firefly®+ Agilent SureSelect Max DNA Library Preparation with Enzymatic Fragmentation & Target Enrichment using Fast Hybridization

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Overview

The Agilent SureSelect Max DNA Library Preparation with Enzymatic Fragmentation Kit is a next-generation sequencing (NGS) library preparation solution designed to convert genomic DNA into sequencing-ready libraries, using enzyme-based fragmentation.

Agilent SureSelect Max Target Enrichment with Fast Hybridization is a hybrid-capture–based target enrichment method used in NGS workflows to selectively enrich genomic regions of interest prior to sequencing. The method is optimized for use with SureSelect Max DNA ibraries, on Illumina and Element Aviti Cloudbreak Freestyle sequencers, and incorporates an accelerated hybridization chemistry that significantly reduces total workflow time while maintaining high capture efficiency and uniformity.

SPT Labtech firefly+ protocols have been developed to run Agilent SureSelect Max DNA Library Preparation with Enzymatic Fragmentation and SureSelect Max Target Enrichment using Fast Hybridization. The protocols run in 96 well plate format and utilize the on-deck thermocycler (ODTC) on firefly+ to allow walkaway workflows. Flexible run configurations allow a user to run from 1 to 12 columns of library preparation and 1 to 2 columns of pre-capture pools or individual libraries through Target Enrichment. Multiple different target enrichment panels can be run together on the same run using the same hybridization conditions.

Here, we demonstrate how these workflows have been automated on firefly+. We provide details on the steps performed in each protocol, together with data to demonstrate the performance of these protocols in generating high quality libraries for sequencing.

The Agilent SureSelect Max DNA Library Preparation with Enzymatic Fragmentation and Target Enrichment using Fast Hybridization firefly+ protocols are available to download and run from the SPT Labtech firefly community.

Protocol highlights

- Full walkaway library preparation protocol
- Flexibility to process from 1 to 12 columns of library preparation in a 96 well plate format. Generating fully adapted molecules ready for sequencing. These libraries can be further enriched with various panels.
- Target Enrichment is split into 2 walkaway protocols, before and after amplification of captured libraries
- Flexibility to run either 1 to 2 columns of pools or samples through the target enrichment workflow downstream of library preparation
- The ODTC on firefly+ allows all incubation and thermocycling steps to take place on the firefly+ - including probe hybridization and hot wash steps

firefly+ protocols

Protocol name	firefly+ run time
SureSelect Max DNA Library Preparation	3 hours 50mins (96 sample run)
SureSelect Max Target Enrichment part 1	4 hours 35mins (8 pool run)
SureSelect Max Target Enrichment part 2	1 hour 12mins (8 pool run)

Table 1. firefly+ protocol names and run times

Protocol overview

SureSelect Max DNA Library Preparation

The firefly+ protocol is based on the manual workflow outlined in the Agilent SureSelect Max DNA Library Preparation with Enzymatic Fragmentation For Illumina Platform NGS Protocol (Version A0 September 2024).

The firefly+ protocol (SureSelect Max DNA Library Preparation) automates the following steps from chapter 2 of the manual protocol, after which an optional QC can be performed:

- **Step 2.** Fragment, end-repair, and 3'-dA-tail the DNA (Frag/A-Tail)
- **Step 3.** Ligate the adaptor
- **Step 4.** Purify libraries using magnetic purification beads
- **Step 5.** Amplify and index the libraries
- **Step 6.** Purify amplified libraries using magnetic purification beads

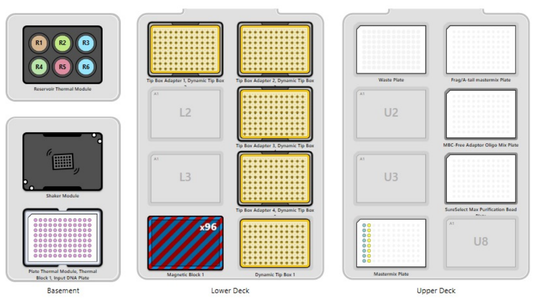

Figure 1. firefly starting deck layout for a 96-sample library preparation run. Deck Layout – Basement: Reservoirs R1 – SureSelect Max Purification Beads, R2 – Nuclease Free Water, R3+R6 - 70% Ethanol, R4 - Amplification Master Mix, R5 - Ligation Master Mix, Plate Thermal Module - 96 sample Thermo Adapter Block and Input DNA Plate. Lower Deck: L1, L5, L6, L7 - 100µL 96 format ATL tips on ATL 35-125µL Tip Stands, L8 - 100µL 96 format ATL tips, L4 – Alpaqua Magnum FLX 96 sample Magnetic Block. Upper Deck: U1 - Waste Plate, U4 Mastermix Plate, U5 Frag/Atail mastermix Plate, U6 – MBC Free Adapter Oligo Mix Plate, U7 – SureSelect Max Purification Bead Plate

