

BRIEF REPORT

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## Direct bisulphite conversion of cervical samples for DNA methylation analysis

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### ABSTRACT

Sodium bisulphite conversion of DNA to separate methylated from unmethylated cytosines is a standard for methylation analysis. This study evaluated a direct cell conversion protocol on cervical samples as alternative to isolated genomic DNA as input.

Clinician-collected cervical samples (n = 120) were subjected to a direct conversion protocol, or genomic DNA was isolated with a fixed amount used for subsequent bisulphite conversion. Converted samples were compared for *ACTB* control gene and methylation of *FAM19A4* and *miR124-2* genes using quantitative methylation-specific PCR (QIASure Methylation Test).

Direct conversion resulted in a high success rate, i.e., 119/120 (99.2%) samples reported a valid test result.  $\Delta\Delta Cq$  values of *FAM19A4* and *miR124-2* were significantly correlated between both protocols (Spearman Rho 0.708 and 0.763, respectively, all p-values = 0.000). Agreement between both the bisulphite protocols was demonstrated by Bland–Altman plots.

A direct cell conversion protocol shows good technical and analytical performance and offers a streamlined workflow for methylation analysis.

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Sodium bisulphite treatment; high-throughput; host-cell DNA methylation; HPV triage; cervical screening; liquid-based cytology

## INTRODUCTION

Cervical cancer is the fourth most commonly occurring cancer in women [1]. The usually slow progression from cervical high-risk human papillomavirus (HPV) infection to cancer via precancerous changes called cervical intraepithelial neoplasia (CIN) provides opportunities for prevention and early detection [1]. Cytology-based screening has been a cornerstone of cervical cancer prevention strategies for decades. In recent years, HPV-based screening has been adopted in several countries given a better protection against cervical cancer and precancer than cytology [2]. With the implementation of HPV-based screening, triage testing has become important to increase specificity and positive predictive value, while retaining accurate identification of women with high-grade CIN, who require follow-up management. Epigenetic biomarkers have a strong potential to be implemented as molecular triage tool. At present, the most widely studied epigenetic alteration is the methylation of DNA at CpG dinucleotides

(5-methylcytosine), which are usually highly concentrated in CpG islands within the promoter regions of human genes [3]. Studies have reported a gradual increase in DNA methylation of specific host-cell genes with higher grade of CIN, reaching highest levels in cervical cancer [4–6]. A well-studied methylation marker panel with host-cell genes *FAM19A4* (currently known as *TAF4*) and *miR124-2* showed good triage performance in HPV-positive women [7–9], as recently validated in a large cross-sectional, multicentre European cohort study across four different countries [10].

A commonly used approach for DNA methylation analysis is genomic DNA isolation from a clinical specimen followed by sodium bisulphite treatment prior to PCR amplification. Treating DNA with sodium bisulphite converts unmethylated cytosines to uracil, while methylated cytosines remain unchanged. This conversion thus generates specific changes in the DNA sequence that depend on the methylation status of the individual cytosines and can be measured by

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subsequent quantitative methylation-specific PCR (qMSP) [11,12]. This protocol could be made more efficient by bisulphite conversion directly on cells, without the need for prior genomic DNA isolation and normalization of DNA input. Here, we evaluated a direct cell conversion protocol on a series of clinician-collected cervical samples. We compared results of *ACTB* control gene and methylation of *FAM19A4* and *miR124-2* genes on bisulphite-treated DNA derived from direct cell conversion to those from a protocol involving prior DNA isolation and normalization (reference protocol). Direct cell conversion protocols could further improve efficiency and considerably enhance the practicability and operations of methylation analysis in diagnostic and screening settings.

