Evaluation of silica spin-column and magnetic bead formats for rapid DNA methylation analysis in clinical and point-of-care settings

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Received January 17, 2024; Accepted May 20, 2024

DOI: 10.3892/br.2024.1800

Abstract. Late-stage cancers lack effective treatment, underscoring the need for early diagnosis to improve prognosis and decrease mortality rates. Molecular markers, such as DNA methylation, offer promise in early cancer detection. The present study compared commercial kits for analyzing DNA from cervical liquid cytology samples in cancer screening. Rapid bisulfite conversion kits using silica spin-columns and magnetic beads were assessed against standard DNA extraction and bisulfite conversion methods for profiling DNA methylation using quantitative methylation-specific PCR. *β-actin* amplification indicated the suitability of small sample volumes for methylation studies using either the pellet or supernatant (cell-free DNA) parts. Comparison of Bisulfite Conversion Kit-Whole Cell (Abcam), Methylamp Bisulfite Modification (Epigentek), EpiTect Fast LyseAll Bisulfite Kit (Qiagen GmbH) and EZ DNA Methylation-Direct Kit (Zymo Research Corp.) showed no significant differences in *β-actin* cycle threshold values. EZ-96 DNA Methylation-Lightning MagPrep (Zymo Research Corp.), a hybrid kit in a 96-well plate format, exhibited swift turnaround time and similar amplification efficiency. Automation with magnetic bead kits increased throughput without compromising amplification efficiency in open PCR systems. Cost analysis favored direct kits over the gold standard manual protocol. This comparison aids in selecting cost-effective DNA methylation diagnostic tests. The present study confirmed comparable kit performance in methylation-based analysis, highlighting the adequacy of cytology samples and the potential of bodily fluids as alternatives for liquid biopsy.

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Key words: molecular marker, cervical cancer, DNA methylation, liquid cytology, human papillomavirus, screening method, quantitative methylation-specific PCR, methylation kit, premalignant lesion, precision medicine

Introduction

Effective management and treatment options for late-stage cancers is limited. Early detection of cancer is crucial for improving survival (1). Molecular markers facilitate detection of cancer at the earliest signs of carcinogenesis before it progresses to more difficult-to-treat stages (2).

Despite recent growth in the genome-wide characterization of abnormal patterns in many types of cancer, there is a lack of sufficiently sensitive and specific cancer biomarkers for early detection (3-6). In addition, reliable DNA isolation and testing from available clinical samples must be optimized for analytical validation. Hence, molecular triage methods with biomarker panels are in active development to predict risk of progression to cancer in the point-of-care setting. For example, DNA methylation tests are being actively examined to triage patients into colposcopy-driven biopsies or ablative treatment across cervical cancer screening clinics worldwide (7-11).

Cervical cancer (CC) is the fourth most frequently diagnosed cancer and the fourth leading cause of cancer death in female patients, with an estimated 604,000 new cases and 342,000 deaths worldwide in 2020 (12); most of these were in developing countries (13). In 2024, in the USA, ~14,000 new cases of invasive cervical cancer are expected to be diagnosed, and an estimated 4,360 patients will die of this disease (14).

Persistent infection by oncogenic human papillomavirus (HPV) is an etiological factor for cervical squamous cell carcinoma. HPV infection is associated with oncogenic progression of cervical intraepithelial neoplasia lesions Grades 1, 2, and 3 (CIN1-3) to carcinoma *in situ* and cervical cancer (5). However, more than 90% of HPV infections resolve without clinical consequence over several years (15,16), and ~30% of CIN3 progress to invasive cancer within 30 years of initial diagnosis (17).

Screening with the Papanicolaou test (Pap test) and the HPV tests has led to a reduction in cervical cancer mortality rates worldwide, mostly in developed countries. However, the death rate has not changed much over the past 10 years (18). Nevertheless, CC is preventable and curable if detected early and managed effectively (19). Currently, there is only one FDA-approved cytology triage test, CINtec PLUS Cytology (Roche), which determines patients most at risk of developing cervical cancer. This test helps identify those who would benefit from more immediate follow-up and those at low risk who can be given more time to clear the infection on their own. However, this is a new test without much market traction yet. Consequently, clinicians do not have a tool to triage HPV-positive patients into colposcopy-driven biopsies, most of which are unnecessary (20,21). Moreover, there is a great need for the development of self-collection kits, especially in low- and middle-income settings, to expedite the use of effective screening methods such as DNA methylation analysis. This approach to detect and prevent the progression of CC is emerging as a breakthrough (22).

Several studies show the feasibility of DNA methylation signatures for effective risk stratification and prognosis in cancer (21,23-25). DNA methylation-based tests can discriminate between patients with early lesions that will progress to become aggressive, consequential disease, from patients with indolent lesions that lead to inconsequential disease (26-28). Moreover, relative accessibility of biological samples using minimally invasive procedures (before, during and after cancer treatment) demonstrates the viability of these liquid biopsy approaches using epigenetic-based markers (29-32). Several studies show the feasibility of DNA methylation signatures for effective risk stratification and prognosis in various types of cancer (21,23-25). Liquid cytology is the standard of care for cervical epithelium samples sent by clinicians to clinical laboratories for co-testing with the Pap and HPV test in. Promoter methylation of *ZNF516* and *FKBP6* can discriminate between normal, premalignant and cancer samples using precision DNA methylation detection by quantitative methylation-specific PCR (qMSP) (33).

To the best of our knowledge, there is no established workflow for early cancer detection in biofluids using precision DNA methylation by qMSP. The gold standard workflow for DNA methylation analysis requires DNA extraction and bisulfite treatment prior to DNA methylation detection by qMSP, pyrosequencing, methylation array or next-generation sequencing. A new generation of kits performs bisulfite conversion in samples without requiring prior DNA extraction. These kits provide workflow improvements, both in terms of conversion efficiency and time to results, while considering the limited amount of input samples available for analysis in the clinical setting (34,35).

