**NextPrep-Mag™ cfDNA
Automated Isolation Kit**

A start-to-finish cfDNA extraction and sequencing automation experience from plasma/serum
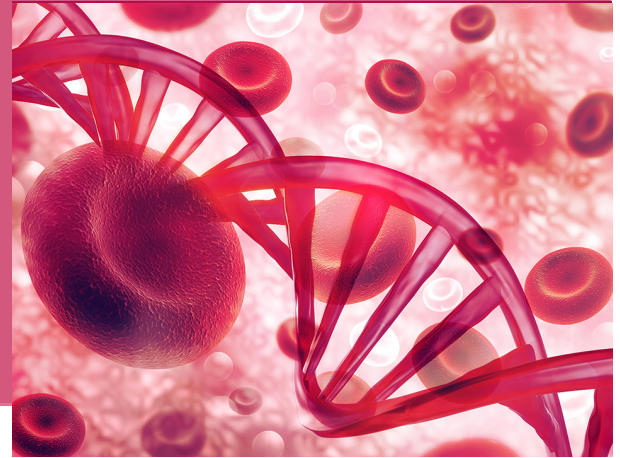
Introduction

There has been a surge of interest in the analysis of DNA recovered from cell-free biological fluids. Cell free DNA (cfDNA) is a sample type of choice due to its quick, very minimally-invasive method of collection that is amenable to analyzing multiple time points and multiple samples at a time when compared to more time-intensive, invasive methods such as tumor biopsy/fine-needle aspiration in cancer testing research or chorionic villus sampling (CVS), or amniocentesis in prenatal testing research.

Circulating DNA can be highly fragmented and present at low concentrations, especially in healthy individuals, which may pose a number of challenges that need to be overcome to achieve reliable cfDNA extraction. This has led to a need for more efficient methods for extracting cfDNA. For plasma and serum, a substantial fraction of cfDNA is recovered as fragments of approximately 170 bp and multimers thereof, which is thought to reflect the association of cfDNA with the histone proteins that comprise nucleosomes. Larger fragments may also be recovered, even after removal of contaminating cells.

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For the Better

A reliable, automated solution for cfDNA extraction is critical for high-throughput cancer research workflows requiring high quality, clean cfDNA as input for downstream applications. The bead-based NextPrep-Mag™ cfDNA extraction workflow accommodates a wide range of plasma and serum input volumes and increases throughput through automation, which is not possible with column or filter-based methods which require a vacuum pump. Hands-on-time is minimized to approximately 30 minutes for 8 manual extractions, which is much faster than filter-based methods*. By automating the kit on the chemagic™ instruments, throughput can be increased to 24 extractions per run requiring only 15 minutes of hands-on-time and approximately 1 hour and 15 minutes of run time. With the automated cfDNA extraction protocol, labs can expect a simple and streamlined workflow that produces reproducible yields every time. The chemagic™ instrument with a 24-rod head processes up to 24 samples of up to 5 mL inputs per run while a 96-rod head chemagic™ instruments can isolate cfDNA from 96 samples at a time from plasma or serum inputs ranging from 1.5 or 4.5 mL per run. Furthermore, the NextPrep-Mag™ cfDNA extraction kit reduces contaminating high molecular weight DNA delivering high-quality cfDNA which is an important consideration for downstream applications like next-generation sequencing (NGS). Resulting cfDNA eluates extracted using the NextPrep-Mag™ isolation kits have been performance-validated using the NEXTFLEX® library preparation kits automated on the Sciclone® and Zephyr® G3 NGS/NGSx workstations for a convenient and streamlined, start-to-finish cfDNA extraction and library prep solution.

