An Automated, Turn-Key cfDNA Extraction Workflow Showcasing Low Frequency Variant Detection

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Introduction

Ongoing research has demonstrated the potential of cell-free DNA (cfDNA) as a universal biomarker for cancer detection, non-invasive prenatal testing (NIPT), and transplant rejection monitoring through its ability to reflect physiological and pathological conditions in the body. cfDNA are a challenging analyte, as they are found in low abundance and in a background of contaminating genomic DNA. Reliable, reproducible, and high throughput extraction methodology is imperative for utilizing the full potential of cfDNA in a clinical setting. Streamlining processes from prefilling reagents to plug-and-play scripting not only enhances speed and accuracy but also allows for improved productivity, precision, and reproducibility. The objective of this application note is to equip researchers with a simple, pre-scripted workflow for automating cfDNA extraction on the Hamilton MagEx STAR using traceable, pre-filled reagent reservoirs. Suitability for use downstream was evaluated using droplet digital PCR (ddPCR) for detecting ultra-low variants with high specificity.

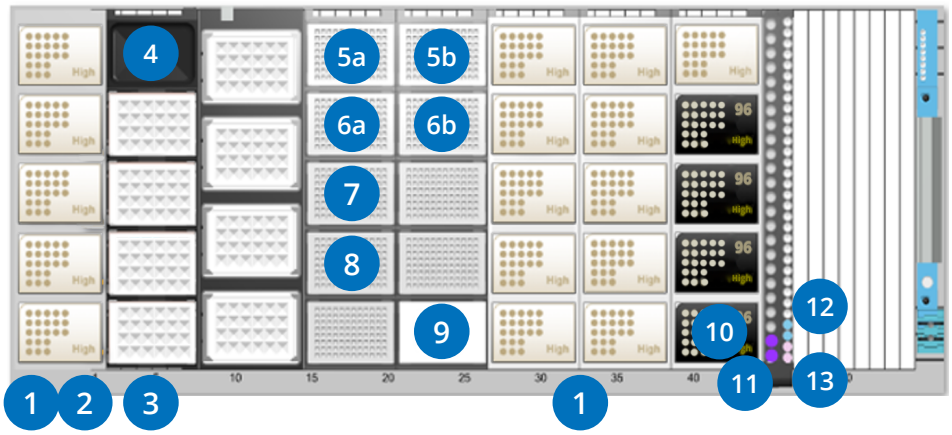
cfDNA extraction was achieved by employing Omega Bio-tek's Mag-Bind® cfDNA LSP Kit (PS3298-1-96PF, **Figure 1**) automated on the Hamilton MagEx STAR (**Figure 2**). Here, LSP stands for Load, Scan, Purify; reagents come in prefilled, barcoded reservoirs and tubes that allow scientists to simply unseal and load the components onto the automation deck without worrying about proper component location. The barcodes allow the position of the component to be dynamically written by the platform, ensuring proper placement, and eliminating user-errors.

Each Mag-Bind® cfDNA LSP Kit has enough reagents to extract cfDNA from 96 samples with a 4 mL sample input arrayed in four 24-well plates. When automated on Hamilton MagEx STAR, 4 x 24 samples can be processed in ~3 hours and 20 minutes. In this application note, we present this workflow for extraction of cfDNA from 4 mL working volume filled in one 24-well plate with supporting cfDNA yield information, as well as further demonstrate the use of extracted cfDNA in ddPCR application for detection of EGFR and NRAS genes at different allelic frequencies.

Foil-Sealed Reagents Included in Mag-Bind® cfDNA LSP Kit

Figure 1. The Mag-Bind® cfDNA LSP Kit (PS3298) contains prefilled reagent reservoirs for cfDNA purification. Some reagents come in sealed tubes, rather than reservoirs, to reduce dead volumes.

Hamilton MagEx STAR Deck Layout for Automating Mag-Bind® cfDNA LSP Kit



Components
1. Hamilton 1000 µL filtered tips
2. 96-well Isolator Tip Plates
3. 24-well Sample Plates containing sample
4. Gravity Waste
5a, 5b. Elution Buffer
6a, 6b. JSB Buffer
7. GT7 Buffer v1.1
8. eSPW Buffer
9. Elution Plate
10. MPH Tip Adapters
11. DS Buffer
12. Proteinase K (x2)
13. Mag-Bind® Particles CH (x2)

Figure 2. Hamilton MagEx STAR deck layout and components for extraction of cfDNA from 4 mL plasma samples.

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24-well Plate Layout

	1	2	3	4	5	6
A	WT	Elution Buffer	WT	WT	Plasma	WT
B	5%	Elution Buffer	5%	5%	Plasma	5%
C	1%	Elution Buffer	1%	1%	Plasma	1%
D	0.1%	Elution Buffer	0.1%	0.1%	Plasma	0.1%

Figure 3. 24-well plate layout depicting expected allelic frequencies based on sample input.

