Practical Laboratory Medicine 12 (2018) e00108

Contents lists available at ScienceDirect

Practical Laboratory Medicine

journal homepage: www.elsevier.com/locate/plabm

Biotinylated amplicon sequencing: A method for preserving DNA samples of limited quantity

Karen Cravero[a], Arielle Medford[a], Aparna Pallavajjala[b], Jenna Canzoniero[c], Natasha Hunter[a], David Chu[a], Rory L. Cochran[a], Ian Waters[a], Eric S. Christenson[a], Kelly Kyker-Snowman[a], Berry Button[a], Alex J. Cole[a], Ben Ho Park[a,d,*]

[a] *The Sidney Kimmel Comprehensive Cancer Center, Department of Oncology, The Johns Hopkins University School of Medicine, Baltimore, MD, United States*
[b] *The Sidney Kimmel Comprehensive Cancer Center, Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, MD, United States*
[c] *Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD, United States*
[d] *The Whiting School of Engineering, Department of Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD, United States*

ARTICLE INFO

Keywords:
Next generation sequencing
Plasma DNA
Droplet digital PCR (ddPCR)
Targeted amplicon sequencing

ABSTRACT

Background: Genomic testing is often limited by the exhaustible nature of human tissue and blood samples. Here we describe biotinylated amplicon sequencing (BAmSeq), a method that allows for the creation of PCR amplicon based next-generation sequencing (NGS) libraries while retaining the original source DNA.

Design and methods: Biotinylated primers for different loci were designed to create NGS libraries using human genomic DNA from cell lines, plasma, and formalin-fixed paraffin embedded (FFPE) tissues using the BAmSeq protocol. DNA from the original template used for each BAmSeq library was recovered after separation with streptavidin magnetic beads. The recovered DNA was then used for end-point, quantitative and droplet digital PCR (ddPCR) as well as NGS using a cancer gene panel.

Results: Recovered DNA was analyzed and compared to the original DNA after one or two rounds of BAmSeq. Recovered DNA revealed comparable genomic distributions and mutational allelic frequencies when compared to original source DNA. Sufficient quantities of recovered DNA after BAmSeq were obtained, allowing for additional downstream applications.

Conclusions: We demonstrate that BAmSeq allows original DNA template to be recovered with comparable quality and quantity to the source DNA. This recovered DNA is suitable for many downstream applications and may prevent sample exhaustion, especially when DNA quantity or source material is limiting.

1. Introduction

The use of patient samples for cancer research has led to the development of new diagnostics and therapeutics for the treatment of human cancers. In particular, tissue specimens have been essential for understanding the mutational landscapes of cancer [1], and

Abbreviations: BAmSeq, Biotinylated amplicon sequencing; NGS, Next generation sequencing; FFPE, Formalin-fixed paraffin embedded; cfDNA, Circulating cell-free DNA; gDNA, Genomic DNA; pDNA, Plasma DNA; qPCR, Quantitative polymerase chain reaction; ddPCR, Droplet digital PCR

* Correspondence to: 1650 Orleans Street, Room 151, Baltimore, MD 21287, United States.
E-mail address: bpark2@jhmi.edu (B.H. Park).

https://doi.org/10.1016/j.plabm.2018.e00108
Received 22 April 2018; Received in revised form 6 July 2018; Accepted 8 August 2018

DNA extraction from primary and metastatic tumors is now routine practice [2]. Similarly, circulating cell free DNA (cfDNA) or "liquid biopsy" is an emerging analyte for many applications in clinical oncology and cancer research [3–6]. The utility of cfDNA for cancer mutation detection with digital PCR and NGS has been shown for a number of clinically relevant oncologic issues including response to therapy [7,8], detection of driver and resistance mutations [9,10] and measurement of residual disease burden [11]. Unfortunately, a limitation of both tissue samples and cfDNA is their exhaustible nature [12], limiting the number of tests and assays that can be performed. This is especially relevant when performing NGS on a limited number of loci, where often the entire sample is used to maximize the chance of detecting a rare mutation within many wild type DNA molecules.

Techniques such as Safe-SeqS [13] and Duplex Sequencing [14] have been developed for increasing the sensitivity of targeted amplicon sequencing [15]. These techniques incorporate barcoding strategies to distinguish PCR and NGS errors from true rare mutant alleles. However, the ultimate sensitivity of these methods depends on the starting amount of DNA, often necessitating the use of the entire sample. To address this problem, we describe a novel modification for amplicon NGS termed Biotin Amplicon Sequencing or BAmSeq. This modification of amplicon sequencing enables recovery of the sample DNA template for future use in other molecular assays, and importantly does not compromise the quantitative integrity of the original source DNA. The use of BAmSeq allow for recycling of DNA for samples of limited quantities.