* times varies depending on user

Methods

cfDNA extraction

For plasma sample preparation (5 mL input) using the 24-rod head system on the chemagic™ 360 instrument, blood from Donors 1-10 were collected in K2EDTA tubes while Donors 11 and 12 were collected in Serum Separating Tubes (SST). Samples were extracted using the NextPrep-Mag™ cfDNA automated isolation kit and quantified using the NGS 3K assay on the PerkinElmer LabChip® GX Touch™ nucleic acid analyzer and the Thermo Scientific® Qubit® assay as described in the legend. For preparation of plasma samples (1.5 mL input) using the 96-rod head system on the chemagic™ 360 instrument, blood was collected by a commercial vendor from healthy donors into bags containing K2EDTA anti-coagulant. Plasma was separated from RBCs and WBCs by centrifugation at 1,600xg for 10 minutes, carefully removed, and shipped to PerkinElmer on wet ice. Plasma was further spun at 4,000xg for 20 minutes. The double-spun plasma was removed and pooled where necessary. Donors 1 and 4 are pools of various donors while Donors 2 and 3 are individual donors.

cfDNA extraction and library prep for targeted sequencing using commercial standards

An equal volume of synthetic plasma spiked with commercially available cfDNA standards, which included mutations in several cancer-associated genes present at 5%, 1%, and 0.1%, was extracted using either the NextPrep-Mag™ kit or commercial reagent Q in a manual format due to low sample availability of synthetic plasma. cfDNA yields were analyzed using a commercially available Alu qPCR assay. Extraction yields as quantified by Alu qPCR were used to normalize input by mass to render 20 ng input for library preparation. A commercially available kit targeting the cancer mutations in EGFR and KRAS/NRAS was used to prepare amplicon libraries for sequencing and subsequent analysis. Note that cfDNA library preparation does not include a DNA fragmentation step since the DNA is naturally fragmented to a size of approximately 170 bp. The yield after library preparation was evaluated with the Roche® KAPA® library quantification kit which only detects ligated products functionally evaluating library yield.

Library preparation for whole genome sequencing

An equal volume (32 µL) of extracted cfDNA using the chemagic™ 360 instrument equipped with a 24-rod head was transferred to a 96-well hard-shelled PCR plate and used as input for whole genome library preparation on the Sciclone® G3 NGS workstation. Libraries were made using the NEXTFLEX® cell free DNA-seq kit for Illumina® platforms, with barcoded adapters diluted 1:8. Libraries were analyzed for yield and size distribution on the LabChip® GX Touch™ HT nucleic acid analyzer.

Next-generation sequencing

The resulting libraries from the amplicon panel was taken through sequencing performed on the Illumina® MiSeq® platform as a 2x150 sequencing run and analyzed. Analysis was conducted to detect variants down to the rate of 0.1%. With Trimmomatic, reads were trimmed of primers and adapters, and any reads short than 70 bp were filtered. Trim reads were aligned to the amplicon references with Bowtie2; variant detection was conducted with Samtools and Python script scripting.

Results

The purity and quality of the resulting cfDNA is an important consideration. The cfDNA eluates resulting from the NextPrep-Mag™ cfDNA isolation kits results in low contaminating high-molecular weight gDNA and minimal proteins in the elution compared to commercial reagent Q (Figure 1).
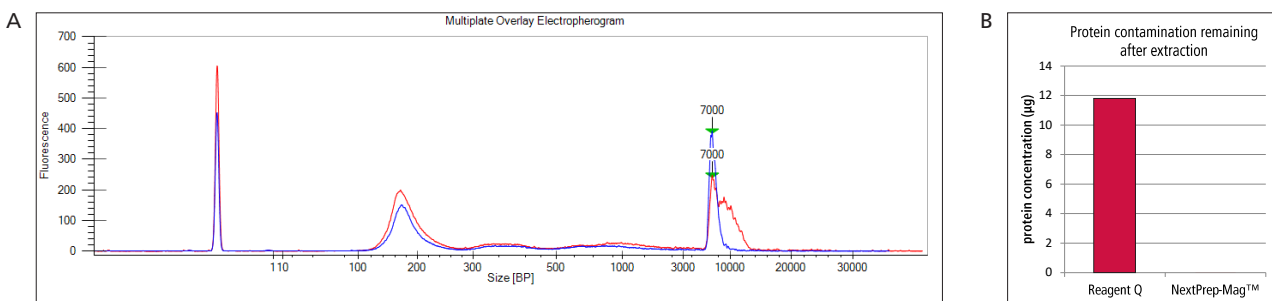

Figure 1. (A) A representative trace of an extracted cfDNA sample on the LabChip® GX Touch™ nucleic acid analyzer instrument (Blue: NextPrep-Mag™ kit, Red: Reagent Q). (B) Healthy donor plasma was extracted using the NextPrep-Mag™ kit or Reagent Q, and protein concentration in eluates were measured and analyzed on the Thermo Scientific™ Qubit® instrument.