Materials and Methods

Sample
Multiplex I cfDNA in Synthetic Matrix II (HD917) obtained from Horizon Discovery was used to mimic a clinical sample. 4 vials containing 8 onco-relevant mutations at different allelic frequencies, 5%, 1%, 0.1%, and 0% (100% Wildtype (WT)), were provided with the reference set to measure extraction efficiency and assay performance. 4 mL K3EDTA Plasma served as the positive control, while 4 mL elution buffer served as the negative control.

Extraction Kit
Omega Bio-tek's Mag-Bind® cfDNA LSP Kit was used for the extraction of cfDNA from 4 mL input volume. Briefly, 1 mL from each of the 4 vials of Multiplex I cfDNA in Synthetic Matrix was combined with 3 mL of elution buffer included with the kit to bring the starting sample volume to 4 mL. Each allelic frequency, as well as positive and negative controls, were run in quadruplicate in a 24-well plate (**Figure 3**). The reagents come in prefilled, foil-sealed reservoirs and capped tubes; Hamilton MagEx STAR deck layout is as shown in **Figure 2**. Each reagent reservoir and tube is barcoded for identification, allowing reagents to be placed in any position and preventing user-error in placement.

Extraction Methodology
The pre-filled components were loaded on the Hamilton MagEx STAR and the pre-scripted program for the Mag-Bind® cfDNA LSP Kit was selected and ran on the instrument. cfDNA was eluted in 100 µL volume.

Yield and Quality
The yield and quality of the cfDNA was evaluated using the Cell-Free DNA ScreenTape Assay on the Agilent TapeStation 4150 system and its fraction relative to the total DNA extracted was assessed.

Droplet Digital PCR
cfDNA containing oncogenes at 4 allelic frequencies (5%, 1%, 0.1%, and 0%) was subjected to droplet digital PCR on a Bio-Rad QX200 Droplet Digital PCR System. Two assays were developed by detecting EGFR and NRAS mutations using ddPCR utilizing FAM probe for the mutant allele and SUN probe for the wildtype. 15 ng of cfDNA was combined with Bio-Rad's ddPCR Supermix for Probes (No dUTP) (Bio-Rad, Hercules, CA, USA) in a total reaction volume of 300 µL, according to the manufacturer's protocol. The droplets were generated using Bio-Rad's QX200 Droplet Generator following manufacturer's instruction and subjected to amplification on the C1000 Touch Thermal Cycler with adjustments of annealing temperature for each probe. The droplets were scanned using QX200 Quanta Soft Droplet Reader (Bio-Rad) and data was analyzed using Bio-Rad's QuantaSoft Analysis Pro Software. ddPCR work and data analysis was performed externally at Emory University, Atlanta, GA.

Results and Discussion

TapeStation analysis using the Cell-Free DNA ScreenTape Assay was performed to evaluate the yield and quality of extracted cfDNA from sample inputs containing 4 different allelic frequencies (n = 4). Dark bands concentrated between 150 and 200 base pairs indicate high yielding, high quality extracted cfDNA for each sample (**Figure 4**). Here, 4 mL K3EDTA Plasma was the positive control, and 4 mL elution buffer was the negative control. The remaining samples are labeled according to the allelic frequency (5%, 1%, 0.1%, and 0%) of the reference standard used. The cfDNA yields for all experimental extractions

