Clinical Chemistry 64:10
1522–1533 (2018)

Evaluation of Preanalytical Conditions and Implementation of Quality Control Steps for Reliable Gene Expression and DNA Methylation Analyses in Liquid Biopsies

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BACKGROUND: Liquid biopsy provides important information for the prognosis and treatment of cancer patients. In this study, we evaluated the effects of preanalytical conditions on gene expression and DNA methylation analyses in liquid biopsies.

METHODS: We tested the stability of circulating tumor cell (CTC) messenger RNA by spiking MCF-7 cells in healthy donor peripheral blood (PB) drawn into 6 collection-tube types with various storage conditions. CTCs were enriched based on epithelial cell adhesion molecule positivity, and RNA was isolated followed by cDNA synthesis. Gene expression was quantified using RT-quantitative PCR for *CK19* and *B2M*. We evaluated the stability of DNA methylation in plasma under different storage conditions by spiking DNA isolated from MCF-7 cells in healthy donor plasma. Two commercially available sodium bisulfite (SB)-conversion kits were compared, in combination with whole genome amplification (WGA), to evaluate the stability of SB-converted DNA. SB-converted DNA samples were analyzed by real-time methylation-specific PCR (MSP) for *ACTB*, *SOX17*, and *BRMS1*. Quality control was assessed using Levey–Jennings graphs.

RESULTS: RNA-based analysis in CTCs is severely impeded by the preservatives used in many PB collection tubes (except for EDTA), as well as by time to analysis. Plasma and SB-converted DNA samples are stable and can be used safely for MSP when kept at −80 °C. Downstream WGA of SB-converted DNA compensated for the limited amount of available sample in liquid biopsies.

CONCLUSIONS: Standardization of preanalytical conditions and implementation of quality control steps is extremely important for reliable liquid biopsy analysis, and a prerequisite for routine applications in the clinic.

Liquid biopsy provides noninvasive real-time monitoring of tumor evolution and therapeutic efficacy for cancer patients, and has received enormous attention because of its obvious clinical implications for personalized medicine *(1)*. Circulating tumor cell (CTC)[2] analysis offers a unique tool to provide deeper understanding of metastasis biology and therapy resistance *(2)*, whereas circulating tumor DNA (ctDNA) analysis enables the identification of genomic alterations, treatment response, and early detection of disease progression *(3, 4)*. Moreover, DNA methylation analysis in CTCs and ctDNA has strong potential to provide another valuable source of circulating epigenetic biomarkers *(5, 6)*. We were the first to report epigenetic changes in CTCs *(7, 8)* and demonstrated a close correlation between DNA methylation in primary tumors, CTCs, and paired plasma ctDNA *(8–10)*. The molecular characterization of CTCs at the DNA, RNA, and protein level is challenging because these cells are extremely fragile and rare, and the amount of available sample is limited *(2)*.

However, despite all the important information that liquid biopsy can provide, it has not been implemented yet into routine clinical practice. Standardization of the procedures used at both the preanalytical and analytical levels is important to achieve this goal *(1, 2)*. Preanalytical conditions such as delays in blood processing, storage temperature of peripheral blood (PB), and even agitation of the sample during shipment of blood can affect the integrity of highly fragile CTCs, and control of these conditions is essential for reliable downstream CTC anal-

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Received May 20, 2018; accepted June 28, 2018.
Previously published online at DOI: 10.1373/clinchem.2018.292318

[2] Nonstandard abbreviations: CTC, circulating tumor cell; ctDNA, circulating tumor DNA; PB, peripheral blood; SB, sodium bisulfite; WGA, whole genome amplification; cfRNA, cell-free RNA; BCT, blood collection tube; cfDNA, cell-free DNA; qPCR, quantitative PCR; gDNA, genomic DNA; MSP, methylation-specific PCR.

ysis *(11, 12)*. Lack of such control could potentially explain many discrepancies reported in the literature *(13)*. Because CTC-mRNA is extremely fragile, gene expression studies in CTCs may be affected by a variety of factors. One important preanalytical factor is the time interval between PB draw and CTC isolation, as the preservatives used in PB collection tubes can severely affect the stability of CTC-mRNA. However, the effect of preanalytical parameters on gene expression in CTCs has not been studied in detail so far. Numerous studies have already evaluated parameters potentially affecting ctDNA concentration and fragmentation and have described the optimal preanalytical handling conditions for ctDNA analysis *(4, 13–20)*. However, the effects of preanalytical parameters on DNA methylation analysis in liquid biopsy have not been studied yet.

The aim of our study was to evaluate in detail (*a*) the effect of preanalytical conditions on gene expression analysis in CTCs, (*b*) the stability of DNA methylation in plasma, (*c*) the stability of sodium bisulfite (SB)-converted DNA samples under different storage conditions, and (*d*) the accuracy and precision of whole genome amplification (WGA) protocols for downstream DNA methylation analysis. We present for the first time a quality control procedure to ensure the reliability of gene expression and DNA methylation analysis in liquid biopsies.

Materials and Methods

RNA-BASED CTC ANALYSIS

Preparation of spiked PB samples. PB samples collected from healthy donors were drawn into 6 different types of tubes in triplicate (10 mL): (*a*) K_2EDTA (BD Vacutainer), (*b*) cell-free RNA (cfRNA) blood collection tube (BCT)(Streck), (*c*) cell-free DNA (cfDNA) BCT (Streck), (*d*) BD/ACD-A in combination with Adnatubes (Qiagen), (*e*) Cellsave (Menarini-Silicon Biosystems), and (*f*) Transfix (Cytomark). In all cases, the first 5 mL of blood was discarded to avoid skin epithelial cell contamination. In each tube, MCF-7 cells (100 cells enumerated in a Malassez hemocytometer) were spiked into PB (10 mL), mixed immediately after spiking by inverting gently 10 times, and then stored at room temperature. Samples were further processed at 3 different time points: (*a*) immediately (T_0), (*b*) after 24 h (T_{24}), and (*c*) after 48 h (T_{48}).