Reproducibility of extraction is another core benefit to an automated extraction kit. The NextPrep-Mag™ cfDNA automated isolation kit run on the chemagic™ instrument shows reliable and reproducible yields using both the 24-rod head and 96-rod head format (Figure 2 and Figure 3).
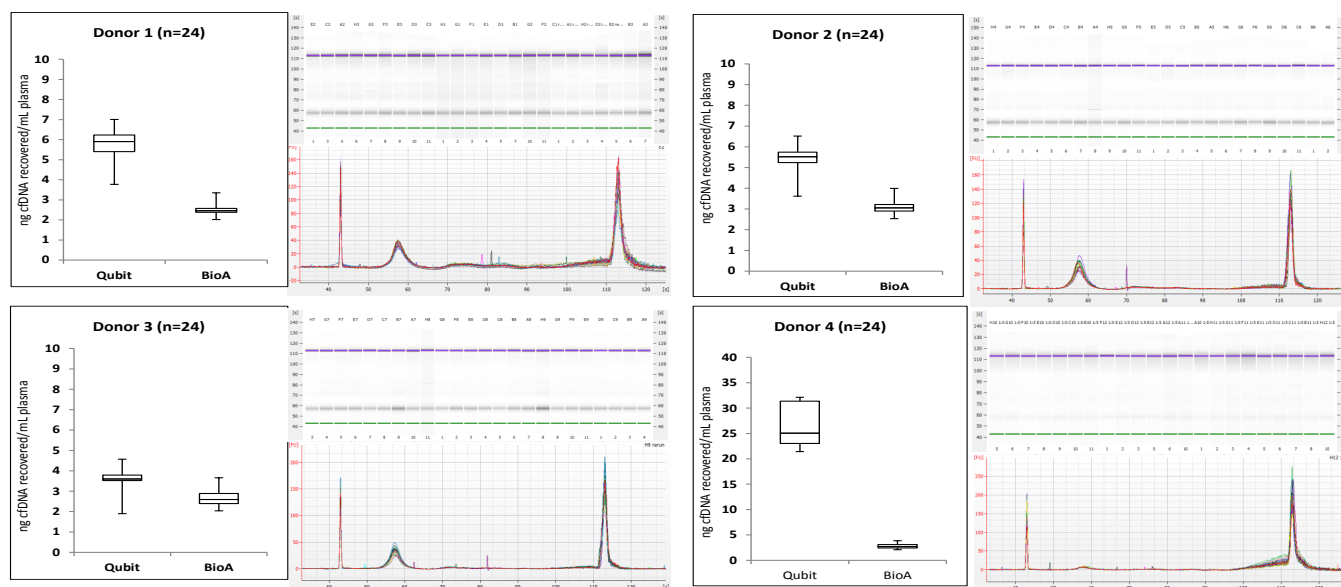

