Verification of performance of a direct fluorescent assay for cell-free DNA quantification, stability according to pre-analytical storage conditions, and the effect of freeze-thawing

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Abstract. A simple fluorescence-based cell-free DNA (CFD) assay has been previously developed that can directly measure nucleic acids without prior DNA extraction and amplification. However, studies on fluorescence-based CFD are lacking. In particular, there is no known information regarding the stability with regard to pre-analytical storage conditions in relation to time and temperature, or on the influence of freeze-thawing. Plasma was directly assayed to measure CFD using PicoGreen™ reagent. Standard linearity and accuracy were confirmed using salmon sperm DNA. Whole blood was left at room temperature (RT) and at 4˚C, and then plasma was separated. The CFD was also measured using thawed plasma after 1 week of freezing. As a correlation with a sperm DNA concentration, CFD demonstrated linearity over a wide range of concentrations, with a 0.998 correlation coefficient. The CFD level showed a change of up to 2.5 μg/ml according to pre-analytical storage time, and the changes were not consistent over time. The CFD values at RT after 1 h were similar to the baseline values, and the relative standard deviation was lowest under this condition. The CFD values between 4˚C and RT were similar over all time periods assessed. After freeze-thawing, the change in CFD value was reduced compared to that before freezing. The present study showed that CFD measurements using plasma processed within 1 h were optimal. Additionally, the effects of substantial changes according to storage conditions were reduced after freeze-thawing, and thus studies using stored samples is viable and relevant.

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Introduction

Cell-free DNA (CFD) can originate from the release of DNA from a cell undergoing apoptosis or necrosis, or from the release of intact cells in the bloodstream and their subsequent lysis (1-4). Several previous studies have reported elevated CFD levels in patients with cancer (2-7). The potential for measuring CFD has been increasingly recognized as a tool for a variety of activities, including diagnosis, monitoring of treatment response and prognosis determination for several different types of cancer (2,4,8-10).

Until recently, quantitative measurement of CFD has been primarily assessed using PCR. However, there are two main problems with PCR. First, it is complex and labor-intensive, particularly with regard to DNA extraction and PCR amplification with specific primers (11). Thus, it is not only inconvenient in terms of cost and time, but also can impede comparison of data between laboratories due to differences in the specifics of the various protocols and differences in the reagents used. Second, there is a lack of clear information on the changes in CFD values according to pre-analytical storage temperature and duration for whole blood before separating the plasma (11). There are other methods for measurement of CFD levels, including droplet digital PCR and the MassARRAY® system (12-14), these methods however, require advanced technology, so may be not easily accessible. CFD values may increase with storage time due to cell lysis (4); conversely, it may be decreased by DNA degradation related to nuclease activity in the blood (15). The stability of CFD values based on storage temperature and time from whole blood sampling to processing by centrifugation is an important issue for application of CFD assays in the hospital. Although there is a general consensus on the value of CFD measurements in a several types of cancer (3,16), CFD assays have been confined to research laboratories.

A fluorescence-based CFD assay was developed that could directly measure DNA using a simple and inexpensive method without prior DNA extraction and amplification (17). The results of fluorescence-based CFD assays are significantly correlated to the CFD level measured using the PCR method (18), and significant changes in CFD levels according to the efficacy of anticancer treatment has been confirmed (4,19). As standardization of fluorescence-based CFD assays is relatively easy (18), inter-researcher and inter-laboratory differences are expected to be small. However, information on basic performance, including accuracy and reproducibility of fluorescence-based CFD assays is insufficient. In addition, pre-analytical storage conditions prior to plasma processing should be clarified for clinical utility. Finally, even when conducting studies using fluorescence-based CFD assays in a research setting, the assays are generally performed using freeze-thawed plasma specimens. Thus, the effect of freeze-thawing on CFD levels should be determined, to improve confidence in the results. The aim of the present study was to examine the basic performance of fluorescence-based CFD assays as measured by accuracy and stability, and assess the potential effects of pre-analytical storage conditions, and the influence of freezing-thawing.

