Application Note

A Simple One-Step Library Prep Method To Enable AmpliSeq Panel Sequencing on Illumina Platforms

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Introduction

Targeted amplicon sequencing is a cost-effective, convenient and rapid method for variant detection. Focusing the sequencing capacity on certain genes of interest eliminates the need for sequencing and analyzing the entire genome. Researchers are not only using targeted sequencing for samples with large amounts of high-quality DNA, but also for more challenging sample types such as formalin-fixed paraffin-embedded (FFPE) tissue, circulating cell-free DNA (cfDNA) from blood, and fine-needle aspirates. In any research, the application of orthogonal sequencing methods is important to ensure confidence. Here we present a protocol using the new QIAseq 1-Step Amplicon Library Kit for the sequencing and verification of the AmpliSeq targeted sequencing assays on Illumina sequencing instruments. By combining end-repair and ligation, the QIAseq 1-Step Amplicon Library Kit offers a fast and efficient 30-minute procedure for the preparation of high-quality, artifact-free Illumina libraries from any PCR amplicons, including AmpliSeq Panels. This application note will outline a straightforward workflow that uses the QIAseq 1-Step Amplicon Library kit to verify AmpliSeq targeted sequencing assays on the Illumina sequencing instruments.

Materials and Methods

The amplicon sequencing workflow is shown in the flowchart in Figure 1. The above workflow was tested with an FFPE DNA Standard Sample with defined mutations at defined frequencies (Quantitative Multiplex FFPE Reference Standard, Horizon Discovery)[1].

Figure 1. Analysis of multiplex PCR products following AmpliSeq PCR and NGS library following 1-Step library prep.

The following components were added for 25 µl of target enrichment PCR reaction: 12.5 µl QIAGEN Multiplex PCR Master Mix (2X, QIAGEN), 5 µl Ion AmpliSeq Cancer Hotspot Panel v2 Primer Pool (5x, Thermo Fisher), 4 µl diluted FFPE DNA (diluted to 10 ng/µl in RNase-free water), and 3.5 µl RNase-free water (QIAGEN). The standard QIAGEN Multiplex PCR cycling conditions were used: 95°C, 15 min for initial denaturation and activation of the HotStarTaq®; 21 cycles of 94°C, 30 sec; 60°C, 90 sec; and 72°C, 90 sec; and a final extension of 72°C, 10min.

Following PCR, amplicons were purified with Agencourt® AMPure® XP beads (Beckman Coulter) with the protocol listed in Appendix B: Amplicon Preparation and Quality Control of the QIAseq 1-Step Amplicon Library Preparation Handbook.

23 µl of purified PCR product was used for library construction following the standard 30-minute, benchtop QIAseq 1-Step Amplicon Library Kit protocol. NGS libraries were sequenced on a MiSeq® instrument with a v2 reagent kit (2x150 bp) (Illumina). Sequencing data were analyzed with the Targeted Amplicon Sequencing (TAS) workflow of Biomedical Genomics Workbench (QIAGEN) using the CHP2.20131001.hotspots_BED file for the AmpliSeq Cancer Hotspot Panel v2 as reference to identify and annotate variants.

Results

We first evaluated whether the standard protocol of QIAGEN Multiplex PCR Mix would be compatible with the primer sets from AmpliSeq Cancer Hotspot Panel v2. We reduced the PCR volume from 50 µl to 25 µl to better resemble the standard AmpliSeq target amplification reaction volume (20 µl). Libraries with expected size distribution (200-300 bp) were generated with the workflow described in Figure 1. No adaptor dimer or other non-specific product was detected.

The final library was sequenced on a MiSeq (Illumina) and sequencing data were analyzed with the Biomedical Genome Workbench (QIAGEN). Coverage uniformity and specificity was high (Table 1), with over 92% bases covered at 0.2X mean coverage or above, and over 97% of mapped reads in targeted region.

Table 1. Target region coverage

Sample multiplexing	12 Samples
Average depth of coverage	4624x
Uniformity	92.34%
Specificity (on-target reads)	97.94%

The sequencing results were analyzed with the CLC Biomedical Genome Workbench and average coverage, coverage uniformity (percentage of bases with 0.2X of mean coverage of above), and specificity (percentage of mapped reads in targeted regions) were calculated.

The genomic regions where the expected variants reside all showed sufficient sequencing coverage (>700) and average quality (>38), for high confidence variant detection (Table 2). Furthermore, all expected 11 mutants from the FFPE reference sample could be accurately detected at the expected frequency (Figure 2).