The objective of the present study was to evaluate rapid spin-column and magnetic bead kits for precision DNA methylation analysis in clinical and point-of-care settings using qMSP. A comprehensive evaluation of commercially available kits was used to develop an optimized workflow for precision DNA methylation quantification of premalignant lesions in liquid cytology samples from cervical epithelium. The performance of spin-column and magnetic bead kits was compared in terms of turnaround time, efficiency and cost. Additionally, we compared the pellet and supernatant (cell-free DNA) parts of the samples to assess their suitability for DNA methylation analysis. The results may enable the use of affordable, PCR-based, precision DNA methylation workflows for early detection of pre-malignant and malignant lesions in biofluids. These workflows may be optimized to create cost-effective diagnostic and screening tests for other tumor sites where readily available non-invasive samples can be collected to perform precision DNA methylation detection of pre-malignant lesions, such as oral, oropharyngeal, and laryngeal epithelium premalignant lesions in saliva, bladder epithelium lesions in urine, vaginal and ovarian epithelium premalignant lesions in self-collected vaginal samples and anal, sigmoid and colon cancer epithelium premalignant lesions in self-collected anal swabs.

Materials and methods

Study design. To evaluate silica spin-column kits, we utilized 100 μl of discarded cervical epithelium liquid cytology samples. These samples were subdivided into five 20 μl aliquots. One aliquot followed the standard protocol, involving traditional DNA extraction using the QIAamp DNA Mini kit, followed by bisulfite treatment with the EpiTecT Bisulfite kit. The remaining four aliquots were designated for technical comparison. Each 20 μl sample underwent centrifugation, separating it into pellet and supernatant fractions, resulting in two distinct samples: 'pellet' and 'supernatant' (S). The supernatant part, considered to contain cell-free DNA (cfDNA), was included to assess its suitability for DNA methylation analysis alongside the cellular DNA in the pellet fraction (Fig. 1A). For the evaluation of magnetic beads kits, we used 48 μl of liquid cytology samples, which were divided into four 12 μl parts. One part was processed using the EZ 96 DNA Methylation Direct™ MagPrep Kit-Automated (A), and another part using the EZ 96 DNA Methylation Direct™ MagPrep Kit-Manual (M). The remaining 24 μl were split equally, with 12 μl processed using the EZ DNA Methylation-Lightning™ Kit Hybrid-Automated (A) and 12 μl processed using the EZ DNA Methylation-Lightning™ Kit Hybrid-Manual (M) (Fig. 1B).

Cohort and samples. Anonymized discarded liquid cytology samples (n=106) from clinical laboratories providing Pap and HPV testing (PathAdvantage Laboratory, Dallas, Texas; and

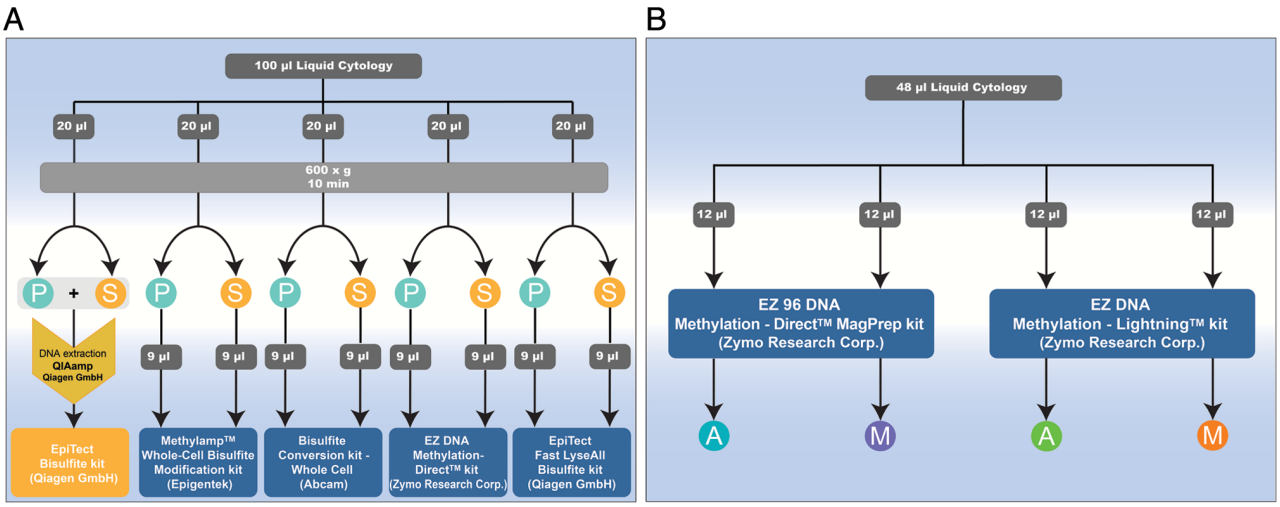

Figure 1. Overview of the study design. (A) A total of 100 µl discarded cervical epithelium liquid cytology samples were subdivided into five 20 µl aliquots. One aliquot followed the standard protocol, involving traditional DNA extraction using the QIAamp DNA Mini kit, followed by bisulfite treatment using the EpiTecT Bisulfite kit. The remaining four aliquots were used for technical comparison. (B) A total of 48 µl liquid cytology samples was divided into 12 µl parts. One part was processed using EZ 96 DNA Methylation Direct™ MagPrep and EZ DNA Methylation-Lightning™ kit Hybrid. A, automated; B, manual; P, pellet; S, supernatant.

CorePlus and Lab Noy laboratories in San Juan, Puerto Rico), were received and processed, beginning in August 2020 until December 2021. Clinicopathological data were not obtained as the objective was to streamline the bisulfite conversion workflow needed to use precision DNA methylation assays in Clinical Laboratory Improvement Amendments-approved clinical laboratories. The present study (# IRB00231112) was approved by the Johns Hopkins School of Medicine Institutional Review Board Baltimore, Maryland, USA. The requirement for informed consent was waived due to the anonymized nature of samples.

Gold standard workflow for bisulfite conversion. The gold standard workflow for bisulfite conversion used the QIAamp DNA Mini kit for DNA extraction (cat. no. #51304), prior to treating genomic DNA (gDNA) with sodium bisulfite using the EpiTecT Bisulfite kit (both Qiagen GmbH; cat. no. #59104), according to the manufacturer's protocols (Appendix S1). All kits are listed in Table SI.