## METHODS

### Cervical samples

Clinician-collected cervical samples in PreservCyt solution (Hologic Inc., San Diego, CA, USA) were obtained from the Scottish HPV Archive of the University of Edinburgh, Scotland, according to the Research Tissue Bank status as approved by the Lothian NRS BioResource (15/ES/0094). Aliquots of 2–4 mL were available for this study. In light of the HPV triage application, the series consisted of cervical samples of 120 HPV-positive women, comprising of 14 women with histologically confirmed CIN3 (median age 38; range 32–45; IQR 32–45), 10 women with histologically confirmed CIN2 (median age 33.5; range 25–54; IQR 27.8–42.3) and 96 women defined as controls (median age 35; range 22–63; IQR 28–44), who either had histologically confirmed CIN0 ( $n = 28$ ) or CIN1 ( $n = 10$ ) or were considered to have no evidence of CIN2 or worse (CIN2+) as they displayed HPV clearance combined with normal cytology in follow-up ( $n = 58$ ).

### DNA isolation

Extraction of DNA from 1 mL PreservCyt sample was performed using an automated extraction system (NucleoMag 96 tissue kit, Macherey-Nagel GmbH&Co. KG, Düren, Germany) and

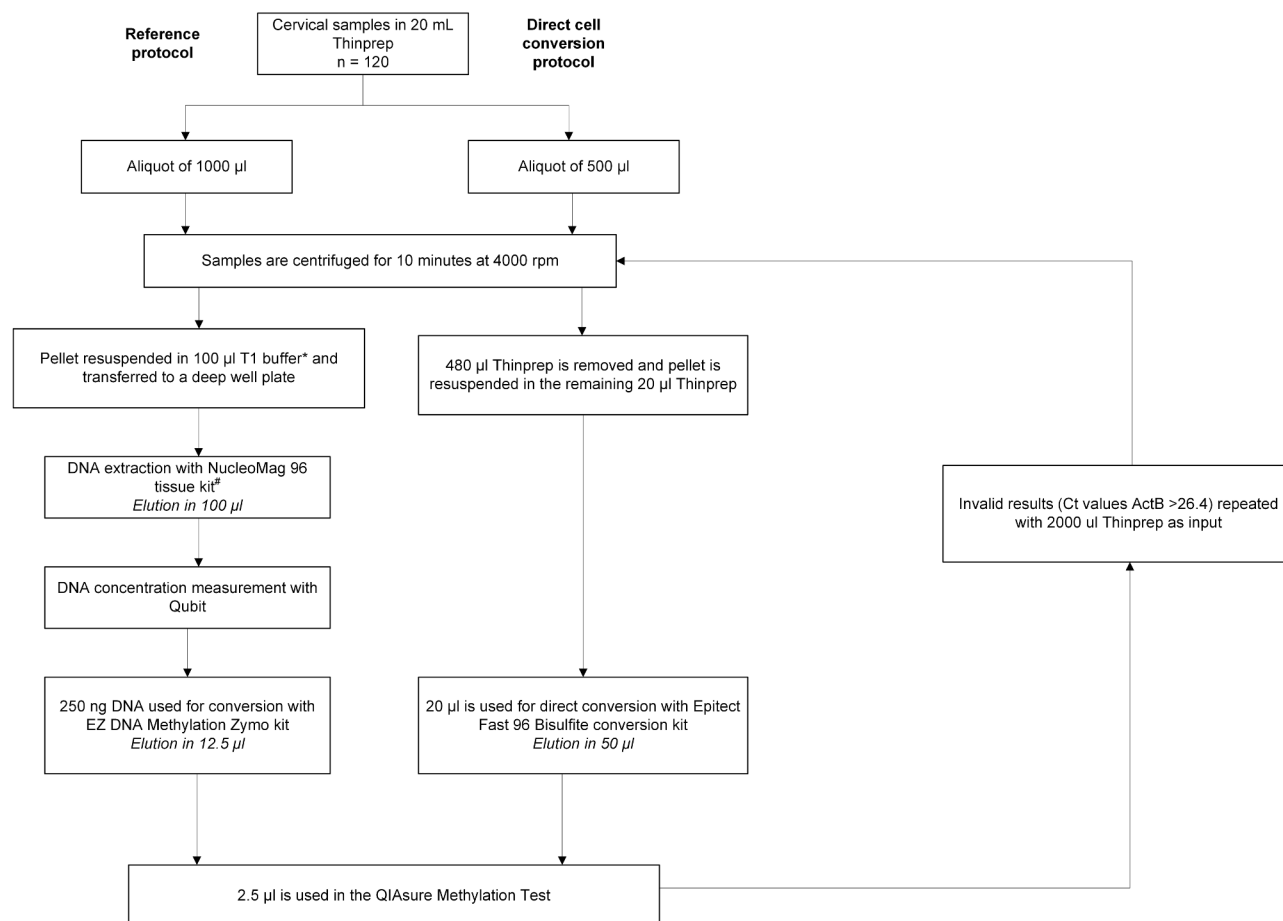
a Microlab Star robotic system (Hamilton, Gräfelfing, Germany) according to manufacturer's protocol. The concentration of extracted DNA was measured using a Qubit fluorometer (Qubit, ThermoFisher Scientific, MA, USA).

