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Improved next-generation sequencing pre-capture library yields and sequencing parameters using on-bead PCR

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ABSTRACT

Tumor DNA sequencing results can have important clinical implications. However, its use is often limited by low DNA input, owing to small tumor biopsy size. To help overcome this limitation we have developed a simple improvement to a commonly used next-generation sequencing (NGS) capture-based library preparation method using formalin-fixed paraffin-embedded-derived tumor DNA. By using on-bead PCR for pre-capture library generation we show that library yields are dramatically increased, resulting in decreased sample failure rates. Improved yields allowed for a reduction in PCR cycles, which translated into improved sequencing parameters without affecting variant calling. This methodology should be applicable to any NGS system in which input DNA is a limiting factor.

LAY ABSTRACT

The results of tumor DNA sequencing can have important clinical implications. However, limited DNA input owing to small tumor size frequently results in a failed analysis. By incorporating a simple modification to the standard sample preparation method, we show that DNA yields can be greatly increased, allowing for more samples to be successfully sequenced. Further, the methodology used also results in a subsequent improvement in sequencing parameters, with no loss in the ability to detect mutations.

METHOD SUMMARY

To improve the library yield for low DNA input tumor samples subjected to NGS, we adopted a simple modification of the standard KAPA Hyper library preparation procedure. Genomic DNA libraries were subjected to a solid-phase reversible mobilization bead-based cleanup; however, they were not eluted from the beads prior to pre-capture PCR. Post-PCR cleanup was performed using a 20% PEG, 2.5M NaCl buffer, resulting in a dramatic yield increase with no loss of sequencing fidelity.

KEYWORDS

DNA library • next-generation sequencing • solid-phase reversible immobilization beads

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Tumor DNA analysis using next-generation sequencing (NGS) has revolutionized many aspects of cancer diagnosis, prognosis, and therapeutics [1]. Two major factors, DNA quality and quantity, influence the proportion of successfully sequenced tumor samples. The quality issue is attributed to the use of formalin-fixed paraffin-embedded (FFPE) tumor tissue, which results in highly fragmented DNA that is chemically modified, whereas the low quantity results from small tumor biopsy sizes. In our experience, using the KAPA Hyper Prep Kit for library preparation (Roche, Basel, Switzerland), in conjunction with a SureSelect^{XT} targeted capture (Agilent, CA, USA), we found that approximately 15% (61/432) of the tumor samples failed to produce a sufficient yield of pre-capture library. Therefore, we modified the standard protocol by performing the pre-capture PCR 'on-bead'. Solid-phase reversible immobilization (SPRI) bead-based DNA purification [2] has become the favored method of DNA purification in many protocols owing to its ease of use, cost-effectiveness, ability to size-select, and suitability for automation. Following genomic DNA (gDNA) fragmentation and adapter ligation, NGS capture-based libraries typically undergo SPRI bead-based purification in which the DNA eluted from the beads is used for PCR enrichment. This elution step inevitably results in the loss of DNA. This study was designed to determine whether performing the PCR directly on the bead/eluate mixture resulted in improved pre-capture library yields when using FFPE-derived tumor samples.

A detailed protocol for library preparation can be found in the Supplementary data file. Tumor DNA was extracted from FFPE sections using a QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) and was fragmented using a LE220 focused-ultrasonicator (Covaris, MA, USA). DNA libraries were then prepared using the KAPA Hyper Prep Kit, per the manufacturer's instructions (Roche) with some modifications. Following DNA end-repair and A-tailing, SureSelect adapters (Agilent) were ligated and SPRI (AMPure, Beckman Coulter, IN, USA) bead purification was performed. However, no final elution of purified DNA was performed. Instead, the entire bead/elution buffer mixture was added to the KAPA PCR mixture where it underwent normal PCR cycling. Therefore, amplification of the pre-capture gDNA library occurred in the presence of the beads. This process is illustrated in Figure 1. Following PCR, the amplified library was purified by adding 90 µl of 20% PEG/2.5M NaCl [2], instead of the recommended 90-µl SPRI bead mixture. Its addition "reactivates" the beads that are already present, and bead-based purification can continue as normal. Following purification, pre-capture libraries were analyzed for yield and average fragment size (range 100–1000 bp) using a 4200 TapeStation System (Agilent). SureSelect^{XT} hybridization using a custom 386 gene, 2.34 Mb probe set was used for target enrichment. The capture of hybridized fragments and their washes and final purification was performed as per the standard SureSelect^{XT} protocol. Pooled libraries were sequenced on an Illumina NextSeq500 (Illumina, CA, USA) using paired 75 bp reads.