Table 2. High confidence variant detection. The coverage and sequencing quality for the bases where the annotated variants were detected

Gene	Variant	Coverage	Average Quality
BRAF	**V600E**	3561	38.24
KIT	**D816V**	10733	38.12
EGFR	**ΔE746-A750**	13731	37.71
EGFR	**L858R**	14302	38.55
EGFR	**T790M**	786	38.60
EGFR	**G719S**	5566	38.25
KRAS	**G13D**	2971	38.43
KRAS	**G12D**	2987	38.62
NRAS	**Q61K**	29483	38.65
PIK3CA	**E545K**	2977	38.07
PIK3CA	**H1047R**	3297	38.70

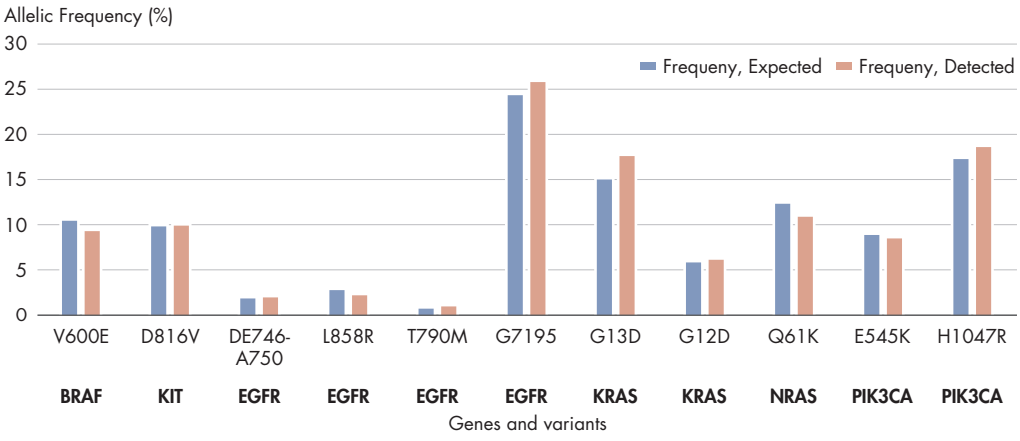

Figure 2: Accurate mutation detection from FFPE reference. The detected variants were annotated and allelic frequency was calculated with the TAS pipeline of the QIAGEN CLC Biomedical Genomics Workbench. The annotation results were then compared with the expected variants in the Quantitative Multiplex FFPE Reference Standard.

Discussion

The QIAseq 1-Step Amplicon Library Kit offers a faster and more efficient alternative to traditional and laborious NGS library preparation methods that typically take at least 2 to 3 hours. By utilizing a novel combined end-repair and ligation reaction, the QIAseq 1-Step Amplicon Library Kit streamlines the entire NGS library preparation process to a single step requiring only 30 minutes at room-temperature. We used the AmpliSeq assays and QIAGEN Multiplex PCR Mix for target amplification, followed by the QIAseq 1-Step Amplicon protocol to generate amplicon libraries that can be sequenced on the Illumina sequencing platforms. Libraries generated with this combined workflow demonstrated high target specificity and coverage uniformity, which are both ▷

required for reliable detection of the variants with efficient use of sequencing capacity. Using an FFPE reference sample with defined mutations, we further verified the ability of the combined protocol to quantitatively and accurately detect gene variants. With 12 samples multiplexed on one MiSeq flow cell we were able to achieve an average coverage of 4624x and were able to detect variants with an allelic frequency of as low as 1% (Figure 2).

References

1. Quantitative Multiplex Reference Standard, Formalin-Compromised DNA, Cat. HD-C749, Horizon Discovery, **https://www.horizondiscovery.com/reference-standards/q-seq-hdx/quantitative-multiplex-reference-standard-hd-c749**

Ordering Information

Product	Contents	Cat. no.
QIAseq 1-Step Amplicon Library Kit (12)	For 12 reactions: 1-Step Amplicon Enzyme Mix and 4x 1-Step Amplicon Buffer, Illumina Library Amplification primer, HiFi PCR Master Mix, RNase-Free Water	180412
QIAseq 1-Step Amplicon Library Kit (96)	For 96 reactions: 1-Step Amplicon Enzyme Mix and 4x 1-Step Amplicon Buffer, 96-plex Illumina Adapter Plate, Illumina Library Amplification primer, HiFi PCR Master Mix, RNase-Free Water	180415

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Visit **www.qiagen.com/QIAseq-1-Step-Amplicon** for more information!