

# Cell-free DNA detection workflows utilizing the Monarch® Mag Cell-free DNA (cfDNA) Extraction Kit



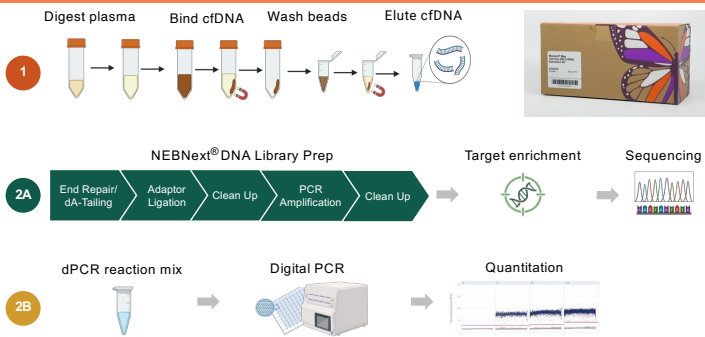
Anagha Kadam, Maggie Heider, Fabrizio Colosimo, Jian Sun, Lucy Foulston  
New England Biolabs, Inc.

## Introduction

- Plasma cell-free DNA (cfDNA) detection is helping advance various aspects of disease assessment through liquid biopsy. Applications include early detection of disease, continued monitoring, and therapy selection, while also enabling improved patient follow-up and economic efficiency over traditional tissue biopsies.
- Several technical and analytical challenges** exist in the field of liquid biopsy that remain a topic of continuous improvement. These include the choice of blood collection tubes, sample stabilization, low concentrations of cfDNA, extraction challenges, as well as the need for higher sensitivity in downstream applications.
- A crucial step in the sample-to-result workflow is the ability to achieve efficient and **reproducible extraction of cfDNA** from the starting sample. The quality and quantity of the cfDNA extracted from the sample can inevitably influence the downstream analytics and signal interpretation.
- In this study, we demonstrate a **sample-to-result workflow** for detecting tumor-specific mutations utilizing contrived samples and highlight our optimal cfDNA extraction solution, Monarch Mag Cell-free DNA (cfDNA) Extraction Kit (NEB #T4070), compatible with sequencing and amplification applications.
- Extracted cfDNA was analyzed via two approaches: (1) sequencing library preparation using an optimized workflow to maximize conversion efficiency, and (2) digital PCR for quantitative assessment of mutation detection sensitivity.

## Methods

### Sample-to-result workflow for cell-free DNA detection



### Sample preparation

- Blood from consenting, self-proclaimed healthy donors was drawn into blood collection tubes.
- EDTA and Streck Nucleic Acid BCT were used to assess the effects of collection tubes.
- Collected blood was processed for plasma within 2 hours of collection.
- Mimix™ OncoSpan, cfDNA Reference Standard (Horizon Discovery) was spiked-in as a reference.
- Unspiked controls were processed in parallel in sample to result workflow.

### cfDNA Library Prep

- For cfDNA library prep, NEBNext® Ultra II™ DNA Library Prep Kit with a modified protocol was used.
- The NEBNext Ultra II DNA Library Prep Kit is compatible with up to 50 µl input volume, further minimizing the need for cfDNA concentration after extraction.
- Modified ligation conditions improve library conversion efficiency.

### Digital PCR

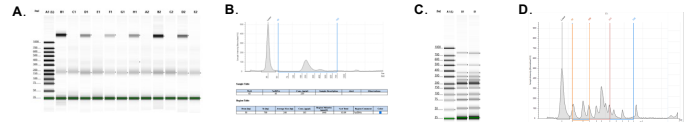
- Digital PCR reactions were performed using the QIAcuity dPCR system (Qiagen), employing the QIAcuity Probe PCR Kit and 26k nanoplates, in accordance with the manufacturer's instructions.
- A duplex assay was developed to enable simultaneous detection of the PIK3CA p.E545K mutation and its corresponding wild-type (WT) allele.
- Primers were sourced from Alvarez-Garcia et al. (2018), and custom hydrolysis probes specific to the E545K mutation and WT sequence were designed using Geneious Prime® version 2025.2.1.
- All reactions were run in duplicate and included appropriate controls, including a positive control and a no-template control (NTC), to ensure assay reliability and specificity.

### Monarch® solution for Cell-free DNA Extraction

- Cell-free DNA from 2 ml plasma was extracted using Monarch Mag Cell-free DNA (cfDNA) Extraction Kit.
- Extracted cfDNA, eluted in 30 µl, was application-ready and subjected to library prep and digital PCR.
- Key features of Monarch cfDNA extraction solution include:
  - Recover small fragments of circulating cfDNA, as low as 50 bp
  - Elute in low volumes without loss in recovery, eliminate additional concentration steps
  - Integrate seamlessly with NEB downstream solutions