### Bisulphite treatment

For bisulphite conversion of DNA, the reference protocol (EZ DNA Methylation Zymo kit; Zymo Research, CA, USA) was performed according manufacturer's recommendation using 250 ng of isolated genomic DNA as input material [13]. This bisulphite-conversion kit is verified for use with the QIASure Methylation Test [14], but not compatible with direct cell input. A direct conversion protocol on the cervical cells used the Epiect Fast 96 Bisulphite conversion kit (QIAGEN, Hilden, Germany) [15]. Cervical cells from 500  $\mu$ L (1/40<sup>th</sup> of the original sample) PreservCyt sample was used as input. For invalid samples, an input of 2 mL (1/10<sup>th</sup> of the original sample) PreservCyt sample was used. Samples were centrifuged and the cell pellet was resuspended in 20  $\mu$ L PreservCyt prior to bisulphite conversion, performed according to manufacturer's instructions, except for elution in 50  $\mu$ L Elution Buffer and omission of carrier RNA. Details on both protocols are outlined in Figure 1.

### DNA methylation analysis

*FAM19A4/miR124-2* methylation analysis was performed using the QIASure Methylation Test (QIAGEN, Hilden, Germany) according to the manufacturer's instructions [14]. Sample input in the assay, for both the reference and direct conversion protocol, is 2.5  $\mu$ L bisulphite-converted DNA. The assay was performed on a Rotor-Gene Q Mdx 5plex HRM instrument (QIAGEN, Hilden, Germany). AssayManager software (QIAGEN, Hilden, Germany) controls amplification as well as data analysis and reporting using a fixed assay profile. The housekeeping gene  $\beta$ -Actin (*ACTB*) was used to verify DNA quality and successful bisulphite conversion. A sample was considered



**Figure 1.** Flowchart of the protocols used.

to have a valid test result when Cq value of *ACTB* was below 26.4.  $\Delta Cq$  values were calculated for each target separately (i.e., *FAM19A4* and *miR124-2*) as the difference between the Cq value of the target and the Cq value of the reference (*ACTB*). This  $\Delta Cq$  is a relative quantitative value for the promoter methylation level of the *FAM19A4* or *miR124-2* gene. For normalization, the  $\Delta Cq$  value of a calibrator sample that is included in the QIASure Methylation Test was subtracted from the  $\Delta Cq$  of the target resulting in a  $\Delta\Delta Cq$  value. The calibrator is a standardized low-copy plasmid DNA sample with known copy number of the three targets (i.e., *FAM19A4*, *miR124-2* and *ACTB*). A lower  $\Delta\Delta Cq$  value corresponds to a higher methylation level of the respective target.

