Evaluation of An Alternative Fragmentation Method in High Throughput NGS Sample Testing of Minimal Residual Disease in Hematological Malignancies

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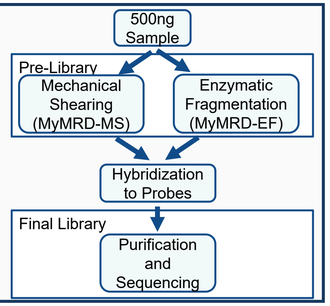
Introduction

Invivoscribe® collaborates with leading pharmaceutical companies testing large batches of samples using target capture-based next-generation sequencing (NGS) assays such as MyMRD® and MyAML® to identify clinically actionable variants. The Invivoscribe MyMRD assay is a multi-gene targeted NGS panel that covers 23 gene targets. Mechanical shearing is the standard method of DNA fragmentation for the assay, which requires specialized equipment and can be time-consuming. Enzymatic fragmentation of DNA does not require specialized equipment, and can easily be scaled for automation. While mechanical fragmentation has long been the standard for fragmenting DNA in target capture-based NGS workflows, alternative methods such as enzymatic fragmentation can reduce sample handling time and dramatically increase throughput. We compared enzymatic fragmentation (using the KAPA™ HyperPlus kit) to our standard mechanical shearing protocol (KAPA™ HyperPrep kit) in the context of the MyMRD assay. In this presentation, the two methods will be referred to as MyMRD-MS (Mechanical Shearing, KAPA™ HyperPrep), and MyMRD-EF (Enzymatic Fragmentation, KAPA™ HyperPlus).
We present similar performance between enzymatic fragmentation and mechanical shearing in both wet lab metrics (yield, average size) and dry lab metrics (single nucleotide variant, or SNV/indel limit of detection or LoD, linearity). We also present limitations in the detection of structural variants (SVs), as MyMRD-EF was less sensitive to the detection of SVs than MyMRD-MS.

Materials & Methods

Genomic DNA was enzymatically fragmented using the KAPA™ HyperPlus kit (Roche) or mechanically fragmented using a Covaris® M220 Focused-Ultrasonicator. Fragmented DNA was then ligated to Illumina adapters to generate NGS libraries ("Pre-Libraries") which were quality checked by LabChip® (PerkinElmer®) or Bioanalyzer (Agilent). These libraries were checked for minimum criteria of percent purity and average size. Yield was obtained using Qubit™ (ThermoFisher™) for DNA quantitation.
These Pre-Libraries are then hybridized to oligonucleotide probes that target the 23 genes in the MyMRD assay.
Finally, these hybridized "Final Libraries" are purified and prepared for sequencing using Illumina platforms (MiSeq®). Sequencing data was analyzed using proprietary Invivoscribe MyInformatics® software. SNV, indel, and SV calls made from the sequencing data were analyzed.



Results

I: Optimization of fragmentation time

To validate enzymatic fragmentation as robust DNA fragmentation method, we first sought to establish a suitable fragmentation method that would produce usable fragments for the MyMRD assay. To compare the two methods, we used Pre-Library data from a previous study in which we validated the MyMRD-MS method. The top panel in Figure 1 illustrates titration of fragmentation time from 5 minutes to 15 minutes. We used average size as the primary output. As expected, longer fragmentation times produced shorter fragments, with the 15' incubation fragments nearing the 500bp average size threshold for the MyMRD assay. 8 minutes was selected as the optimal fragmentation time to reproducibly obtain fragments >500bp. We also analyzed yield and percent purity at the MyMRD Pre-Library stage. Percent purity was defined as the amount of useful product in the 300-700bp range. A minimum of 250ng Pre-Library yield and purity of >30% is required for the MyMRD assay. All four fragmentation time periods (5', 8', 10', and 15') produced sufficient yield and purity for the assay (Figure 1).

