BRIEF COMMUNICATION

Effects of Prolonged Storage of Whole Plasma or Isolated Plasma DNA on the Results of Circulating DNA Quantification Assays

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Analysis of molecular markers in biological fluids has been proposed as a tool for early detection and monitoring of cancer. Circulating plasma DNA concentrations have been found to be higher in cancer patients than in cancer-free control subjects, but little is known about the effect of specimen storage on plasma DNA concentrations. Here we investigated the impact of long-term storage of both plasma samples and purified plasma DNA on the reproducibility of plasma DNA quantification as determined using real-time polymerase chain reaction analysis. The analysis was performed on samples from a subset of 34 lung cancer patients and 28 matched control subjects selected from 200 subjects in our previously published case–control study and from 117 cancer-free smokers enrolled in a lung cancer screening program. Two samples of plasma and isolated DNA were assessed for each patient, with a median of 41 months between the first and second assessments for participants in the case–control study and 9 months for participants in the screening study. DNA levels declined substantially between the two assessments at an average rate of approximately 30% per year. These data provide valuable information for the rational planning of retrospective studies of banked series of biological samples, particularly if collected over a long period of time, as can occur in large clinical trials. [J Natl Cancer Inst 2005;97:1848–50]

Several studies have demonstrated the presence of substantially higher amounts of free circulating DNA in the plasma or serum of cancer patients than in healthy control subjects *(1–5)*. We developed a TaqMan-based real-time quantitative polymerase chain reaction (qPCR) assay for the human telomerase reverse transcriptase (hTERT) gene in which absolute quantification of DNA is obtained by interpolation of the experimental data on a standard curve generated with reference genomic DNA *(6)*. Using this assay we recently reported elevated circulating DNA levels in 69% of 100 lung cancer patients versus only 2% of 100 matched control subjects. When quantification of plasma DNA level was used as a diagnostic test for lung cancer in this study sample, the area under the receiver operating characteristic (ROC) curve was 0.94 *(6)*, indicating high sensitivity and specificity. Given the sensitivity and specificity of the qPCR test for hTERT DNA, we have suggested that it could be used together with spiral computed tomography (SCT) in early lung cancer detection programs *(6)*.

For a test based on DNA levels to be clinically applicable, the reproducibility of DNA measurements must be considered carefully. Several recently published articles have shown that various factors related to blood sampling and processing can affect the concentration of circulating DNA *(7–10)*. It has been shown that DNA concentration is approximately 3- to 24-fold higher in serum than in plasma *(7–9)*, but the influence of time delay in blood processing for plasma and serum has been analyzed in few studies *(9,10)*. Jung et al. *(9)* found no changes in DNA concentration in plasma samples stored at room temperature or at 4 °C for up to 24 hours. However, this time delay and the storage temperature of blood before centrifugation had a substantial impact on the DNA concentration in serum. We recently reported satisfactory reproducibility of a DNA quantification assay (DNA DipSticks, Invitrogen) between independent measurements, a lack of variability in the within-subject classification, and measurement concordance of cell-free DNA levels after over 3 months of storage of DNA aliquots isolated from plasma *(10)*.

However, the issue of whether prolonged storage of whole plasma or isolated plasma DNA might affect the results of DNA quantification assays has not been comprehensively explored, to our knowledge. Here we investigated the effects of prolonged storage of whole plasma or isolated plasma DNA aliquots on the plasma DNA quantification assay by hTERT qPCR by comparing results of measurements performed after many months of storage. Two sets of subjects were investigated. Set 1 included 34 lung cancer patients and 28 matched control subjects selected from among the 200 subjects in our case–control study *(6)* on the basis of the availability of both isolated DNA and never-thawed plasma aliquots for analysis. Set 2 included 117 heavy smokers out of the 1035 volunteers of the Milan SCT pilot study *(11)*; these subjects were chosen because the DNA concentration in their initial plasma sample was close to the value of 10 ng/ml, which corresponds to a 90% specificity in discriminating patients with lung cancer from cancer-free subjects as computed by the ROC curve in our previous study *(6)*.

Collection of blood and separation of plasma fractions have been described previously *(6)*. Isolated DNA was stored at −20 °C, whereas plasma aliquots were kept at −80 °C. DNA was isolated from stored plasma as previously described *(5)*. Descriptive analyses of plasma DNA concentrations were conducted by calculating medians, minimums, maximums, and 25% and 75% percentiles for each stratum as defined by the set of subjects, their classification (i.e., as case patients or control subjects in set 1), and the assessment (i.e., whether it was the first or second assessment within the same subject). Because the distribution of DNA concentrations was positively skewed,

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all of the analyses were carried out after logarithmic transformation of DNA concentrations.

