Application Note

Genomics

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Comparison of Manual and Automated NGS Library Preparation Using the Magnis Dx NGS Prep System with Cancer All-In-One Assay on FFPE Samples

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Introduction

The introduction of hybridization capture-based next-generation sequencing (NGS) methods into clinical practice helps laboratories obtain good quality data even from highly compromised samples such as DNA from formalin-fixed, paraffin-embedded (FFPE) or plasma samples. However, before these technologies can be implemented, molecular diagnostics laboratories need simplified, standardized, and more reproducible library preparation protocols. This study shows how one such method, the Agilent SureSelect HS Cancer All-In-One Lung assay, can be used to accurately confirms variants in non-small cell lung cancer (NSCLC) FFPE samples with known mutations. The SureSelect assay also enables the detection of mutations in clinical cases where the genetic profile is unknown. Moreover, a direct comparison of manual library preparation with automated preparation using the Agilent Magnis Dx NGS Prep system reveals that automation can provide greater yield and increased library complexity compared to manual methods.

Materials and Methods

Sample Selection

23 NSCLC FFPE samples were selected for this study. They had already been well-characterized in prior validation studies and included key variant types including point mutations, deletions, and structural rearrangements (Figure 1). Two control samples were included, consisting of Agilent female control gDNA.

DNA Extraction, Quantification, and Qualification

Genomic DNA (gDNA) extraction and purification was performed with the QIAamp DNA FFPE Tissue Kit for sections taken from FFPE tissues. gDNA was qualified with the Agilent Genomic DNA ScreenTape assay on an Agilent 4200 TapeStation system (Figure 2). The observed DNA quality from these samples was heterogeneous, both in terms of concentration and degradation index (measured by the DNA Integrity Number (DIN)), with DIN values for these samples ranging between 1.9 and 8.1.

DNA Shearing

gDNA samples were normalized where possible to a final concentration of 7 to 8 ng/µL. This allowed 50 ng of gDNA to be sheared in 7 µL following the manufacturer's protocol for the Agilent SureSelect Enzymatic Fragmentation kit. Where concentrations were lower than 7 ng/µL, all material available in 7 µL was loaded and sheared. Shearing conditions were identical regardless of DIN value.

Library Preparation and Qualification

Libraries were prepared using the Agilent Magnis Dx NGS prep system following the manufacturer's protocol for the Agilent SureSelect Cancer All-In-One Lung assay. 12 PCR cycles were used for pre-capture amplification and 14 PCR cycles were used for post-capture amplification. Samples were processed in three independent runs. For the manual library preparations, the SureSelect HS protocol for FFPE samples was followed with the same number of pre- and post-capture cycles (12 and 14, respectively). Library preparations were identical regardless of DIN value.

Sequencing

Libraries obtained from both the Magnis Dx NGS Prep system and manual preparation were quantified with the Agilent High Sensitivity D1000 ScreenTape assay on a 4200 TapeStation system. Based on these results, libraries were then normalized and sequenced on two lanes of an Illumina HiSeq 2500 system.

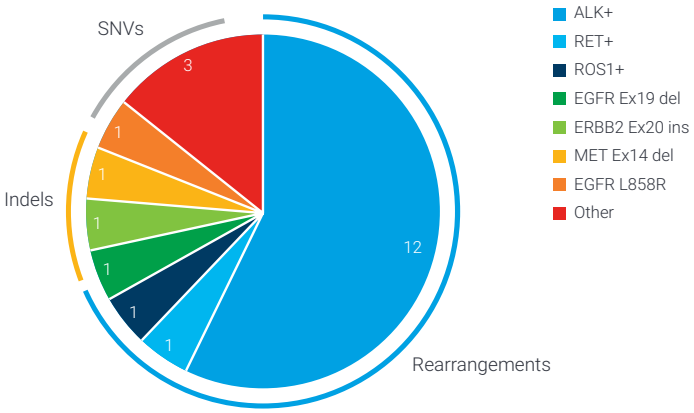

Figure 1. Sample distribution for the genetic variants assayed. Of the 22 NSCLC samples included in this study, over 50% included an *ALK+* mutation. The remaining variants were evenly divided among several other genes.

