# Enzymatic fragmentation vs sonication for the production of cfDNA reference standards for liquid biopsy assays

Aldo Mele[1], Hannah Child[1], Katarzyna Wilczynska[1], Christian Paar[2] and Julie Wickenden[1]
*[1] Horizon Discovery, [2] Kepler University Hospital, Austria*

## Introduction

Liquid biopsies hold great promise to revolutionize the field of clinical oncology testing. Cell-free DNA (cfDNA) can be extracted from a routine patient blood sample and used to determine the genetic profile of a solid tumor located elsewhere within the body. This facilitates more informative disease management for the clinician, without the need for invasive surgery for the patient. With new cfDNA NGS assays being able to detect variants from as little as 2-10ng DNA, assay validation to ensure sufficient accuracy has never been so critical. Reference materials that closely mimic real cfDNA samples are essential to support this effort. Here we present results from a comparative study of DNA fragmentation methods applied during the production of cfDNA reference standards. We show a comparison between enzymatic fragmentation and mechanical shearing (sonication), and the benefit of including a size selection step for data accuracy and performance of NGS gene panel workflow.

## Methods

DNA extracted from engineered cancer cell lines, representing the Multiplex 1 blend at 5% or 0.1%, was fragmented by mechanical or enzymatic shearing. In addition, a size selection step was included to obtain a fragment size distribution profile that closely mimics real cfDNA samples. The allele frequency of specific variants was confirmed by ddPCR. The eight-sample cfDNA material experimental set was externally tested on the Illumina TruSight Tumor 15 (TST-15) panel to assess library preparation and variant calling performance.

**NGS:** Sequencing was performed on the MiSeqDx system in RUO mode. Filter settings for analysis with VariantStudio (and automatic analysis) were: Read depth >500 and MAF >2%.

## Conclusion

Results show good performance of both sonicated and enzymatically sheared cfDNA material, where all variants present above the LOD could be detected on the Illumina TST-15 assay.

Whilst enzymatically sheared cfDNA did not show any increase in NGS library yield, it did display slightly more accurate variant calling, in addition to a more defined tapestation profile – centred around 168bp, which was further enhanced when combined with a size selection step (*Fig 1A yellow trace*).

This highlights the potential of these alternative techniques to produce cfDNA that is highly commutable to patient samples and suitable for the validation of ddPCR and NGS liquid biopsy assays.

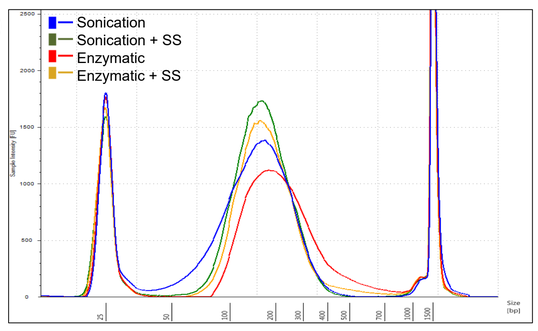
## Results

Tapestation analysis confirmed that both sonication and enzymatic fragmentation produced cfDNA with a peak size in line with real clinical samples (*Fig 1*). Proof of principle variant detection by ddPCR confirmed the presence of three of the eight mutations across 2 of four genes (*EGFR, KRAS, NRAS* and *PIK3CA*) at either 0.1% or 5% variant allele frequency *(Fig 2)*. Library preparation using the Illumina TST-15 gene panel showed good library yield across all eight samples, as assessed by Qubit *(Fig 3).* NGS sequencing results showed good and comparable variant calling ability between both sonicated and enzymatically sheared samples (*Fig 4*). *(Due to the Limit of Detection (LOD) of the assay used, no variants were detected in any sample at 0.1% AF (even with filter settings removed, data not shown.)*

| Library Pool A | | ng/µL |
|---|---|---|
| 1 | 5% sonic. _ 31097 | 27.6 |
| 2 | 5% sonic. + SS _ 31168 | 49.3 |
| 3 | 5% enzymatic _ 31116 | 44.7 |
| 4 | 5% enzymatic + SS _ 31119 | 38.5 |
| 5 | 0,1% sonic. _ 29637 | 50.5 |
| 6 | 0,1% sonic. + SS _ 31169 | 49.7 |
| 7 | 0,1% enzymatic _ 31117 | 42.7 |
| 8 | 0,1% enzymatic + SS _31120 | 57.2 |
| **Library Pool B** | | **ng/µL** |
| 1 | 5% sonic. _ 31097 | 49.8 |
| 2 | 5% sonic. + SS _ 31168 | 53.1 |
| 3 | 5% enzymatic _ 31116 | 44.5 |
| 4 | 5% enzymatic + SS _ 31119 | 40.5 |
| 5 | 0,1% sonic. _ 29637 | 58.7 |
| 6 | 0,1% sonic. + SS _ 31169 | 52.5 |
| 7 | 0,1% enzymatic _ 31117 | 45.1 |
| 8 | 0,1% enzymatic + SS _31120 | 47.8 |