CTC enrichment. After adding 30 mL of red cell lysis buffer (containing NH_4Cl, 155 mmol/L; $KHCO_3$, 10 mmol/L; and EDTA, 0.1 mmol/L, pH = 7.3), samples were incubated (20 min) at room temperature, mixing occasionally by gentle inversion; after centrifugation (530*g*, room temperature, 20 min), the supernatant was removed and subsequently red cell lysis buffer (30 mL) was added. After a second centrifugation (530*g*, room temperature,10 min), the supernatant was removed and red cell lysis buffer (10 mL) was added. Capture beads, coated with the monoclonal antibody BerEP4 against the human epithelial antigen EpCAM (Dynabeads® Epithelial Enrich, Life Technologies), were further used for CTC enrichment *(21)*.

RNA extraction, cDNA synthesis, and RT-qualitative PCR. Total RNA was isolated from the EpCAM-positive CTC fraction using TRIZOL-LS (ThermoFisher), and cDNA synthesis was performed as previously described *(21)*. Total RNA concentration was measured in a NanoDrop-1000 spectrophotometer, calibrated with the recommended CF-1 standard solution. Total RNA (100 ng/μL) isolated from MCF-7 cells was used as a positive control for cDNA synthesis. For gene expression, we used our previously developed and analytically validated RT-qualitative PCR (qPCR) assays for *B2M*[3] and *CK19 (21, 22)*.

DNA METHYLATION ANALYSIS IN LIQUID BIOPSY

ctDNA isolation from plasma. PB was collected from 2 healthy donors into 10-mL K_2EDTA tubes. Plasma was isolated within 2 h from PB by centrifugation (530*g*, room temperature, 10 min). Once isolated, plasma samples were centrifuged again (2000*g*, room temperature, 10 min) and pooled before transferring into clean 2-mL tubes. Plasma samples were maintained at room temperature until spiking. Genomic DNA (gDNA) was isolated from MCF-7 and SKBR3 (10^6 cells) using the QIAamp DNA Micro Kit (Qiagen) and dissolved in 30 μL of Tris-EDTA buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH = 8) (ThermoFisher). DNA concentration was measured in a NanoDrop-1000 spectrophotometer and calibrated with the recommended CF-1 standard solution. DNA (1000 ng) from MCF-7 cells was sonicated for 30 min and subsequently spiked into 4 tubes containing 2 mL of pooled plasma. Each tube was stored at −80 °C and further analyzed at T_0: 0 days, T_1: 1 months, T_3: 3 months, and T_8: 8 months. The QIAamp Circulating Nucleic Acid Kit (Qiagen) was used to isolate ctDNA from 2 mL of plasma.

SB conversion. ctDNA isolated from spiked plasma samples and gDNA isolated directly from MCF-7 and SKBR3 cells were accordingly SB-converted. For the stability study of DNA methylation in plasma and SB-converted DNA samples under different storage conditions, we used the EZ-DNA Methylation Gold Kit

[3] Human genes: *B2M*, beta-2-microglobulin; *CK19*, cytokeratin 19; *ACTB*, actin beta; *SOX17*, SRY-box 17; *BRMS1*, transcriptional repressor and anoikis regulator; *PD-L1*, programmed death-ligand 1; *AR-V7*, androgen receptor splice variant-7.

(ZYMO Research). SB-converted DNA from MCF-7 (spiked in plasma) was stored at −80 °C until use, whereas SB-converted DNA from SKBR3 was stored in aliquots at −20 °C and −80 °C. For downstream WGA studies, we also used the EpiTect Fast Bisulfite Kit (Qiagen); the bisulfite reaction volume was set up for both high (1 ng to 2 μg) and low (1–500 ng) DNA concentrations, according to manufacturer's instructions, and the eluted placental and 100% methylated SB-converted DNA standards were stored at −80 °C.

WGA. WGA of SB-converted DNA was performed using the EpiTect Whole-Bisulfitome Kit (Qiagen). This protocol is optimized for the amplification of >50 ng of SB-converted DNA, diluted with nuclease-free water to a final volume of 10 μL. The amplification was performed in a thermal cycler (Mastercycler® pro, Eppendorf) (28 °C/8 h, 95 °C/5 min, and 4 °C until storage; lid temperature set to 70 °C). After WGA, SB-converted DNA was diluted (1:10 and 1:5) for further downstream real-time methylation-specific PCR (MSP) analysis.

Real-time MSP. The quality of SB-converted DNA was first checked by real-time MSP for β-actin (*ACTB*), and then our previously described real-time MSP for *SOX17* and *BRMS1 (10)* was used for downstream evaluation of SB-converted DNA stability and WGA performance. The stability of DNA methylation in plasma was evaluated using real-time MSP for *SOX17*, whereas the stability of SB-converted DNA was evaluated using real-time MSP for *BRMS1.* WGA performance for SB-converted DNA was evaluated using real-time MSP for *SOX17.* Human placental gDNA (Sigma-Aldrich) was used as control after SB conversion. Universal Methylated Human DNA Standard (ZYMO Research) was used as fully methylated positive control.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS statistics version 23. A P value ≤0.05 was considered significant. Graphics were generated with MS Excel 2010 (Microsoft), and Levey–Jennings graphs were created with QI Macros: SPC Software for Excel (KnowWare International).

Results

RNA-BASED CTC ANALYSIS

Quality control. Systematic quality control was performed for all steps involved in the procedure to ensure the quality and reproducibility of results (Fig. 1). Positive controls were used for monitoring assay variation over time according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines *(23)*. The specific steps follow.

RNA-extraction step. To evaluate the accuracy and reproducibility of the whole procedure, in each series of experiments we used a control for the RNA-extraction step. For this purpose, we aliquoted in 23 different vials 100 MCF-7 cells, lysed with TRIZOL-LS and stored at −80 °C. From Levey–Jennings graphs (Fig. 2A, part 1) derived from 23 individual RNA-extraction procedures and performed over 5 months, the RNA-extraction step was highly reproducible. None of the quality control criteria was violated.

cDNA synthesis step. In each cDNA synthesis experiment, we included a cDNA synthesis control. For this purpose, we aliquoted in 23 different vials 100 ng/μL of MCF-7 RNA and stored them at −80 °C. From the Levey–Jennings graph (Fig. 2A, part 2) derived using the *B2M* assay, 19 of 23 (82.6%) Cq values were within ±1 SD of the mean value, whereas 3 of 23 values were within ±2 SD and 1 was within ±3 SD. For the positive control of cDNA synthesis, using the *CK19* assay, 19 of 23 (82.6%) Cq values were within ±1 SD, whereas 3 of 23 values were within ±2 SD. In 1 case (day 134), the Cq value exceeded the control limit, indicating a control random error; thus, the relationship between this value and the other results within the current and previous assays was examined. Because the Cq value of the RNA-extraction control, analyzed on the same day, was within the limit of ±2 SD from the mean value, the final result was accepted.