Materials and methods

Fluorescence-based CFD assay. Fluorescence-based CFD was directly assayed using Quant-iT PicoGreen™ dsDNA Reagent (cat. no. P7584; Invitrogen; Thermo Fisher Scientific, Inc.) without DNA extraction or amplification. The CFD in plasma was measured after being diluted with TE buffer (cat. no. V6231; Promega Corporation). All standards and samples were deposited in a 100 μl volume in each well of a 96-well microplate. For staining, 100 μl PicoGreen reagent was added volume to each sample in black 96-well plates for 3 min at room temperature (~20˚C; cat. no. SPL30296; SPL Life Sciences Co., Ltd.), and the mixture was diluted 400-fold. Fluorescence intensity was measured with a black 96-well microplate reader (Spark; Tecan Group, Ltd.) at an emission wavelength of 535 nm and an excitation wavelength of 485 nm. All assays were performed twice, and the average value was used.

Verification of fluorescence-based CFD. To assess linearity and accuracy, DNA standards were prepared using salmon sperm DNA (10 mg/l; cat. no. 15632-011; Thermo Fisher Scientific, Inc.) that was diluted to 1,000, 750, 500, 250, 100, 10, 1 and 0.2 ng/l. A total of 16 independent experiments were performed to analyze the correlation between the mean of the fluorescence intensity and DNA concentration value, and the final measurement range was used after a quantitative standard linearity test to confirm the accuracy at each concentration. To identify an appropriate dilution ratio without interference effects, plasma samples were diluted to 1/2, 1/10, 1/20, 1/50 and 1/100 using TE buffer. Salmon sperm DNA was spiked into the diluted plasma at a concentration of 10 ng/ml to evaluate the recovery rate for the known amount of DNA.

Stability of CFD according to storage conditions and influence of freeze-thawing. To assess the impact of various storage conditions, whole blood of 5 volunteers were collected for use in the present study. These volunteers did not have acute or chronic disease and were not taking any medication. The group was composed of 2 women and 3 men with a median age of 34 (range, 29-45). A volume of 22 ml peripheral blood drawn from each participant; 2 ml blood was placed in each of the 11 EDTA tubes using a 20 gauge needle. Whole blood samples in each tube were stored at 4˚C or room temperature for various periods of time (0, 1, 2, 3, 4 or 6 h) prior to centrifugation. Subsequently, plasma samples were obtained by centrifuging the peripheral blood at 3,000 x g for 10 min at room temperature, and the plasma samples were collected as the upper supernatant layer. The CFD value was measured for each condition (temperature and incubation time). The plasma samples obtained for each storage condition were stored in frozen aliquots at -80˚C and thawed after a month to assess the potential impact of freeze-thawing and to assess reproducibility. The research protocol was approved by the Institutional Review Board of Pusan National University Yangsan Hospital (Yangsan, Republic of Korea; approval no. 04-2019-024), and written informed consent was obtained from all participants.

Statistics. Standard linearity of fluorescence-based CFD was evaluated using simple linear regression analysis. Relative standard deviation (RSD), which is the standard deviation divided by mean, was used to identify the precision between the averages of the sequentially analyzed results. With regard to analysis of storage conditions and the freeze-thawing effect, the differences in CFD amongst the groups and follow-up periods were compared using repeated measures ANOVA and Bonferroni post hoc tests. Statistical analyses were performed using SPSS version 19.0 (IBM Corp.).

Results

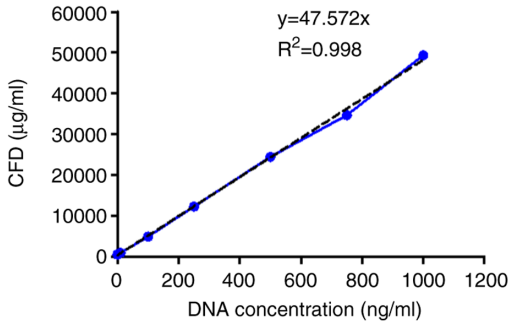
Basic performance of the fluorescence-based CFD assay. As a result of analyzing the average fluorescence intensity correlation with the sperm DNA concentration, quantification of plasma CFD demonstrated linearity over a wide range of concentrations (1-1,000 ng/ml) with a strongly positively correlated standard curve (R^2=0.998) (Fig. 1). The 1/50 diluted plasma showed a 114% recovery rate at 11.4 ng/ml, whereas the other diluted ratios showed a recovery rate of ≥200%, and a notable difference between the spiked DNA concentration. Considering interference effects and recovery, the appropriate dilution ratio was determined to be 1/50 (Table I).

