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Comparison of methods for the isolation of cell-free DNA from cell culture supernatant

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Abstract

In vitro characterization of cell-free DNA using two-dimensional cell culture models is emerging as an important step toward an improved understanding of the physical and biological characteristics of cell-free DNA in human biology. However, precise measurement of the cell-free DNA in cell culture medium is highly dependent on the efficacy of the method used for DNA purification, and is often a juncture of experimental confusion. Therefore, in this study, we compared six commercially available cell-free DNA isolation kits for the recovery of cell-free DNA from the cell culture supernatant of a human bone cancer cell line (143B), including two magnetic bead-based manual kits, one automated magnetic bead-based extraction method, and three manual spin-column kits. Based on cell-free DNA quantitation and sizing, using the Qubit dsDNA HS assay and Bioanalyzer HS DNA assay, respectively, the different methods showed significant variability concerning recovery, reproducibility, and size discrimination. These findings highlight the importance of selecting a cell-free DNA extraction method that is appropriate for the aims of a study. For example, mutational analysis of cell-free DNA may be enhanced by a method that favors a high yield or is biased toward the isolation of short cell-free DNA fragments. In contrast, quantitative analysis of cell-free DNA in a comparative setting (e.g. measuring the fluctuation of cell-free DNA levels over time) may require the selection of a cell-free DNA isolation method that forgoes a high recovery for high reproducibility and minimal size bias.

Keywords

Liquid biopsy, cell-free DNA, circulating tumor DNA, cell-free DNA isolation, cell culture, in vitro

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Introduction

Through various pathways, such as apoptosis, necrosis, and regulated extrusion, the majority of cell types in the human body shed fragmented segments of their genome, or specific parts thereof, into the circulatory system (i.e. blood) and other adjacent body fluids (e.g. urine, stool, and cerebrospinal fluid).[1,2] These cell-free DNA (cfDNA) molecules conserve the unique genetic and epigenetic codes that characterize the cells from which they originate, and as such exhibit unparalleled specificity as biomarkers for the detection of genomic aberrations that are normally difficult to investigate with non-invasive techniques, in particular solid tumors[3,4] and fetal genetic disorders.[5] To date, three cancer management tests have been approved by the U.S. Food and Drug Administration (FDA) for use in routine clinical practice,[6–10] while many Clinical Laboratory Improvement Amendments (CLIA) labs offer services for the characterization of cfDNA mutational profiles in cancer patients. Moreover, numerous non-invasive prenatal testing (NIPT) facilities worldwide offer a range of diagnostic cfDNA-based tests.[11]

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However, barring these exciting developments, thousands of studies attest that the development of clinically meaningful cfDNA-based tests is complicated significantly by a lack of knowledge concerning the molecular origin and physical features of cfDNA.[3,12] This poor understanding is mainly a result of the difficulty to study the biological properties of specific cfDNA molecules within a highly complex and nucleic acid-heterogeneous biospecimen such as blood—a direct consequence of the inherent complexity of an in vivo system. Therefore, owing to the relative simplicity of two-dimensional (2D) cell culture models and the high level of variable control that such in vitro experiments offer, some researchers are exploring this approach as an alternative or auxiliary avenue for elucidating the biological properties of cfDNA.[13–19]

One of the most prominent physical differences between cfDNA molecules of different origins occurs at the level of fragment size. For example, due to the interplay between structure and mechanisms of biogenesis and degradation, cfDNA originating from apoptosis (~166 bp), necrosis (~10,000 bp), active extrusion (~1000–3000 bp), extracellular vesicles (~150–6000 bp), and mitochondria (~40–300 bp) displays different sizes (reviewed in Bronkhorst et al.[3]). In addition, cfDNA originating from normal versus cancer cells shows different fragment sizes,[20,21] where the latter is often characterized by shortening. This emphasizes the importance of selecting an extraction procedure that maximizes the precision of cfDNA measurements.

However, there are currently more than 40 commercially available cfDNA extraction methods, including kits for both manual and automated isolation. Within this menu of methods, there are approaches that differ in principle, generally based either on the binding of DNA molecules to magnetic particles, silica gel membranes, or organic chemicals. Consequently, as demonstrated by several plasma-based comparative studies, there is often a high level of variability among different methods with respect to their cfDNA recovery efficiency, size discrimination, and reproducibility,[22–26] often to a very large extent. This makes it difficult to identify an optimal approach for cfDNA isolation. While numerous studies have compared methods for the isolation of cfDNA from various human body fluids, no study has to our knowledge yet performed a direct comparison of methods for the isolation of cfDNA from cell culture supernatant. In order to address this shortcoming, we used the cell culture supernatant from the human bone osteosarcoma cell line (143B) as a source of cfDNA to compare six commonly used and commercially available cfDNA extraction kits.