RT-qPCR step. To evaluate the accuracy and reproducibility of RT-qPCR in each RT-qPCR run, we included in all runs the same cDNA as a positive control. For this purpose, we aliquoted cDNA from MCF-7 cells and then stored these 23 different aliquots at −80 °C. According to Levey–Jennings graphs (Fig. 2A, part 3), none of the quality control criteria was violated.

Stability of CTC-mRNA. We evaluated the effect of type of BCTs and storage conditions on the stability of CTC-mRNA for downstream gene expression studies (Fig. 3). When PB was stored in K_2EDTA tubes, *CK19* mRNA was readily detectable at all time points (T_0, T_{24}, and T_{48}). In cfDNA BCT tubes, *CK19* mRNA was detected at all time points but at a higher Cq, indicating a significant degradation of CTC-mRNA in these tubes. In ACD-A/Adnatubes, *CK19* mRNA was detected at T_0 and T_{48}; however, at T_{48}, far fewer copies were observed. In cfRNA BCT, Cellsave, and Transfix tubes, *CK19* mRNA was detected only at T_0 but not at T_{24} or T_{48}, indicating a significant degradation of CTC-mRNA in these tubes. *B2M* expression was significantly lower in cfRNA BCT, Cellsave, and Transfix tubes when compared with K_2EDTA tubes at all time points. Our data suggest that CTC-mRNA analysis is severely affected by the preservatives used in these tubes (Fig. 4, A and B).

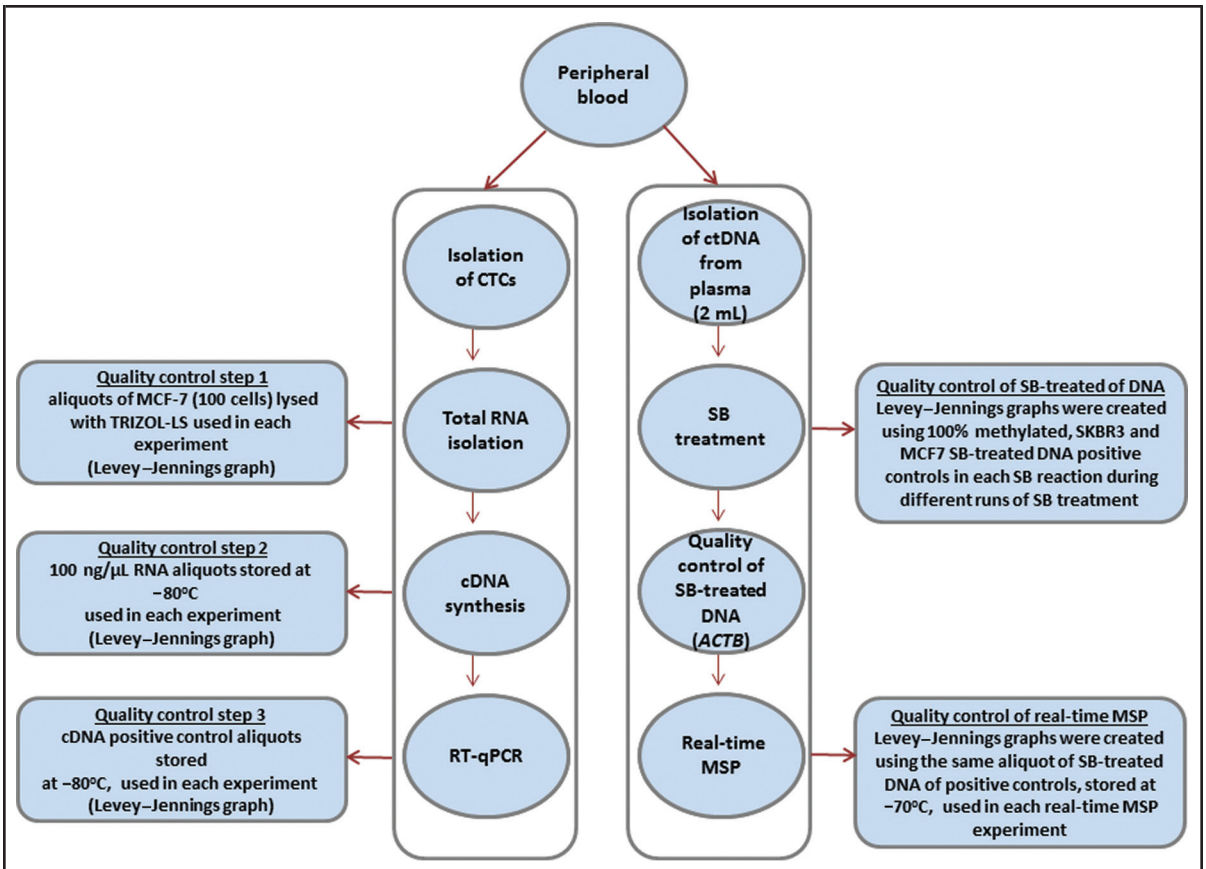

Fig. 1. Experimental flowchart of quality control steps for gene expression and DNA methylation analysis in liquid biopsy.

Recovery rates at all time points were estimated by comparing our results with those obtained using MCF-7 cells analyzed the same way but not subjected to the PB spiking and downstream isolation steps. For this purpose, 10, 100, 1000, and 10 000 MCF-7 cells were lysed at T_0 and processed to mRNA isolation, cDNA synthesis, and RT-qPCR using identical protocols. A calibration curve based on *CK19* RT-qPCR for a known number of MCF-7 cells run in triplicate for each concentration (Fig. 4C) was used for the estimation of the percentage recovery in each PB collection tube at 3 specific time points (T_0, T_{24}, and T_{48}) (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol64/issue10). The mean recovery at T_0 was 86% in K_2EDTA, 50% in cfDNA BCT, 32% in cfRNA BCT, 107% in ACD-A/Adnatubes, 11% in CellSave, and 15% in Transfix tubes (Fig. 4D). At T_{24}, the mean recovery was 44% in K_2EDTA and 9% in cfDNA BCT tubes. At T_{48}, the mean recovery was 36% in K_2EDTA and 6% in ACD-A/Adnatubes.