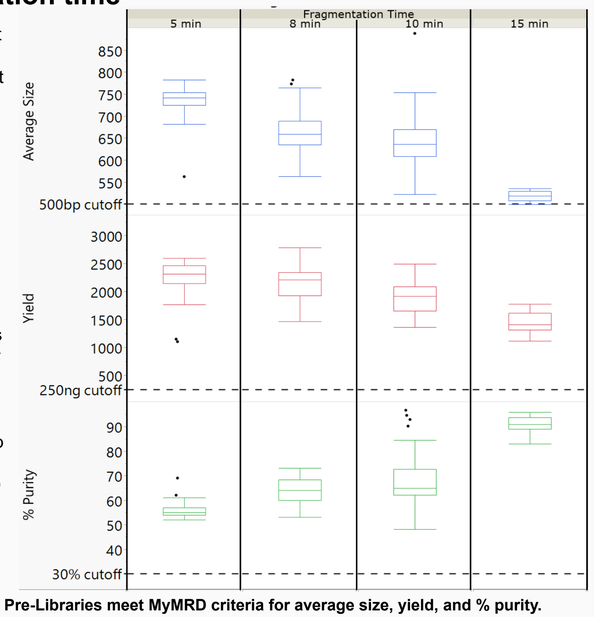


Figure 1: Enzymatically fragmented Pre-Libraries meet MyMRD criteria for average size, yield, and % purity.

Results

II: Comparison of mechanically and enzymatically fragmented libraries

Since fragmentation only involves the Pre-Library stage of library construction, we set out to establish whether enzymatically fragmented DNA (using KAPA™ HyperPlus) was equivalent to mechanically fragmented DNA (using KAPA™ HyperPrep). Using a 500ng DNA input (standard for MyMRD), we compared the average size, yield, and % purity as measured by Qubit and Bioanalyzer / LabChip between both methods. Figure 2 displays performance between historical data from mechanical shearing (N=204) and our entire dataset obtained using enzymatic fragmentation (N=188). These data demonstrate the equivalence at the Pre-Library stage between MyMRD-EF and MyMRD-MS.

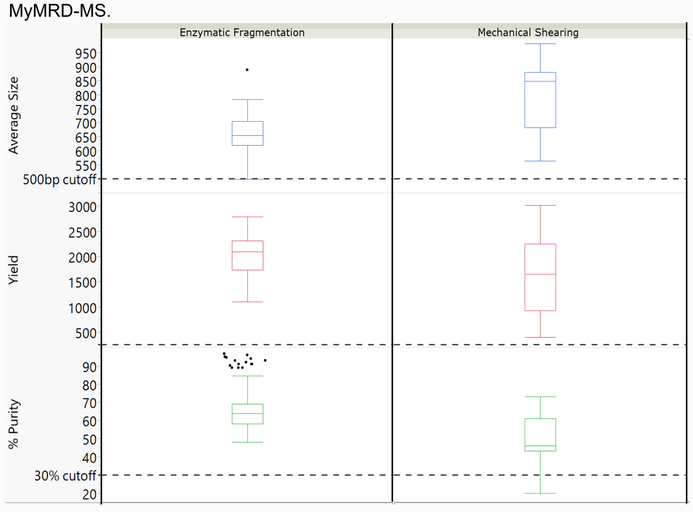


Figure 2: Enzymatically fragmented Pre-Libraries are similar to mechanically sheared Pre-Libraries with respect to average size, yield, and % purity.

III: Expected and Observed Read Frequency display excellent linearity

A series of contrived linearity samples that were previously run on the MyMRD-MS assay were re-run using the MyMRD-EF workflow to demonstrate equivalence in linearity using the alternate library prep kit. We compared expected variant VAFs to observed VRFs and noted excellent linearity between expected and observed read frequency (R^2=0.948). These data demonstrate equivalent linearity between MyMRD-EF and MyMRD-MS.

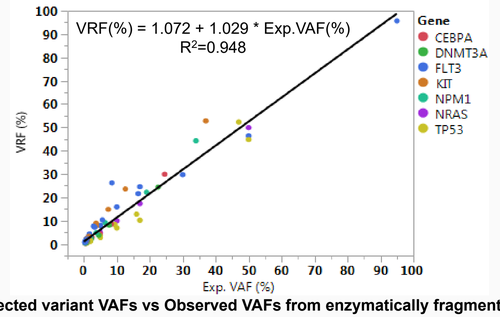


Figure 3: Expected variant VAFs vs Observed VAFs from enzymatically fragmented samples.