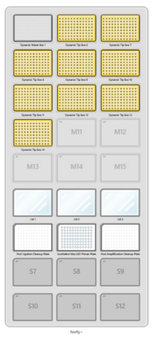

Figure 2. firefly+ starting layout for a 96-sample library preparation run. Deck Layout M1 – Empty ATL tip box, M2-M10 100µL 96 format ATL tips, S1-S3 Plate Lids, S4 – Post Ligation Cleanup Plate, S5 – SureSelect Max UDI Primer Plate, S6 – Post Amplification Cleanup Plate

SureSelect Max Target Enrichment

The firefly+ protocols are based on the manual workflow outlined in the Agilent SureSelect Max Target Enrichment using Fast Hybridization For NGS using the Illumina Platform Protocol (Version A0 September 2024). These protocols support both the post-capture pooling workflow and the pre-capture pooling workflow.

SureSelect Max Target Enrichment part 1

The first firefly+ Target Enrichment protocol automates the following steps from chapter 2 of the manual protocol:

- **Step 2.** Hybridize libraries to the SureSelect probe
- **Step 3.** Prepare streptavidin beads and buffers for capture
- **Step 4.** Capture the hybridized libraries

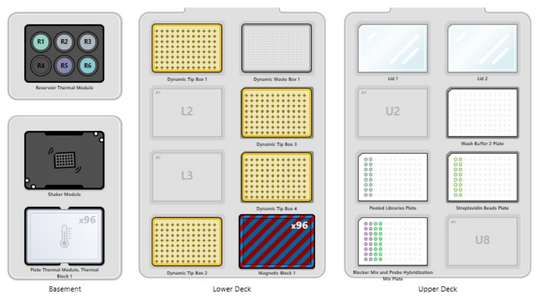

Figure 3. firefly starting deck layout for a 16 sample Target Enrichment run (Part 1). Deck Layout – Basement: Reservoirs R1 – SureSelect Bead Binding Buffer, R2+R3 – Wash Buffer 2, R5 – Nuclease-Free Water, R6 – Wash Buffer 1. Lower Deck: L1+L4+L6+L7 - 100µL 96 format ATL tips, L4 – Empty ATL tip box, L8 - Alpaqua Magnum FLX 96 sample Magnetic Block. Upper Deck: U1+U5 – Plate Lids, U3 – Pooled Libraries Plate, U4 – Blocker Mix and Probe Hybridisation Mix Plate, U6 – Wash Buffer 2 Plate, U7 – Streptavidin Beads Plate

Figure 4 firefly+ starting layout for a 16 sample Target Enrichment run (Part 1). Deck Layout M1 - 100µL 96 format ATL tips, M15 – Waste Plate, S3+S4+S5 – Plate Lids on Plates

SureSelect Max Target Enrichment part 2

The second firefly+ Target Enrichment protocol follows on and automates the following steps from chapter 2 of the manual protocol:

- **Step 5.** Amplify the captured libraries
- **Step 6.** Purify the final libraries using magnetic purification beads

QC and quantification of the final libraries can then be performed.

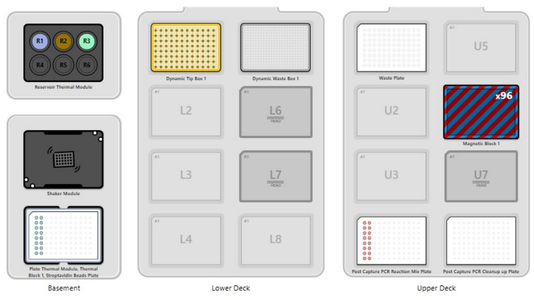

Figure 5. firefly starting deck layout for a 16 sample Target Enrichment run (Part 2). Deck Layout – Basement: Reservoirs R1 – 70% Ethanol, R2 – SureSelect Max Purification Beads, R3 – Low TE Buffer, Plate thermal Module - 96 sample Thermo Adapter Block and Streptavidin Beads Plate. Lower Deck: L1 - 100µL 96 format ATL tips, L4 – Empty ATL Tip Box. Upper Deck: U1 – Waste Plate, U4 – Post Capture PCR Reaction Mix Plate, U6 - Alpaqua Magnum FLX 96 sample Magnetic Block, U8 – Post Capture PCR Cleanup Plate.

Figure 6. firefly+ starting layout for a 16 sample Target Enrichment run (Part 2). Deck Layout – S1 – Plate Lid.