Materials and methods

Cell culturing and sample processing

The human bone cancer (osteosarcoma) cell line 143B was obtained from the American Type Culture Collection (ATCC® CRL-8303™). Cells were grown in Dulbecco's modified Eagle's medium (HyClone DMEM/high glucose; cat #11965092, lot #2081862) containing 4 mM L-glutamine, 4500 mg/L glucose, and sodium pyruvate. It was further fortified with 10% fetal bovine serum (PAN Biotech; cat #P30-3302) and 1% penicillin/streptomycin (Lonza; cat #DE17-02E, lot #7MB159). Cells were incubated in humidified atmosphere containing 5% CO_2 at 37°C. Cells were seeded in 6 × 175 cm^2 flasks (Thermo Fisher Scientific; cat #159920, lot #159573) at 30% density in 30 mL growth medium. Following 12 h of incubation, the cell culture medium was removed and replaced with 30 mL fresh medium (this is to ensure that dead cells and DNA originating from the initial shock of seeding are not present in the final sample). After another 24 h of incubation, medium was collected in 50 mL tubes (this time was selected because cells are then at the exponential growth phase, and is also when the most cfDNA is present in the medium).[19] Collected samples were centrifuged at 1000*g* for 10 min, and then the medium of two flasks was pooled in 75 cm^2 flasks (Thermo Fisher Scientific; cat #156472, lot #158491) to create three biological replicates (i.e. 60 mL × 3). For each of the replicates, 8 mL of medium was aliquoted into seven 15 mL nuclease-free conical tubes (CELLSTAR®, Greiner Bio-One; cat #1882714, lot #E16103T6), respectively, and then stored at −80°C. Thus, each aliquot corresponds to sample material for quadruplicate extractions (i.e. four replicates) using one extraction kit, with one aliquot to spare.

Isolation of cfDNA

CfDNA was isolated directly from cell culture supernatant using six commercially available cfDNA extraction kits (summarized in Table 1). Except where indicated, cfDNA was extracted according to the specifications of the manufacturer. Prior to cfDNA extraction, cell culture supernatants were thawed at 37°C in a temperature-controlled water bath, vortexed and spun-down briefly to collect droplets. For each of the three biological replicates (i.e. three experimental repeats), cfDNA was extracted in quadruplicate from 1.7 mL of cell culture supernatant for each of the extraction kits, except the NucleoSpin® Plasma XS (Macherey-Nagel) kit for which only 0.6 mL was used for isolation, as the latter kit is designed specifically for smaller input

Table 1. CfDNA extraction kits compared in this study.

Kit	Code	Isolation volume (mL)	Elution volume (μL)	Denaturing agent	Principle
cfPure® Cell-Free DNA Extraction Kit (BioChain)	A	1.7	20	No	Magnetic bead
MagMAX™ Cell-Free DNA Isolation Kit (Thermo Fisher Scientific)	B	1.7	30	No	Magnetic bead
MagNA Pure 24 System (Roche)	C	1.7	100	Yes	Magnetic bead (automated)
QIAamp Circulating Nucleic Acid Kit (Qiagen)	D	1.7	20	Yes	Spin-column
NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel)	E	1.7	20	No	Spin-column
NucleoSpin® Plasma XS (Macherey-Nagel)	F	0.6	20	No	Spin-column

volumes. The volume of buffer used to elute DNA in each protocol is shown in Table 1. Last, in the case of the NucleoSpin® Gel and PCR Clean-up protocol (Macherey-Nagel), binding buffer NTB (Macherey-Nagel; cat #740595.150, lot #1803/001) was used instead of the kit-provided buffer NTI, since buffer NTB allows the extraction of cfDNA from larger sample volumes. Isolated cfDNA was stored at −20°C until analysis.