DNA METHYLATION ANALYSIS IN LIQUID BIOPSY

Quality control. Positive and negative controls were included in all steps to ensure the quality and reproducibility of results (Fig. 1).

SB-conversion step. We evaluated the accuracy and reproducibility of the whole procedure over time and during different time points and different runs of SB conversion of DNA samples by including 100% methylated, SKBR3, and MCF-7 SB-converted DNA as positive controls and creating Levey–Jennings graphs. As can be seen in Fig. 2B, parts 1 through 3, none of the quality control criteria was violated.

Real-time MSP. To establish a quality control procedure for each analytical run, we created a Levey–Jennings graph including all positive controls used in real-time MSP assays. For the 100% methylated positive control, approximately all Cq values fall within ±1 SD, indicating that the analytical process is within control. When SB-converted DNA from MCF-7 cells was used as a control, 66.7% of total Cq values were within ±1 SD,

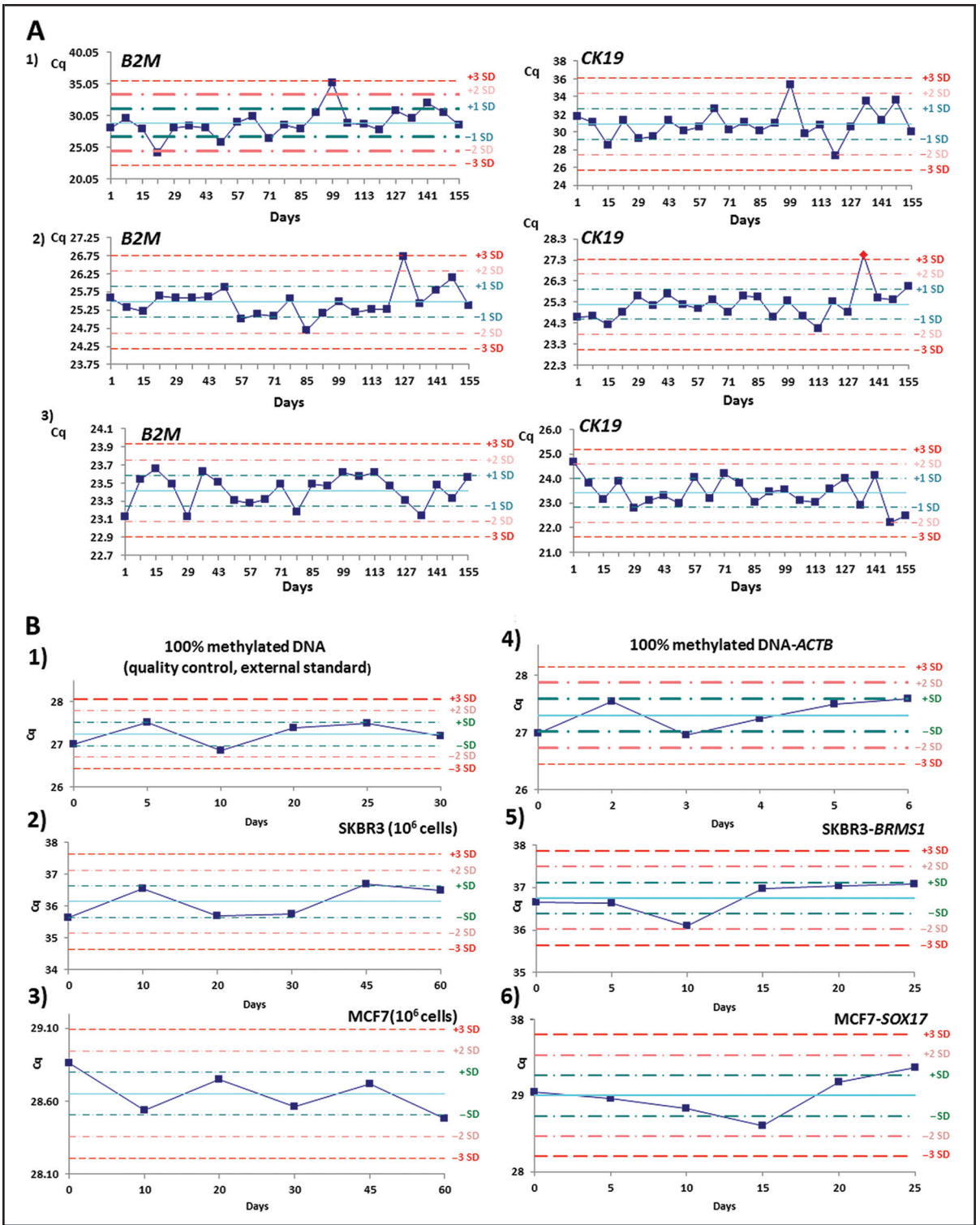

Fig. 2. Quality control analysis and steps.

(A), Quality control of CTC-RNA analysis (Levey–Jennings graphs): (1) whole procedure, (2) cDNA synthesis, and (3) RT-qPCR. (B), Levey–Jennings graphs. Quality control steps for the SB-converted DNA: (1) 100% methylated DNA (quality control, external standard), (2) MCF7 (10^6 cells), (3) SKBR3 (10^6 cells), and for real-time MSP assays: (4) 100% methylated DNA (*ACTB*), (5) SKBR3 (*BRMS1*), and (6) MCF7 (*SOX17*).