Figure 2. Highly reproducible cfDNA extractions on chemagic™ 360 instrument. cfDNA was extracted from 5 mL of doubly-spun EDTA plasma from four healthy donors in four automated runs (24 replicates of a single donor/run). Reproducible yields are shown (1) qualitatively by gel and electropherogram and (2) quantitatively for both total DNA (Thermo Scientific™ Qubit® HSDNA assay) and for mononucleosome peak (Agilent® Bioanalyzer® 100-275 bp region). For Donors 1-4, %CVs were 10, 10, 12, and 14% on the Agilent® Bioanalyzer® instrument and 12, 7, 9, and 18% on the Thermo Scientific™ Qubit® instrument, respectively. Donor 4 showed significant contamination with gDNA, resulting in much higher Thermo Scientific® Qubit® yields for that donor. Each automation run takes 75 minutes, with 15 minutes of hands-on time. On graph, Qubit = Thermo Scientific™ Qubit® HSDNA assay and BioA = High Sensitivity DNA kit on the Agilent® Bioanalyzer® 2100 platform.
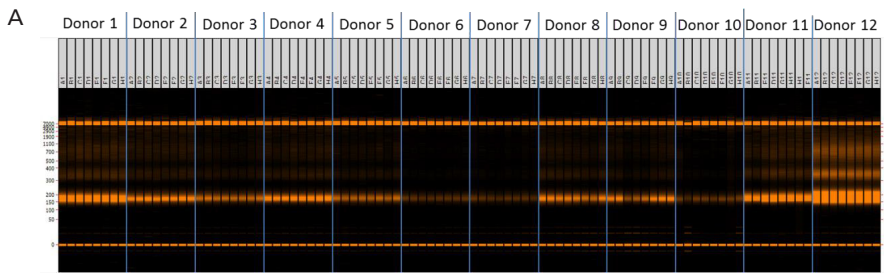
Figure 3. Assay Reproducibility. Blood from Donors 1-10 were collected in K2EDTA tubes while Donors 11 and 12 were collected in Serum Separating Tubes (SST). (A) LabChip® gel image of cfDNA extracted from all 96 samples in a single run. Replicates of each donor are sectioned off by vertical bars. (B) Box and whisker plots of cfDNA yields of samples shown in (A). Quantification of cfDNA derived from mono-nucleosomes (100-275 bp region) was determined using LabChip® Reviewer software (upper) while total cfDNA recovery was quantified by Thermo Scientific™ Qubit® assay (lower). The y-axis is in ng of cfDNA recovered/mL of plasma or serum extracted.
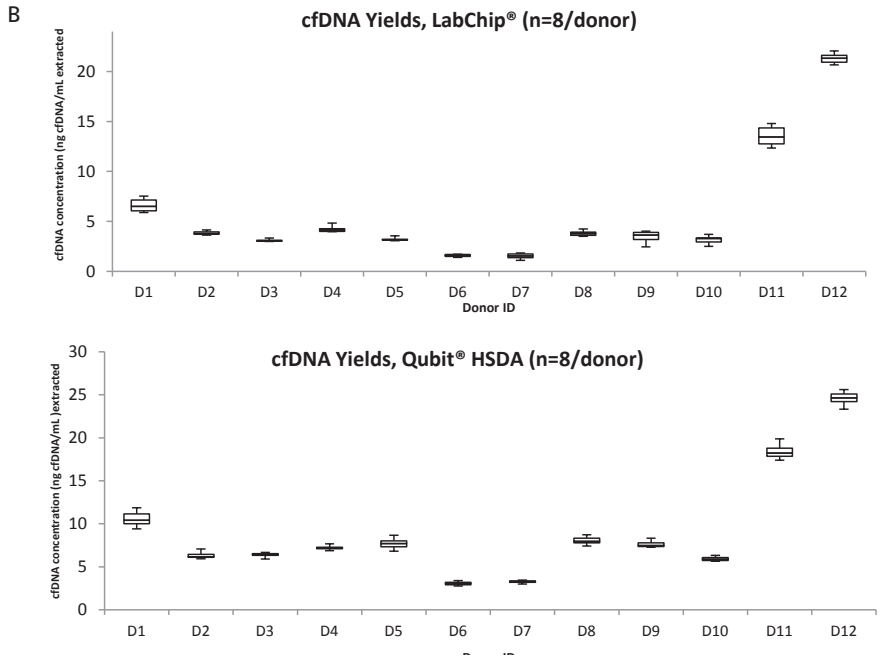
A simplified, automated workflow for both extraction and library prep can facilitate operational burden on a labs' processing throughput power and provide confidence in reproducible results from sample to sample while minimizing risk of operator error. NGS libraries constructed with the NEXTFLEX® cell free DNA-seq kit automated on the PerkinElmer Sciclone® G3 NGSx workstation using cfDNA isolated with the 96 rod head system on the chemagic™ 360 instrument have consistent library yields (Figure 4).
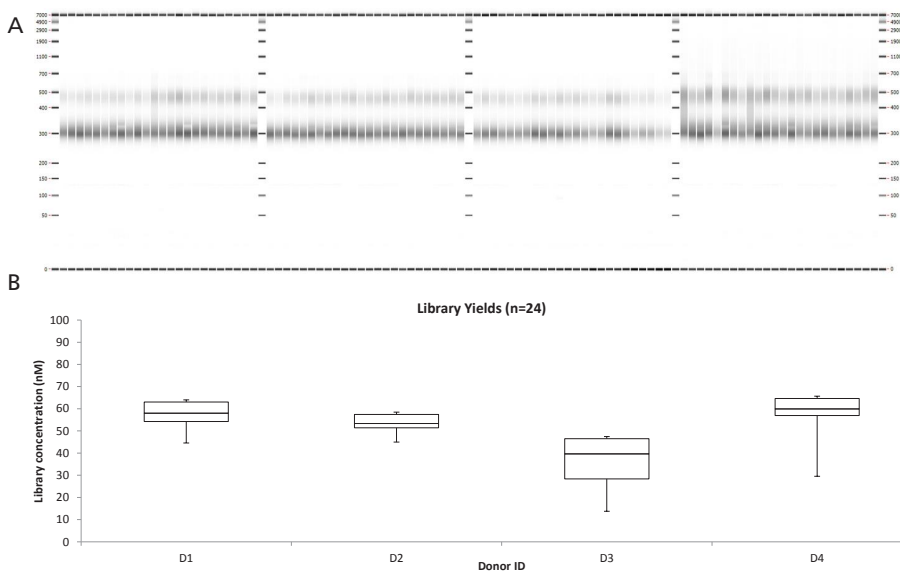