Quantification of cfDNA

Quantification of cfDNA was performed using the Qubit® fluorometer 3.0 (Invitrogen, Life Technologies) in combination with the Qubit™ dsDNA HS Assay Kit (Invitrogen, Life technologies; cat #Q32851, lot #1724782). As per the manufacturer's instructions, a standard curve was prepared using the zero and 10 ng/μL Qubit DNA standards provided in the kit. For all cfDNA extractions, 3 μL of sample was diluted in 197 μL Qubit working solution before measurement. Following normalization to the volume of sample used for isolation as well as the volume used for cfDNA elution for each protocol, quantitative measurements of cfDNA are expressed as the total mass of cfDNA (ng) present in an 8 mL aliquot of cell culture supernatant. For this study, the Qubit™ dsDNA HS Assay was selected for cfDNA quantification based on the results from previous work.[27]

Size determination of cfDNA

Sizing of cfDNA was performed by capillary electrophoresis (CE) using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc.) equipped with Expert 2100 software, in combination with a High Sensitivity DNA microchip (Agilent Technologies; cat #5067-4627, lot #WG23BK50) and High Sensitivity DNA kit (Agilent Technologies; cat #5067-4627, lot #1834). The assay was performed according to the instructions provided by the manufacturer. After cfDNA fragments are separated by CE, they are normalized to a ladder and two DNA markers, which are then represented as a virtual band. The software then automatically calculates the size and concentration of each band. In order to assess the cfDNA fragment size discrimination of each extraction kit, the percentage of cfDNA fragments that lie within five selected fragment size ranges was calculated using the size-gating function of the Expert 2100 software. The five selected size ranges were as follows: (a) 50–250 bp, (b) 250–450 bp, (c) 450–650 bp, (d) 650–3000 bp, and (e) 3000–10,000 bp, respectively.

Statistics

All statistics were performed using the GraphPad Prism software version 5.0 and Microsoft Excel. Differences between group means were calculated using one-way analysis of variance (ANOVA), followed by pairwise comparison using a post hoc Tukey test. Outliers were identified and omitted using the Grubbs test. p values smaller than 0.05 were considered statistically significant.

Results and discussion

In this study, we compared six methods for the isolation of cfDNA from cell culture supernatant, including three spin-column methods based on the binding of cfDNA to silica-based membranes (QIAamp Circulating Nucleic Acid Kit (Qiagen), NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel), and NucleoSpin® Plasma XS kit (Macherey-Nagel)), two manual methods based on the binding of cfDNA to magnetic beads (cfPure® Cell-Free DNA Extraction Kit (BioChain) and MagMAX™ Cell-Free DNA Isolation Kit (Thermo Fisher Scientific)), and one automated magnetic bead-based cfDNA extraction method (MagNA Pure 24 System (Roche)).

Based on cfDNA quantification measurements using the Qubit dsDNA HS Assay, all methods were able to recover cfDNA. However, the average cfDNA yield showed wide variability across the different methods (ANOVA, $p < 0.0001$) (Figure 1). Based on a multi-

