Automating Archer's FUSION*Plex*™-HT and VARIANT*Plex*™-HT Library Prep Workflows on Hamilton Microlab STAR Liquid Handlers for High-Throughput Next-Generation Sequencing

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Introduction

With expanded utilization and reduced costs of Next-Generation Sequencing (NGS), it has become increasingly critical for laboratories to adapt their manual library preparation workflows for use with Automated Liquid Handling (ALH) devices. ALHs have many benefits including increased sample throughput, decreased operator hands-on time, and improved sample preparation consistency. We have developed, tested, and optimized a fully walk away solution for Archer's FUSION*Plex*™-HT (FP) and VARIANT*Plex*™-HT (VP) Anchored Multiplex PCR (AMP™) liquid chemistry which meets all these requisites, while maintaining library quality.

Methods

Here we demonstrate the performance of our internally developed and liquid class optimized method for Hamilton's Microlab STAR NGS instruments. We used a range of input masses and commercially-available reference material input of varying qualities to generate Archer FP and VP libraries across six automated runs. Archer catalog panels used in this study range in size, targeting anywhere from 17-185 genes. All libraries were sequenced using an Illumina® NextSeq 2000 System and data analysis was performed using the Archer Analysis v7 bioinformatics pipeline. Plates were laid out as seen below to confirm minimal edge, batch, and time effects on library quality. FFPE reference inputs were extracted using Qiagen AllPrep DNA/RNA FFPE Kit. All extracted nucleic acid was diluted to the appropriate concentration for library preparation. Inputs were all quantified in advance with Invitrogen™ Qubit™ Quantification Assays appropriate for the input type and quality.

	1	2	3	4	5	6	7	8	9	10	11	12
A	5ng FFPE RNA	50ng FFPE RNA	10ng FFPE RNA									
B	10ng FFPE RNA	5ng FFPE RNA	50ng FFPE RNA									
C	50ng FFPE RNA	10ng FFPE RNA	5ng FFPE RNA									
D	5ng FFPE RNA	50ng FFPE RNA	10ng FFPE RNA									
E	10ng FFPE RNA	5ng FFPE RNA	50ng FFPE RNA									
F	50ng FFPE RNA	10ng FFPE RNA	5ng FFPE RNA									
G	5ng FFPE RNA	50ng FFPE RNA	10ng FFPE RNA									
H	10ng FFPE RNA	5ng FFPE RNA	50ng FFPE RNA									

EMPTY

	1	2	3	4	5	6	7	8	9	10	11	12
A	5ng FFPE RNA	50ng FFPE RNA	10ng FFPE RNA									
B	10ng FFPE RNA	5ng FFPE RNA	50ng FFPE RNA									
C	50ng FFPE RNA	10ng FFPE RNA	5ng FFPE RNA									
D	5ng FFPE RNA	50ng FFPE RNA	10ng FFPE RNA									
E	10ng FFPE RNA	5ng FFPE RNA	50ng FFPE RNA									
F	50ng FFPE RNA	10ng FFPE RNA	5ng FFPE RNA									
G	5ng FFPE RNA	50ng FFPE RNA	10ng FFPE RNA									
H	10ng FFPE RNA	5ng FFPE RNA	50ng FFPE RNA									

EMPTY

Figure 1/2. FUSION*Plex*™-HT, FFPE RNA: Inputs consisted of 5ng, 10ng, and 50ng of SeraCare Seraseq® FFPE Tumor Fusion RNA v4 Reference Material RNA. Libraries were prepared with the FUSION*Plex*™-HT Lung v2 and FUSION*Plex*™-HT Pan Solid Tumor v2 panels for small and large panel compatibility assessment, respectively.