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Results

IV. Limit of Detection: SNVs, Indels, SVs

Gene	Variant	Type	Expected VAF	Observed VAF
FLT3	126 bp ITD	Indel	0.33%	0.74%
	c.2503G>T,p.D835Y	SNV	0.17%	0.65%
	c.2504A>T,p.D835V	SNV	30%	29.72%
	c.2503G>T,p.D835Y	SNV	0.85%	2.34%
	c.2503G>T,p.D835Y	SNV	2.89%	7.64%
	21bp ITD	Indel	95%	95.69%
	30bp ITD	Indel	1.00%	1.40%
	126 bp ITD	Indel	1.65%	3.93%
	30bp ITD	Indel	5.00%	7.95%
NPM1	c.860_863dupTCTG,p.W288Cfs*12	Indel	0.38%	0.59%
	c.860_863dupTCTG,p.W288Cfs*12	Indel	0.76%	0.59%
TP53	c.406dupC,p.Q136Pfs*13	Indel	0.94%	0.46%
	c.743G>A,p.R248Q	SNV	1.00%	0.64%
	c.406dupC,p.Q136Pfs*13	Indel	4.70%	3.55%
	c.743G>A,p.R248Q	SNV	5.00%	3.23%
NPM1	c.860_863dupTCTG,p.W288Cfs*12	Indel	3.80%	4.87%
DNMT3A	c.2644C>T,p.R882C	SNV	0.45%	0.35%
	c.2644C>T,p.R882C	SNV	2.25%	2.36%
KIT	c.2466T>A,p.N822K	SNV	0.74%	1.95%
	c.2466T>A,p.N822K	SNV	3.70%	8.36%
NRAS	c.182A>T,p.Q61L	SNV	1.00%	0.94%
	c.182A>T,p.Q61L	SNV	5.00%	4.79%
CEBPA	c.402G>A,p.A134A	SNV	0.49%	0.57%
	c.402G>A,p.A134A	SNV	2.45%	2.84%

Table 1: Indel and SNV detection using the MyMRD-EF workflow. MyMRD-MS and MyMRD-EF are equivalent in indel and SNV detection.

Previous studies using the MyMRD-MS method revealed the limit of detection (LoD) for indels and single nucleotide variants (SNVs) to be 0.34% and 0.5%, respectively. These studies defined LoD as the lowest VAF for which the variant is detected in all replicates (4/4 replicates). To confirm LoD using enzymatic fragmentation, 4 replicates of contrived samples from the MyMRD-MS study were re-run using the MyMRD-EF workflow. Detection of variants in the MyMRD panel with varying expected VAFs for indels and SNVs are listed in Table 1 below. All expected indels and SNVs were detected down to 0.33% and 0.17%, respectively. These data establish equivalency between MyMRD-MS and MyMRD-EF in indel and SNV detection.

Gene	Variant	Detected	Expected VAF
KMT2A/AFF1	t(4;11)	0 of 4	0.12%
		0 of 4	0.60%
	t(11;4)	0 of 4	0.51%
		2 of 4	2.55%
RUNX1/CBFA2T1 (*AML1/ETO*)	t(8;21)	1 of 4	0.36%
		4 of 4	1.80%
	t(21;8)	4 of 4	0.43%
		4 of 4	2.15%

Table 2: SV detection using the MyMRD-EF workflow. MyMRD-EF failed to detect SVs previously detected by MyMRD-MS.

For structural variants (SVs), the MyMRD-MS LoD was found to be 1.8%. Using the MyMRD-EF workflow, we were able to detect all (4 of 4) *RUNX1/CBFA2T1* translocations at the 1.8% level as expected. However, only 2 of 4 *KMT2A/AFF1* translocations were detected at 2.55% expected VAF. Detection failure was traced to low coverage resulting from inversion artifacts detected in the sequencing data. These inversions were not present in mechanically fragmented samples and varied in size, position, and frequency, indicating these artifacts are exclusive to enzymatic fragmentation. These data suggest MyMRD-EF is not compatible with structural variant calling.

Conclusions

With the notable exception of inversion artifacts, enzymatic fragmentation using the KAPA™ HyperPlus kit with the MyMRD assay produced libraries comparable to those from mechanically fragmented genomic DNA. Both methods detected the same set of SNVs and indels, resulting in equivalence in linearity and concordance in SNV/indel detection. We observed reduced sensitivity for structural variant detection in enzymatically fragmented samples; we were unable to detect SVs in enzymatically fragmented samples that were detected using MyMRD with mechanical shearing. Incorporating enzymatic fragmentation to current and future assays could provide a cost-effective way to process large batches of samples and enable automated construction of NGS libraries; however, mechanical shearing is still required for specimens requiring structural variant detection at low prevalence.