Protocol performance

SureSelect Max DNA Library Preparation with Enzymatic Fragmentation

A 96 sample SureSelect Max DNA Library Preparation with Enzymatic Fragmentation run was performed on firefly+. The run included 90 high-quality genomic DNA samples – 45 replicates of Coriell NA12878 and 45 replicates of Coriell NA24385. 200ng was used as the starting input amount. 6 No Template Controls (NTCs) were also included and spread across the plate.

The run included a 10-minute enzymatic fragmentation time and 7 cycles of library amplification and had a run time of 3 hours 50 minutes. The resulting libraries were then diluted 1 in 10 and ran on the Agilent Fragment Analyzer (Agilent, DNF-474 HS NGS Fragment Kit). The average library size across the 90 DNA input samples was 341 base pairs (bp), with a %CV of 3.0%.

The resulting libraries were quantified by qPCR to determine their size adjusted concentration (nM) using a LightCycler 480 System (Roche, KAPA Library Quantification kit). The average library concentration across the plate was 4490nM with a CV of 9.5%. No significant contamination was observed in the 6 NTCs, with the average quantification value being 0.2nM across the NTCs, 0.004% of the average concentration shown by the positive samples.

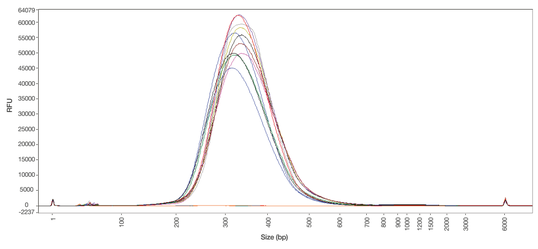

Figure 7. Example Fragment Analyzer trace overlays for 16 samples from rows C and F of the 96-sample run, including the 6 NTCs. NTCs show no visible library peak.

SureSelect Max Target Enrichment

	SureSelect Human All Exon V8 (SSEL V8)	SureSelect Cancer CGP
Sample Type	gDNA, FFPE, FF	gDNA, FFPE, FF
Input amount	10 – 200ng	10 – 200ng
Targets	Human All Exon: RefSeq, GENCODE, CCDS	679 genes
Total Capture Size	35.1 Mb	2.7 Mb
Hybridization	Fast, 90-minutes	Fast, 90-minutes
Part number	5191-6878	5280-0037

Table 2. Catalog Panels used for Target Enrichment performance assessment

Following library preparation, target enrichment was performed on firefly+ with SureSelect Max Target Enrichment part 1 and part 2. SureSelect Max Target Enrichment part 1 includes hybridization and capture. SureSelect Max Target Enrichment part 2 amplifies captured libraries followed by clean-up to generate enriched libraries ready for sequencing. Hybridization was performed at 66ºC. Captured libraries were amplified by 10 cycles of Post-Capture PCR. Single captures (1-plex) and multiplex captures were tested with SureSelect XT HS Human All Exon V8 (Exome V8) and SureSelect Cancer CGP panels. Exome single captures (3x NA12878 and 3x NA24385 libraries) and CGP single captures (7x NA12878 and 7x NA24385 libraries) were performed with 1000ng library input into each 1-plex capture.

Multiplex captures were tested in duplicate. For multiplex captures, pre-capture pools were created with equal number of NA12878 and NA24385 libraries, with 4000ng total library input into each pre-capture pool. An 8-plex capture was tested with Exome V8 and a 16-plex capture was tested with SureSelect Cancer CGP.

Following the Target Enrichment runs on firefly+ the libraries were ran on the Agilent Fragment Analyzer (Agilent, DNF-474 HS NGS Fragment Kit) and qPCR (LightCycler 480 System - Roche, KAPA Library Quantification kit) to determine sizing and concentration respectively.

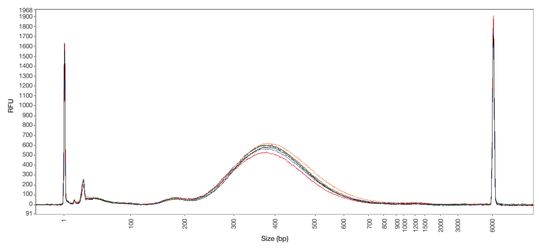

Figure 8. Example Fragment Analyzer trace overlays for the 6 single plex Exome samples post Target Enrichment

Sample type	Samples run	HYB Input (ng)	Average Size (bp)	Average concentration (nM)
Exome single plex	6	1000	381	3.82
Exome 8-Plex Pool	2	4000	391	14.35
CGP Single Plex	14	1000	396	0.92
CGP 16-Plex Pool	2	4000	387	4.10

Table 3. Fragment Analyzer Sizing (bp) and qPCR Library Concentration (nM) of saamples following Target Enrichment

Sequencing results

Sequencing of the Target Enriched libraries prepared on the firefly+ was performed using the AVITI Cloudbreak Freestyle sequencing kit (Element Biosciences). Table 4 and Figures 9-11 summarizes the sequencing data.