2. Materials and methods

2.1. BAmSeq

A 5' biotin molecule and subsequent streptavidin pull down step was added to the overall methodology of the Safe-SeqS technique [13]. Safe-SeqS was developed to increase the sensitivity of mutation detection by tagging amplicons of interest using a random sequence of 12–14 nucleotides called "unique identifiers". The protocol barcodes DNA strands during the first PCR step followed by a secondary PCR in which Illumina adapter sequences are added to the tagged template. In the BAmSeq approach described in Fig. 1, first, a 5' biotin molecule is attached to the amplicon of interest by using biotinylated forward and reverse primers during the first PCR step. PCR reactions were performed in a 50 μL final volume with final concentrations of 1x Phusion® HF Buffer, 200 μM dNTP's, 5% DMSO, 0.5 μM forward and reverse primers, and 2 units of Phusion® Hot Start Polymerase (New England Biolabs) using the following cycling conditions: an initial denaturation at 98 °C for 30 s (s), followed by one cycle of 98 °C for 10 s, 64 °C for 15 s, and 72 °C for 30 s. Subsequent cycles (2–5 depending on the amplicon) maintained the same temperatures and times for denaturation and elongation steps, while the annealing temperature was changed from 64 °C to 61 °C. Upon completion of the first PCR step, the PCR products are now covalently linked to biotin molecules. Second, the sample was cleaned using AMPure XP PCR Purification magnetic beads (Agencourt) per the manufacturer's recommendation. Third, the sample was eluted into 20 μL of water and incubated in equal volume of Dynabeads® MyOne™ Streptavidin (ThermoFisher Scientific) to extract all the biotin-tagged DNA strands, including residual primers. Prior to the incubation, Streptavidin beads were washed to remove buffers and preservatives as specified in the manufacturer's protocol. An equal volume of cleaned streptavidin beads was added to the eluent and incubated for at least 1 h at room temperature with mild shaking to maintain the beads in suspension. After incubation, the tubes were placed in a magnetic stand to separate the supernatant (containing the original source DNA) and the magnetic beads (bound to amplicon of interest), until the solution appears clear (10–15 min). Subsequently, the two samples collected are:1) the supernatant containing the recovered DNA and residual buffers. This sample was carefully removed and collected in a microcentrifuge tube, cleaned using AMPure XP beads, and either stored at – 20 °C, or used for further applications, and 2) the streptavidin-biotin-amplicon complex. The sample was washed twice with 1X B & Q buffer and once with distilled water, then resuspended in 22 μL of water as per the manufacturer's recommendations.

The eluted streptavidin-biotin-DNA complex beads are used as template for the second PCR step, in which Illumina-specific adapters are added, as well as indexes (unique 6 bases) for multiplexing. Because the entire complex can be used directly as template for the PCR reaction, denaturation of the streptavidin-biotin complex is not necessary. The following thermocycling conditions were used for this amplification step: 25–35 cycles (depending on initial DNA concentration used) at 98 °C for 10 s (s), 66 °C for 30 s, and 72 °C for 40 s. Upon PCR conclusion, the entire reaction is placed on a magnet and allowed to separate (10–15 min). Without disturbing the pellet, the supernatant (sequencing library) is collected, and the magnetic beads stored at – 20 °C or discarded. The newly isolated sequencing library then undergoes an additional clean-up step using the AMPure XP beads. Cleaning is followed by quantification using the KAPA Library quantification kit, a qPCR assay by Bio-Rad®, and subjected to NGS with the Illumina platform. A schematic of the protocol is shown in Fig. 1, and a complete list of all primers used in this study can be found in Supplemental Table S1.

2.2. Patient and sample collection

All patients were consented and enrolled in an IRB protocol at the Johns Hopkins Sidney Kimmel Comprehensive Cancer Center (JHSKCCC; Baltimore, MD) approved for collection and genomic analysis of tissue and bodily fluids from breast cancer patients for use in research. FFPE normal tissue samples and plasma DNA from four patients (two patients with breast cancer and two normal plasma samples) were collected and used for the study.