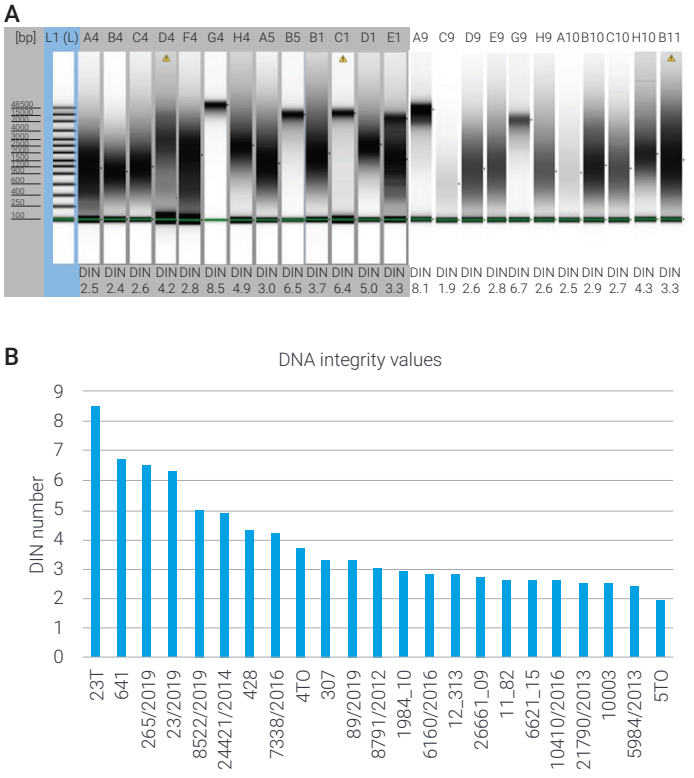

Figure 2. DNA quality metrics assayed by the 4200 TapeStation system. Figures depict a digital electropherogram (panel A) and DNA integrity numbers (panel B) of samples used in this study.

Analysis

FASTQ data were analyzed with the Agilent Alissa Align & Call software using the All-In-One module. The analysis focused on confirming previously identified mutations and potential new ones with a pathogenic effect. Agilent Alissa Interpret software assessed the pathogenicity of the variants that were found.

Sequencing Metrics

Sequencing read length was 100 bp and the number of paired reads were normalized for each sample to ensure the same number of reads were used for both manual and automated preparations. Analysis was performed on library preparation metrics and variant calling accuracy.

Results

Library Preparation and Yields

Each sample was prepared twice, with both manual and automated protocols, using the SureSelect All-In-One (AIO) Lung assay. This approach allowed us to compare recoveries and sequencing metrics. The AIO Lung panel enables the detection of different types of variants at the DNA level including copy number variants (CNVs), single-nucleotide variants (SNVs), insertions and deletions, and translocations. Figure 3 shows the list of genes present in the AIO Lung assay. Manual samples were processed in two batches. Samples processed on the Magnis Dx NGS Prep system were run in four batches on three different instruments.

Metrics: Comparison of Manual versus Automated Methods

Sequencing metrics obtained were compared between libraries prepared either manually or with the Magnis Dx NGS Prep system. Compared to manual processing, Magnis Dx-prepared libraries generally showed a greater yield and library complexity (indicating more sequencing information per sample). Also, libraries prepared on the Magnis Dx NGS Prep system demonstrated less variability than manually prepared libraries, with a %CV of 42% and 52%, respectively. A summary of these metrics can be seen in Figure 4.

SureSelect Cancer AIO lung assay				
AKT1 ●	DDR2 ●	FGFR3	NRAS ●	RET ◆
ALK ● ◆	EGFR ●	KRAS ●	NTRK1 ◆	ROS1 ◆
BRAF ●	ERBB2 ● ■	MEK1 ●	PIK3CA ●	STK11 ●
CD274 ■	FGFR1 ■	MET ● ■	PTEN ● ■	TP53 ●

- One assay, 20 genes, all actionable lung cancer genes, 225 Kb panel design
- 5 fusion/TL target genes: ALK, ROS1, RET, FGFR3, NTRK1
- 5 CNV target genes: ERBB2, CD247/PDL-1, MET1, FGFR1, PTEN

Figure 3. SureSelect Cancer All-In-One Lung assay gene content.

Figure 4. Library yield and complexity for manual versus Magnis Dx-prepared NGS libraries. Libraries prepared on the Magnis Dx NGS Prep system exhibited both greater yield (Panel A) and library complexity (Panel B) compared to manual preparations.