***Figure 3: NGS Library Yield***

**A)**




**B)**

| Sample | Peak (bp) | Region [35-950bp] Average Fragment Length (bp) | % of Total |
|---|---|---|---|
| Sonication | 178 | 170 | 95.08 |
| Sonication + SS | 171 | 180 | 96.54 |
| Enzymatic | 187 | 243 | 94.95 |
| Enzymatic + SS | 168 | 196 | 95.58 |

***Figure 1: Size Distribution***
***(A) TapeStation analysis of size distribution of all sample conditions (B) Table listing peak size and average fragment length***

| | | 5% AF samples | | | 0.1% AF samples | | |
|---|---|---|---|---|---|---|---|
| | Mutation | Expected AF (%) | Acceptance Criteria (%) | Measured AF (%) | Expected AF (%) | Acceptance Criteria (%) | Measured AF (%) |
| Sonication | EGFR L858R | 5 | 3.5 - 6.5 | 4.5 | 0.10 | 0.05-0.15 | 0.13 |
| | EGFR V769-D770insASV | 5 | 3.5 - 6.5 | 4.4 | 0.10 | 0.07 - 0.20 | 0.11 |
| | NRAS Q61K | 6.3 | 4.4 - 8.2 | 6.3 | 0.13 | 0.07-0.20 | 0.14 |
| Sonication + SS | EGFR L858R | 5 | 3.5 - 6.5 | 4.6 | 0.10 | 0.05-0.15 | 0.13 |
| | EGFR V769-D770insASV | 5 | 3.5 - 6.5 | 4.2 | 0.10 | 0.07 - 0.20 | 0.10 |
| | NRAS Q61K | 6.3 | 4.4 - 8.2 | 6.4 | 0.13 | 0.07-0.20 | 0.12 |
| Enzymatic | EGFR L858R | 5 | 3.5 - 6.5 | 4.7 | 0.10 | 0.05-0.15 | 0.12 |
| | EGFR V769-D770insASV | 5 | 3.5 - 6.5 | 4.5 | 0.10 | 0.07 - 0.20 | 0.10 |
| | NRAS Q61K | 6.3 | 4.4 - 8.2 | 6.6 | 0.13 | 0.07-0.20 | 0.13 |
| Enzymatic + SS | EGFR L858R | 5 | 3.5 - 6.5 | 4.7 | 0.10 | 0.05-0.15 | 0.13 |
| | EGFR V769-D770insASV | 5 | 3.5 - 6.5 | 4.3 | 0.10 | 0.07 - 0.20 | 0.10 |
| | NRAS Q61K | 6.3 | 4.4 - 8.2 | 6.5 | 0.13 | 0.07-0.20 | 0.14 |

***Figure 2: Representative ddPCR QC analysis on three of the eight mutations at either 0.1% or 5% variant AF***

| | Mutations | | | | | | | Sonication (31097) | Sonication + Size Selection (31168) | Enzymatic (31116) | Enzymatic + Size Selection (31119) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Expected Variant AF (%) (ddPCR) | Gene | Variant | Type | Protein Position | AA Change | Exon | Consequence | Variant AF (%) as detected on Illumina TST15 NGS gene panel | | | |
| 5.0 | EGFR | ΔE746 - A750 | deletion | 0 | KELREA/K | 19/28 | inframe-deletion | 7.1 | 8.2 | 4.7 | 5.5 |
| 5.0 | EGFR | L858R | snv | 858 | L/R | 21/28 | missense | 4.4 | 2.7 | 5.9 | 6.1 |
| 5.0 | EGFR | T790M | snv | 790 | T/M | 20/28 | missense | 5.6 | 3.6 | 5.9 | 4.2 |
| 5.0 | EGFR | V769 - D770insASV | insertion | 766 | M/MASV | 20/28 | inframe-insertion | 5.9 | 4 | 4.3 | 3.8 |
| 6.3 | KRAS | G12D | snv | 12 | G/D | 2/6 | missense | 7.7 | 7.9 | 6.5 | 5.8 |
| 6.3 | NRAS | Q61K | snv | 61 | Q/K | 3/7 | missense | 9.2 | 5.2 | 6.5 | 6.8 |
| 6.3 | NRAS | A59T | snv | 59 | A/T | 3/7 | missense | 7 | 8.3 | 6.2 | 6.9 |
| 6.3 | PIK3CA | E545K | snv | 545 | E/K | 10/21 | missense | 5.3 | 6.3 | 6.5 | 6.7 |

***Figure 4: NGS results from 5% AF cfDNA test samples run on the Illumina TST-15 assay***

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