Silica spin-column technology for bisulfite conversion. Paired pellet and supernatant samples were compared with kits using spin-column technology, with minor modifications to the manufacturer's instructions as described below (Fig. 1A).

Methylamp Whole Cell Bisulfite Modification (kit #3). The pellet and supernatant were resuspended in 9 µl W3 solution. Next, 1 µl W1/W2 solution was added to PCR tubes including samples and placed in a thermocycler at 65˚C for 45 min. Post-incubation, 110 µl W4/W5/W6 solution was added to each tube and subjected to thermocycling at 99˚C for 20 min, 65˚C for 90 min and 99˚C for 10 min. Following PCR, 200 µl W7 was added to all the spin-columns, which were placed in collection tubes. The sample and 100 µl 100% isopropanol were added to the column containing W7, incubated at room temperature for 2 min and centrifuged at 13,000 x g for 20 sec. The flowthrough was discarded and 200 µl 70% ethanol was added and centrifuged at 13,000 x g for 25 sec. Then, 50 µl W6/ethanol solution was added to columns, incubated at room temperature for 10 min and centrifuged at 13,000 x g for 20 sec. The flowthrough was discarded, and 200 µl 90% ethanol was added twice and centrifuged at 13,000 x g for 20 and 40 sec, respectively. The columns were transferred to 1.5 ml tubes and 18 µl W8 was added. Finally, the columns were centrifuged at 13,000 x g for 1 min to elute the modified DNA.

Bisulfite Conversion kit-Whole Cell (kit #4). The pellet was resuspended in 9 µl Cell Collection Buffer and combined with the supernatant (9 µl), then transferred into a 0.2 ml PCR tube. To each PCR tube, 1 µl Final Digestion Solution was added and placed in a thermocycler at 65˚C for 45 min, followed by vortexing at 2,500 rpm. until the solution was clear or saturated (about 2 min) at room temperature. Next, 110 µl DNA Modification Solution was added, and thermocycling was performed at 99˚C for 20 min, 65˚C for 90 min and 99˚C for 10 min. Spin-columns were loaded with 200 µl DNA Binding Solution and placed in collection tubes. Samples were added to the columns, along with 100 µl 100% isopropanol. After a 2 min room temperature incubation, centrifugation was performed at 13,000 x g for 20 sec and the flowthrough was discarded. The columns were washed with 200 µl 70% ethanol, centrifuged at 13,000 x g for 25 sec, 50 µl DNA Cleaning solution was added, incubated at room temperature for 10 min and centrifuged at 13,000 x g for 20 sec. This step was followed by two washes with 200 µl 90% ethanol, each centrifuged at 13,000 x g for 20 and 40 sec, respectively. The columns were placed into new 1.5 ml tubes. Lastly, 18 µl Modified DNA Elution was added to columns and centrifuged at 13,000 x g for 1 min to elute the modified DNA.

EZ DNA Methylation-Direct kit (kit #5). The pellet was resuspended in 9 µl PBS, and 9 µl supernatant was used. Samples were transferred to a 0.2 ml PCR tube along with 10 µl 2X M-Digestion Buffer and 1 µl Proteinase K, then incubated

at 50˚C for 20 min. After centrifugation at 13,000 x g for 5 min, 20 µl supernatant was combined with 130 µl CT Conversion Reagent and subjected to thermocycling at 98˚C for 8 min, followed by 64˚C for 3.5 h. Post-PCR, 600 µl M-Binding Buffer was added to spin-columns placed into collection tubes. Samples were loaded into columns containing M-Binding Buffer, centrifuged at 13,000 x g for 30 sec and the flow-through was discarded. Subsequently, 100 µl M-Wash Buffer was added to the columns and centrifuged at 13,000 x g for 30 sec. Columns were treated with 200 µl M-Desulfonation Buffer at room temperature for 20 min and centrifuged at 13,000 x g for 30 sec. Then, two washes with 200 µl M-Wash Buffer were performed; each wash was centrifuged at 13,000 x g for 30 sec. Columns were then placed into 1.5 ml tubes and eluted with 18 µl M-Elution Buffer via centrifugation at 13,000 x g for 1 min to obtain DNA.

EpiTect Fast LyseAll Bisulfite kit (kit #6). The pellet was resuspended in 9 µl distilled water, and 9 µl supernatant was used. Samples were transferred to 0.2 ml PCR tubes. Then, 15 µl Lysis Buffer FTB and 5 µl Proteinase K were added. After centrifugation, samples were incubated at 56˚C for 30 min. Then, 85 µl Bisulfite Solution and 15 µl DNA Protect Buffer were added and thermocycling was performed with denaturation at 95˚C for 5 min and incubation at 60˚C for 20 min. The denaturation and incubation steps were repeated once. The samples were transferred to microcentrifuge tubes with 310 µl Buffer BL containing 10 µg/ml carrier RNA and 250 µl 100% ethanol. Centrifugation was performed at full speed (14.000 x g) for 1 min, and the samples were loaded into columns placed in collection tubes, followed by centrifugation at full speed (14.000 x g) for 1 min. After adding 500 µl Buffer BW, the samples were centrifuged at full speed (14.000 x g) for 1 min. Next, 500 µl Buffer BD (desulfonation buffer) was added to each spin column and incubated for 15 min at room temperature (15-25˚C). After centrifugation at 14.000 x g, another wash with 500 µl Buffer BW was performed. The columns were eluted with 250 µl 100% ethanol, and the residual liquid was removed by centrifuging at full speed (14.000 x g) for 1 min. Following incubation at 60˚C for 5 min, the columns were transferred to new microcentrifuge tubes and 18 µl Buffer EB was added and centrifuged at 14.000 x g to elute DNA.

Magnetic beads technology for bisulfite conversion. Supernatant samples were compared with kits using magnetic beads technology (Fig. 1B).