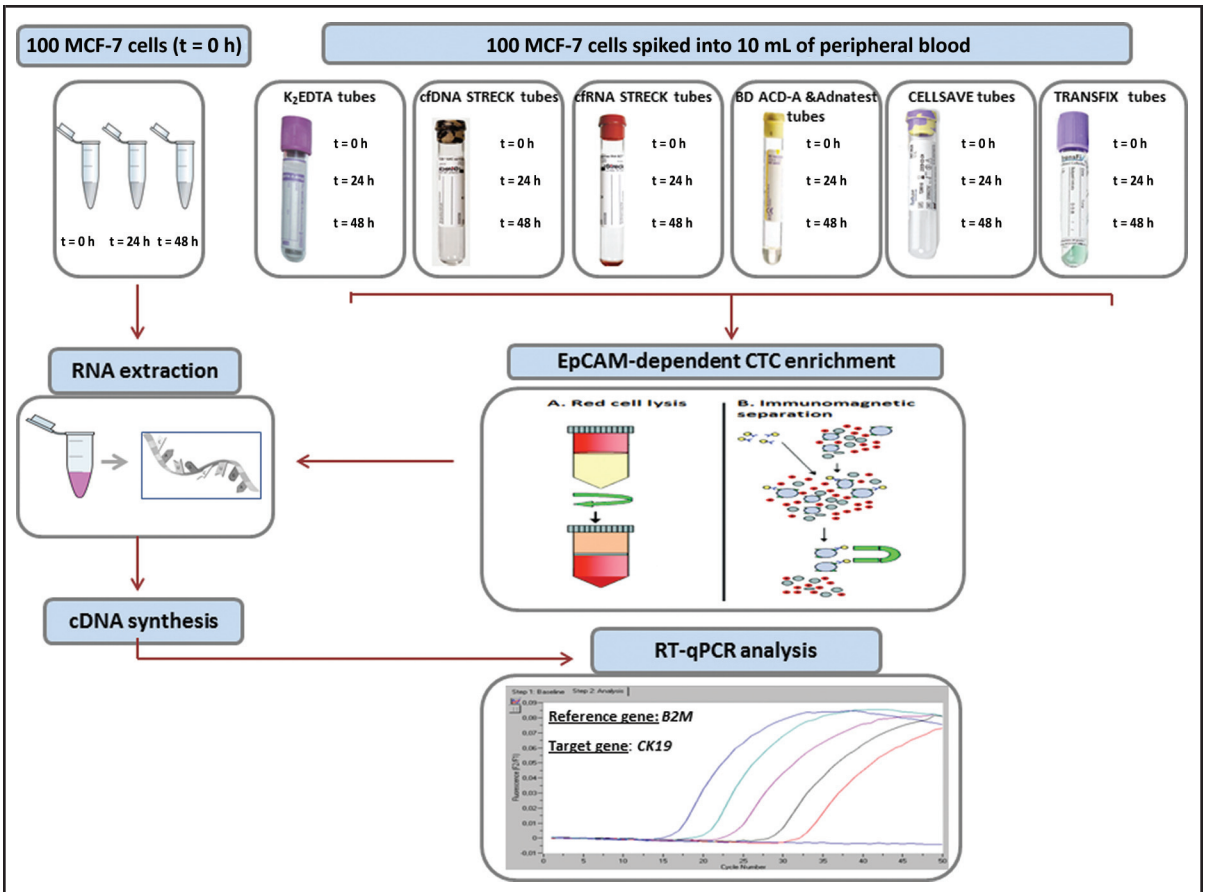

Fig. 3. Evaluation of stability of CTC-mRNA upon collection and storage of PB.

whereas 2 values were within ±2 SD of the mean value, pointing out a process within control limits. Finally, when SB-converted DNA from SKBR3 cells was used as a control, none of the Westgard rules was violated, as 83.3% of Cq values were within ±1 SD and 1 value was within ±2 SD (Fig. 2B, parts 4–6).

Stability of DNA methylation in plasma. Plasma spiked with MCF-7 DNA was separated in 4 aliquots and further used to evaluate the stability of DNA methylation during storage. One aliquot was immediately processed (T_0), whereas the remaining 3 aliquots were stored at −80 °C and analyzed at 3 different time points (T_1, T_3, T_8) in a total period of 8 months (Fig. 5A). Each sample was analyzed in triplicate, and the average Cq (mean ± SD) values over time are presented in Fig. 5B. When performing a 2-tailed paired *t*-test between Cq values at day 0 and each different time point, our results indicate that plasma storage at −80 °C for a period of up to 3 months does not significantly affect ctDNA methylation analysis; however, for both genes tested, we observed a statistically significant differentiation ($P < 0.05$) when plasma was stored for 8 months.

Stability of SB-converted DNA. We further evaluated the stability of SB-converted DNA over time. gDNA was isolated from 10^6 SKBR3 cells, SB-converted, split into 12 aliquots, and stored at −20 °C and −80 °C. Single aliquots were defrosted at different time points (T_0, T_1, T_3, T_6, T_{12}) for a total period of 1 year, and the stability of SB-converted DNA was evaluated by real-time MSP for *ACTB* and *BRMS1*. The effect of multiple freeze–thaw cycles on Cq differences was assessed in parallel by analyzing 1 SB-converted DNA sample that was defrosted in each time point (Fig. 6A). Each sample was analyzed in triplicate, and the Cq (mean ± SD) values are shown in Fig. 6B. After performing a 2-tailed paired *t*-test between Cq values at day 0 and at each different time point, we conclude that SB-converted DNA is stable under storage at −20 °C and −80 °C for up to 1 year. Moreover, repetitive freeze–thaw steps do not affect stability of SB-converted DNA. Thus, SB-converted DNA samples stored at either −20 °C or −80 °C can be used safely for downstream molecular analysis using real-time MSP assays.

Combination of SB conversion with downstream WGA. We further compared the efficacy of 2 different SB-