TapeStation Analysis of Purified cfDNA

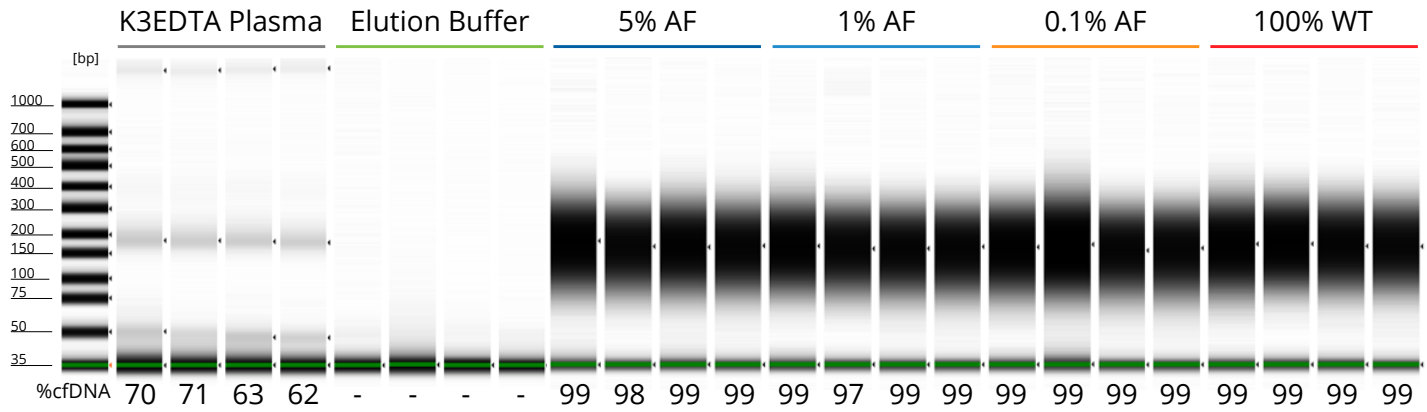

Figure 4. Evaluation of cfDNA extracted using the Mag-Bind® cfDNA LSP Kit automated on the Hamilton MagEx STAR. This workflow purifies high yields of high-quality cfDNA. WT: Wildtype. AF: Allelic Frequency.

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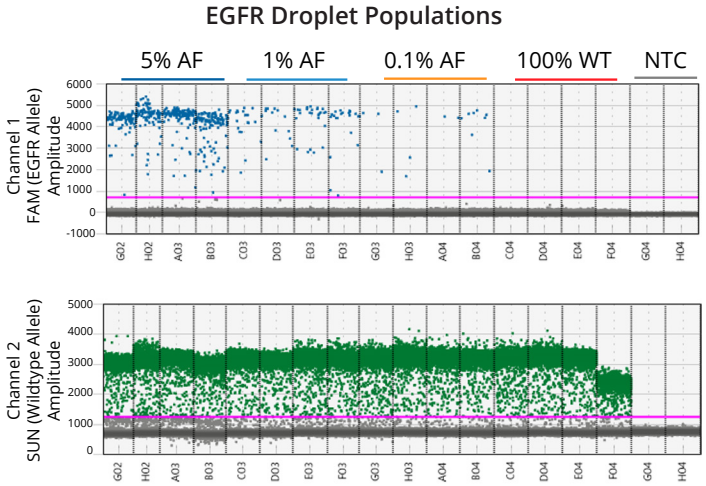

Figure 5. Visualization of the positive and negative droplet populations of the EGFR Assay at different allelic frequencies. The threshold is indicated with a pink horizontal line.

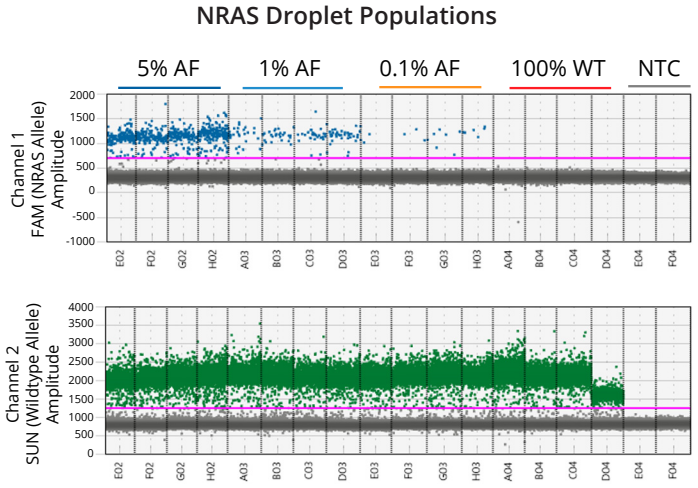

Figure 6. Visualization of the positive and negative droplet populations of the NRAS Assay at different allelic frequencies. The threshold is indicated with a pink horizontal line.

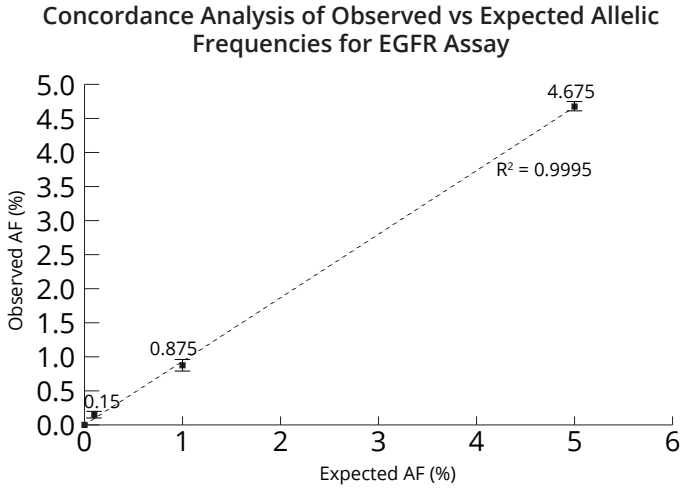

Figure 7. Concordance analysis of EGFR gene (Variant L858R) at allelic frequencies determined by ddPCR. The shaded region represents the accepted range of observed allelic frequencies. Mutant alleles detected at frequencies as low as 0.1%. AF: Allelic Frequency.