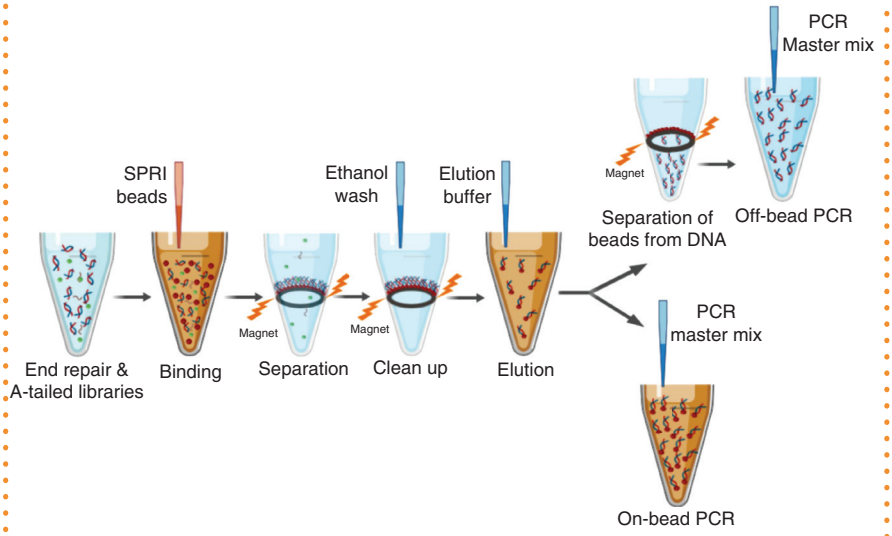

Figure 1. Overview of the methods used in the study.
SPRI: Solid-phase reversible immobilization.

We first sought to determine whether pre-capture library yields were affected by using the on-bead PCR method. One high-quality cell line-derived DNA (NA12878, Coriell Institute) and one FFPE-derived tumor DNA were used, each at two different input amounts. The standard 'off-bead' and modified 'on-bead' methods were performed in parallel for the four samples. The results revealed a dramatic increase in pre-capture library yield in each case, with an average increase of 4.15-fold (Figure 2A).

We next compared pre-capture library yields between the off-bead method using the standard 10 PCR cycles and the on-bead method using nine PCR cycles. Our rationale was that if sufficient on-bead yields could still be generated with one less PCR cycle, the sequencing read duplicates would be reduced, leading to an improved total read depth for these samples. To test this

