illumina®

Analytical performance of the AmpliSeq™ for Illumina Focus Panel with FFPE samples

Accurate, sensitive, and precise variant detection in tumor samples.

Introduction

Next-generation sequencing (NGS) has transformed cancer research, enabling variant detection in numerous genes in a single assay. By focusing on genes related to a particular disease or pathway, targeted sequencing provides the advantage of higher depth of coverage. This increased analytical sensitivity may be particularly important for detection of low frequency variants in heterogenous tumor tissues.

The AmpliSeq for Illumina Focus Panel was developed for biomarker analysis in both DNA and RNA concurrently. The panel enables highly accurate mutation detection in 52 genes with known relevance to solid tumors, including lung, colon, breast, ovarian, melanoma, and prostate (Table 1). Researchers can use the panel to investigate single nucleotide variants (SNVs), insertion/deletions (indels), and copy number variants (CNVs) in DNA samples, or gene fusions in RNA samples. The AmpliSeq for Illumina Focus Panel allows rapid and accurate assessment of genomic variation for translational and clinical research studies (Figure 1).

This application note demonstrates the high analytical sensitivity and precision of the AmpliSeq for Illumina Focus Panel for variant detection with reference standards and formalin-fixed, paraffin-embedded (FFPE) tumor samples using the Eppendorf ep*Motion*® in an automated library preparation workflow.

Table 1: AmpliSeq for Illumina Focus Panel gene content

DNA			RNA	
Hotspot genes		CNVs	Fusion drivers	
AKT1	*JAK1*	*ALK*	*ABL1*	*PPARG*
ALK	*JAK2*	*AR*	*ALK*	*RAF1*
AR	*JAK3*	*BRAF*	*AKT3*	*RET*
BRAF	*KIT*	*CCND1*	*AXL*	*ROS1*
CDK4	*KRAS*	*CDK4*	*BRAF*	
CTNNB1	*MAP2K1*	*CDK6*	*EGFR*	
DDR2	*MAP2K2*	*EGFR*	*ERBB2*	
EGFR	*MET*	*ERBB2*	*ERG*	
ERBB2	*MTOR*	*FGFR1*	*ETV1*	
ERBB3	*NRAS*	*FGFR2*	*ETV4*	
ERBB4	*PDGFRA*	*FGFR3*	*ETV5*	
ESR1	*PIK3CA*	*FGFR4*	*FGFR1*	
FGFR2	*RAF1*	*KIT*	*FGFR2*	
FGFR3	*RET*	*KRAS*	*FGFR3*	
GNA11	*ROS1*	*MET*	*MET*	
GNAQ	*SMO*	*MYC*	*NTRK1*	
HRAS		*MYCN*	*NTRK2*	
IDH1		*PDGFRA*	*NTRK3*	
IDH2		*PIK3CA*	*PDGFRA*	

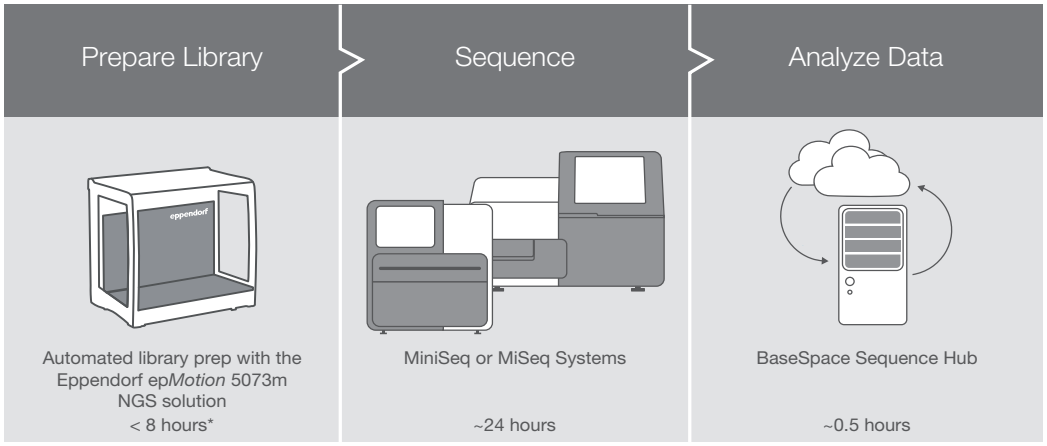

Figure 1: Demonstrated AmpliSeq for Illumina Focus Panel workflow—The AmpliSeq for Illumina Focus Panel is part of an integrated, streamlined NGS workflow that is compatible with automated library preparation, sequencing, and data analysis.
*Indicates time required for sequencing-ready DNA and RNA combined library preparation.