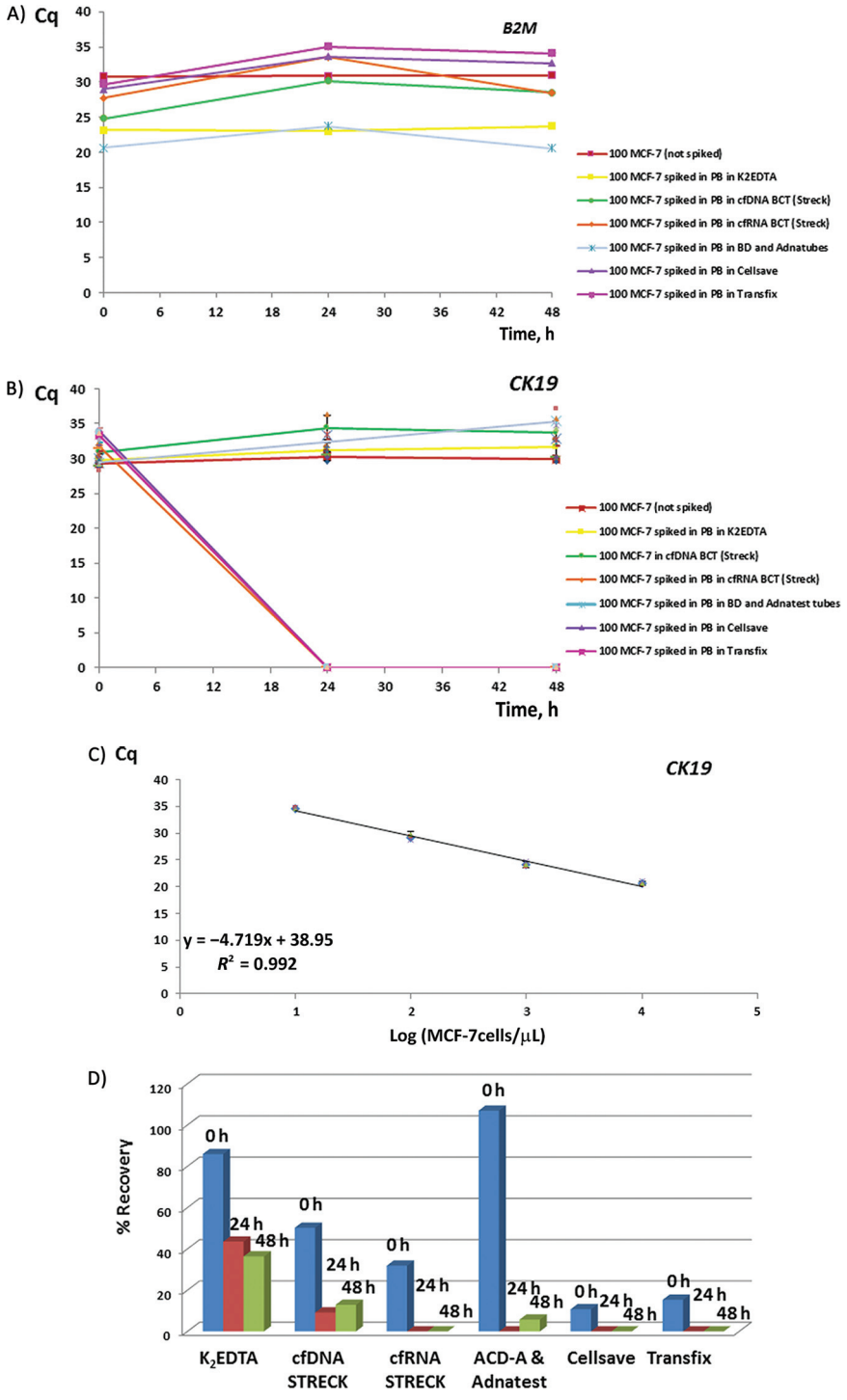

Fig. 4. Evaluation of CTC-RNA stability in 6 different commercially available BCTs at different time points and under different storage conditions.

In all, 100 MCF7 cells were used as recovery control (100%), and results are expressed as Cq values (RT-qPCR). *B2M* (A) and *CK19* (B) RT-qPCR; for *CK19* standard curve (C): Cq plotted vs log (cells/μL), as measured in triplicate, and percentage recovery of *CK19* mRNA transcripts as quantified by RT-qPCR at different time points (D).