Figure 4. Automated library prep of extracted cfDNA. 32 μL of extracted cfDNA shown in Figure 3 was used as a template for library preparation using the NEXTFLEX® cell free DNA-seq kit on the PerkinElmer® Sciclone® G3 NGSx Workstation. 96 samples were processed in a single run. (A) LabChip® GX Touch™ gel image of 96 libraries. DNA libraries were diluted 1:8 and run on a HSDNA chip. (B) Nanomolar concentrations of the ~300 bp region of the library (corresponding to mononucleosomal cfDNA) was quantified using the LabChip® GX Reviewer software. Box and whisker plots were generated for each donor. %CVs were 11, 9, 28, and 14% for donors 1-4, respectively.

In the realm of liquid biopsy, most NGS applications revolve around targeted sequencing. We used commercially available cfDNA standards spiked into synthetic plasma and extracted the cfDNA using the NextPrep-Mag™ cfDNA automated isolation kit and commercial reagent Q. The subsequent cfDNA eluates were used to construct libraries with a commercially available cfDNA amplicon panel targeting several oncogenic mutations and sequenced as described in methods. Figure 5 shows libraries constructed using cfDNA isolated using the NextPrep-Mag™ cfDNA kit had higher library prep yields and proper mutation detection.
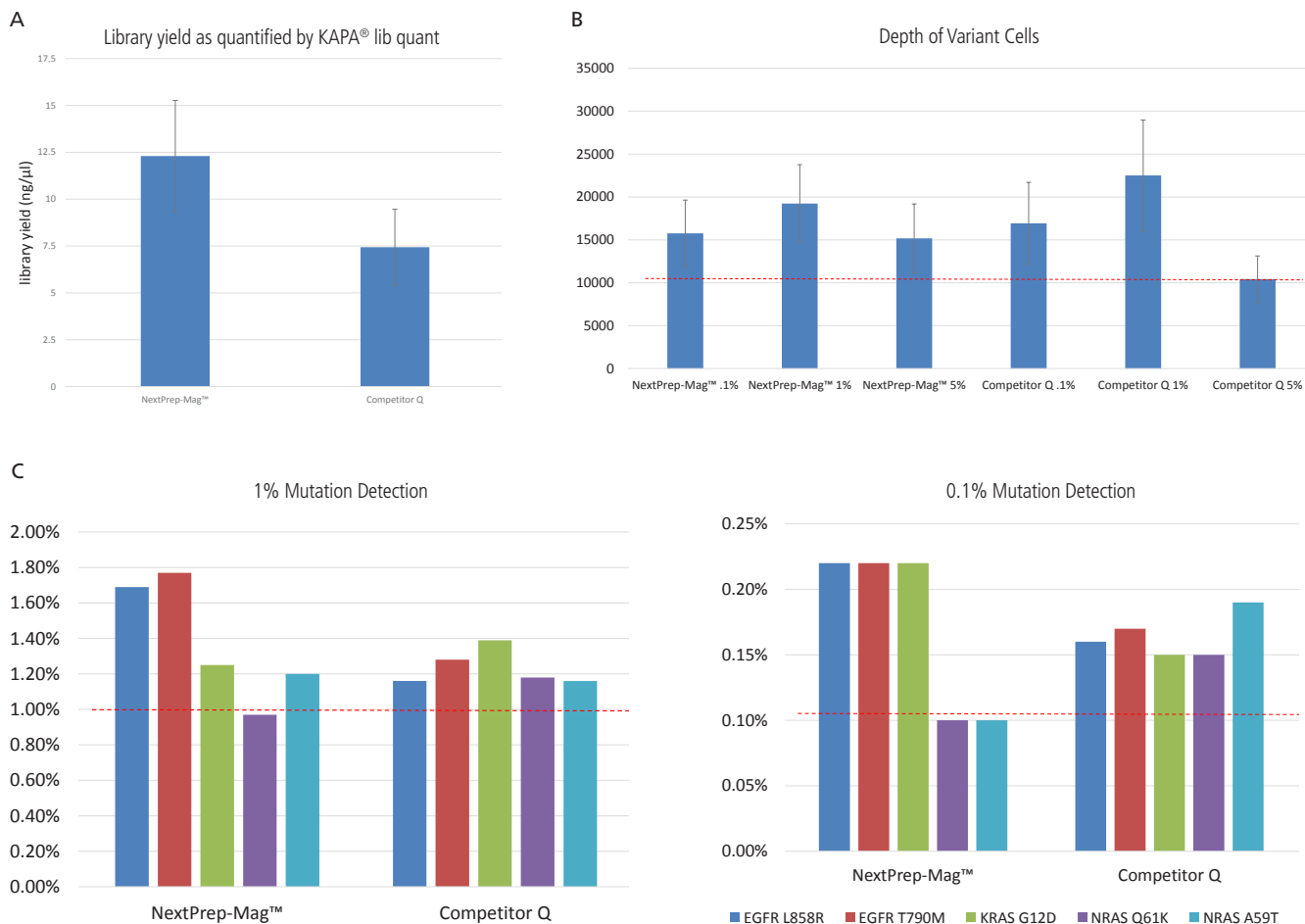

Figure 5. Targeted amplicon panel performance using cfDNA mutation standards in plasma extracted via a manual filter column-based method or the NextPrep-Mag™ cfDNA automated isolation magnetic bead-based chemistry. (A) Targeted amplicon