### Data and statistical analysis

The direct cell bisulphite conversion protocol was compared with the reference protocol for results of *ACTB* control gene (Cq values) and methylation of *FAM19A4* and *miR124-2* genes ( $\Delta\Delta Cq$  values). Paired evaluation of  $\Delta\Delta Cq$  values of *FAM19A4* and *miR124-2* for both bisulphite protocols was done by Spearman rank analysis. Agreement between  $\Delta\Delta Cq$  values derived from the two protocols was visualized using Bland–Altman plots. In these plots, the difference of two methods is plotted against the average of both methods. We computed the mean of the differences (i.e., bias), the standard deviation (SD), and the 95% limits of agreement (=bias  $\pm 1.96 \times$  SD) [16].

All statistical analyses were performed in IBM SPSS Statistics version 26.0 (International Business

Machines Corp., Armonk, New York, USA), and p-values are two-sided with 0.01 as significance threshold.

## RESULTS

To assess whether bisulphite conversion directly on cervical cells performs equally well as the reference protocol, 120 cervical samples were subjected to both protocols. For the reference protocol, genomic DNA was isolated and DNA concentrations measured. DNA yield of the samples varied between 0.18  $\mu\text{g}$  and 9.75  $\mu\text{g}$ . Accordingly, 108/120 (90.0%) samples reached the normalized input of 250 ng for bisulphite conversion. All 120 samples had a valid test result, with *ACTB* Cq values ranging from 22.53–26.11.

With 1/40<sup>th</sup> sample input volume in the direct conversion protocol, 114/120 (95.0%) had a valid test result, with *ACTB* Cq values ranging from 20.67 to 26.26. Of the six initially invalid samples (*ACTB* Cq values 26.41–31.77), five samples had sufficient material available for repeat testing with 1/10<sup>th</sup> sample input volume, which all generated valid test results, with *ACTB* Cq values ranging from 24.48 to 25.68.

$\Delta\Delta\text{Cq}$  values of *FAM19A4* and *miR124-2* were significantly correlated between both protocols (Figures 2a and 2b; Spearman Rho 0.708  $p = 0.000$  and 0.763,  $p = 0.000$ , respectively), with particularly strong correlation in CIN2+ (Spearman's Rho 0.965,  $p = 0.000$  for *FAM19A4*, and 0.889,  $p = 0.000$  for *miR124-2*). In further investigation of the agreement between methylation results from each protocol, Bland–Altman plots were prepared (Figures 2c and 2d). Overall, there was a good agreement between  $\Delta\Delta\text{Cq}$  values obtained using the direct cell conversion protocol and the reference protocol for both *FAM19A4* (Figure 2c) and *miR124-2* (Figure 2d), with a few outliers observed mainly at higher pairwise average  $\Delta\Delta\text{Cq}$  values.