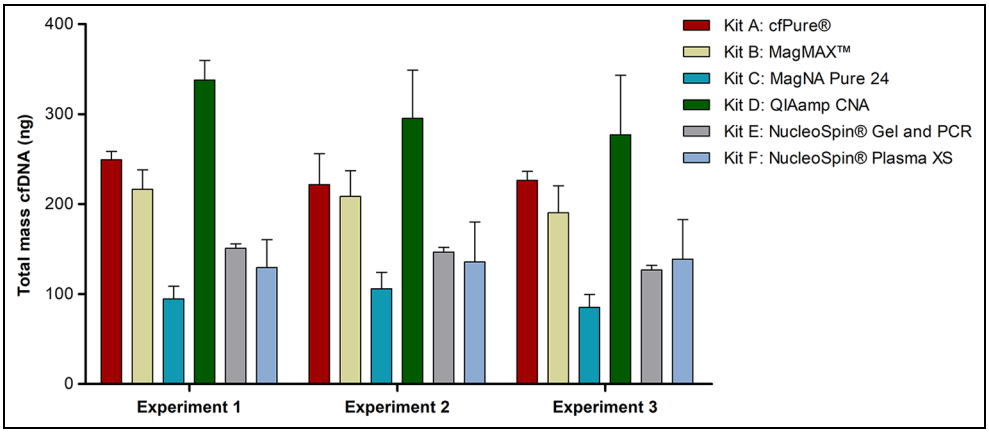

Figure 1. Comparison of cfDNA yields obtained by six different extraction methods. Kits A, B, and C use magnetic beads, while kits D, E, and F use spin-columns. For each extraction kit, four individual extractions were performed from 1.7 mL of 143B cell culture supernatant, except for kit B (n = 4 extractions from 0.6 mL). The experiment was repeated three times using aliquots pooled from three biological replicates (i.e. for each experiment, 8 mL aliquots for each extraction kit were made from 60 mL cell culture supernatant, which was obtained by pooling two 30 mL samples from two 175 cm^2 cell culture flasks, respectively). After factoring in differences in both elution volumes and sample input volume used for different kits, quantitative measurements of cfDNA (left y-axis) are expressed as the total mass of cfDNA (ng) present in an 8 mL aliquot of cell culture supernatant. Error bars indicate standard deviation. In all experiments, the average amount of cfDNA recovered by the extraction kits differed significantly (ANOVA, $p < 0.0001$), while there were statistically significant differences in cfDNA yield between several individual kits, as shown by Tukey's multiple comparison test (results summarized in Table 2). Coefficient of variation percentages for each of the kits in each of the experiments are summarized in Table 3.

Table 2. Multi-comparison of cfDNA recovery by different extraction kits.

Kits compared	$p < 0.05$ (yes/no)
A vs. B	No
A vs. C	Yes
A vs. D	Yes
A vs. E	Yes
A vs. F	Yes
B vs. C	Yes
B vs. D	Yes
B vs. E	Yes
B vs. F	Yes
C vs. D	Yes
C vs. E	Yes
C vs. F	No
D vs. E	Yes
D vs. F	Yes
E vs. F	No

cfDNA: cell-free DNA.

comparison test, the average cfDNA yield showed statistically significant differences between all kits, except between (1) Kit A (cfPure® Cell-Free DNA Extraction Kit) versus Kit B (MagMAX™ Cell-Free DNA Isolation Kit), (2) Kit C (MagNA Pure 24 System) versus Kit F (NucleoSpin® Plasma XS), and (3) Kit E (NucleoSpin® Gel and PCR Clean-up) versus Kit F (NucleoSpin® Plasma XS) (Table 2). Interestingly, Kit D (QIAamp Circulating Nucleic Acid Kit) delivered statistically significant higher yields than all other methods, while Kit C (MagNA Pure 24 System) delivered statistically significant lower yields than all methods except Kit F (NucleoSpin® Plasma XS) (Table 2 and Figure 1). The QIAamp Circulating Nucleic Acid Kit has consistently demonstrated superior recovery efficiency over various other methods in a number of comparative studies,[22,23,28–31] while automated cfDNA extraction methods generally show lower recovery efficiency than manual methods.[32,33] Although the QIAamp Circulating Nucleic Acid Kit delivered the highest yield in our study, it did not, between replicate extractions, deliver the most consistent yields among the tested kits (Table 3). Interestingly, the least expensive kit (NucleoSpin® Gel and PCR Clean-up) showed the highest reproducibility for the recovery of cfDNA between experimental replicates (Table 3). While other similar studies have also observed variation in reproducibility between kits,[28,30] it is conceivable that this variation would even out when each extraction protocol is mastered through repeated application and kit-specific standardization of pre-extraction procedural variables.

In order to evaluate the bias of the different methods toward the extraction of different cfDNA fragment sizes, the fraction of cfDNA fragments that lie within five different size ranges was calculated, including (a) 50–250 bp, (b) 250–450 bp, (c) 450–650 bp, (d) 650–3000 bp, and (e) 3000–10,000 bp. No statistically significant difference was

Table 3. Reproducibility of cfDNA recovery of different kits.

Kit	Code	CV %			Average CV %
		Experiment 1	Experiment 2	Experiment 3	
cfPure® Cell-Free DNA Extraction Kit (BioChain)	A	3.69	15.48	4.41	7.86
MagMAX™ Cell-Free DNA Isolation Kit (Thermo Fisher Scientific)	B	10	13.64	15.84	13.16
MagNA Pure 24 System (Roche)	C	14.93	17.28	16.85	16.35
QIAamp Circulating Nucleic Acid Kit (Qiagen)	D	6.45	18.08	23.88	16.14
NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel)	E	3.23	3.66	4.13	3.67
NucleoSpin® Plasma XS (Macherey-Nagel)	F	24.06	32.58	31.71	29.45

cfDNA: cell-free DNA; CV: coefficient of variation.

Table 4. Multi-comparison of cfDNA size discrimination by different extraction kits.