panel library yields from cfDNA mutation standards as described in methods. cfDNA isolated using the NextPrep-Mag™ kit showed higher library yield, despite slightly lower extraction yields (data not shown). (B) Analysis of adequate coverage of each library sequenced where the horizontal dotted line at 10,000x coverage is the threshold indicating the depth needed to quantify a variant at 0.1% rate (10 variants / 10,000 total reads). (C) Rates of detection for five variants were analyzed at 1% and 0.1%.

Discussion

cfDNA is a powerful sample type that has become popular in the past years in different applications, including liquid biopsy clinical research. We presented here the reliability and reproducibility of the extraction and library prep workflows that automation provides, as well as the robustness of both the extraction and library prep chemistries. By incorporating the NextPrep-Mag™ cfDNA isolation kit, which is automated on the chemagic™ series of instruments, labs can expect higher quality cfDNA eluates with minimal contaminating high-molecular weight gDNA and salts when compared to the more traditional and cumbersome column filter-based commercial alternatives that are not amenable to automation. By leveraging a start-to-finish automated extraction and cfDNA-seq library prep using the NEXTFLEX® cell free DNA-seq kit on the PerkinElmer Sciclone® or Zephyr® G3 NGS workstations, labs can maximize their operational processivity while minimizing risk of operator error.

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