EZ 96 DNA Methylation Direct™ MagPrep kit (manual; kit #7). Bisulfite conversion of DNA was performed using Zymo Research EZ 96 DNA Methylation Direct™ MagPrep kit, as per manufacturer's instructions. Briefly, 13 µl M-Digestion Buffer, 12 µl sample and 1 µl Proteinase K were used for sample digestion. Samples were incubated for 20 min at 50˚C. A total of 130 µl CT Bisulfite Conversion Reagent was added to 26 µl digested sample solution. Samples were transferred to a thermocycler to perform bisulfite conversion as follows: 98˚C for 8 min and 64˚C for 3.5 h. Samples were placed on a heating element at 55˚C for 30 min to dry the beads. Finally, 25 µl M-Elution Buffer was added for elution.

EZ 96 DNA Methylation Direct™ MagPrep kit (automated; kit #8). Bisulfite conversion of DNA was performed using Zymo Research EZ 96 DNA Methylation Direct™ MagPrep kit, as per manufacturer's instructions. Briefly, 13 µl M-Digestion Buffer, 12 µl sample and 1 µl Proteinase K were added for sample digestion. Samples were incubated for 20 min at 50˚C. A total of 130 µl CT Conversion Reagent was added to 26 µl digested sample solution. Samples were transferred to a thermocycler to perform bisulfite conversion as follows: 98˚C for 8 min and 64˚C for 3.5 h. A total of 600 µl M-Binding Buffer was added to a KingFisher 96 deep-well plate with 10 µl MagBinding Beads. A total of three M-Wash plates was prepared using 400 µl M-Wash Buffer twice. A total of 200 µl M-Desulfonation Buffer was added into a new plate. A total of 50 µl M-Elution Buffer was added to an elution plate.

EZ DNA Methylation-Lightning™ Kit (manual) Hybrid (kit #9). DNA was treated with bisulfite. The Zymo Research EZ-96 DNA Methylation-Lightning™ MagPrep kit was used, but the manufacturer's instructions were adjusted to include a digestion step (D5044) prior to bisulfite conversion, instead of extracting DNA beforehand. Briefly, 13 µl M-Digestion Buffer, 12 µl sample and 1 µl Proteinase K were used for sample digestion. Samples were incubated for 20 min at 50˚C. A total of 130 µl Lightning Bisulfite Conversion Reagent was added to 26 µl DNA sample. Samples were transferred to a thermocycler for DNA denaturation and bisulfite conversion as follows: 98˚C for 8 min and 54˚C for 60 min. Samples were transferred to a new deep-well plate containing 600 µl M-Binding Buffer and 10 µl MagBinding Beads (previously vortexed), following a 5 min incubation step at room temperature. A total of 400 µl Wash Buffer was added, the plate was placed on magnetic stand and supernatant was discarded after beads were pelleted (supernatant was discarded after each wash). A 20 min incubation at room temperature was performed using the magnetic stand for desulfonation. A 30-min incubation at 55˚C was performed to dry the beads. The beads were incubated with 25 µl Elution Buffer for 4 min at 55˚C. Next, the plate was then placed on a magnetic stand for 1 min, allowing the beads to pellet. The supernatant was carefully transferred to a clean elution plate.

EZ DNA Methylation-Lightning™ kit (automated) Hybrid (kit #10). DNA was treated with bisulfite according to a modified protocol. The Zymo Research EZ-96 DNA Methylation-Lightning™ MagPrep kit was used, but the manufacturer's instructions were adjusted to include a digestion step (D5044) prior to bisulfite conversion, instead of extracting DNA beforehand. Briefly, 13 µl M-Digestion Buffer, 12 µl sample and 1 µl Proteinase K were used for sample digestion. Samples were incubated for 20 min at 50˚C. A total of 130 µl Lightning Conversion Reagent was added to 26 µl DNA sample. Samples were transferred to a thermocycler for DNA denaturation and bisulfite conversion with the following program: 98˚C for 8 min and 54˚C for 60 min. The desulfonation and washing steps) of the converted DNA were performed while the DNA was bound to the MagBinding Beads, using the KingFisher Flex instrument (Thermo Fisher Scientific, Inc.).

DNA methylation analysis. DNA methylation was assessed by qMSP analysis of bisulfite-modified genomic DNA, optimized for QuantStudio 6-Flex Real-Time PCR (Thermo Fisher Scientific, Inc.). Primers and probes were previously designed (33) to amplify the CpG-rich regions located in the promoters of *ZNF516* (Zinc finger protein 516) and *FKBP6* (FKBP prolyl isomerase family member 6). *β-actin*, the reference gene, was used to assess DNA input. Each reaction contained methylated bisulfite-converted DNA as positive control and unmethylated bisulfite-converted DNA as negative control. Standard curves were created using the EpiTect PCR Control DNA Set (Qiagen GmbH, cat. no. #59695) for quantification in silica-based column kits, or the Zymo Methylated DNA Control kit (cat. no. #D5014) for quantification in magnetic bead kits. Non-template controls were added to guarantee the absence of contamination. Bisulfite-converted DNA primers and probe sequences and annealing temperatures are provided in Table SII. Bisulfite treated DNA primers/probe sequences did not overlap with genomic DNA sequences of the same genomic region using NCBI primer blast check because genomic DNA sequences are altered during bisulfite treatment (36).

Each plate included DNA samples, positive and negative control and multiple water blanks as non-template controls. Serial dilutions (0.001, 0.010, 0.1, 1, and 10 ng) of DNA were used to generate a standard curve for each plate. *β-actin* was used to measure sample cellularity and judge sample adequacy. The relative levels of methylated DNA for each gene in each sample were determined as a ratio of the amplified gene quantity to the quantity of *β-actin* (mean value of duplicates of gene of interest/mean value of the triplicates of *β-actin*). The performance of each kit in amplifying the *β-actin* gene was comparable since the same amount of sample input was used. As the amount of DNA template decreases, cycle threshold (Cq) value will increase (37). Therefore, the best performing kit will amplify *β-actin* at lower Cq values.

Fluorogenic PCR reactions were performed in duplicate for samples and triplicate for controls in a reaction volume of 10 µl that contained 1 µl bisulfite-modified DNA, 600 nM forward and reverse primer, 200 nM probe, 0.6 U platinum Taq polymerase (Invitrogen; Thermo Fisher Scientific, Inc.), 200 µM each dATP, dCTP, dGTP and dTTP and 2.5 mM $MgCl_2$. Amplifications were performed as follows: 95˚C for 5 min, followed by 50 cycles at 95˚C for 15 sec and 58˚C for 1 min in a QuantStudio 6-Flex and were analyzed using QuantStudio™ Real-Time PCR Software (Applied Biosystems; Thermo Fisher Scientific, Inc.; version 1.3).