Kits compared	$p < 0.05$ (yes/no)				
	50–250 bp	250–450 bp	450–650 bp	650–3000 bp	3000–10,000 bp
A vs. B	No	No	No	No	No
A vs. C	No	No	Yes	No	Yes
A vs. D	No	No	Yes	No	No
A vs. E	No	No	No	Yes	Yes
A vs. F	No	No	No	Yes	No
B vs. C	No	No	No	No	No
B vs. D	No	No	No	No	No
B vs. E	No	No	No	Yes	No
B vs. F	No	No	No	Yes	No
C vs. D	No	No	No	No	No
C vs. E	No	No	No	Yes	No
C vs. F	No	No	Yes	Yes	No
D vs. E	No	No	No	Yes	Yes
D vs. F	No	No	Yes	Yes	No
E vs. F	No	No	No	Yes	No

cfDNA: cell-free DNA.

found between kits for the recovery of cfDNA fragments that range between (a) 50–250 bp ($p = 0.1145$) and (b) 250–450 bp ($p = 0.0574$), while a statistically significant difference was found between kits for the recovery of cfDNA fragments that range between (c) 450–650 bp ($p = 0.0066$), (d) 650–3000 bp ($p < 0.0001$), and (e) 3000–10,000 bp ($p = 0.0074$) (Figures 2 and 3(a)–(e)). Moreover, based on post hoc pairwise comparison tests for cfDNA recovery in the latter size ranges, there were statistically significant differences between several kits for the recovery of cfDNA with specific lengths (Table 4).

To test the bias of magnetic bead versus spin-column–based kits toward the recovery of short versus long fragments, we calculated the average fraction of cfDNA fragments ranging between the two size groups of 50–250 bp and 250–10,000 bp, respectively, that were collectively recovered by all magnetic bead-based extraction methods combined (kits A, B, and C) versus all spin-column–based methods combined (kits D, E, and F). Interestingly, extraction methods based on the purification of cfDNA using magnetic beads recovered a higher fraction of short cfDNA fragments (i.e. 50–250 bp) versus extraction methods based on the binding of cfDNA to silica-based membranes ($p < 0.05$) (Figure 3(f)). In contrast, extraction methods based on the binding of cfDNA to silica-based membranes recovered a higher fraction of longer cfDNA fragments (i.e. 250–10,000 bp) versus extraction methods based on the binding of cfDNA to magnetic beads ($p < 0.05$) (Figure 3(f)). The same observation has recently been made by two studies that have compared methods for the isolation of cfDNA from human plasma samples.[23,34] Interestingly, accumulating evidence indicates that cancer-associated DNA mutations are generally enriched in short cfDNA fragments, while wild-type cfDNA fragments tend to be longer.[35–39] Therefore, the sensitivity of mutation-based assays may be enhanced by the selection of a cfDNA extraction method that favors the recovery of short cfDNA fragments. However, it is important here to clarify that, although