Methods

Samples

Various reference and tumor FFPE samples were procured for use in this study. Reference samples included Quantitative Multiplex Reference Standard genomic DNA (gDNA) (Horizon Discovery Group, Cat no. HD701), OncoSpan gDNA (Horizon Discovery Group, Cat no. HD827), ALK-RET-ROS RNA fusion (Horizon Discovery Group, Cat no. HD784), Structural Multiplex Reference Standard gDNA (Horizon Discovery Group, Cat no. HD753), NA12878 (Coriell Institute for Medical Research), NCI-H2228 (American Type Culture Collection (ATCC), Cat no. CRL-5935), HL-60 (ATCC, Cat no. CCL-240), and the Seraseq Fusion RNA Mix v3 (SeraCare, Cat no. 0710-0431). DNA and RNA extracts from FFPE samples of varying tissue type and quality were obtained from the Illumina Biological Specimen Inventory (BSI) Systems repository (Table 2).

Table 2: Normal and tumor FFPE samples

DNA		RNA	
Sample source	No. of samples	Sample source	No. of samples
Bone	1	Bladder	1
Brain	1	Brain	3
Breast	4	Breast	4
Colon	7	Colon	3
Esophagus	2	Lung	7
Lung	15	Ovary	1
Pancreas	2	Prostate	3
Prostate	2	Skin	3
Skin	1	Stomach	1
Stomach	3	Thyroid	3
Cell lines	8	Unknown	1
Total	46	Total	30

Automated library preparation

Libraries were prepared from 10 ng of input RNA or DNA (or otherwise specified amounts), in accordance with the AmpliSeq for Illumina Focus Panel User Guide. Library preparation in this application note followed an automated protocol using the Eppendorf ep*Motion* 5073m NGS solution. This system is capable of supporting up to 32 samples in a single run (16 DNA and 16 RNA samples). Automating library prep reduces potential variation and human error from multiple, repeated pipetting steps and provides less on-bench time. Reagents from the AmpliSeq for Illumina Library PLUS (96 Reactions, Cat no. 20019102), AmpliSeq for Illumina CD Indexes Set A (Cat no. 20019105), and AmpliSeq for Illumina Library Equalizer (Cat no. 20019171) were loaded on the robot as recommended by the automated methods. Resulting libraries were normalized (manually or automated with the Equalizer Kit) and pooled before loading on to a flow cell for sequencing, as recommended by the MiSeq or MiniSeq System Denature and Dilute Libraries Guide (Document # 15039740 v03 and Document # 1000000002697 v07, respectively).

Sequencing

Prepared libraries were sequenced at 2 × 151 bp on the MiSeq System (Cat no. SY-410-1003) using the MiSeq Reagent Kit v3 (600 cycles, Cat no. MS-102-3003). Libraries were also run on the MiniSeq System (Cat no. SY-420-1001) using the MiniSeq High Output Kit (300 cycles, Cat no. FC-420-1003).

Data analysis

Resulting data was analyzed with the DNA Amplicon App, RNA Amplicon App, or the DNA + RNA Amplicon App, all available in BaseSpace™ Sequence Hub. The DNA + RNA Amplicon App provides integrated analysis of DNA and/or RNA libraries through the DNA Amplicon app for somatic and germline variant calling, OncoCNV caller app for CNV detection, the RNA Amplicon App for fusion calling, and Pindel for structural variant detection. Confirmation of variants was conducted using digital droplet PCR (ddPCR) assay (BioRad) or an orthogonal NGS assay covering similar targets.