Stability of fluorescence-based CFD according to storage conditions. At both RT and 4˚C, the CFD values increased after 1 h, decreased at 2 h, increased after 3 h and then decreased after 6 h (Fig. 2). The changes in CFD values were significant according to the time course (F=481.038, P<0.001); however, the changes did not show consistency over time. Nevertheless, considering that the CFD values at RT for 1 h were similar to the baseline CFD values and the RSD values at 1 h were the most stable. These results suggest that plasma processed from whole blood within 1 h at RT is optimal. CFD values between 4˚C and RT were similar over all time periods (F=1.004, P=0.346), showing that temperature was not a major factor of storage conditions within 6 h. The mean baseline CFD level was 8.4 μg/ml with a standard deviation of 0.2 μg/ml.

Table I. Interference effect and recovery of fluorescence based cell free-DNA.

Sample dilution	Spiking, ng/ml	ng/ml	Internal control[a]	Recovery, %
Sample 1/10	0	282.8	-	-
Sample 1/20	0	199.8	-	-
Sample 1/50	0	123.8	-	-
Sample 1/100	0	87.2	-	-
Internal control 1/10	10	264.2	-18.6	-185.6
Internal control 1/20	10	226.4	26.6	265.7
Internal control 1/50	10	135.2	11.4	114
Internal control 1/100	10	108.9	21.7	216.8

[a]Internal control refers to the difference in the measured values between sample spiking and the sample at the same dilution concentration.



No.	Concentration (ng/ml)	Average	RSD (%)	Accuracy (%)
1	0	521.4	12.0	
2	0.2	481.0	1.9	
3	1	566.4	11.8	93.2
4	10	893.1	19.1	77.0
5	100	4858.9	16.6	89.8
6	250	12236.1	13.8	97.0
7	500	24402.0	12.7	98.9
8	750	34613.4	10.7	94.1
9	1000	49323.6	6.9	101.1

Figure 1. Correlation between the direct fluorescent assay and PCR method for measurement of CFD. As a result of analyzing the average fluorescence intensity correlation with the sperm DNA concentration, quantification of plasma CFD demonstrated linearity over a wide range of concentrations (1-1,000 ng/ml) with a correlating standard curve (R^2=0.998). RSD, relative standard deviation; CFD, cell free DNA.

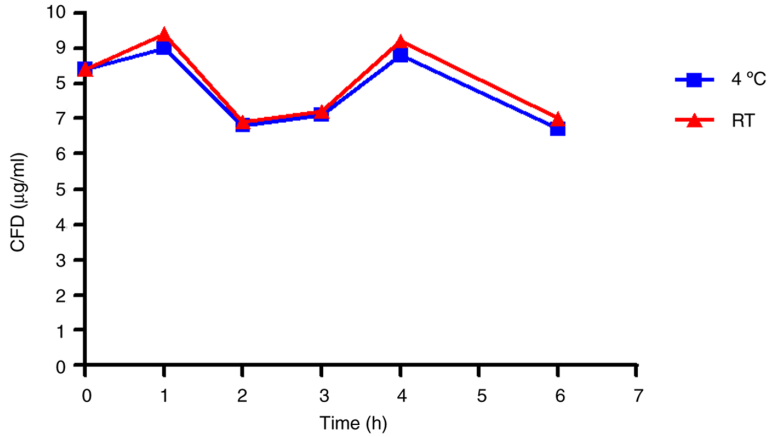
Influence of freeze-thawing on the fluorescence-based CFD assay. Plasma samples were stored as aliquots at -80˚C and thawed after 1 month to assess the influence of freeze-thawing and reproducibility. There was a significant difference in CFD values after freeze-thawing with regard to storage time (F=3.387, P=0.007; Fig. 3); however, the changes in CFD values were reduced compared to those before freezing (fresh sample, F=481.038; samples after freezing, F=3.387). There was no significant difference in CFD values after freeze-thawing with regard to the previously exposed temperature (F=1.342; P =0.280).