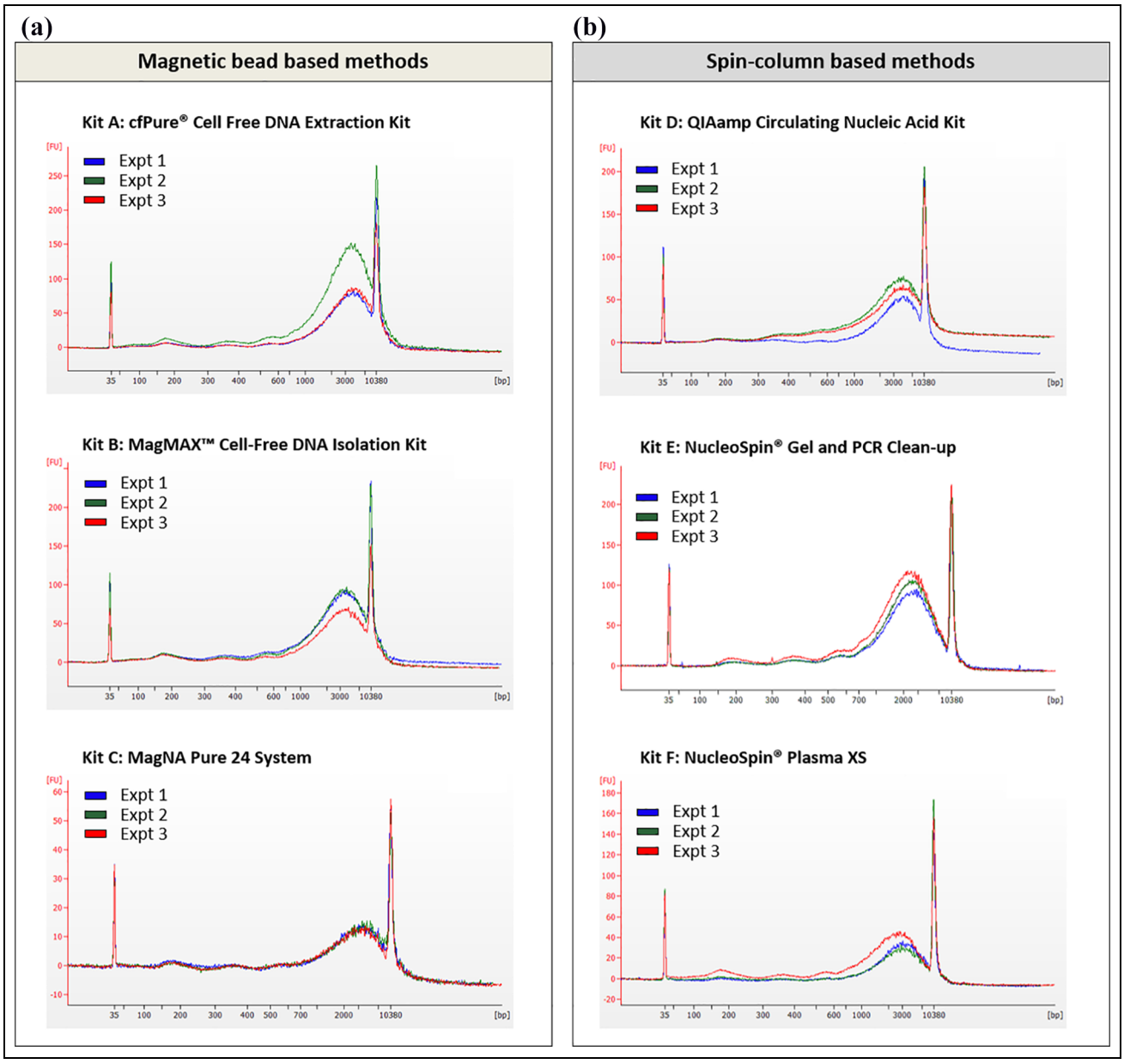

Figure 2. Comparison of cfDNA size profiles obtained by six different extraction methods. Capillary electropherograms show the DNA fragment size profile obtained following isolation of cfDNA from the cell culture supernatant of 143B cells using six different methods. For each extraction kit, one cfDNA sample was randomly selected from each of the three experimental repeats and subject to size analysis. Column A illustrates the cfDNA size profiles obtained by cfDNA isolation methods based on the binding of DNA to magnetic beads. Column B illustrates the cfDNA size profiles obtained by cfDNA isolation methods based on the binding of DNA to silica gel membranes. In each electropherogram, two major peaks can be seen, one at 35 bp and one at approximately 10,000 bp. These peaks correspond to the two size markers. The relative fluorescence of these markers is used to calculate the size of the unknown samples. Thus, any deviation from the baseline, excluding the markers, indicates the size of cfDNA. Further statistical analyses on these size profiles are given in Figure 3.

magnetic bead-based methods seem to be more biased than silica gel column-based methods toward the extraction of short cfDNA fragments, it is not yet clear which approach delivers the overall highest yield and purity of short cfDNA molecules and tumor-derived cfDNA molecules. In addition, the cell line used in this study seems to be characterized by the extracellular presence of cfDNA with relatively high molecular weight (i.e. the majority of cfDNA fragments range in size between 1000 and 5000 bp). It is likely that a similar study conducted on a cell line that is characterized by an increased proportion of short cfDNA fragments