Results

Performance with FFPE samples

To demonstrate the analytical performance of the AmpliSeq for Illumina Focus Panel with FFPE tumor samples, 68 total FFPE samples (DNA and RNA) of varying quality obtained from BSI Systems were evaluated. After library prep and sequencing on the MiSeq System, analysis resulted in high performing sequencing metrics (Table 3, Figure 2). The AmpliSeq for Illumina Focus Panel provided highly accurate variant calling in FFPE samples, indicated by predictive metrics such as positive predictive value (PPV), negative predictive value (NPV), recall, sensitivity, and others (Table 4).

Table 3: Sequencing metrics[a] for FFPE samples

	DNA		RNA	
Metric	Mean	StDev	Mean	StDev
On-target	96.4%	4.8%	68%	8%
Coverage uniformity	93.8%	8.9%	—	—
Mean amplicon coverage[b]	3631	1371	2836	560

a. Sequencing metrics are from a representative MiSeq run.
b. The thresholds for metrics for RNA samples were 40% for on-target percentage and 1779 for mean amplicon coverage.

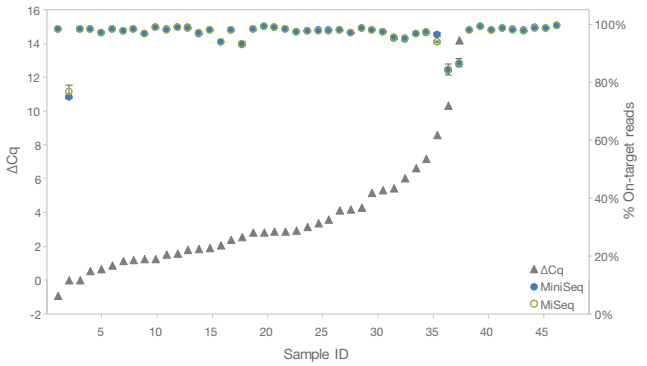

Figure 2: FFPE DNA aligned reads by sample quality—FFPE DNA sequenced on the MiSeq and MiniSeq Systems resulted in high percentage of on-target reads (blue dots and green rings) regardless of sample quality, measured by ΔCq (triangles).

Table 4: Predictive value with FFPE samples

	SNV	Indel	CNV	Fusion[a]
True positive (TP)	19	10	16	3
False positive (FP)	0	0	0	0
True negative (TN)	17	18	13	5
False negative (FN)	0	0	0	0
Total	36	28	29	8
Positive predictive value (PPV) = TP/(TP+FP)	100%	100%	100%	100%
Negative predictive value (NPV) = TN/(TN+FN)	100%	100%	100%	100%
Recall = TP/(TP+FN)	100%	100%	100%	100%
Accuracy = (TP+TN)/(TP+FP+FN+TN)	100%	100%	100%	100%
False positive rate (FPR) = FP/N	0%	0%	0%	0%
False negative rate (FNR) = FN/(TP+FN)	0%	0%	0%	0%
Sensitivity = TP/(TP+FN)	100%	100%	100%	100%
Specificity = TN/(FP+TN)	100%	100%	100%	100%

a. FFPE RNA samples above alignment threshold were analyzed for fusion variants. Results showed confirmed detection of *NTRK1-TPM3*, *EML4-ALK*, and *TMPRSS2-ERG* in the qualified samples. Samples absent for *EML4-ALK* fusion were confirmed negative.

Variant calling accuracy and precision

To demonstrate the high accuracy of variant detection in DNA samples, references HD701 and HD827 were evaluated with the AmpliSeq for Illumina Focus Panel. Results show significant correlation between observed and expected variants above 5% variant allele frequency (VAF) threshold (official AmpliSeq for Illumina VAF cut-off), with 100% of variants (SNV and indels) detected for both standards (Figure 3).

To demonstrate the precision of the AmpliSeq for Illumina Focus Panel, intra-assay variability (correlation between replicates for the same user) and inter-assay variability (correlation between users) were determined using reference variants in HD701 and HD827 samples (Table 5). Repeatability and reproducibility ranged from R^2 correlation coefficient of 0.98 to 1.00 value.