Median DNA levels at the first and second assessments were compared by using the Student's *t*-test. Data from the subjects in set 1 were used to determine the diagnostic discrimination allowed by DNA levels by nonparametric estimation of the area under the ROC curve *(12)*. To obtain an overall estimate of DNA decay since time from blood draw, multiple linear regression models were fit to the combined data for both sets of participants. In these models, DNA levels (after storage of isolated DNA or extraction of stored plasma samples) were regarded as the dependent variable; time from blood draw, subject classification (as case patient or control subject), and the set of subjects were included as predictors; and the subject was modeled as a random factor to account for the correlation between repeated measurements (i.e., the first and second assessments within the same subject). For exploratory purposes, suitable interaction and nonlinear terms (quadratic or cubic transformations of the time from blood drawing) were also included in the models and used to check whether DNA level decay with time from blood drawing deviated from linearity or changed in the two sets of patients or between case patients and control subjects; if these terms were not statistically significant, they were dropped from final models. The analyses were carried out using SAS software (SAS Institute, Inc., Cary, NC). Two-sided *P* values below .05 were regarded as statistically significant.

Descriptive statistics of DNA concentration distribution at the two assessments are shown in Table 1. In the set 1 participants, plasma samples were quantified at a median time from blood draw of 10.5 months (range = 4 to 15 months) and of 50 months (range = 45 to 56 months), with a median interval between assessments of 41 months (range = 38 to 43 months). For both isolated DNA and plasma samples, median levels at the second assessment were systematically lower than those at the first assessment for both case patients and control subjects. The reductions were statistically significant ($P<.001$), with the exception of stored plasma in control subjects ($P = .063$) (Table 1). For the initial DNA plasma levels, the area under the ROC curve calculated with this set of case patients and control subjects was 0.963, a value very close to that obtained for the entire series of 200 subjects (0.94) *(6)*. When the DNA levels at the second assessment were used to generate ROC curves for the set 1 participants, the areas under the ROC curves were 0.958 and 0.904 for stored isolated DNA and DNA isolated from stored plasma, respectively (data not shown). Thus, the good diagnostic performance of the quantification assay was maintained after prolonged storage of whole plasma and isolated DNA, despite the dramatic reduction in circulating DNA levels.

Table 1. DNA concentrations (ng/ml) in subjects stratified by set and case/control status at the first and second assessments*

Source of DNA	Assessment	Median	Minimum	25th percentile	75th percentile	Maximum	P†
Isolated DNA							
Set 1 case patients	First	23.4	1.2	15.3	36.9	823.0	
	Second	6.4	0.6	4.6	11.2	150.9	<.001
Set 1 control subjects	First	2.4	0.3	0.9	4.5	18.6	
	Second	0.6	0.0	0.4	1.4	4.3	<.001
Set 2	First	10.3	8.0	9.1	12.1	117.9	
	Second	7.9	1.6	6.2	10.7	97.2	<.001
Stored plasma							
Set 1 case patients	First	23.4	1.2	15.3	36.9	823.0	
	Second	5.6	1.3	3.3	11.1	24.8	<.001
Set 1 control subjects	First	2.4	0.3	0.9	4.5	18.6	
	Second	1.8	0.1	1.1	2.9	4.0	.063
Set 2	First	10.3	8.0	9.1	12.1	117.9	
	Second	5.1	0.5	3.6	7.1	33.3	<.001

*Median time between assessments was 41 months for set 1 participants and 9 months for set 2 participants.

†*P* value from Student's *t* test for comparing median DNA levels at the first and second assessments.

For the subjects in set 2, DNA content in plasma samples was quantified at median times from blood draw of 16 months (range = 4 to 24 months) for the first assessment and of 24 months (range = 17 to 41 months) for the second assessment, with a median interval between assessments of 9 months (range = 4 to 29 months). Median plasma DNA concentrations, whether assayed in stored isolated DNA or in DNA from stored plasma, were statistically significantly (both $P<.001$) lower at the second assessment than at the first assessment (Table 1).

For stored isolated DNA, the yearly decay rate estimated from the set 1 and set 2 combined data by means of a linear regression model was 30.5% (95% confidence interval = 28.2% to 32.7%). The yearly degradation rate for DNA from stored plasma (30.7%) was similar to that observed for isolated DNA, but regression analysis also revealed some nonlinear behavior of DNA levels, which was heterogeneous between control subjects in set 1 and the subjects in set 2 and between case patients and control subjects in set 1. These findings should be explored in a different kind of study, in which multiple measurements spread over time are available within the same subject. Such a study will help to clarify the kinetics of DNA degradation in stored plasma.

Our results indicate that prolonged storage of both isolated DNA and whole plasma samples leads to substantial DNA degradation as measured by real-time PCR-based assays. This observation should be taken into account during planning and execution of large prospective clinical trials that involve the analysis of circulating plasma DNA or when stored plasma specimens are used for retrospective analyses that involve comparison of amounts of plasma DNA or detection of biomarkers in plasma DNA. Whether similar storage-related degradation also occurs for other molecules (e.g., RNA and protein) circulating in the bloodstream remains to be determined.

Finally, future studies of plasma DNA biomarkers should involve quality control programs to determine the influence of both preanalytic factors (i.e., factors related to blood sampling and processing) and analytic factors (i.e., the

use of different procedures to quantify circulating DNA) on the variability of quantitative assays.

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Notes

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