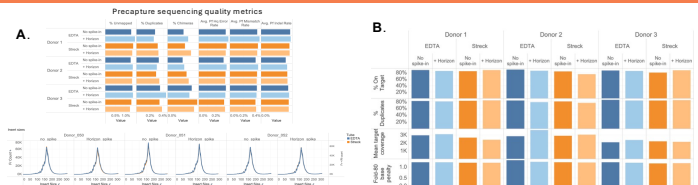
## Results

### NEB's Monarch extraction workflow enables high quality cell-free DNA extraction

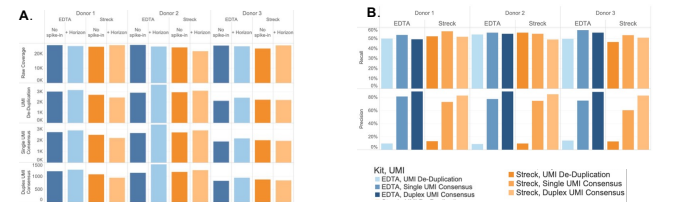


**Figure 1: Extracted cfDNA is high quality, concentrated and recovers different size fragments present in the sample.** (A) Cell-Free DNA TapeStation profile with adjacent lanes showing plasma extracted EDTA tube and Streck tube. (B) Representative electropherogram of extracted cfDNA. (C) Extracted cfDNA from plasma spiked with 50-800 bp ladder. (D) Electropherogram showing recovery of all fragments of the ladder, including as low as 50 bp.

### NEBNext DNA Library prep solution enables sensitive variant detection from cfDNA

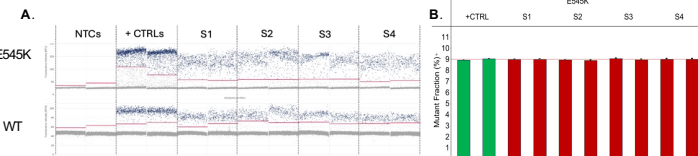


**Figure 2: Extracted cfDNA produces high quality WGS and hybrid capture libraries.** 45 µl of purified cfDNA (ranging from 5-25 ng input on average) +/- spike-in (Mimix™ OncoSpan, cfDNA Reference Standard) was used to prepare libraries using the NEBNext Ultra II DNA Library Prep Kit with XGen Duplex Seq Adapters (Integrated DNA Technologies) and 9 PCR cycles. (A) Libraries were sequenced on the Illumina® NovaSeq® 6000 2x100 bp and downsampled to 2M read pairs. Reads were trimmed using fastp (v.0.20.0), aligned to GRCh38 using Bowtie2 (v2.5.0), duplicates marked using Picard Mark Duplicate (v2.20.0), and library quality metrics assessed using Picard Alignment Summary Metrics (v1.56.0). Insert size was calculated using Picard Insert Size Metrics (v1.56.0). (B) Approximately 2,000 ng of library was used as input for hybrid capture using a custom cancer panel (Twist Bioscience). Captured libraries were sequenced on the Illumina® NovaSeq 6000 2x150 bp and downsampled to 20M read pairs. Reads were trimmed using fastp (v.0.20.0), aligned to GRCh38 using bwa-mem (v.0.7.17), UMIs processed using fgbio (v.2.3.0), and capture quality metrics assessed using Picard HS metrics (v.2.18.29).



**Figure 3: High variant calling precision using extracted cfDNA.** Captured libraries were sequenced on the Illumina NovaSeq 6000 2x150 bp and downsampled to 20M read pairs. Reads were trimmed using fastp (v.0.20.0), aligned to GRCh38 using bwa-mem (v.0.7.17), UMIs processed using fgbio (v.2.3.0). (A) Coverage was calculated for each analysis approach using mosdepth (v.0.2.6), with effective coverage decreasing with each successive consensus approach used. High coverage was obtained with duplex consensus indicating high library complexity. (B) Somatic variant calling was performed using VarDict (v.1.8.3). Libraries show high precision with use of consensus sequence. Due to spike-in level, Horizon variants were expected to be observed at VAF ranging from <0.1% to 1%, with >75% falling at or below VAF 0.1%, with only stochastic detection at the coverage obtained.

### Monarch cfDNA workflow enables maintenance of allele frequency of mutations



**Figure 4. Monarch cfDNA extraction enables accurate allele frequency quantitation via dPCR.** (A) Representative fluorescence plots showing positive partitions for the PIK3CA p.E545K mutation (FAM™) and wild-type (WT) allele (CY5™), demonstrating strong signal-to-noise (S/N) ratios and robust assay performance. The positive control (+CTRL) consisted of 10 ng per reaction of Mimix™ OncoSpan cfDNA Reference Standard with 9% allelic frequency (AF). No Template Control (NTC) reactions confirmed the absence of non-specific amplification. (B) Quantification of E545K mutation fraction (%) across duplicate reactions. Monarch cfDNA extraction yields consistent and accurate allele frequency measurements, supporting its suitability for sensitive mutation detection using duplex dPCR assays.

## Conclusions

- Our optimized extraction method enables isolation of high quality cfDNA compatible with multiple downstream applications.
- We present a streamlined workflow showing the effect of different blood collection tubes, reproducible extraction, and concentrated cfDNA that enables improved sequencing data quality, high conversion efficiency, and high sensitivity for detecting spike-in mutations.
- Key features of the Monarch extraction solution include low elution volumes and recovery of small fragments.
- Integration with NEB's downstream solutions enables a user-friendly application of sample-to-results solutions.

NEB's Monarch and NEBNext products are for Research Use Only.

## References

- Figures created in <https://www.biocompare.com/>
- Medina JE, Dracopoli NC, Bach PB, Lau A, Scharpf RB, Meijer GA, et al. Cell-free DNA approaches for cancer early detection and interception. *Journal for Immunity Therapy of Cancer*. 2023;11:e006913. <https://doi.org/10.1136/jit-2022-006913>
- Alvarez-Garcia, Virginia, et al. "A simple and robust real-time qPCR method for the detection of PIK3CA mutations." *Scientific Reports* 8.1 (2018): 4290.