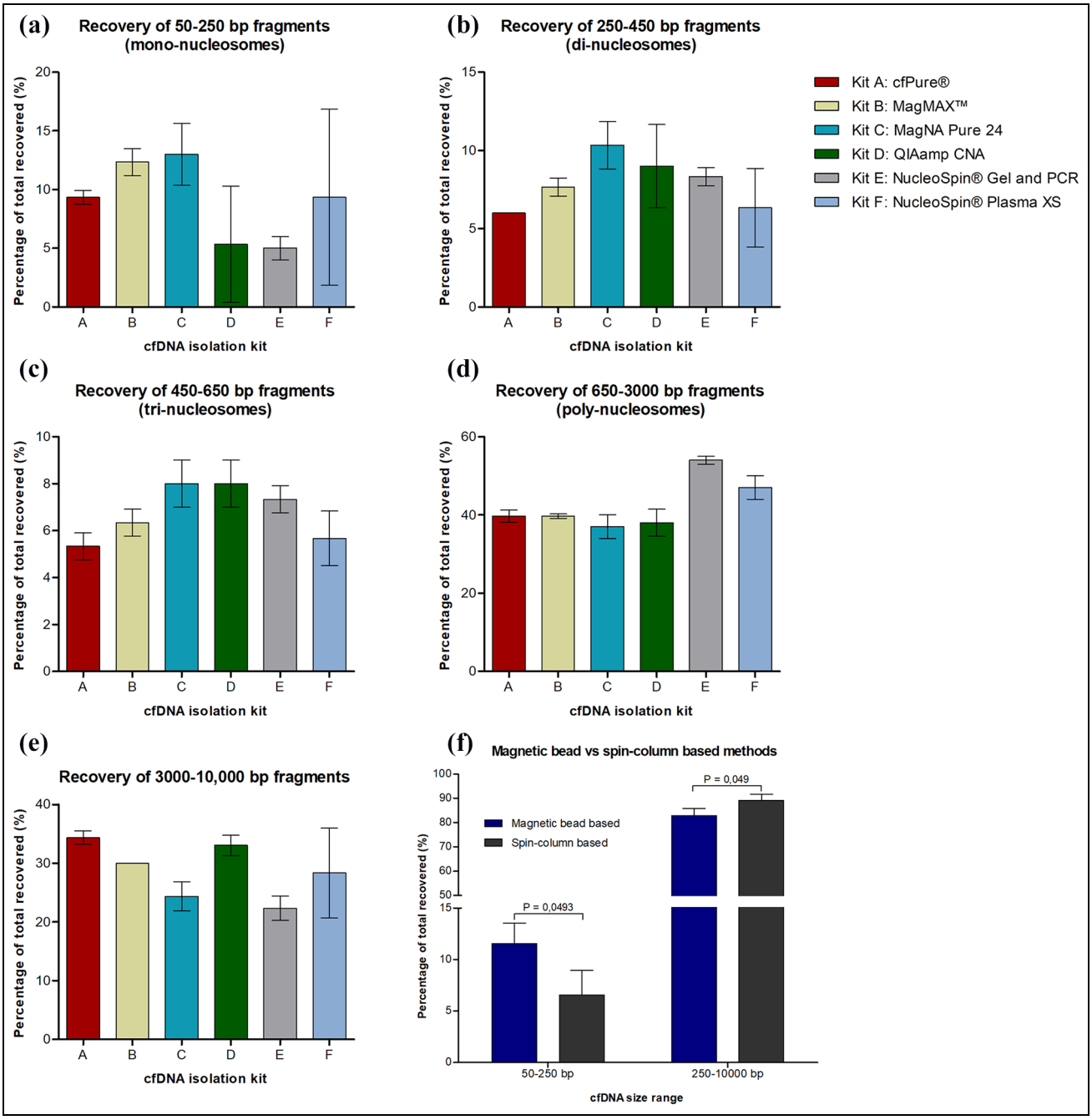

Figure 3. Recovery of different cfDNA fragment size populations by six different extraction methods. In order to evaluate the bias of each of the six different extraction kits toward the extraction of different cfDNA fragment sizes, the fraction of cfDNA fragments that lie within five different size ranges was calculated using the size-gating function of the Agilent Bioanalyzer software. As determined by one-way ANOVA, no statistically significant difference was found between kits for the recovery of cfDNA fragments that range between (a) 50–250 bp (ANOVA, $p = 0.1145$) and (b) 250–450 bp (ANOVA, $p = 0.0574$), while a statistically significant difference was found between kits for the recovery of cfDNA fragments that range between (c) 450–650 bp (ANOVA, $p = 0.0066$), (d) 650–3000 bp (ANOVA, $p < 0.0001$), and (e) 3000–10,000 bp (ANOVA, $p = 0.0074$). Results from post hoc pairwise comparison tests are summarized in Table 4. (f) In order to evaluate the bias of magnetic bead versus spin-column kits toward the recovery of short versus long fragments, the average fraction of cfDNA fragments ranging between the two size groups 50–250 bp and 250–10,000 bp, respectively, that were collectively recovered by magnetic bead (kits A, B, and C) versus spin-column–based (kits D, E, and F) methods was evaluated and found to differ significantly ($p < 0.05$). For each extraction kit, one cfDNA sample was randomly selected from each of the three experimental repeats and subject to size analysis ($n = 3$). Error bars indicate standard deviation. p values smaller than 0.05 indicate statistically significant differences.