Discussion

The present study showed the basic performance of a simple fluorescence-based CFD assay as an accurate method for measuring CFD. Whilst, CFD values exhibited large variability over all time periods depending on storage time during the pre-analytical phase. If processing of blood was not consistently performed at the same time, the CFD values were not reliable. Thus, strict management of pre-analytical storage conditions based on predefined guidelines is necessary. The present study showed that CFD values measured in plasma samples processed within 1 h at RT were similar to the baseline values, and the RSD was lowest. Hence, pre-analytical conditions of 1 h at RT were deemed to be optimal for fluorescence-based CFD assays. On the other hand, CFD values were consistent regardless of pre-analytical storage conditions after freeze-thawing, indicating the reliability of results of the fluorescence-based CFD assay using stored plasma, such as that from biobanks.

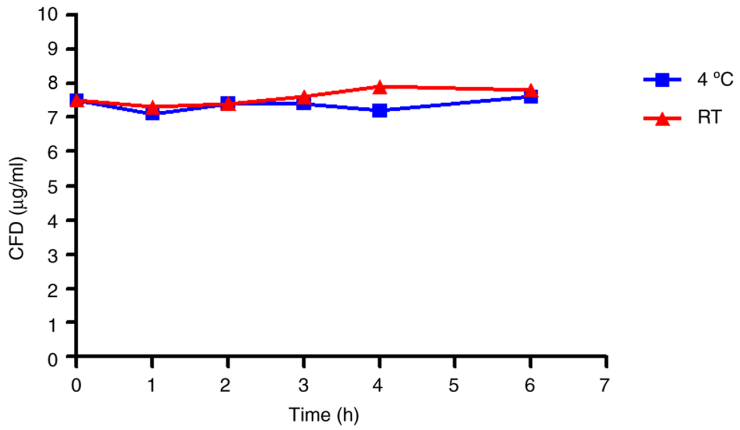
The fluorescence-based CFD assay using PicoGreen™ is a convenient and cost-effective method for direct assay of CFD. This method is free from technical issues such as DNA extraction, so there are fewer issues, such as inter-tester-laboratory variations due to the test itself. The PCR assay is expensive and difficult to standardize due to the relative complexity of the methods, exhibiting low reliability due to large variability depending on time of testing and tester (11).

Although the fluorescence-based CFD assay can reduce technical issues, a clear standard for the pre-analytical phase has to be defined. As mentioned above, the present study showed that it is necessary to measure CFD with whole blood processing within 1 h. Prior studies showed that plasma must be separated from whole blood samples within a certain period of time to prevent factitious CFD variations (20). In addition, the present study showed an increase or decrease in CFD levels with an irregular tendency over a storage period of 6 h. This result was presumed to be due to the influence of cell lysis or changes in DNase activity/levels over time. Deregulation of caspases occurs during cancer development and progression, releasing DNA or nucleosome into the circulated blood (21), which can increase CFD in patients with cancer. Conversely, DNA is rapidly degraded and hydrolyzed from the blood circulation by DNases, and the half-life of CFD in blood seems to be short (22), which can cause reductions in the levels of CFD. Stability of CFD derived from cancer cells is more fragile than that of DNA from healthy cells, and CFD from cancer cells is more fragile than that of healthy



	Time (h)	Baseline	1 h	2 h	3 h	4 h	6 h	Sources	F	P-value
Mean ± SD (μg/ml)	4°C group	8.4 ±0.2	9.4 ±0.5	6.9 ±0.3	7.2 ±0.3	9.2 ±0.5	7.0 ±0.3	Time	481.038	<0.001
								Group	1.004	0.346
	RT group		9.0 ±0.2	6.8 ±0.5	7.1 ±0.3	8.8 ±0.5	6.7 ±0.5	Time x Group	2.375	0.090
RSD (%)	4°C group	4.0	4.8	4.5	4.7	5.1	4.0	–	–	–
	RT group		2.7	4.6	3.7	5.3	7.5	–	–	–

Figure 2. Fluorescence-based CFD according to varying storage condition. At both RT and 4˚C, the CFD values increased after 1 h, decreased at 2 h, increased after 3 h and then decreased after 6 h. The changes in CFD values were significant according to time course. F=481.038, P<0.001. The changes were not consistent over time. RSD, relative standard deviation; CFD, cell free DNA; RT, room temperature.