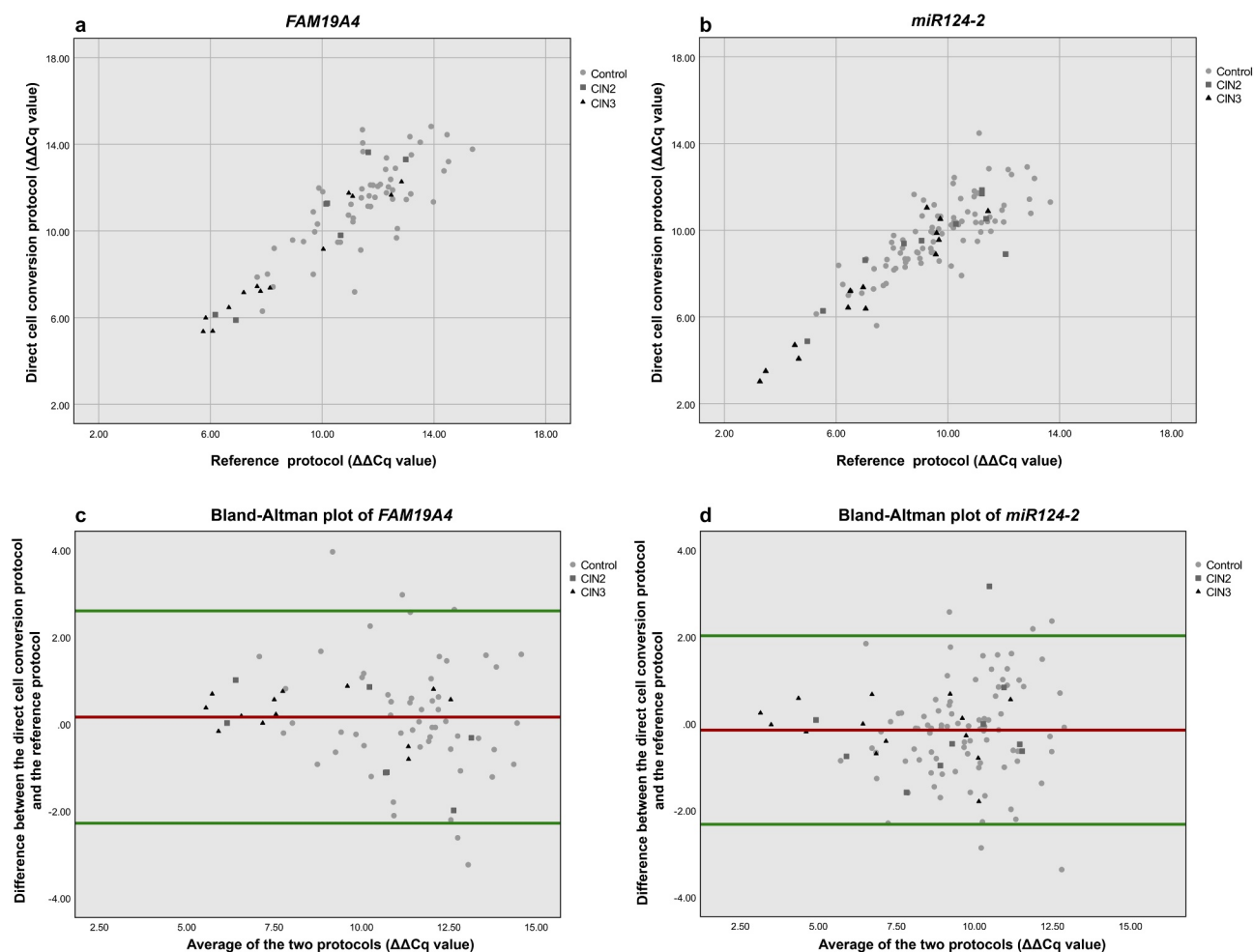
## DISCUSSION

In this study, we report on a direct cell bisulphite conversion protocol that can be performed on cervical samples, without the need of prior

genomic DNA isolation and input normalization. The direct cell conversion protocol had a high valid rate and performed analytically equally well on cervical samples as the reference protocol using normalized DNA as input.

Bisulphite conversion is an essential step in many DNA methylation assays. A direct cell conversion protocol may overcome some laborious steps in the workflow of DNA methylation analysis, i.e., genomic DNA isolation, DNA concentration measurement, and standardization of DNA input, and thereby is of great interest for implementation of DNA methylation assays in service laboratories that have a cervical screening and/or diagnostic remit. Clinician-collected cervical samples are taken under relatively standardized conditions and in that way likely well suited for protocols with standardized input volume, as is also the case in many HPV test platforms. With the introduction of more sample-to-result HPV assays, a direct cell conversion protocol is highly beneficial as a physical DNA isolate may not be generated as a result of the standard HPV testing workflow in cervical screening. Automated protocols could further improve efficiency and considerably enhance the practicability of methylation analysis in diagnostic and screening settings associated with high-throughput. Of note, an automated solution for preparation of purified bisulphite-converted DNA from direct conversion of plasma and urine has been reported [17]. Our findings provide the basis for development of streamlined and automated workflows for methylation analysis.