Cost calculation and time assessment for sample preparation and bisulfite treatment. The cost calculation for the sample preparation process was conducted using the unit price of each item divided by the number of samples per kit, excluding laboratory technician costs. This calculation included all necessary consumables, such as tubes, tips, and reagents. Detailed costs for sample preparation, excluding the bisulfite modification kits, were outlined with each cost item measured in terms of cost per sample and cost per plate (83 samples). For PCR, the price was calculated for the amplification of *β-actin*, *ZNF516*, and *FKBP6* in singleplex reactions. The time required to generate bisulfite-treated DNA was recorded from the beginning of the procedure to the preparation of ten samples. Hands-on time for the spin-column kits was calculated by including the time for centrifuging ten samples, dividing them into pellet and supernatant, and all incubation steps. The total preparation time for 10 samples was compared between direct kits and the gold standard kit, with each process performed in triplicate.

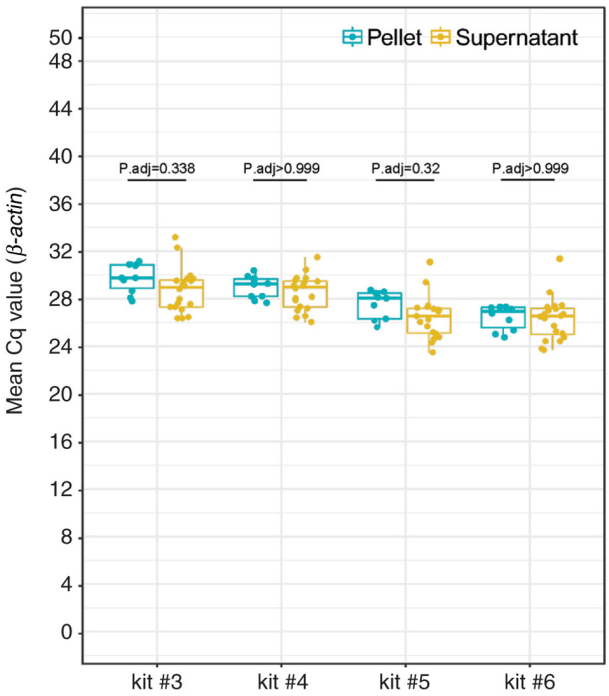

Figure 2. *β-actin* mean Cq values using direct silica-based kits, comparing pellet and supernatant (cell-free DNA) samples.

Statistical analysis. PCR data were analyzed, interpreted, and exported as .xlsx files using QuantStudio Software (Applied Biosystems; Thermo Fisher Scientific, Inc.; version 1.7.1). The output files were converted to .csv, and boxplots were prepared to visualize Cq values data using R software (R Core Team; version 4.0.0). A paired t-test with Bonferroni correction was used to compare Cq values of pellet and supernatant, as proxy for cellularity. One-way ANOVA with Tukey's HSD post hoc test was used for kit comparisons. P<0.01 was considered to indicate a statistically significant difference.

Results

Comparison between direct kits that use spin-column technology. Summary statistics and interquartile range (IQR) of *β-actin* Cq values are listed in Table SIII. There was no significant difference in *β-actin* mean Cq values between pellet (n=10) and supernatant (n=20; Fig. 2). There was no significant difference in mean Cq values for pellets when comparing kits #5 and #6. However, mean Cq values for pellets were significantly higher for kits #4 and #3 compared with #6. There was no statistical difference in mean Cq values for supernatant samples between kits #5 and #6. Nevertheless, mean Cq values for supernatant were significantly higher for kits #4 and #3 compared with #6 (Figs. S1 and S2).