Automated and manual processing resulted in similar sequencing coverage across samples with a mean of more than 100X coverage of on-target reads for 94% of the manually prepared library versus 96% for automated libraries (Figure 5).

Variant Analysis Results

The AIO module of Alissa Align & Call easily displayed variant types grouped into three main categories: SNVs, CNVs, and fusions/translocations.

SNVs: Both Magnis Dx-based and manual methods gave comparable SNV results for variants detected and their variant allele frequency (VAF) with a regression of 0.9933% (Figure 6). Among the variants that were confirmed, *MET* exon 14 skipping was detected with a VAF of 20.5 and 27% (Figure 7). Similarly, a low-frequency deletion with a VAF of 5.8 and 5.9% in exon 19 of *EGFR* was confirmed (Figure 8).

Fusions: We were able to detect fusions in *ALK, ROS*, and *RET* genes. Except for one *ALK* variant, all of the expected fusions were confirmed in both manually prepared and Magnis Dx-prepared samples (Table 1). In one sample the *ALK* translocation even was detected only in the Magnis Dx-processed sample. This sample had overall higher sequencing coverage in the Magnis Dx processing which likely lead to the fusion detection.

Table 1. List of fusions detected.

Target gene	Samples tested	Detected manually	Detected with Magnis Dx	Fusions identified
ALK	12	11	12	EML4-ALK (11)
ROS1	1	1	1	CD74-ROS1
RET	1	1	1	KIF5B-RET

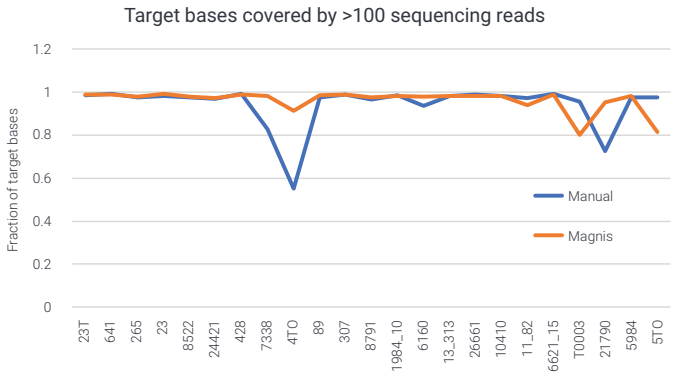

Figure 5. Percentage of target bases that exhibited greater than 100X coverage. Both automated and manual systems exhibited similar coverage with 96 and 94%, respectively, of bases having greater than 100X coverage.

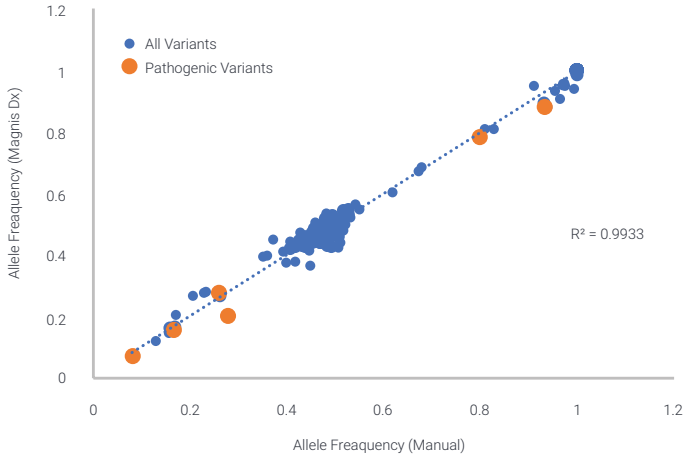

Figure 6. Correlation of VAFs obtained from manual and Magnis Dx-prepared libraries. Both automated and manual VAFs exhibited a high degree of correlation.

Conclusion

Translocations, SNVs, and indels were successfully detected in routine clinical samples using the Lung Cancer All-in-One assay in libraries prepared with both manual and automated methods. There was a high concordance in variants detected between manual and automated methods, although slightly better recovery was observed in the libraries prepared with the Magnis Dx NGS Prep system system (particularly for the worst quality DNA samples)-(data not shown).

