A rapid library preparation method with custom assay designs for detection of variants at 0.1% allelic frequency in liquid biopsy samples

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ABSTRACT

Cancer is one of the leading causes of death worldwide and was responsible for 8.8 million deaths in 2015. This number is expected to continue to increase and options for early detection are critical. Non-invasive liquid biopsies from blood samples can be used to examine cell free DNA and RNA derived from tumor cells.

Herein, we describe a new research method for library preparation using the Ion AmpliSeq™ HD Library Kit with custom assay designs from Ion AmpliSeq HD Panels for detection of low level variants from liquid biopsy samples. This method includes incorporation of molecular tags that enable 0.1% Limit of Detection (LOD) in cell free DNA (cfDNA) and dual barcodes for sample identification. This method is also applicable to formalin-fixed paraffin embedded (FFPE) samples. The libraries can be prepared in as little as 3 hours and are compatible for analysis with the Ion GeneStudio™ S5 system.

Custom assay panels were designed for DNA targets, synthesized, and used to generate libraries with control cfDNA-like samples and FFPE samples containing known variants at 0.1% and 1% LOD respectively. Variants could be detected by sequencing with sensitivity of ≥80% and specificity of ≥98% for cfDNA samples, and sensitivity ≥90% and specificity ≥80% for FFPE DNA samples. Panels were also designed for RNA fusion assays and tested with control samples containing known fusion and exon skipping variants.

We developed a research method to generate libraries from custom assay designs for liquid biopsy blood samples.

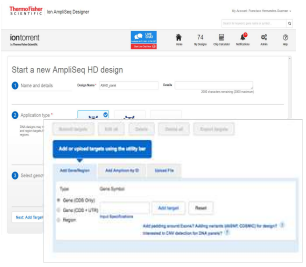
INTRODUCTION

Cancer is one of the leading causes of death worldwide and was responsible for 8.8 million deaths in 2015[1]. Non-invasive liquid biopsy from blood samples is rapidly becoming a growing area of interest for diagnosis and monitoring of disease progression as well as recurrence [2, 3, 4]. Early identification of circulating cell free tumor DNA could potentially lead to a better outcome for disease treatment.

Next generation sequencing provides a tool to evaluate multiple targets of interest to identify low level variants as markers of disease. Low sample input, flexibility in assay content, and high sensitivity are critical features needed for analysis of liquid biopsy samples. Here we present the Ion AmpliSeq Designer and Ion AmpliSeq™ HD Library Kit technology designed to meet these needs.

MATERIALS AND METHODS

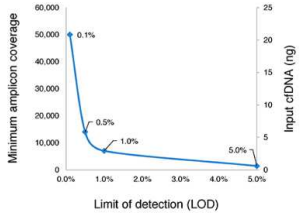
Desired DNA target sequences of interest were used to design custom AmpliSeq HD assays through the Ion AmpliSeq Designer. Desired RNA fusion assays of interest were selected from a menu of available RNA Gene Fusion Designs within the Ion AmpliSeq Designer.



An AmpliSeq Oncology Panel and the equivalent AmpliSeq HD format were also designed and tested.

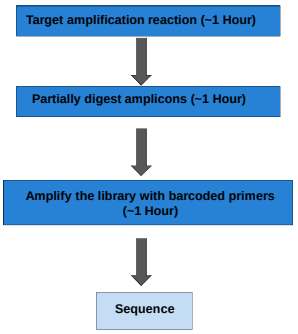
Oligos were synthesized and pooled together to use as primer panels for generating libraries with the Ion AmpliSeq™ Library Kit or Ion AmpliSeq™ HD Library Kit as described in the respective User Guides. Control DNA and RNA samples were used for evaluation with 20 ng input. Resulting libraries were templated on the Ion Chef™ Instrument and sequenced on the Ion S5™ Systems on Ion 530™ or Ion 540™ Chips. Data analysis was performed using the Torrent Suite™ Software and the Ion Reporter™ Software.

Example data is shown below to compare amplicon read coverage required to limit of detection and cfDNA input.



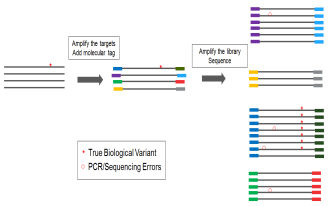
RESULTS

Figure 1a. Ion AmpliSeq HD Library Preparation Workflow



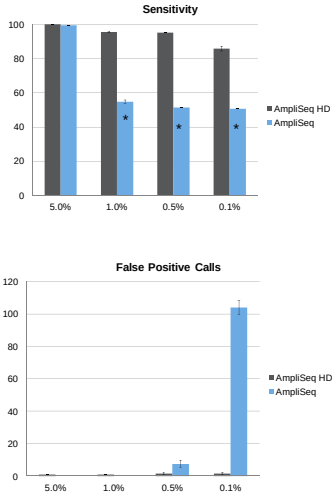
Library preparation work flow can be performed with a total of 45 minutes hands on time. Starting from DNA through data analysis, the total workflow can be completed in less than 2 days.

Figure 1b. Ion AmpliSeq HD Families



Starting molecules are uniquely tagged and result in families of molecules. Counting families accurately quantifies the input DNA (or RNA) profile and allows detection of true low frequency variants.

Figure 2. Comparison of an AmpliSeq and AmpliSeq HD Oncology Panel



An AmpliSeq Oncology Panel and the equivalent AmpliSeq HD format were evaluated with a control DNA with known frequency variants of 0.1%, 0.5%, 1%, and 5%. Sensitivity and false positive (FP) metrics were compared. AmpliSeq HD performance showed improved results for low frequency variant calls (sensitivity) and reduced false positive calls.

*Variant calling workflows for <5% for AmpliSeq samples are not standard analysis workflows.

Table 1. Performance of Custom AmpliSeq HD DNA Assay Panels, On Target and Uniformity Metrics

	Target Length	Primer Pairs	On Target	Base Uniformity
Panel A	75-100 bp	183 in 1 pool	94.8%	99.1%
Panel B	125-175 bp	223 in 2 pools	97.5%	96.6%

Two custom AmpliSeq HD DNA Assay Panels were designed and synthesized for evaluation with the Ion AmpliSeq HD library preparation method. Panel A was designed with 75-100 bp targets and tested with a cfDNA-like control sample. Panel B was designed with 125-175 bp targets and tested with FFPE DNA control sample from Horizon Diagnostics, Structural Multiplex FFPE Reference Standard, Cat# HD789.

Table 2. Performance of Custom AmpliSeq HD DNA Assay Panel A

Sample	Number of Runs	Sensitivity (%)	Average number of FP (Hotspot)
20 ng 0.5%	6	94.2	0
20 ng 0.1%	6	81.3	0.17
10 ng 0.5%	6	91.7	0.17

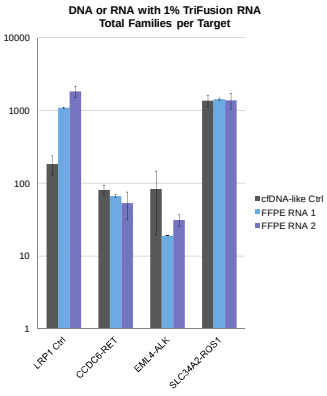
Evaluation of sensitivity and false positive (FP) metrics with Panel A using a cfDNA-like control sample with variants of 0.1% or 0.5% frequency.

Table 3. Performance of Custom AmpliSeq HD DNA Assay Panel B

Sample	Number of Runs	Sensitivity (%)	Average number of FP (Hotspot)
Normal FFPE DNA	4	NA	0
FFPE DNA with 1% Variants	4	100	0

Evaluation of sensitivity and false positive (FP) metrics with Panel B using a FFPE DNA control sample with variants of 1% frequency and a normal liver FFPE DNA sample.

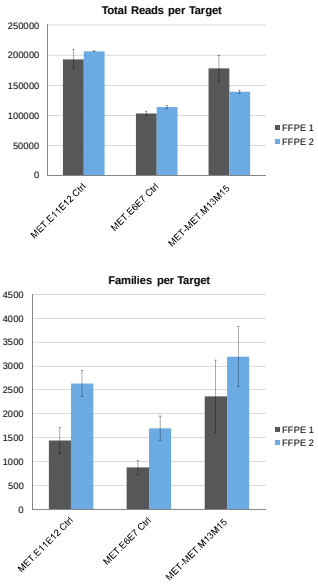
Figure 3. Performance of AmpliSeq HD RNA Assay Panel using Pre-designed RNA Gene Fusion Designs.



One custom AmpliSeq HD RNA Assay Panel was generated from pre-designed RNA Gene Fusion designs and synthesized for evaluation with the Ion AmpliSeq HD library preparation method. A cfDNA-like control and two purified FFPE RNA research samples were combined with a TriFusion control RNA at 1% final composition. The TriFusion RNA contains three fusions, one each for RET, ALK, and ROS1 fusion drivers. LRP1 Ctrl indicates LRP1 gene expression control assay. CCDC6-RET, EML4-ALK, and SLC34A2-ROS1 indicate combined family numbers for one or more assays present in the panel that can target each gene fusion.

Using Ion AmpliSeq HD library preparation and sequencing on the Ion S5 Instrument resulted in identification of all three fusions.

Figure 4. Detection of a MET Exon 14 Skipping Variant in FFPE Research Samples.



The generated custom AmpliSeq HD RNA Assay Panel together with two purified FFPE RNA samples were used for Ion AmpliSeq HD library preparation. Following sequencing and data analysis, the results confirmed the presence of a MET Exon 14 skipping variant in each sample. MET.E11E12 and MET.E6E7 indicate the expression control assays. MET-MET.M13M15 indicates the MET 14 exon skipping target assay.

CONCLUSIONS

We developed a research method to generate libraries from custom assay designs for liquid biopsy blood samples for detection of low frequency variants. This method is compatible with low sample input from cfDNA, FFPE DNA, and FFPE RNA samples. It can also be used for detection of RNA gene fusion variants and exon skipping variants.

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TRADEMARKS/LICENSING

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