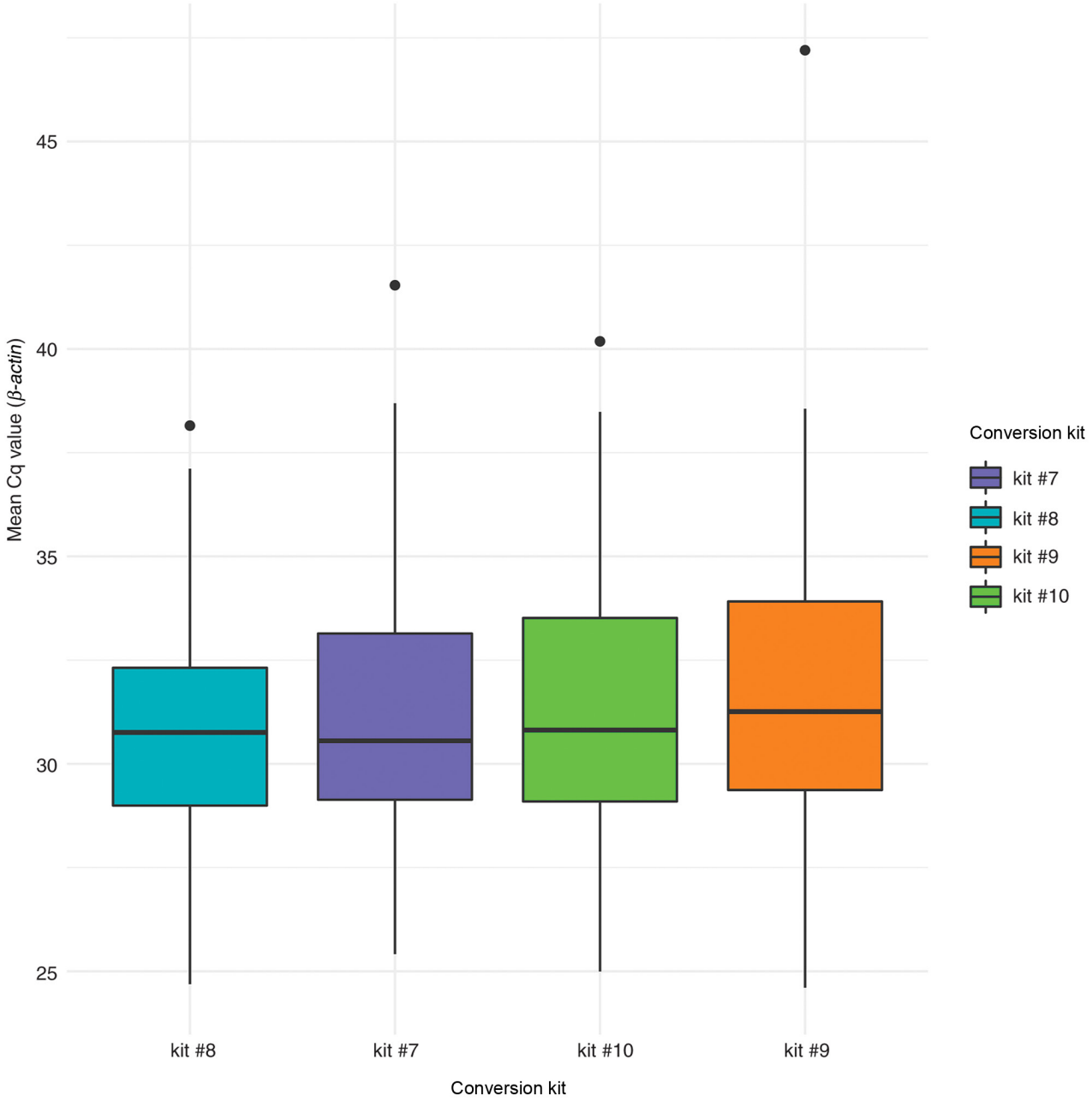

Figure 3. *β-actin* mean Cq values using magnetic beads kits.

Comparison between direct kits that use magnetic beads technology. *β-actin* Cq values are listed in Table SIV. Kits #7 (manual) and #10 (automated) presented the same mean values, while kit #8 had slightly higher values for automated sample processing. Kit #7 (manual) had the lowest median (29.7), and kit #9 (hybrid manual) had the highest (32.3). There is a one-cycle difference in medians for automated sample processing. Both automated kits had the lowest IQRs, with kit #10 at 3.3 and kit #8 at 3.9, respectively. There was no statistically significant difference when comparing mean Cq values for manual and automated sample processing (Fig. 3).

Amplification of CpG-rich regions. Amplification of *ZNF516* and *FKBP6* after bisulfite modification with spin-column or magnetic beads was successful (Fig. 4). There were no differences between pellet and supernatant (Fig. 4A), nor between the gold standard protocol (kits #1 + #2) with kits #3 and #4. *ZNF516* methylation levels were significantly different between the standard protocol and kits #5 and #6 (Fig. 4B). There were no significant differences in *ZNF516* and *FKBP6* methylation comparing manual and automatic bisulfite conversion of samples with magnetic bead technology in 96-well plates (Fig. 5).

Costs and time for sample preparation. Costs of bisulfite conversion kits using spin-column and magnetic bead technology were compared with gold standard EpiTect kit (November 2023 prices). Gold standard cost/sample was US$7.04, while direct kits were more affordable, ranging from $2.18 (kit #7 & #8) to $6.88 (kit #4; Table SV). For PCR, the price was calculated for the amplification of *β-actin, ZNF516* and *FKBP6* in singleplex reactions (Table SVI).