Table 5: Reproducibility and repeatability for reference samples

HD701 reference sample							
	User 1	User 2	User 3		Run 1	Run 2	Run 3
User 1				Run 1			
User 2	0.99			Run 2	0.98		
User 3	0.99	1.00		Run 3	0.99	0.99	

HD827 reference sample							
	User 1	User 2	User 3		Run 1	Run 2	Run 3
User 1				Run 1			
User 2	1.00			Run 2	0.99		
User 3	0.99	1.00		Run 3	0.99	0.99	

Values represent correlation coefficient (R^2) of comparison groups.

Gene fusion detection

To demonstrate the ability of the AmpliSeq for Illumina Focus Panel to recognize structural variants within RNA transcripts, reference samples HD784, NCI-H2228, and the Seraseq Fusion RNA Mix v3 were evaluated. Results showed a 100% call rate for gene fusions within these samples (data not shown).

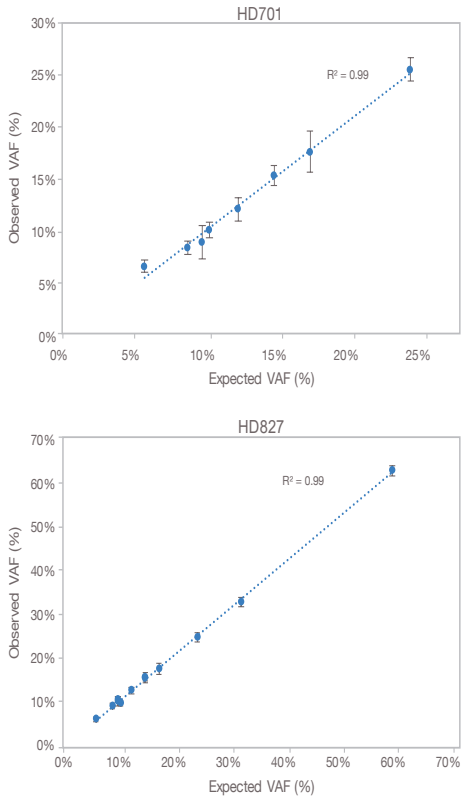

Figure 3: Somatic variant calling above 5% threshold in reference samples—High correlation (R^2 > 0.99) was seen between expected and observed VAF for reference samples (n=20 per data point) with standard deviation.

Assay sensitivity

To demonstrate analytical sensitivity of the AmpliSeq for Illumina Focus Panel, a limit of detection (LOD) experiment was conducted with reference sample HD753. The amount of input gDNA used for library prep was titrated down from 20 ng to 1 ng. Resulting libraries were sequenced and assayed for variant calling across four replicates of

Table 7: Limit of detection for variants in reference HD753

Variant type[a]	Chromosome no.	Gene	Variant	20 ng	10 ng	5 ng[b]	1 ng[b]
				% detected (replicates)			
SNV high GC	chr. 19	*GNA11*	Q209L	100% (4/4)	100% (4/4)	100% (4/4)	100% (4/4)
SNV high GC	chr. 14	*AKT1*	E17K	100% (4/4)	100% (4/4)	100% (4/4)	100% (4/4)
SNV low GC	chr. 3	*PIK3CA*	E545K	100% (4/4)	100% (4/4)	100% (4/4)	100% (4/4)
Long insertion	chr. 7	*EGFR*	V769_D770ins ASV	100% (4/4)	100% (4/4)	100% (4/4)	100% (4/4)
Long deletion	chr. 7	*EGFR*	ΔE746–A750	100% (4/4)	100% (4/4)	100% (4/4)	100% (4/4)
CNV	chr. 7	*MET*	amplification	100% (4/4)	100% (4/4)	75% (3/4)	0% (0/4)
CNV	chr. 2	*MYC-N*	amplification	100% (4/4)	100% (4/4)	100% (4/4)	100% (4/4)

a. Variant information was provided by Horizon Discovery Group as part of the known genotype of this reference sample.
b. The AmpliSeq for Illumina Focus Panel assay recommends 10 ng sample input.

each input amount. With the exception of a CNV amplification in the *MET* gene, all variants assayed were called for all replicates across all dilutions (Table 7).