	1	2	3	4	5	6	7	8	9	10	11	12
A	50ng Moderate	50ng Moderate	10ng Severe	50ng Moderate	10ng Severe	200ng Moderate						
B	10ng Severe	50ng Severe	50ng Moderate	50ng Severe	10ng Moderate	200ng Severe						
C	50ng Moderate	10ng Moderate	50ng Severe	200ng Moderate	50ng Severe	10ng Severe						
D	50ng Severe	10ng Severe	1200ng Severe	200ng Severe	50ng Moderate	10ng Moderate						
E	10ng Severe	50ng Moderate	200ng Moderate	50ng Moderate	10ng Severe	50ng Severe						
F	10ng Severe	50ng Severe	10ng Moderate	50ng Severe	10ng Moderate	50ng Severe						
G	200ng Severe	10ng Moderate	50ng Severe	10ng Moderate	50ng Severe	10ng Severe						
H	200ng Moderate	10ng Severe	50ng Moderate	10ng Severe	50ng Moderate	10ng Moderate						

EMPTY

	1	2	3	4	5	6	7	8	9	10	11	12
A	50ng Moderate	50ng Moderate	10ng Severe									
B	10ng Severe	50ng Severe	50ng Moderate									
C	50ng Moderate	10ng Moderate	50ng Severe									
D	50ng Severe	10ng Severe	200ng Severe									
E	10ng Moderate	200ng Moderate	200ng Moderate									
F	10ng Severe	50ng Severe	10ng Moderate									
G	200ng Severe	10ng Moderate	50ng Severe									
H	200ng Moderate	10ng Severe	50ng Moderate									

EMPTY

Figure 3/4. VARIANT*Plex*™-HT, FFPE DNA, Moderately and Severely Compromised: Inputs consisted of 10ng, 50ng, and 200ng of SeraCare Seraseq® Compromised FFPE Tumor DNA Reference Material (moderate) interleaved with Horizon Quantitative Multiplex Reference Standard fcDNA (severe) at the same input masses. Libraries were prepared with the VARIANT*Plex*™-HT Solid Tumor Focus v2 (top) and VARIANT*Plex*™-HT Pan Solid Tumor (bottom) panels for small and large panel compatibility assessments, respectively.

	1	2	3	4	5	6	7	8	9	10	11	12
A	50ng Myc. DNA	50ng rGiaB	50ng mGiaB	50ng Myc. DNA	50ng rGiaB	50ng mGiaB	50ng Myc. DNA	50ng rGiaB	50ng mGiaB	50ng Myc. DNA	50ng rGiaB	50ng mGiaB
B	50ng mGiaB	50ng rGiaB	50ng Myc. DNA	50ng mGiaB	50ng rGiaB	50ng Myc. DNA	50ng mGiaB	50ng rGiaB	50ng Myc. DNA	50ng mGiaB	50ng rGiaB	50ng Myc. DNA
C	50ng rGiaB	50ng mGiaB	50ng rGiaB	50ng rGiaB	50ng mGiaB	50ng rGiaB	50ng mGiaB	50ng rGiaB	50ng mGiaB	50ng Myc. DNA	50ng rGiaB	50ng mGiaB
D	50ng mGiaB	200ng Myc. DNA	50ng mGiaB	50ng mGiaB	200ng Myc. DNA	50ng mGiaB	50ng Myc. DNA	50ng rGiaB	50ng mGiaB	50ng Myc. DNA	50ng rGiaB	50ng mGiaB
E	50ng Myc. DNA	50ng rGiaB	50ng rGiaB	50ng Myc. DNA	50ng rGiaB	50ng rGiaB	200ng Myc. DNA	50ng mGiaB	50ng mGiaB	200ng Myc. DNA	50ng mGiaB	50ng mGiaB
F	50ng rGiaB	50ng mGiaB	50ng mGiaB	50ng rGiaB	50ng mGiaB	50ng mGiaB	50ng rGiaB	50ng mGiaB	50ng mGiaB	50ng mGiaB	50ng mGiaB	50ng Myc. DNA
G	50ng mGiaB	50ng rGiaB	50ng mGiaB	50ng rGiaB	50ng mGiaB	50ng rGiaB	50ng mGiaB	50ng rGiaB	50ng mGiaB	50ng mGiaB	50ng Myc. DNA	50ng mGiaB
H	50ng rGiaB	50ng Myc. DNA	50ng rGiaB	50ng mGiaB	50ng Myc. DNA	50ng rGiaB	50ng rGiaB	50ng Myc. DNA	50ng rGiaB	50ng mGiaB	50ng Myc. DNA	50ng rGiaB

