylation trigger of the glutathione-S-transferase gene (GSTP1) in prostate cancer cells. *Oncogene.* 2002 Feb 7;21(7):1048-61.
9. Kerstens HM, Robben JC, Poddighe PJ, Melchers WJ, Boonstra H, de Wilde PC, Macville MV, Hanselaar AG. AgarCyto: a novel cell-processing method for multiple molecular diagnostic analyses of the uterine cervix. *J Histochem Cytochem.* 2000 May;48(5):709-18.
10. Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, Shibata D, Danenberg PV, Laird PW. MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res.* 2000 Apr 15;28(8):E32.
11. Widschwendter M, Siegmund KD, Muller HM, Fiegl H, Marth C, Muller-Holzner E, Jones PA, Laird PW. Association of breast cancer DNA methylation profiles with hormone receptor status and response to tamoxifen. *Cancer Res.* 2004 Jun 1;64(11):3807-13.
12. Ogino S, Kawasaki T, Brahmandam M, Cantor M, Kirkner GJ, Spiegelman D, Makrigiorgos GM, Weisenberger DJ, Laird PW, Loda M, Fuchs CS. Precision and performance characteristics of bisulfite conversion and real-time PCR (MethylLight) for quantitative DNA methylation analysis. *J Mol Diagn.* 2006 May;8(2):209-17.
13. Arnold CN, Goel A, Niedzwiecki D, Dowell JM, Wasserman L, Compton C, Mayer RJ, Bertagnolli MM, Boland CR. APC promoter hypermethylation contributes to the loss of APC expression in colorectal cancers with allelic loss on 5q. *Cancer Biol Ther.* 2004 Oct;3(10):960-4.
14. Prall F, Weirich V, Ostwald C. Phenotypes of invasion in sporadic colorectal carcinomas related to aberrations of the adenomatous polyposis coli (APC) gene. *Histopathology.* 2007 Feb;50(3):318-30.
15. Shen L, Kondo Y, Rosner GL, Xiao L, Hernandez NS, Vilaythong J, Houlihan PS, Krouse RS, Prasad AR, Einspahr JG, Buckmeier J, Alberts DS, Hamilton SR, Issa JP. MGMT promoter methylation and field defect in sporadic colorectal cancer. *J Natl Cancer Inst.* 2005 Sep 21;97(18):1330-8.
16. Suehiro Y, Wong CW, Chirieac LR, Kondo Y, Shen L, Webb CR, Chan YW, Chan AS, Chan TL, Wu TT, Rashid A, Hamanaka Y, Hinoda Y, Shannon RL, Wang X, Morris J, Issa JP, Yuen ST, Leung SY, Hamilton SR. Epigenetic-Genetic Interactions in the APC/WNT, RAS/RAF, and P53 Pathways in Colorectal Carcinoma. *Clin Cancer Res.* 2008 May 1;14(9):2560-9.
17. Hashimoto K, Shimizu Y, Suehiro Y, Okayama N, Hashimoto S, Okada T, Hiura M, Ueno K, Hazama S, Higaki S, Hamanaka Y, Oka M, Sakaida I, Hinoda Y. Hypermethylation status of APC inversely correlates with the presence of submucosal invasion in laterally spreading colorectal tumors. *Mol Carcinog.* 2008 Jan;47(1):1-8.
18. Bastian PJ, Ellinger J, Wellmann A, Wernert N, Heukamp LC, Muller SC, von Ruecker A. Diagnostic and prognostic information in prostate cancer with the help of a small set of hypermethylated gene loci. *Clin Cancer Res.* 2005 Jun 1;11(11):4097-106.
19. Brock MV, Hooker CM, Ota-Machida E, Han Y, Guo M, Ames S, Glockner S, Pian-tadosi S, Gabrielson E, Pridham G, Pelosky K, Belinsky SA, Yang SC, Baylin SB, Herman JG. DNA methylation markers and early recurrence in stage I lung cancer. *N Engl J Med.* 2008 Mar 13;358(11):1118-28.
20. Cote RJ, Peterson HF, Chaiwun B, Gelber RD, Goldhirsch A, Castiglione-Gertsch M, Gusterson B, Neville AM. Role of immunohistochemical detection of lymph-node metastases in management of breast cancer. International Breast Cancer Study Group. *Lancet.* 1999 Sep 11;354(9182):896-900.
21. Pagliarulo V, Hawes D, Brands FH, Groshen S, Cai J, Stein JP, Lieskovsky G, Skinner DG, Cote RJ. Detection of occult lymph node metastases in locally advanced node-negative prostate cancer. *J Clin Oncol.* 2006 Jun 20;24(18):2735-42.

LIGHTCYCLER and HRM are trademarks of Roche.

SYBR is a registered trademark of Molecular Probes, Inc.

Other brands or product names are trademarks of their respective holders.

License Disclaimer:

Parts of the Software used for the LightCycler® 480 System are licensed from Idaho Technology Inc., Salt Lake City, UT, USA. This product is covered by one or more of U.S. 6,197,520, 6,303,305, 6,387,621, 6,503,720, 6,730,501 and corresponding claims in their non-U.S. counterparts, owned by Roche Diagnostics GmbH and/or licensed from Idaho Technology, Inc. This LightCycler® 480 Real-Time PCR System is a real-time thermal cycler licensed for research use only under U.S. Patent No. 6,814,934 and corresponding claims in its non-U.S. counterparts, and under one or more of U.S. Patents Nos. 5,475,610, 5,602,756, 6,703,236, 7,238,517, 7,504,241, 7,537,377 or corresponding claims in their non-U.S. counterparts, owned by Applied Biosystems, LLC. No rights are conveyed expressly, by implication or estoppels to any other patent claims. The LightCycler® 480 Real-Time PCR System is not intended for *in vitro* diagnostic use in the U.S. For further information on purchasing additional rights, contact the Director of Licensing at Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California, 94404, USA.

No right is conveyed expressly, by implication, or by estoppels under any other patent claim, such as claims to apparatus, reagents, kits, or methods such as 5' nuclease methods. License rights to practice PCR methods under the foregoing Roche patents for research and other *non-in vitro* diagnostic applications may be purchased from Applied Biosystems or may be obtained by purchasing licensed reagents from Roche, Applied Biosystems, or other Authorized third party. For information on purchasing licenses for research and other *non-in vitro* diagnostic applications, contact the Director of Licensing at Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

A license to practice PCR methods with real-time detection under patents of F. Hoffmann-La Roche Ltd, and Roche Molecular Systems ("Roche") for *in vitro* diagnostic applications may be purchased from Roche or may be obtained by purchasing *in vitro* diagnostic reagents from Roche or any other Authorized third party. The LightCycler® 480 Real-Time PCR System is not intended for *in vitro* diagnostic use in the U.S.

This product is covered in-part by US 5,871,908 or any foreign equivalents, co-exclusively licensed from Evotec OAI AG. The purchase price includes a license to practice the methods covered by US 5,871, 908 by using the product. Purchase of this product, however, does not convey to the purchaser a license or right to (i) commercially make, have made or sell reagents and/or kits, or (ii) buy or use reagents and/or kits provided by a third party used in conjunction with the product or any other thermocycler to practice the methods covered by US 5,871,908 or any foreign equivalents.

For life science research only.
Not for use in diagnostic procedures.

Published by:

Roche Diagnostics GmbH
Roche Applied Science
Werk Penzberg
82372 Penzberg
Germany

www.roche-applied-science.com

© 2010 Roche Diagnostics GmbH
All rights reserved.