To further demonstrate assay sensitivity, titration experiments were performed in which reference samples with known mutations were titrated into normal (wildtype) reference samples. In the first experiment, gDNA from HL-60, a cell line with an amplification CNV resulting in 20 copies of the *MYC* gene, was titrated into a diploid background (NA12878 reference genome) to create DNA mixtures containing 20, 10, 8, 6, 4 and 2 copies (baseline) of the *MYC* gene. 10 ng of gDNA of each dilution were prepared, sequenced, and analyzed with the OncoCNV caller app. The AmpliSeq for Illumina Focus Panel enabled detection of all copies of the *MYC* gene, with significant correlation to CNV detection via ddPCR (Figure 4).

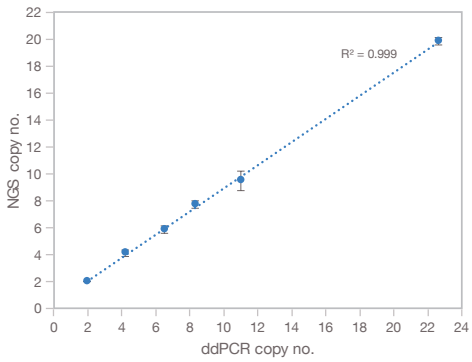

Figure 4: *MYC* CNV limit of detection—Accurate gene copy detection in a dilution series of *MYC* CNV positive DNA into a diploid background with the AmpliSeq for Illumina Focus Panel, with significant correlation to ddPCR.

Two gene fusion LOD experiments were performed. In the first, the amount of input RNA extracted from NCI-H2228, positive for an EML4-ALK fusion, was titrated down from 10 ng to 0.05 ng input into a fusion negative sample (n=4 for each input). After library preparation, sequencing on the MiSeq System, and analysis with the RNA Amplicon app, gene fusion detection was evaluated (Table 8). In the second experiment, NCI-H2228 RNA was heat-degraded to assess fusion detection relative to sample quality as measured by DV150. A known fusion transcript was detected with high senisitvity at 20 copies, which was dependent on read alignment at ≥ 40% (Table 9).

Table 8: Effect of input on *EML4-ALK* fusion detection

Input[a] (ng)	ddPCR copies	No. of expected fusion transcripts	% samples with ≥ 3 called	% samples with ≥ 2 called	% samples with ≥ 1 called
10	5440	3	100%	100%	100%
5	2660	3	100%	100%	100%
0.5	322	3	100%	100%	100%
0.25	130	3	100%	100%	100%
0.1	40	3	0%	50%	100%
0.05	20	3	0%	0%	100%
0	0	0	0%	0%	0%

a. Libraries were prepared using 10 ng total RNA input when combined with a fusion negative sample.

Table 9: Effect of sample quality on *EML4-ALK* fusion detection

DV150[a]	No. of observed fusion transcripts (mean)	Aligned reads	Mean coverage	ddPCR copies
84	3	73%	4377	664
65	3	73%	2478	192
39	2	56%	3166	36
27	1	40%	1779	14.6
16	0	19%	892	2.6
9	0	1%	57	0
9	0.5	0	1	0

a. DV150 is the percentage of RNA fragments that are > 150 nucleotides in size.

Summary

The AmpliSeq for Illumina Focus Panel was developed for biomarker analysis in both DNA and RNA concurrently. The panel enables highly accurate mutation detection in 52 genes with known relevance to solid tumors. This application note demonstrates the high analytical sensitivity and precision of the AmpliSeq for Illumina Focus Panel for FFPE tumor sample variant detection using an automated-compatible workflow.

Learn more

To learn more about the AmpliSeq for Illumina Focus Panel, visit www.illumina.com/products/by-type/sequencing-kits/library-prep-kits/ampliseq-focus-panel.html.

Illumina • 1.800.809.4566 toll-free (US) • +1.858.202.4566 tel • techsupport@illumina.com • www.illumina.com