Figure 5. VARIANT*Plex*™-HT Core Myeloid, Genomic and Myeloid DNA: Inputs consisted of 50ng of Coriell Institute Genome in a Bottle (GiaB) which was loaded to the input plate alternating between male and female GiaB (m/fGiaB). Horizon Myeloid DNA Reference Standard was loaded at 10ng, 50ng, and 200ng input masses distributed among the GiaB inputs to confirm no sample-to-sample contamination. Libraries were prepped with the VARIANT*Plex*™-HT Core Myeloid panel.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10ng Myc. DNA	50ng Ref. RNA	50ng Ref. RNA	10ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	10ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	10ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA
B	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA
C	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA
D	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA
E	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA
F	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA
G	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA
H	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA	50ng Ref. RNA	50ng Myc. RNA	50ng Ref. RNA

Table 6. FUSION*Plex*™-HT Myeloid, Universal Reference and Myeloid RNA: Inputs consisted of 50ng of Takara Reference RNA for real-time qPCR which was loaded to the input plate with 10ng and 50ng of SeraCare Seraseq® Myeloid Fusion RNA Mix distributed among the reference RNA to confirm no sample-to-sample contamination. Libraries were prepped with the FUSION*Plex*™-HT Myeloid panel.

Results – VARIANT*Plex*-HT Solid Tumor Focus V2

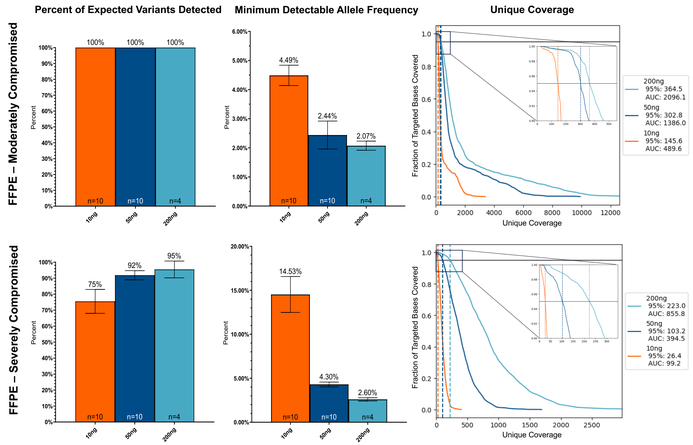

Figure 7. High Sensitivity when Calling Solid Tumor Variants with a Small Catalog Panel in Moderately and Severely Compromised FFPE DNA Inputs. In moderately compromised FFPE DNA inputs (SeraCare), 240/240, 240/240, and 96/96 expected variants were detected in 10ng, 50ng, and 200ng replicates, respectively. In severely compromised FFPE DNA inputs (Horizon), 83/110, 101/110, and 42/44 expected variants were detected in 10ng, 50ng, and 200ng replicates, respectively. The libraries generated had adequate unique fragment coverage to detect variant allele frequency (VAF) down to 2.075% in the moderately compromised inputs, and 2.60% in the severely compromised inputs with 95% confidence. The VP Solid Tumor Focus v2 panel contains 575 primers targeting 20 genes commonly mutated in solid tumors and microsatellite instability (MSI).