The direct conversion protocol with 1/40<sup>th</sup> of the original sample volume as input used basically similar input amount in qMSP, i.e., 1/800<sup>th</sup> of original sample volume, as the protocol with prior DNA isolation, when considering a typical DNA concentration of about 20 ng/ $\mu\text{l}$  as seen in this study. Three of the six initially invalid samples with the direct protocol had *ACTB* Cq values close to the threshold for valid test results (i.e., <0.5 Cq difference). Three corresponded with a low DNA concentration (<5 ng/ $\mu\text{l}$ ) in the reference protocol suggesting a low amount of cells in the original PreservCyt sample. Using four times more input of these samples in the direct cell conversion protocol generated a valid result.



**Figure 2.** Correlation of  $\Delta\Delta\text{Cq}$  values of *FAM19A4* (a) and *miR124-2* (b) between direct cell conversion and reference protocol. For both x- and y-axis: a lower  $\Delta\Delta\text{Cq}$  value represents a higher methylation value. Bland–Altman plot for *FAM19A4* (c) and *miR124-2* (d). The horizontal red line shows the mean of the difference in  $\Delta\Delta\text{Cq}$  value (=bias) between the two protocols, being 0.167 for *FAM19A4* and  $-0.142$  for *miR124-2*. The horizontal green lines show the upper and lower 95% limits of agreement (= bias  $\pm 1.96 \times \text{SD}$ ), being  $-2.274$  and  $+2.608$ , respectively, for *FAM19A4* and  $-2.316$  and  $+2.031$ , respectively, for *miR124-2*.

Overall, our data support congruence between the two different bisulphite protocols. We found a particularly strong correlation of  $\Delta\Delta\text{Cq}$  values of *FAM19A4* and *miR124-2* between both protocols in samples associated with CIN2+. The divergence between results was slightly larger at lower levels of methylation, corresponding to higher Cq values in PCR. A higher variation seen with higher Cq values is inherent to the PCR process, which becomes more stochastic in the presence of less template. Of interest, when we exploratively applied the assay's cut-off to score a sample hypermethylation-positive or -negative [14], the direct cell conversion protocol also showed a high agreement (108/119, 90.8%, Cohen's kappa 0.711) with the reference protocol. The percent agreement and

kappa value are well in line with an earlier study reporting on the intra- and inter-laboratory agreement of the *FAM19A4/miR124-2* methylation test [18]. In that study, an inter-laboratory workflow (i.e., bisulphite conversion and assay combined) agreement of 90.0% and kappa score of 0.76 were found. The data presented herein support further studies to validate the clinical performance of the new protocol [19].

Various bisulphite conversion kits, including those with direct cell input, have been evaluated before [20], but this is the first study comparing a direct cell protocol with a protocol including DNA isolation and normalization, on cervical samples. Preliminary findings also support the direct conversion protocol for use with self-

collected cervico-vaginal samples. Self-sampling is seen increasingly as a credible approach to improve cervical screening coverage and particularly gaining attention in view of the new challenges imposed by the COVID-19 pandemic [21].

In conclusion, we showed that a direct cell conversion protocol demonstrates a high success rate and good analytical performance on cervical samples as compared with a protocol using normalized genomic DNA as input. Direct cell conversion provides a practical workflow, and the results shown here may form the basis for effective high-throughput DNA methylation analysis to support a fully molecular solution to cervical cancer screening.

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## Author contributions

Study design: DAMH, RDMS, AF, AH, CJLMM

Data collection: SD, RB, KC

Data management: LV, AF

Laboratory experiments: SD

Statistical analysis: LV

Data interpretation: LV, DAMH

Writing first draft of manuscript: LV, DAMH

All authors were involved in writing the manuscript and gave final approval of the submitted and published version of the manuscript.

## Data availability

The data that support the findings of our study are available from the corresponding author upon reasonable request.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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