(i.e. 50–250 bp) will yield slightly different results, such as the HMEC-1[27] and HepG2 cell lines.[17] For example, comparison of the same methods using cell culture supernatant that contains high levels of mono-nucleosomes and low levels of longer cfDNA fragments may, for example, show that the cfPure® Cell-Free DNA Extraction Kit delivers a yield more comparable to the QIAamp Circulating Nucleic Acid Kit. Therefore, given the apparent differences in cfDNA fragmentation profiles between cell lines under normal physiological conditions, as well as changes to these profiles induced by exposing cells to various compounds and stressors, the optimal approach may be to establish the most optimal extraction kit for individual cell lines and specific experimental scenarios. While there may be significant similarities between cell lines, such comparative studies may need to be repeated until a clear consensus emerges.

It is perhaps worth noting here that there is still some confusion regarding the molecular origin of different cfDNA species. While the majority of researchers argue that cfDNA fragments that range in size between 1000 and 3000 bp is the product of necrosis or accidental cell lysis, a number of in vitro studies could not correlate both the quantitative and qualitative characteristics of these DNA fragments with contaminating genomic DNA fragments originating from either accidental or regulated cell death.[15,16,19,40] These findings suggest that cfDNA molecules that range between 1000 and 3000 bp (often $\pm$ a couple hundred to thousand base pairs), or at least a significant portion of this population, may originate from actively dividing cells through a mechanism of regulated extrusion. Although these observations are intriguing, more evidence is needed to demonstrate its validity. If these larger cfDNA fragments are indeed released by cells into the circulatory system, their presence may be obscured through rapid degradation via normal metabolic processes, which are typically absent in cell culture supernatant. Similarly, a recent study demonstrated the presence of larger cfDNA fragments (1000–6000 bp) in bile—a body fluid that does not likely possess the same DNA degradation qualities of plasma.[41] Alternatively, intact larger cfDNA fragments may be missed due to the use of DNA extraction methods that are biased toward the isolation of small cfDNA fragments. It is likely that the resolution of such an experimental problem may only be achievable through a method or combination of methods that allow the complete recovery and high resolution differentiation between all cfDNA populations (e.g. sub-nucleosomes, mono-nucleosomes, di-nucleosomes, and high molecular weight DNA) that are present in a given biospecimen.

Taken together, these results demonstrate the importance of selecting a cfDNA isolation method that suits the specific aims of a study. For example, the extraction kit requirements for patient samples (e.g. plasma) versus cell culture supernatant are not necessarily the same. The sensitivity and specificity of assays for profiling plasma cfDNA mutations from cancer patients may be enhanced by the selection of a cfDNA extraction method that favors the recovery of short cfDNA fragments. In contrast, unraveling the biological and physical properties of cfDNA using cell culture models may require accurate sizing and quantitative analysis of cfDNA in comparative settings (e.g. measuring the fluctuation of cfDNA characteristics over time), which may require the selection of a cfDNA isolation method that forgoes a high recovery for high reproducibility and minimal size bias. Similarly, to meet the demands of high capacity conditions, automated cfDNA extraction methods may be ideal when maximum cfDNA recovery is not essential.

Conclusion

In this study, it was demonstrated that six different commercially available cfDNA extraction methods show significant variability with respect to recovery, yield reproducibility, and size discrimination for the isolation of cfDNA from cell culture supernatant. In concurrence with previous comparative studies using plasma as source material, the QIAamp Circulating Nucleic Acid Kit delivered the highest cfDNA yield. The NucleoSpin® Gel and PCR Clean-up Kit delivered the fourth highest cfDNA yield, but showed the highest reproducibility. The MagNA Pure 24 System delivered the lowest yield, which is consistent with recent reports indicating that automated extraction methods generally demonstrate lower recovery efficiency compared to manual kits. Furthermore, the results reported here suggest that magnetic bead methods are more biased than spin-column methods toward the recovery of short cfDNA fragments (50–250 bp), while spin-column methods are more biased than magnetic bead methods toward the recovery of larger cfDNA fragments (250–10,000 bp). While this finding is supported by two recent studies, there is no clear scientific consensus on which approach delivers the overall highest yield of short versus long cfDNA fragments. In conclusion, these results highlight the importance of selecting a cfDNA extraction method that suits the study objectives.

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