Results – VARIANT*Plex*-HT Pan Solid Tumor

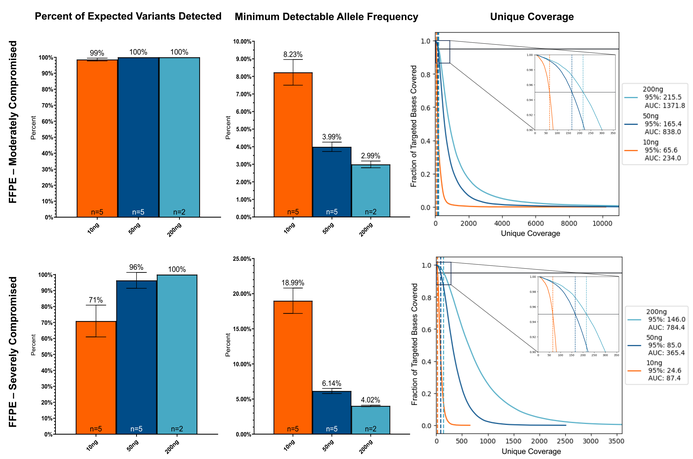

Figure 8. High Sensitivity when Calling Solid Tumor Variants with a Large Catalog Panel in Moderately and Severely Compromised FFPE DNA Inputs. In moderately compromised FFPE DNA inputs (SeraCare), 138/140, 140/140, and 56/56 expected variants were detected in 10ng, 50ng, and 200ng replicates, respectively. In severely compromised FFPE DNA inputs (Horizon), 39/55, 53/55, and 22/22 expected variants were detected in 10ng, 50ng, and 200ng replicates, respectively. The libraries generated had adequate coverage to detect VAF as low as 2.99% in the moderately compromised inputs, and 4.02% in the severely compromised inputs with 95% confidence. The VP Pan Solid Tumor panel contains 9878 primers targeting 185 genes commonly mutated in solid tumors, as well as microsatellite instability (MSI) and tumor mutational burden (TMB) status.

Results – VARIANT*Plex*-HT Core Myeloid

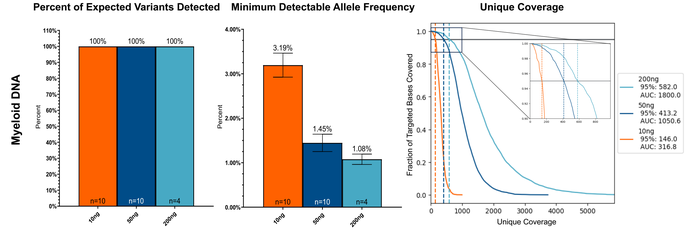

Figure 9. Amazing Ability to Call Variants in Blood. In myeloid variant positive inputs (Horizon) 200/200, 200/200, and 80/80 expected variants were detected in 10ng, 50ng, and 200ng input replicates, respectively. No unexpected variants were detected in myeloid variant negative inputs (m/fGiaB). The high-quality DNA input allowed for great library coverage and VAF detection as low as 1.08%. The VP Core Myeloid panel contains 748 primers targeting 37 gene commonly mutated in myeloid malignancies.

Results – VARIANT*Plex*-HT Mean Fragment Size

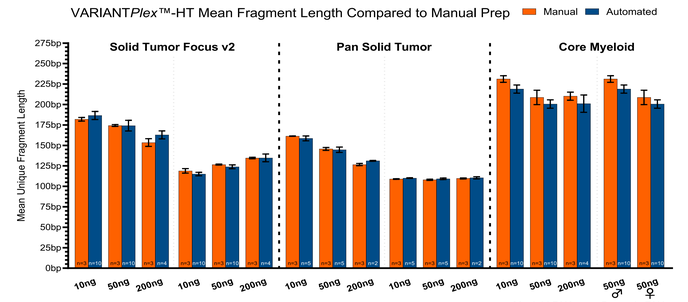

Figure 10. Minimal to No Difference in Library Mean Fragment Sizes Between Manual and Automated Preps. Library fragment lengths correlate with library quality, with longer fragment lengths resulting in better coverage and thus increased confidence for calling variants. DNA isolated from FFPE, especially with more compromised inputs, tend have shorter fragments than genomic DNA isolated from whole blood. Automated applications often struggle to retain larger average fragment size in completed libraries, however, our optimized method produces libraries with a similar mean fragment size to manual preps. Not shown here, median fragment size and skewness is also very similar between manual and automated preps.