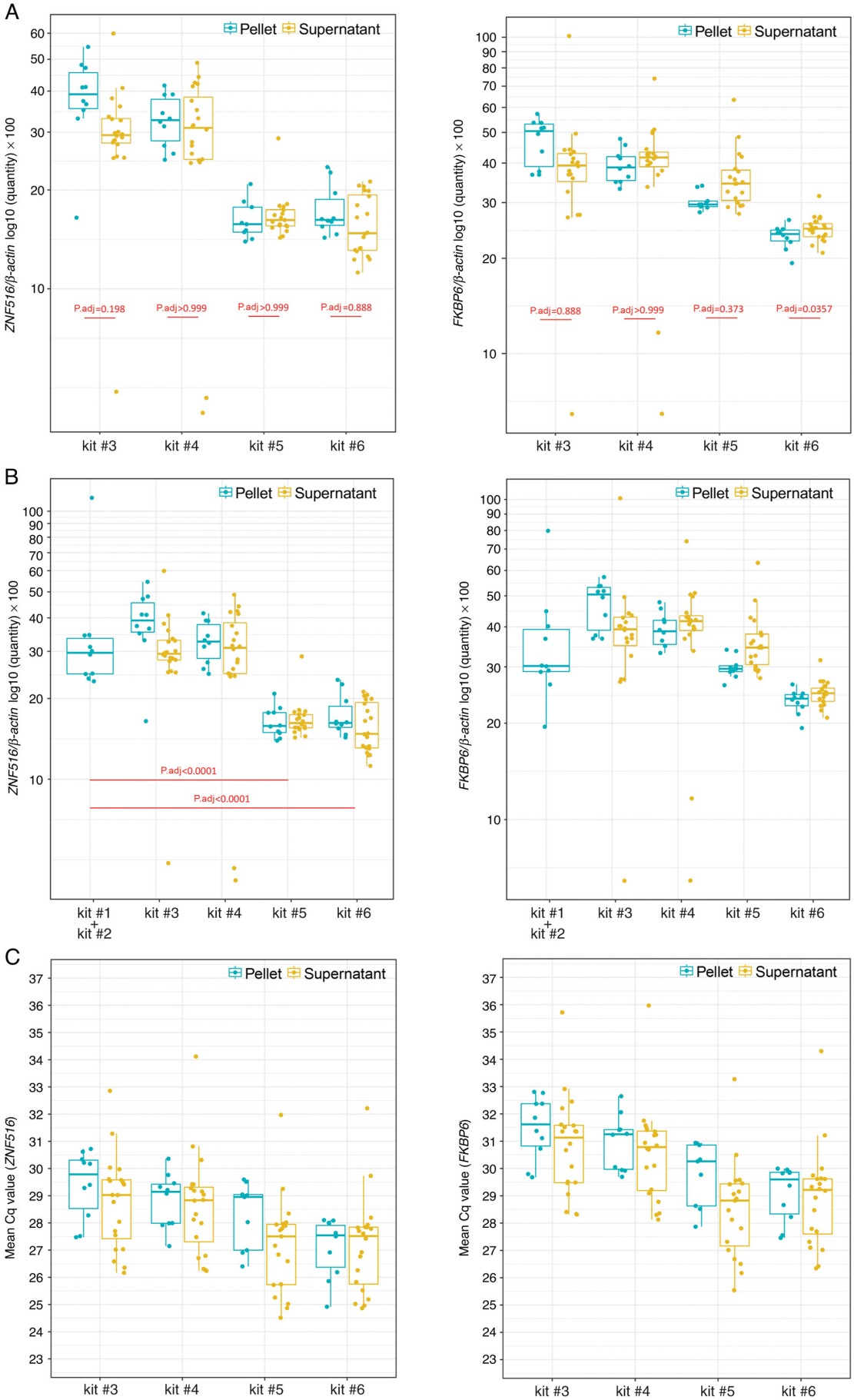

Figure 4. qMSP analysis for *ZNF516* and *FKBP6* using direct silica-based kits. (A) DNA methylation. (B) DNA methylation results by qMSP analysis after direct bisulfite conversion. (C) Mean *ZNF516* and *FKBP6* Cq values. qMSP, quantitative Methylation-Specific PCR; ZNF516, Zinc finger protein 516; FKBP6, FKBP prolyl isomerase family member 6.

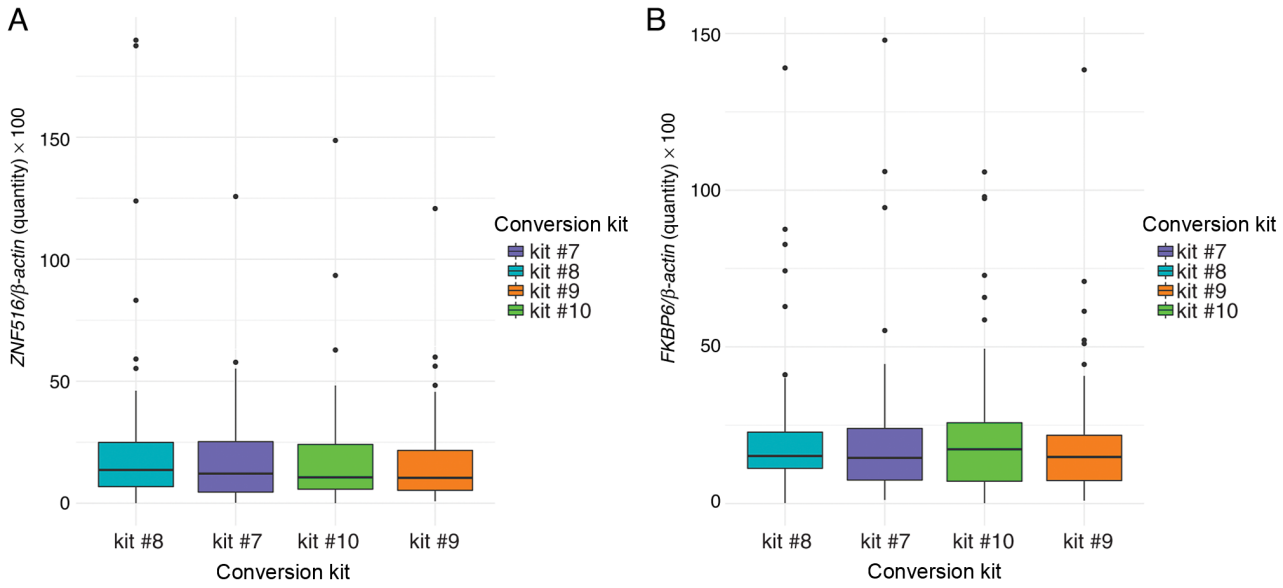

Figure 5. qMSP analyses for genes *ZNF516* and *FKBP6*, using magnetic beads kits. qMSP analysis for (A) *ZNF516* and (B) *FKBP6*. qMSP, quantitative methylation-specific PCR; ZNF516, Zinc finger protein 516; FKBP6, FKBP prolyl isomerase family member 6.

Next, time of sample preparation for 10 samples in triplicate between direct kits and the gold standard kit was compared (Table SVII). Kit #6 ranked better for spin-column kits in sample preparation (180 min). Kit #10 had the lowest hands-on time (105 min) to prepare 83 samples in a 96-well plate format. The longest procedure was the standard protocol (kits #1 + #2; 455 min).

Feasibility of using low volumes of liquid cytology samples for DNA methylation studies. Kit #6 was the best option for assessing DNA in liquid cytology samples because it provided the lowest inter-variability between pellet and supernatant. Moreover, all samples tested showed successful *β-actin* amplification with favorable Cq values (median=28.48, IQR=1.92). These results were comparable to those obtained from swab samples. Thus, it was feasible to use small sample volumes for methylation studies, as 9 μl sample was sufficient for successful amplification.

Discussion

The present study demonstrated that small volumes of liquid cytology samples can be used for efficient rapid bisulfite conversion using magnetic beads prior to DNA methylation analysis with qMSP. Automation increases throughput without compromising amplification efficiency in open PCR systems, while providing swift turnaround times (38). Efficiency and cost analyses favored direct kits over the gold standard manual protocols that require DNA extraction from cell pellets prior to bisulfite conversion. These were obtained from real-world samples. Therefore, these findings are relevant for future prospective clinical trials of DNA methylation assays developed for use in clinical and point-of-care settings.

Different kits have been assessed for quality of converted DNA, primarily in terms of DNA fragmentation, degradation and conversion efficiency, offering a valuable starting point in selecting the most appropriate kit (39-42). For example, Kint *et al* (2018) provided a comprehensive workflow of 12 commercial bisulfite kits by comparing DNA fragmentation, recovery or conversion efficiency using different techniques, such as electrophoresis, quantitative and digital PCR, DNA spectroscopic analysis and next-generation sequencing (39). The kits assessed included suppliers such as Zymo Research Corp., Qiagen GmbH, and Promega. In the aforementioned study, the DNA was extracted from peripheral blood mononuclear cells and the results presented distinct performances between kits. This variability suggests that the most appropriate kit might differ depending on the specific goals of the study, such as whether the priority is minimizing DNA fragmentation or maximizing recovery efficiency.