Data was down sampled to 6Gb (40M 2x150bp) for SSEL V8 and 0.45Gb (3M 2x150bp) for CGP analysis. PCT_SELECTED_BASES, PCT_EXC_DUPE, MEDIAN_TARGET_COVERAGE and Fold80 are reported from analysis with Picard HS Metrics (Broad Institute). Vardict (version 1.8.3) was used for variant calling. Recall/Sensitivity and Precision are reported for GIAB high confidence variants in regions targeted by SSEL V8.
V8 target regions intersected with HG001 or NA12878 covers 18426 total variants (18060 SNPs and 366 Indels).
V8 target regions intersected with HG002 or NA24385 covers 18545 total variants (18190 SNPs and 355 Indels).
Recall = True positive / (True positive + False negative) and Precision = True positive / (True positive + False positive).

Percent On-target

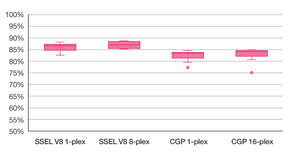

Figure 9. Percent On-target

Coverage

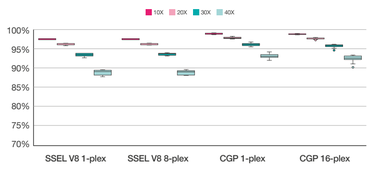

Figure 10. Base coverage at 10X, 20X, 30X and 40X

Variant Calling

SSEL V8

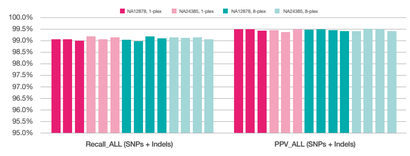

Figure 11. ≥99% Recall and Precision for all samples in single (1-plex) and multiplex (8-plex) captures.

Key Capture Metrics

Description	SSEL V8 1-plex	SSEL V8 8-plex	CGP 1-plex	CGP 16-plex
PCT_SELECTED_BASES	86.1%	87.0%	82.4%	82.7%
PCT_EXC_DUPE	4.7%	8.6%	2.0%	5.1%
MEDIAN_TARGET_COVERAGE	51	50	61	58
FOLD_80_BASE_PENALTY	1.51	1.48	1.48	1.49
Percentage of targeted bases covered by:				
...at least 10 reads	97.5%	97.5%	98.9%	98.8%
...at least 20 reads	96.2%	96.3%	97.9%	97.7%
...at least 30 reads	93.4%	93.6%	96.1%	95.7%
...at least 40 reads	88.8%	88.8%	93.1%	92.4%

Table 4. Key Capture Metrics

Reagents

Protocol	Part name	Part number
SureSelect Max DNA Library Preparation	SureSelect Max Enzymatic Fragmentation Library Prep Kit	G9660A, G9660B
	SureSelect Max Adaptors and UDI Primers Kit for ILM	G9668A G9668B G9668C G9668D
	SureSelect Max Purification Beads	G9962A (5 mL), G9962B (30 mL)
SureSelect Max Target Enrichment	SureSelect Max Fast Hyb Kit	G9689A, G9689B
	SureSelect Max Blockers and Primers Kit for ILM	G9699A, G9699B
	SureSelect Max Purification Beads	G9962A (5 mL), G9962B (30 mL)
	SureSelect XT HS Probe	Human All Exon V8 (5191-6878) Cancer CGP (5280-0037)

Table 5. Agilent kits used in the DNA Library Preparation with Enzymatic Fragmentation and Target Enrichment with Fast Hybridization workflows

Summary

These results demonstrate that the Agilent SureSelect Max DNA Library Preparation with Enzymatic Fragmentation and SureSelect Target Enrichment using Fast Hybridization workflows have been successfully automated on firefly+ to generate sequencing libraries which have been run on the Element AVITI sequencer.

Libraries prepared on firefly+ show consistent fragment sizing and concentration across a 96 well plate. 6 NTCs were run across the plate and showed no well-to-well contamination.

Sequencing of the Target Enriched libraries prepared on firefly+ show high % On-Target rate of >80% and 30X base coverage of >93% for both exome V8 and CGP panels regardless of whether the libraries were captured individually or as part of 8-plex and 16-plex captures. Expected variants were called with Recall and PPV rates at ≥99%.

The Agilent SureSelect Max DNA Library Preparation with Enzymatic Fragmentation and Target Enrichment using Fast Hybridization firefly+ protocols are available to download and run from the SPT Labtech firefly community.