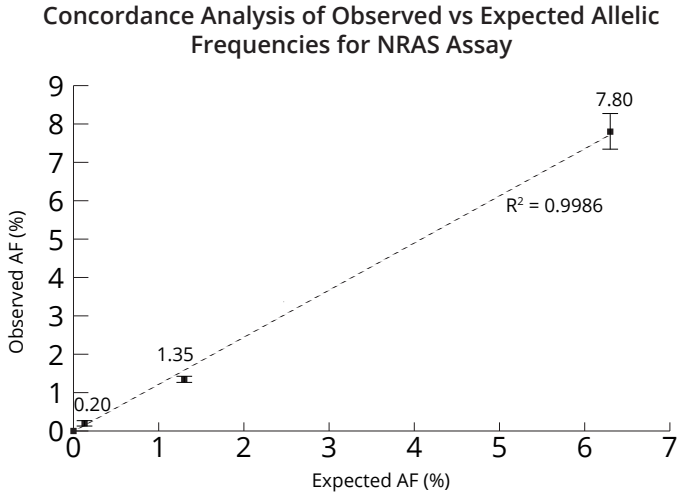

Figure 8. Concordance analysis of NRAS gene (Variant A59T) at allelic frequencies determined by ddPCR. The shaded region represents the accepted range of observed allelic frequencies. Mutant alleles detected at frequencies as low as 0.1%. AF: Allelic Frequency.

ranged between 302-329 ng (50-700 bp on the TapeStation), which is significantly greater than the expected yields reported by Horizon Discovery (120 ng per 1 mL of product using the QIAmp® Circulating Nucleic Acid Kit with 2 mL extraction input). From their certificate of analysis, Horizon Discovery expects 30% extraction efficiency; whereas the Mag-Bind® cfDNA LSP Kit workflow was able to achieve 76-83% extraction efficiency[1].

Each ddPCR assay (EGFR and NRAS) generated a minimum of ~15,000 droplets. The droplet populations at each allelic frequency in the EGFR and NRAS assays are shown in **Figures 5 and 6**, respectively. The threshold for each assay was manually set using 100% Wildtype control to ensure separation between positive and negative populations. The absence of droplets in the No Template Control (NTC) confirms no non-specific amplification. The assay results for EGFR and NRAS genes at each allelic frequency were then interpreted based on the threshold set. Positive droplets were detected for both NRAS and EGFR assays at all the allelic frequencies tested.

The number of positive droplets at each allelic frequency was summed and compared to the expected allelic frequency to check for concordance between actual vs expected. The results of the concordance analysis performed between the average observed AF (%) vs expected AF (%), as reported by Horizon Discovery, for EGFR and NRAS genes are shown in **Figures 7 and 8,** respectively. The expected ratios are 0% for 100% Wildtype, 5%, 1% and 0.1% for EGFR mutant alleles and

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6.3%, 1.3%, and 0.13% for NRAS mutant alleles, as reported by Horizon Discovery. The average observed allelic frequencies for EGFR as well as NRAS mutant genes are in excellent concordance with the expected allelic frequencies for all experimental groups tested (R^2 = 0.99). The results indicate cfDNA extracted using the Mag-Bind® cfDNA LSP Kit (PS3298-1-96PF) on the Hamilton MagEx STAR was capable of detecting mutant alleles at frequencies as low as 0.1% when analyzed by ddPCR.

Conclusions

The pre-scripted workflow utilizing pre-filled reagents from Omega Bio-tek's Mag-Bind® cfDNA LSP Kit (PS3298-1-96PF) resulted in high-quality cfDNA suitable for use in downstream applications like ddPCR and was able to detect mutant alleles at frequencies as low as 0.1%. The traceability and convenience of the pre-filled components, as well as the reliability and efficiency of the pre-scripted extraction workflow make the Mag-Bind® cfDNA LSP Kit automated on the Hamilton MagEx STAR an attractive solution for cfDNA analysis in clinical and research settings.

References

1. Horizon Discovery. Multiplex cfDNA in Synthetic Matrix II Reference Standard: Certificate of Analysis. URL: https://horizondiscovery.com/-/media/Files/Horizon/resources/Certificate-of-analysis/HD917_45462_V2.pdf?sc_lang=en

Disclaimer

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Product Information

Product	Description	Size
Mag-Bind® cfDNA LSP Kit	PS3298-1-96PF	4 x 24 Preps

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