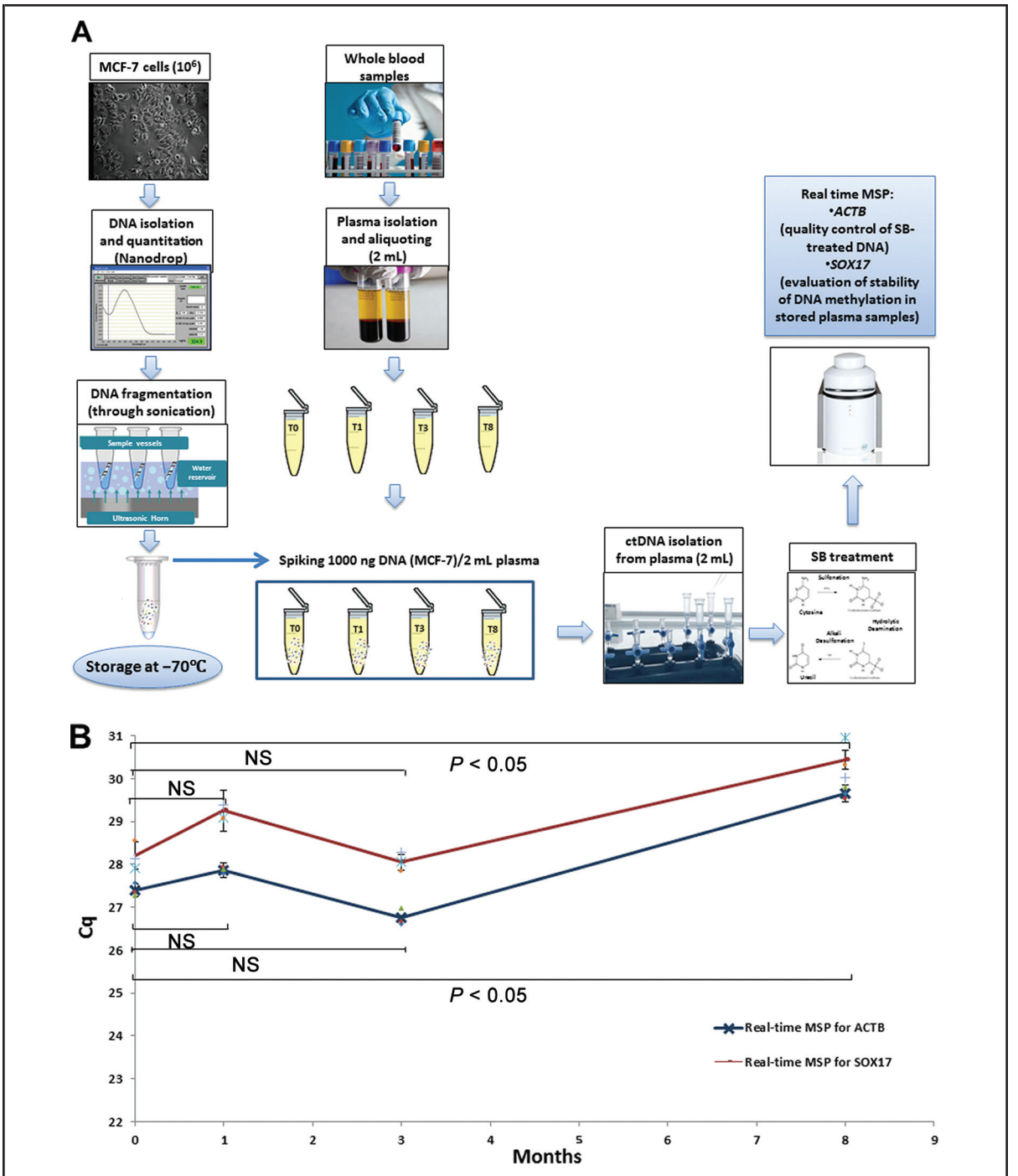

Fig. 5. (A), Experimental outline of evaluation of stability of DNA methylation in plasma; (B), real-time MSP (Cq values) of SB-converted DNA for *ACTB* and *SOX17* during plasma storage at −80 °C for up to 8 months (all in triplicate).

conversion kits (EpiTect Fast Bisulfite Kit and EZ-DNA Methylation Gold Kit), in combination with downstream WGA using the EpiTect Whole-Bisulfitome Kit (Qiagen) that is specifically designed for real-time MSP applications. When the EpiTect Fast Bisulfite Kit was used, best results were obtained when setting the SB conversion for high concentration samples and diluting the SB-converted and amplified DNA 1:10 before MSP.

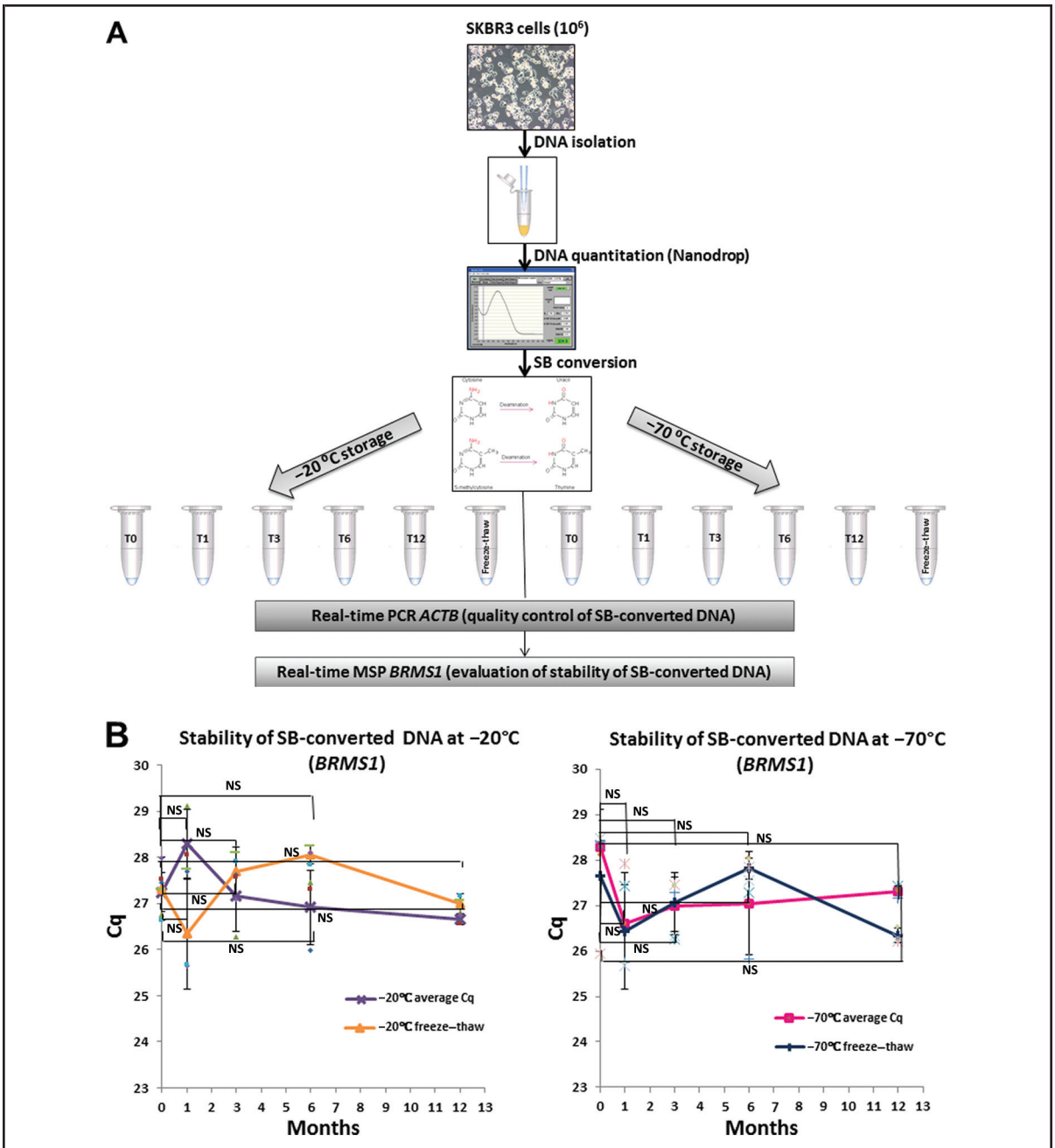

Fig. 6. (A), Experimental outline of evaluation of stability of SB-converted DNA during storage at −20 °C and −80 °C; (B), real-time MSP (Cq values) of SB-converted DNA for *BRMS1* (all in triplicate).

Real-time MSP results before and after amplification, both for placental DNA and 100% methylated DNA, were similar only when samples were diluted 1:10 and used as a target for real-time MSP. In every other case, discrepancies were observed. When the EZ-DNA Methylation Gold Kit was combined downstream with the EpiTect Whole-Bisulfitome Kit, our results revealed that samples should not be diluted for MSP after the amplification reaction because it was only in this case that Cq values before and after amplification were almost the same. In contrast, when amplified SB-converted samples were diluted (1:5 or 1:10), a significant loss in WGA performance was observed (see Table 2 in the online Data Supplement).