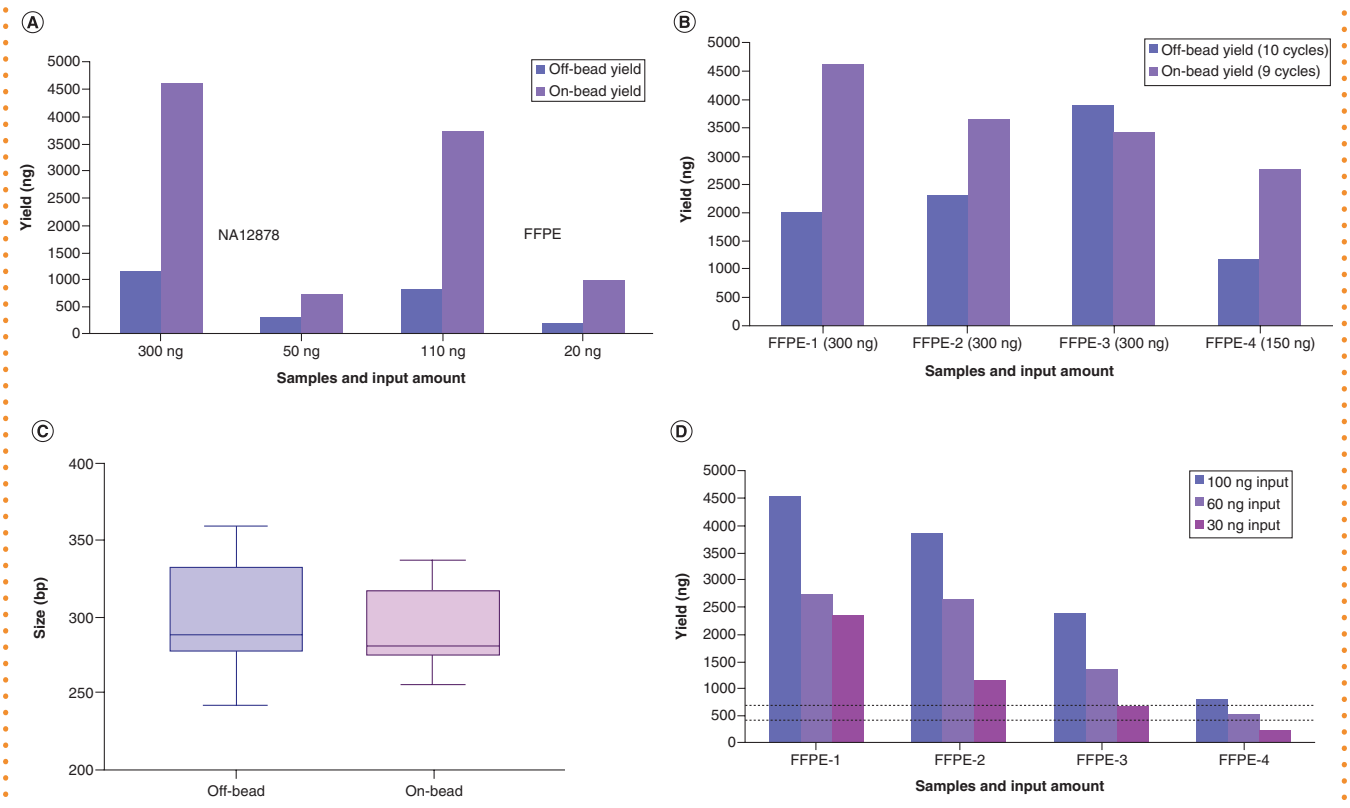

Figure 2. Analysis of off-bead versus on-bead pre-capture library preparations. (A) Library yields for one control and one FFPE tumor sample performed at two different DNA input amounts. All four sample preparations were performed in parallel. Average yield increase using on-bead PCR was 4.15-fold. (B) Off-bead versus on-bead yield comparisons for four FFPE tumor samples, with off-bead samples undergoing 10 PCR cycles and on-bead samples undergoing nine cycles. On-bead yields increased an average of 1.8-fold despite the reduction in cycles. (C) Box plots of average fragment sizes for the eight comparisons used in (A & B). On-bead PCR did not appear to significantly impact on library fragment size (Paired t-test, $p = 0.122$, $t = 1.76$, $df = 7$). (D) Analysis of DNA input limits required to generate a pre-capture library yield sufficient for hybridization when using nine cycles on-bead PCR. Recommended (750 ng) and lower limit (500 ng) yields are shown by dashed horizontal lines. Results show that 30 ng of input generates sufficient library in two cases, a borderline yield in one case and a failed library in one case.
FFPE: Fomalin-fixed paraffin-embedded.

Table 1. Selected NGS parameters from four sample pairs.

Sample_ID	FFPE-1 Off	FFPE-1 On	FFPE-2 Off	FFPE-2 On	FFPE-3 Off	FFPE-3 On	FFPE-4 Off	FFPE-4 On
Mean coverage	209.7	383.0	355.9	426.4	302.9	439.6	344.5	491.2
% bases above 100	93.5	98.1	93.2	94.9	95.7	97.6	95.7	97.4
% bases above 500	0.6	20.8	22.2	33.9	11.3	30.9	16	43.1
Total reads	34,741,134	36,859,984	41,782,784	36,957,886	31,148,930	35,678,966	39,477,926	37,762,250
% Mapped reads duplicates	38.53	23.76	35.76	23.22	26.39	9	37.38	21.01
Total reads minus duplicates	21,414,277	28,141,701	26,891,969	28,405,461	22,970,169	32,485,748	24,774,921	29,853,605
% Reads on target	35.0	48.0	51.4	56.0	47.2	47.3	48.3	56.5
Mean coverage for target bases	246.6	450.4	462.3	536.8	359.5	511.9	399.8	567.8
Median fragment length	144	142	113	121	137	138	153	153

Note the reduction in '% Mapped read duplicates' attributed to the reduction in cycles for the on-bead samples and the corresponding increase in read depth and coverage statistics. Median fragment length remains unchanged.
FFPE: Formalin-fixed paraffin-embedded; NGS: Next-generation sequencing.

hypothesis, four FFPE tumor DNAs were prepared in parallel. Three sample pairs used 300 ng of input DNA, whereas one used 150 ng. Figure 2B shows that in three of the four pairs the on-bead method, with nine PCR cycles, produced a greater library yield than the standard off-bead method with 10 cycles. The average on-bead increase in yield across the four sample pairs was 1.8-fold. In addition, we compared the average fragment size generated for these four sample pairs, along with the four sample pairs tested in the first analysis (Figure 2A). On-bead PCR did not appear to impact on the library fragment size ($p = 0.12$, paired *t*-test) (Figure 2C).

