Reviewer's report

Title: Quality assessment of DNA derived from up to 20 years old formalin fixed paraffin embedded tissue (FFPE) for PCR-based methylation analysis using SMART-MSP and MS-HRM

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Reviewer: Hongdo Do

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In the present study, the authors used two HRM-based technologies (MS-HRM and SMART-MSP) to assess the utility of DNA extracted from FFPE tissues for methylation studies. The methylation status of two commonly methylated genes, CDKN2A and RARB, was determined in non-small cell lung cancers by the two techniques. This study is novel in that the HRM-based techniques are directly compared to assess the DNA quality of FFPE tissues. It was shown that both MS-HRM and SMART-MSP can be used for quantitative methylation studies of DNA samples extracted from formalin-fixed paraffin-embedded tissues up to 20 years old.

The following issues should be further addressed by the authors.

Major Compulsory Revisions

1. The authors tested the reproducibility of SMART-MSP for the CDKN2A and RARB genes and concluded that the reproducibility of the technique did not decrease with the age of samples. However, the RARB results in Figure 5 show very high intra-sample differences as seen in samples 4, 6, and 30. There are about 10 samples (10/23) having 5-fold or higher difference when the reproducibility is considered within the individual samples. As the same sample and conditions were used in the triplicate testings, it is very unlikely to have 5-fold or higher difference if the assays are reproducible. If the authors fail to verify the reproducibility of a given assay within a sample, how can the reproducibility of samples from different storage time periods be reliably compared? Does high variation in the estimate of methylation indicate that SMART-MSP is not reproducible in this context?

2. Figure 5 should be plotted relative to the absolute values of methylation.

3. In Table 2, estimates of methylation for RARB are up to 186.6%. This should be addressed.

4. The authors’ statement that they developed the methodologies is both true but also misleading. Two of the authors were involved in the development of MS-HRM and SMART-MSP while they were employed at the Peter MacCallum Cancer Centre. It might be inferred that the development was done at the University of Aarhus. For this reason, it is preferable for this statement to be
Minor Essential Revisions

5. Standard dilution series were prepared by mixing of methylated and unmethylated controls. The unmethylated controls were prepared by two rounds of whole genome amplification of DNA extracted from peripheral blood. The authors should give more details to describe how they prepared the series of dilutions.

6. In this study, FFPE DNA was extracted from whole sections without microdissection. As DNA from non-tumour cells is present and thus each sample is likely to be contaminated by normal DNA, the levels of methylation in this study do not represent the methylation levels present in the tumour cells. Information on tumour percentage in each sample should be provided in order to fully interpret the results. It is preferable to test high purity tumour samples for methylation as methylation of both CDKN2A and RARB genes in normal lung tissues has been reported in individuals with NSCLC (Feng, 2008, Cancer Epidemiol Biomarkers Prev). The occurrence of methylation in normal tissue adds an additional degree of complexity in determining methylation levels.

7. The authors are advised to omit Figure 3. It shows very little information except the melting temperature information which is described in the text.

8. The name of the gene studied in Figure 4 should be included in the figure legend.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being published

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests.