The first low input, three-day NGS Ion AmpliSeq™ Methylation Panel and protocol (Poster #142)

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ABSTRACT

Of critical importance to a variety of cancer types is the identification of reliable diagnostic and prognostic DNA methylation biomarkers. Impeding implementation is the absence of streamlined bioinformatics pipelines combined with painless, resolved, flexible multiplexed assays with low input requirements. The recently released Ion AmpliSeq™ Methylation Panel for Cancer Research is the first of its kind and demonstrates the capability to create a targeted panel leveraging the AmpliSeq™ technology on the Ion NGS S5 sequencing platform. The Ion AmpliSeq™ Methylation Panel for Cancer Research combines an easy 3-day protocol with manual or automated library options, low input (~10-20ng starting), and flexible multiplexed approach with quantitative information at single base pair resolution. The bioinformatics analysis has been streamlined into a downloadable plugin providing DNA methylation calls on both Watson and Crick strands and methylated:unmethylated ratios for each CpG. Samples tested to date include standard fresh frozen and FFPE.

INTRODUCTION

A complete workflow has been developed starting from bisulfite conversion and progressing through library construction, template preparation, sequencing and data analysis. The entire end-to-end workflow can be completed in three days (Fig.2). The Ion AmpliSeq™ Methylation Panel for Cancer Research was created using a subset of clinically relevant markers from the BLUEPRINT consortium[1/2]. Amplicons were designed to target both original strands, but only one of the two was selected for the final panel, which contains 40 amplicons, 31 of which are cancer markers associated with colon cancer, prostate cancer, leukemia and lymphoma (Fig.3). The panel was evaluated using two control gDNA samples. The first had an expected average methylation state across all CpGs of >98% and the second <5%. An equal mixture of these two samples was also tested (Fig.4). The analysis report provides the total number of reads covering the target CpGs, and the percentage of those reads that are methylated at both the sample and amplicon level (Fig.6). The AmpliSeq™ methylation technology provided quantitative information with FFPE cancer versus benign samples at base pair resolution, independent of methylation status, compared orthogonally to MSP assay output (Fig.5).
The AmpliSeq™ Methylation Panel for Cancer Research offers a low DNA input, three-day end-to-end workflow with high resolution, targeted and quantitative methylation analysis of clinically relevant targets from low DNA input. The potential exists to design custom methylation panels.

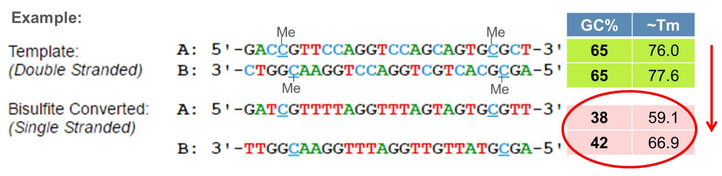
MATERIALS AND METHODS

Consult the bisulfite methylation library construction protocol using the Ion AmpliSeq™ Library Kit Plus and the protocol using the Ion AmpliSeq™ Kit for Chef DL8 for detailed material and methods. The community AmpliSeq™ Methylation Panel for Cancer Research contains one pool at 5x concentration. Sample per Chip: 16 per 530 chip for Chef ExT templating (14 samples + 2 NTC) Genomic DNA or FFPE DNA (Bisulfite converted DNA required for library production) (Fig.2 & 3). Duplicate commercially available control gDNA samples of average methylation state across all CpGs of approximately >98%, <5%, and an equal mixture of the two equaling ~52% methylation states were run (Figure 4). The DNA standards are purified from HCT116 DKO cells that contain genetic knockouts of DNA methyltransferases, DNMT1 (-/-) and DNMT3b (-/-) , and the unmethylated standard has less than 5% methylated DNA (Fig. 4). The human methylated DNA standard has been methylated enzymatically at all CpG dinucleotides by M. SssI methyltransferase (Fig. 4). Per SOP, Unmethylated Lambda DNA was spiked into each sample and primers exist in the panel to determine conversion efficiency with sequencing output files. To analyze the sequencing output from this panel, a Torrent Suite plugin under the name “methylation_analysis” is available on the Themo Fisher Scientific Plugin store. The methylation_analysis plugin performs alignment and methylation calling for amplicons on both the Watson (W) and Crick (C) strands. Each amplicon may have zero, one or more designated CpG targets (hotspots) of interest (Fig. 7). A summary report shows each barcode name along with the sample name, the total number of reads covering the target CpGs, and the percentage of those reads that are methylated (Fig. 6). In addition, for each barcode, text files are generated giving the number of methylated reads, unmethylated reads and percent methylation for each amplicon. There are separate text files for the designated target CpGs, all CpGs in the amplicon insert and all non CpG Cs in the amplicon insert (Fig. 6). This plugin has been tested using the Ion AmpliSeq™ Methylation Panel for Cancer Research only. If you choose to use this plugin for any panel other than the Ion AmpliSeq™ Methylation Panel for Cancer Research you must validate it for your purposes. We can’t attest to the quality of the results with a custom panel. Figure 1 describes the general bisulfite conversion overview.

Figure 1. Bisulfite Conversion Overview

DNA Methylation Bisulfite Conversion Overview:

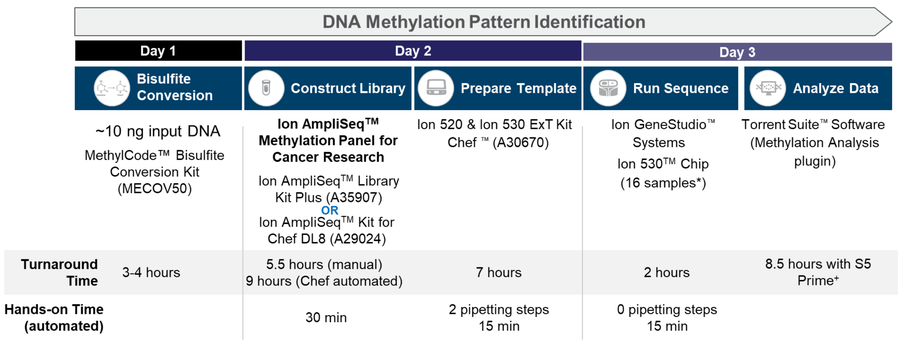
- In humans, DNA methylation occurs at the 5 position of the pyrimidine ring of the cytosine residues within CpG sites to form 5-methylcytosines.
- Non-methyl protected Cs are converted to Ts
- Methyl protected Cs in CpGs are retained as Cs (~1% in humans)
- Bisulfite converted DNA is single stranded and fragmented
- Results in a general decrease in GC%, Tm, and complexity



RESULTS

Figure 2. Ion AmpliSeq™ Methylation Panel for Cancer Research- 3 Day Protocol

High accuracy, ease of use, short time to answer with low DNA Input



Turnaround Time	3-4 hours	5.5 hours (manual) 9 hours (Chef automated)	7 hours	2 hours	8.5 hours with S5 Prime+
Hands-on Time (automated)		30 min	2 pipetting steps 15 min	0 pipetting steps 15 min	

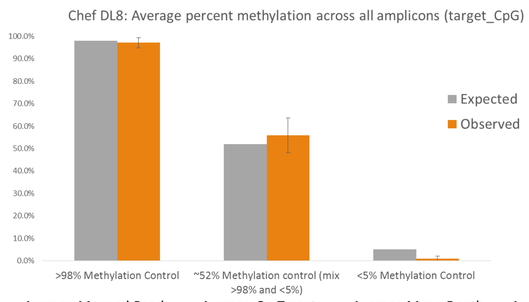
* Includes control. Verified manual workflow with Ion 520™ (4 samples)
+ Analysis time will vary based on S5 and S5 Plus systems, and Ion Chip

Figure 3. Ion AmpliSeq™ Methylation Panel for Cancer Research- Panel Use

No. Pools	Total Amplicons	Target Markers	No. Amplicons	Diseases associated
One Pool	40 amplicons	*Cancer markers*	*31*	*Colon cancer, Prostate Cancer, Leukemia, Lymphoma*
		Technical benchmarking	*7*	-
		Control	*2*	-

- **Sample Type: Human Tissue/Cell Line and FFPE gDNA**
- Recommended **10 to 100ng per sample, ≥ 20ng FFPE**
- **2 Lambda controls** for QC library and sequencing + bisulfite conversion efficiency

Figure 4. Ion AmpliSeq™ Methylation Panel Shows High Correlation With Known Truth



Minimum of **>50 methylation reads total per amplicon**:

- 16 sample barcodes (14 + 2 NTC) on a 530 chip S5
- **5ng input** (SOP 60uL H20 to wells A5 and B5 Chef DL8 protocol)

Average Mapped Reads	Average On Target	Average Mean Depth	Average Uniformity	Average Mean Read Length (bp)
308,500	96.5%	13,242	89.6%	88

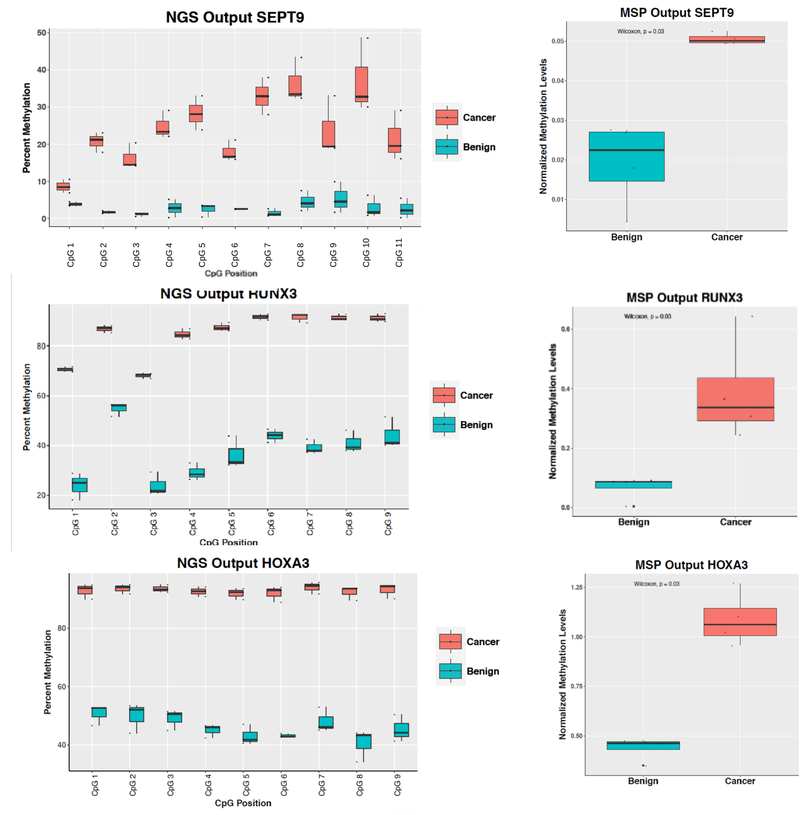
Conversion efficiency % (Based on Lambda spike-in) 99.57%

Duplicate commercially available control gDNA samples of average methylation state across all CpGs of approximately >98%, <5%, and an equal mixture of the two equaling ~52% methylation states were run with the **Ion AmpliSeq™ Methylation Panel for Cancer Research**. This data is based on the data from the control samples of commercially available human methylated & unmethylated DNA standard set. The commercially available DNA standards are purified from HCT116 DKO cells, and the unmethylated standard has less than 5% methylated DNA. The human methylated DNA standard has been methylated enzymatically at all CpG dinucleotides by M. SssI methyltransferase. Per SOP, Unmethylated Lambda DNA was spiked into each sample prior to bisulfite conversion and primers exist in the panel to determine conversion efficiency using sequencing output files. The methylation_analysis plugin performed the alignment and methylation calling

Figure 5. Biomarker and therapeutic target discovery for bladder and prostate cancer

David Berman, MD PhD
Director, Queen’s University Cancer Research Institute

The AmpliSeq™ NGS assay provided quantitative information with FFPE cancer versus benign samples at base pair resolution, independent of methylation status

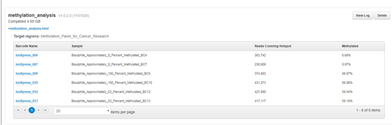


Patel and Pickle et al. 2019 (Unpublished)

Orthogonal testing versus in-lab methylation specific PCR (MSP) was performed with a subset of amplicons from the AmpliSeq™ Methylation Panel for Cancer Research. The AmpliSeq™ Panel subset was used with FFPE cancer versus benign samples (~19-25ng FFPE DNA input) and ran with manual library construction on 530 Chips (Figure 2).

Figure 6. Ion AmpliSeq™ Methylation Panel- Methylation Analysis Plugin

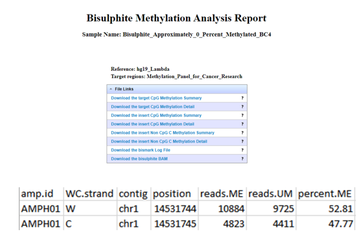
- Summary report provides coverage and % methylation for each barcoded sample



- Watson Coverage Analysis Results
- Crick Coverage Analysis Results
- Download a ZIP report summary.

- Links to coverage analysis results for both strands
- Detailed results for all samples available in zip format

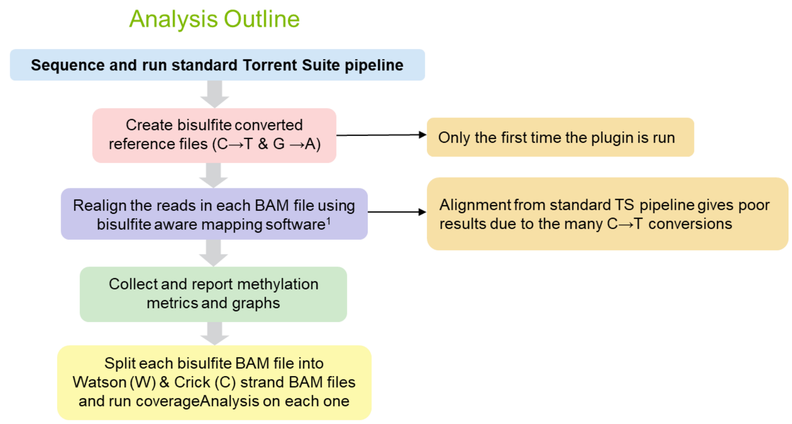
- Number of methylated and unmethylated reads for each amplicon are shown
- Aligned BAM files available for download



amp.id	WC.strand	contig	position	reads.ME	reads.UM	percent.ME
AMPH01	W	chr1	14531744	10884	9725	52.81
AMPH01	C	chr1	14531745	4823	4411	47.77

The target CpG Methylation summary file allows a specific CpG for each amplicon to be chosen and its methylation percentage reported. The insert CpG methylation detail file allows for the methylation percentage of every individual CpG under the amplicon to be individually reported.

Figure 7. Ion AmpliSeq™ Methylation Panel- Methylation Analysis Outline



1. The plugin incorporates a modified version of the bisulfite alignment tools Bismark / TABSAT with tmap

The methylation_analysis plugin is executed after standard Torrent Suite™ analysis pipeline completes. Two bisulfite converted reference files are created during the first use of the plugin only, which will add several extra hours. The plugin realigns all reads using bisulfite aware mapping techniques which are required due to the many C to T conversions in the reads. The output from the plugin is currently a set of text files with methylation calls. The coverageAnalysis plugin is also run separately on the Watson (W) and Crick (C) BAM files generated by the methylation_analysis plugin.

CONCLUSIONS

The first low input, three-day, end-to-end NGS workflow for Ion AmpliSeq™ Methylation panels was showcased here. The streamlined protocol starts at bisulfite conversion and progressing through flexible multiplexed AmpliSeq™ library construction (both manual and automated), template preparation, sequencing and simplified data analysis. The Ion AmpliSeq™ Methylation Panel for Cancer Research was initially tested with commercially available methylation controls and showed high correlation with known truth at low (<5%), middle (~52%) and high (>98%) methylation status. A subset of the panel provided quantitative information with FFPE cancer versus benign samples at base pair resolution, independent of methylation status, and compared orthogonally to MSP assay output. The potential also exists to design custom methylation panels.

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