Sample processing using the fully automated Magnis Dx NGS Prep system gave increased library complexity for challenging samples. This feature led to a discrepancy in the detection of an *EML4-ALK* translocation that was detected in libraries prepared with the Magnis Dx NGS Prep system system, but not with manual library preparation. Manually prepared libraries exhibited a higher variance than could be explained by the drop out alone.

Overall, the Magnis Dx NGS Prep system demonstrated ease of use, reproducibility, and reliability in the detection of different variant types. These benefits make it a viable option for molecular pathology laboratories that perform routine testing, particularly with the automated sample quality control afforded by the TapeStation system.

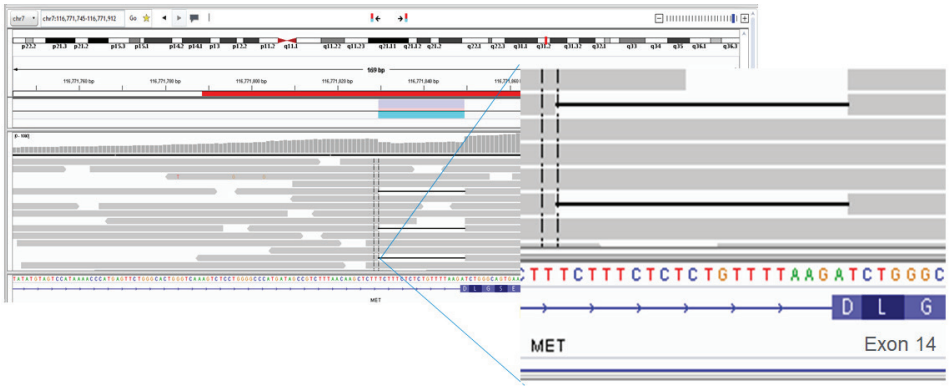

Figure 7. Depiction of a 20 bp deletion of the *MET* exon 14 splice acceptor site. Automated preparation on the Magnis Dx system resulted in a detection rate 6.5% higher (27%) than that of manual methods.

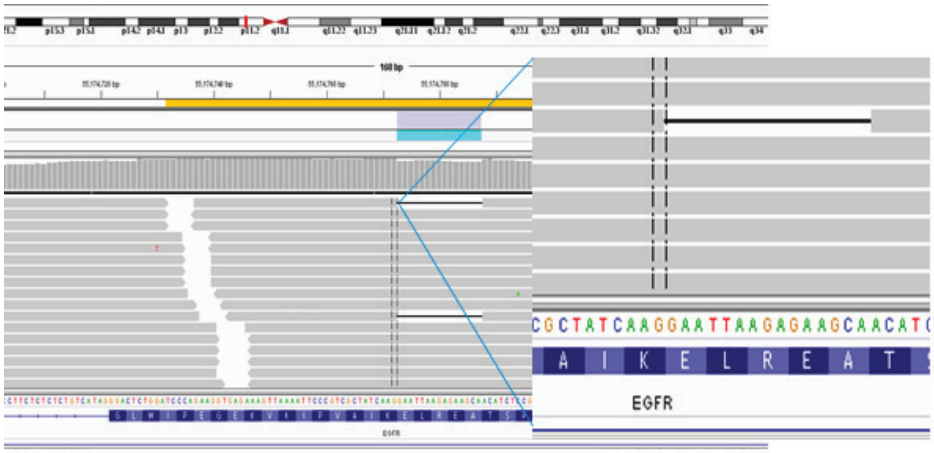

Figure 8. Depiction of a 15 bp in-frame deletion in exon 19 of *EGFR*.

Table 2. The following list of products were utilized in this publication.

Sample QC	
Product Description	**Part Number**
Genomic DNA ScreenTape assay	5067-5365
Genomic DNA Reagents	5067-5366
4200 Tapestation system	G2991BA
D1000 ScreenTape	5067-5582
D1000 Reagents	5067-5583
Library Preparation and Target Enrichment	
Product Description	**Part Number**
Magnis Dx NGS Prep system	K1007AA
SureSelect XT HS Enzymatic Fragmentation kit	5191-4080
Magnis SureSelect XT HS, 1 - 500 kb, ILMN, 96	G9731B (design ID A3097591 All in One Lung Assay)
SureSelect Cancer All-In-One Lung HS, 1 -32	G9706R
Data Analysis and Reporting	
Product Description	**Part Number**
Alissa A&C Tier 1	G5357AA-103
Alissa Interpret Tier 1	K5852AA-103

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