Recently, Hong and Shin (43) tested six bisulfite conversion kits using a multiplex quantitative real-time PCR system. A total of 20 peripheral blood samples with 50 ng gDNA as input was used and three key features of bisulfite conversion were analyzed: Efficiency, recovery and degradation levels (43). The aforementioned study found >99% conversion efficiency in five kits and one performing near 94%. One kit presented a lower degradation level and recovery rates of the kits ranged from 18 to 50%. All the kits used purified gDNA followed by bisulfite treatment. Another study evaluated a DNA hypermethylation marker panel of the six marker regions called GynTect, in which liquid-based cytology media was directly treated by sodium bisulfite with modifications (44). This demonstrates the feasibility and effectiveness of directly treating liquid cytology samples with sodium bisulfite for DNA methylation analysis, which aligns with our approach of using small volumes of liquid cytology samples for efficient rapid bisulfite conversion using magnetic beads prior to DNA methylation analysis with qMSP. This supports the potential for simplified and streamlined workflows in clinical settings.

Holmes *et al* (2014) evaluated nine kits: EpiTect Fast FFPE Bisulfite, EpiTect Bisulfite, EpiTect Fast DNA Bisulfite (Qiagen GmbH), EZ DNA Methylation-Gold, EZ DNA Methylation-Direct, EZ DNA Methylation-Lightning (Zymo

Research Corp.), innuCONVERT Bisulfite All-In-One, innuCONVERT Bisulfite Basic and innuCONVERT Bisulfite Body Fluids kit (Analytik Jena) (45). High yields were obtained using the EZ DNA Methylation-Gold and innuCONVERT Bisulfite kits. All kits yielded high purity DNA suitable for PCR analyses and did not exhibit PCR inhibition, meaning that the bisulfite-treated DNA did not interfere with the PCR amplification process. The innuCONVERT Bisulfite Body Fluids kit allowed the analysis of 3 ml plasma, serum, ascites, pleural effusion and urine. However, to the best of our knowledge, no study has compared methylation kits released in recent years. Since liquid cytology is a valuable sample source, the present study aimed to provide a comprehensive comparison of commercial kits that generate DNA-bisulfite treated sample in a few hours.

Understanding the pros and cons of kits is valuable to pick the right option that fits the purpose of the markers to be tested and the entire workflow. Here, supernatant could be used directly in the bisulfite treatment process without centrifugation, speeding up the entire process.

The costs for this streamlined process need to be considered. Therefore, the present compared kits that perform bisulfite conversion with spin-column and magnetic bead technology, using manual and automatic processing. The price/sample was higher for spin-column kits compared to 96-well format using magnetic beads technology. Including PCR and all the reagents, test cost/sample is $6.24 using kit #7, which is less costly than many other FDA-approved kits currently available in different types of cancer diagnostic.

The present study demonstrated the feasibility of using as 9 μl methanol-based samples with good detection of *β-actin* amplification, with no difference between pellet and supernatant (cfDNA) parts using silica spin-columns. A meta-analysis by Nanda *et al* (2000) on cytology samples showed that despite relatively low sensitivity (51%), it is still used for primary CC screening (46). CC screening program has recommended high-risk HPV (hrHPV) DNA testing over the past decade (47). Moreover, due to a higher sensitivity yet lower specificity than cytology (48), hrHPV test is usually used as a co-test alongside cytology (49). Currently, most samples are methanol-based, and after the screening test, they are discarded. Therefore, the availability of these samples opens opportunity to be further used for molecular testing such as methylation detection since it requires only a few microliters of the discarded sample. In addition, it was not necessary to pellet the samples to obtain methylation profiles comparable to the standard extraction protocol used in the present study.

The Liquid Biopsy Working Group recently emphasized the importance of clinical assay validation of next-generation sequencing assays that use circulating tumor DNA (50). While DNA methylation sequencing is beyond the scope of the present study, the present assay also uses cfDNA. Consequently, technical challenges are similar, including processing time from clinical sample to bisulfite-converted DNA and the clinical assay standardization processes. Next-generation sequencing and qPCR DNA methylation based-tests offer non-invasive insight into tumor genetics and epigenetics, aiding treatment decisions and disease monitoring.

The primary limitation of the present study is that selection of kits was constrained by market availability. Cost and throughput are two primary entry barriers to DNA methylation PCR tests in US clinical laboratories. Consequently, the aim of the present study was not to perform a clinical study but to evaluate differences in performing DNA methylation screening tests with silica columns and magnetic beads on discarded samples from clinical laboratories.

Overall, the present study provided better understanding of the direct methylation kits available in the market using the panel of methylated genes we previously developed to distinguish normal and CC liquid pap smear samples from patients (33). Kits will achieve the highest use if they are commercially available at low cost and easy-to-use format. These kits should provide the best performance and require simple equipment that is readily available at most point-of-care facilities.

Acknowledgements

Not applicable.

Funding

The present study was supported in part by the National Institute of Minority Health and Health Disparities (grant no. R44MD014911), National Cancer Institute (grant no. R44CA254690) and Small Business Innovation Research/Small Business Technology Transfer Matching Grant Program of the Puerto Rico Science, Technology and Research Trust.

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

FTZ and RGP conceived and supervised the study, designed the experiments, visualized data and wrote the manuscript. FTZ and RGP confirm the authenticity of all the raw data. FTZ, EW, ARL, AGN, APW, ACO, ARC and RGP performed experiments and analyzed data. KG performed experiments. FTZ, EW and RGP wrote and revised the manuscript. FTZ, ARL, AGN, APW, ACO, ARC, KG, DS and RGP interpreted data and revised the manuscript. DS and RGP supervised the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was conducted in accordance with the ethical standards as formulated in the Helsinki Declaration (1964) and approved by the Johns Hopkins School of Medicine (approval no. #IRB00231112) and Ponce School of Medicine Institutional Review Board (approval no. #2301130571). The requirement for informed consent was waived due to the anonymized nature of samples.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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