Discussion

Liquid biopsy is based on the analysis of CTCs, ctDNA, circulating microRNA, and tumor-derived extracellular vesicles *(1, 2, 24, 25)*. ctDNA and CTCs have complementary roles as a source of cancer biomarkers in liquid biopsy *(1, 2)*. RNA-based CTC analysis can elucidate the critical signaling pathways involved in cancer biology *(1, 26)* and give important prognostic and predictive information, as studies on *CK19 (21, 22)*, *PD-L1 (27)*, and *AR-V7* have shown *(28)*, whereas ctDNA analysis can be applied as a high-throughput strategy for the assessment of clinical samples, but is limited to point mutations, structural rearrangements, copy number aberrations, and changes in DNA methylation.

Clinical utility of liquid biopsy tests can be evaluated only when based on standardized and strictly controlled procedures. Preanalytical factors, if not properly recognized and controlled, can have a serious effect on sample quality and, thus, on the quality of liquid biopsy analyses. Lack of standard operating procedures is severely impeding the application of CTC-based and ctDNA-based diagnostic tests in the clinic *(29)*. Molecular analysis of CTCs has been limited by the requirement to process fresh blood within a few hours after blood draw *(30, 31)*. To minimize preanalytical errors because of the stability of CTCs in PB during transportation to distant sites, a variety of tubes based on the addition of specific preservatives intended to extend the time between blood draw and CTC isolation has been proposed. Commercially available cfDNA BCTs are claimed to stabilize nucleated blood cells, preventing the release of cellular DNA into plasma and inhibiting degradation of cfDNA *(14–16)*. However, most of these tubes are designed for a downstream analysis of CTCs at the protein level based on imaging approaches, but not for gene expression studies.

In the present study, we first evaluated the effect of preanalytical conditions for gene expression analysis in CTCs. Our data indicate that RNA-based CTC analysis is severely impeded by the preservatives used in most tested PB collection tubes, except when common K_2EDTA tubes are used and analysis is completed within 24 h and, at most, 48 h. Our results are in full agreement with those previously reported for *AR-V7* quantification by droplet digital PCR in CTCs *(32)*. cfDNA BCTs were proposed for CTC analysis in a study using high-definition single-cell analysis *(12)* and in another study using CellSearch *(33)*. However, when we evaluated these cfDNA BCTs for gene expression in CTCs, *CK19* mRNA was detected at all time points but at higher Cq numbers than in K_2EDTA, indicating a significant degradation of mRNA transcripts. The discrepancies between our results and the studies above may be attributed primarily to the different approaches used for detection of CTCs: RNA-based in our case vs enumeration of CTCs and protein-based CTC imaging, respectively *(12, 33)*. When using cfRNA BCTs, we found that *CK19* mRNA was detected only at T_0 but not at T_{24} and T_{48}, whereas *B2M* expression was significantly lower when compared with K_2EDTA tubes at all time points. In Cellsave tubes, we detected *CK19* mRNA only at T_0 but not at T_{24} and T_{48}, indicating a significant degradation of CTC-mRNA, whereas *B2M* expression was also significantly lower than in K_2EDTA tubes at all time points. TransFix tubes have been used to detect CTCs by image analytics after fluorescence scanning microscopy *(34)*; however, according to our results, they are not appropriate for RNA-based studies. When we evaluated CTC-mRNA stability in ACD-A/Adnatubes, *CK19* mRNA was detected at T_0 and T_{48}; however, at T_{48}, far fewer copies were observed for both *CK19* and *B2M*.

We further evaluated the effect of preanalytical conditions on DNA methylation analyses in liquid biopsies, such as the time interval to plasma isolation, storage conditions for SB-converted DNA, and the validity of WGA for SB-converted DNA. Adverse results on the stability of DNA methylation during different storage conditions of whole blood over time have been reported; according to 1 study, storage conditions have no impact on DNA integrity and methylation *(35)*, whereas a significant increase in methylation after 10 months of whole blood storage was reported in another study *(36)*. Our results indicate that DNA methylation information is not preserved when ctDNA extraction is performed in plasma stored for 8 months even at −80 °C. On the contrary, SB-converted DNA can be safely stored before DNA methylation analysis by real-time MSP for 1 year at both −20 °C and −80 °C. When commercially available SB-conversion kits were evaluated, the results varied from study to study *(37–39)*. Our results on the comparison of the efficacy of 2 SB-conversion kits, selected as the best among 12 different SB-conversion kits *(37)*, showed similar values, with a slightly better performance for the EZ-DNA Methylation Gold Kit.

We evaluated for the first time the performance of WGA for DNA methylation studies in liquid biopsy, as this procedure can provide a large amount of DNA for downstream analysis. However, using conventional WGA, DNA methylation information is lost, unless unbiased amplification of SB-converted DNA is achieved *(40)*. Our proposed protocol ensures reliable WGA of SB-converted DNA; addition of this step could compensate for the limited amount of available sample for DNA methylation studies in liquid biopsies.

In the present study, we evaluated the effect of preanalytical parameters on gene expression in CTCs and on the stability of DNA methylation in plasma and SB-converted DNA under different storage conditions. According to our results, standardization of preanalytical

parameters involved in gene expression and DNA methylation analysis is critical to ensure the reliability of results before any clinical application in liquid biopsy, for which the amount of available DNA is limited. Quality control checking should be included in each step of the analysis.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

E.S. Lianidou, administrative support.

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: M. Zavridou, the IMI contract no. 115749 CANCER-ID: "Cancer treatment and monitoring through identification of circulating tumor cells and tumor related nucleic acids in blood" (https://www.cancer-id.eu/); S. Mastoraki, the IMI contract no. 115749 CANCER-ID: "Cancer treatment and monitoring through identification of circulating tumor cells and tumor related nucleic acids in blood" (https://www.cancer-id.eu/), the General Secretariat for Research and Technology (GSRT)/Hellenic Foundation for Research and Innovation (HFRI) in the context of the action 1st Proclamation of Scholarships from ELIDEK for PhD Candidates Scholarship Code: 2162. MCF-7 cells were donated by Prof. O. Tsitsiloni, Department of Biology University of Athens.
Expert Testimony: None declared.
Patents: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or final approval of manuscript.

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