Sequencing libraries for the four sample pairs from Figure 2B were prepared and sequenced in a single run. Table 1 shows nine important sequencing metric comparisons from the four pairs. In each case, the on-bead samples had a lower '% mapped reads duplicates' value, whereas all read depth and coverage statistics were improved despite two of the off-bead samples having a higher 'total reads' value. Median fragment length was unchanged. All four of these tumor samples, plus the tumor sample analyzed in test 1 (Figure 2A), had previously been analyzed with our 386 gene targeted panel under standard conditions. Therefore, we compared the variants detected in our on-bead results with those previously detected. These tumor samples had been selected because of the high number and diversity of their variants. A total of 66 variants had been detected in these five samples. These comprised 60 × SNVs, 1 × 1 bp deletion, 1 × 1 bp insertion, 1 × 8 bp deletion, 1 × 24 bp deletion, 1 × 25 bp deletion, and 1 × gene fusion (*GOLGA4-RAF1*). All 66 variants were detected in our on-bead samples, and variant allele frequencies were similar (*t*-test for two independent means, $p = 0.21$) (Supplementary Table 1).

Finally, we sought to determine the lower limits of DNA input required to produce sufficient pre-capture library for hybridization. The SureSelect^{XT} protocol recommends using 750 ng of pre-capture library, although we found a lower limit of 500 ng is sufficient. Accordingly, we designated pre-capture library yields >750 ng as 'pass', 750–500 ng as 'borderline' and <500 ng as 'fail'. Four FFPE tumor DNAs were used, each at gDNA inputs of 100, 60 and 30 ng. Pre-capture libraries were produced using the on-bead method using nine PCR cycles. Results show that at 100 ng input all samples passed, at 60 ng input three samples passed and one was borderline, and at 30 ng input two samples passed, one sample was borderline, and one sample failed (Figure 2D). We are able to obtain >30 ng of DNA from >95% of our FFPE tumor samples. This represents a significant increase in likely 'passes' compared with our previous results, in which approximately 15% (61/432) of the samples failed to generate sufficient library for hybridization. Note that this is despite a reduction in PCR cycles.

In summary, we demonstrate that the use of on-bead pre-capture library enrichment dramatically increased our library yields. The concept of skipping SPRI eluting steps to gain efficiency has been previously described in detail by Fisher *et al.* [3] and termed 'with-bead SPRI'. Fisher describes the use of with-bead SPRI after end-repair, A-tailing and adaptor ligation. However, using the KAPA Hyper Prep Kit these steps are already optimized in a single tube; our work shows further efficiency gains by directly doing an on-bead PCR. In our laboratory this has resulted in approximately 50% fewer FFPE tumor samples failing sequencing. Furthermore, the protocol enables a decrease in the number of PCR cycles used and this has had a positive effect on the final sequencing results, with less read duplicates, and higher read depths and coverage noted. Crucially, as a diagnostic laboratory analyzing patient tumor samples, it has had no negative effect on variant calling accuracy or variant allele frequency determination. This simple modification of a well-established protocol should be applicable to many other NGS systems and, indeed, many other methodologies where a pre-PCR bead-based clean-up is required on limited amounts of input DNA. ▶

Here, we present a simple yet effective method for improving PCR yields and sequencing parameters, following SPRI bead-based cleanups of NGS pre-capture libraries. This should aid with increasing the numbers of clinical samples that are able to be sequenced and provide subsequent clinical benefit for patients. Although we have used this method in an NGS setting, it should be applicable to any case in which limited DNA is available or higher PCR yields are required.

SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2019-0059

AUTHOR CONTRIBUTIONS

TS, CRM, GM, HX, SBF and APF conceived the experiments. CRM, BY and DYC performed all of the experiments. CRM and GM analyzed the results. CRM wrote the paper with assistance from all co-authors.

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ETHICAL CONDUCT OF RESEARCH

The authors state that they have obtained appropriate institutional review board approval.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

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REFERENCES

1. Sabour L, Sabour M, Ghorbian S. Clinical applications of next-generation sequencing in cancer diagnosis. *Pathol. Oncol. Res.* 23(2), 225–234 (2017).
2. DeAngelis MM, Wang DG, Hawkins TL. Solid-phase reversible immobilization for the isolation of PCR products. *Nucleic Acids Res.* 23(22), 4742–4743 (1995).
3. Fisher S, Barry A, Abreu J *et al.* A scalable, fully automated process for construction of sequence-ready human exome targeted capture libraries. *Genome Biol.* 12(1), r1 (2011).