Results – FUSION*Plex*-HT Detected Fusions

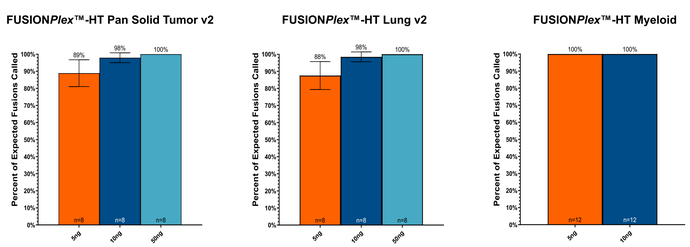

Figure 11. High Sensitivity Calling of Expected Fusions at Low Input Masses Across Several Catalog Panels. In FFPE RNA Inputs (SeraCare), 112/128, 126/128, 128/128 expected fusions were detected in 5ng, 10ng, and 50ng replicates with the Lung v2 panel, respectively. 128/144, 141/144, and 144/144 expected fusions were detected with the Pan Solid Tumor v2 panel for the same input and input masses, respectively. The FP Lung v2 panel contains 323 primers targeting 17 genes commonly mutated in non-small cell lung cancer (NSCLC), and the FP Pan Solid Tumor v2 panel contains 1086 primers targeting 137 genes commonly mutated in solid tumors. In myeloid positive RNA inputs, 96/96 and 96/96 expected fusions were detected in 5ng and 10ng replicates with the FP Myeloid panel, respectively. This panel contains 507 primers targeting 84 genes commonly mutated in myeloid malignancies. No unexpected fusions were detected in myeloid negative inputs (Universal Reference RNA).

Results – Timing

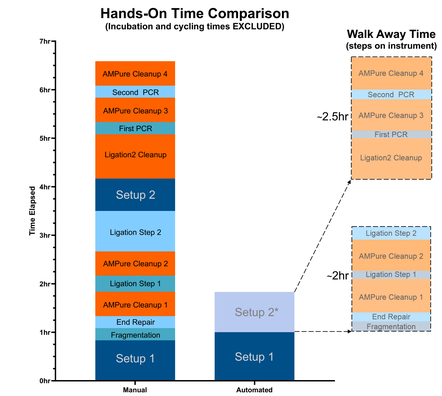

Figure 12. Heavily Reduced Hands-On Time when Compared to an Expert Manual Operator. Automated library prep for a 48 sample VARIANT*Plex*™-HT workflow. Times do not include any incubations or PCR cycling as this would not be considered hands-on time for either workflow. Total elapsed time for each prep condition was approximately 6.5hr, excluding incubation and PCR cycling. No time lost to automated processing.

**Automated workflow was broken up across two days, but CAN be run end-to-end in full walk away mode if desired.*

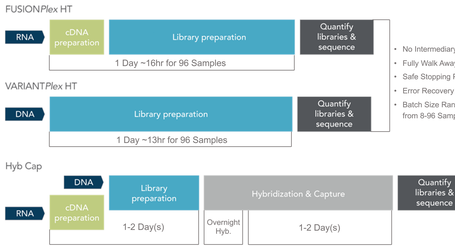

Figure 13. Flexible and Expedient Sample Processing. Either chemistry can be run in a single day, end-to-end, fully walk away, with no touch points beyond the initial instrument setup. Our Hamilton method can run anywhere between 8 and 96 samples at a time for either FUSION*Plex*™-HT or VARIANT*Plex*™-HT chemistries. Total run time is approximately 13 hours for VP and 16 hours for FP when prepping a full 96-well plate. All protocol designed safe stopping points are enabled and available to the user, allowing breakup of the workflow if that is best for your labs sample processing procedure.

Conclusions

Our internally developed method is optimized to produce high quality libraries, using a range of panel sizes, across multiple input types and input masses. Library quality is reflected in the ability to call all expected variants and fusions when using nominal input mass and quality.

Our highly flexible method overcomes many of the typical challenges of automated library preparation, while also focusing on ease-of-use by allowing a large range batch sizes, no unnecessary touchpoints, and the option to do preps end-to-end or in chunks, both with walk-away capability.

This Hamilton method and other developed scripts are currently available for use and installation at your site on your instrument. For more information, follow the QR code below, which takes you to the Archer/IDT Automation intake form.

Scan to request your script

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