	Time (h)	Baseline	1 h	2 h	3 h	4 h	6 h	Sources	F	P-value
Mean±SD (μg/ml)	4°C group	7.5 ±0.1	7.1 ±0.5	7.4 ±0.2	7.4 ±0.2	7.2 ±0.1	7.6 ±0.4	Time	3.387	0.007
								Group	1.342	0.280
	RT group		7.3 ±0.2	7.4 ±0.3	7.6 ±0.4	7.9 ±0.1	7.8 ±0.2	Time x Group	1.643	0.156
RSD (%)	4°C group	9.4	6.1	3.5	4.3	6.4	5.0	–	–	–
	RT group		4.3	5.5	4.3	3.4	2.4	–	–	–

Figure 3. Influence of freeze-thawing in the fluorescence-based CFD assay. Plasma samples were stored frozen as aliquots at -80˚C and thawed after 1 month to assess the influence of freezing, and the reproducibility of the results. There was a significant difference in CFD values after freeze-thawing according to storage time. F=3.387, P=0.007. However, there was no significant difference in CFD values after freeze-thawing based on the previously exposed temperature. F=1.342, P=0.280. RSD, relative standard deviation; CFD, cell free DNA; RT, room temperature.

donors, and is thus easily disrupted (23). Therefore, when fluorescence-based CFD assays are applied to patients with cancer, the pre-analytical period identified in the present study must be strictly controlled.

The fluorescence-based CFD assay is only a quantitative method for assaying CFD, and cannot be applied for individual genomic profile analysis. In addition, the quantitative method does not provide a measure of tumor-specific CFD, but instead both germline and tumor CFD (17,18). Due to these limitations, it cannot be applied to precision medicine using tumor mutation profiling of tumor-specific CFD, such as the Mass-array® system (14). However, tumor-specific CFD assays are well known for their correlation with tumor volume. Abbosh *et al* (24) reported that a tumor volume of 10 cm^3 was required for detection sensitivity of tumor-specific CFD tests, which is considerably larger than the volume of an early stage/asymptomatic tumor (24). Whilst, considering that CFD primarily emerges from tissue in the surrounding environment during cancer development rather than from cancer cells themselves, simple quantitative fluorescence-based CFD may be more useful for roles such as response monitoring, prognostication and early detection. In particular, considering that the cure rate is increasing in the minimal disease status before definitive clinical disease in the era of immunotherapy (25), fluorescence-based CFD quantitative assays may be used to detect early disease, such as minimal residual disease in post-operative settings or for long-term surveillance.

The present study evaluated only the basic performance of the fluorescence-based CFD assay and did not specify any usage scenarios (for example, detection of cancer or response to treatments). Although there are several limitations, the present study laid the foundation for ongoing research into fluorescence-based CFD assays. Additional studies are required to allow the CFD assay to provide a more robust, consistent and informative method for use in clinical settings. It is necessary to reconfirm the results of the present study using a larger cohort of patients with cancer, and conduct future studies to demonstrate clinical relevance, such as a cohort study for cancer detection, evaluating cancer treatment response and detecting recurrence for surveillance.

In conclusion, the fluorescence-based CFD assay proved to be simple and accurate, but was limited due to variations in the pre-analytical storage period. The present study showed that CFD measurements using processed plasma within 1 h is optimal. The effects of substantial changes according to storage conditions were reduced after freeze-thawing, thus, studies using stored samples, such as those from a biobank are viable.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JJK analyzed and interpreted the data, and drafted and revised the manuscript. KP designed the current study, performed the experiments, analyzed and interpreted the data, and drafted and revised the manuscript. YRH, and SHK performed the experiments. SBO, SYO, YJH analyzed and interpreted the data, and drafted the manuscript. MSY assisted with the statistical analysis. JJK and KP confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The research protocol used in the present study was approved by the Institutional Review Board of Pusan National University Yangsan Hospital (Yangsan, Republic of Korea; approval no. 04-2019-024), and written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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