A fully automated walkaway system programmed with a novel library prep target enrichment protocol for detection of ultra-low frequency mutations in liquid biopsy and tumor samples

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AACR 2026

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POSTER # 7262

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Introduction

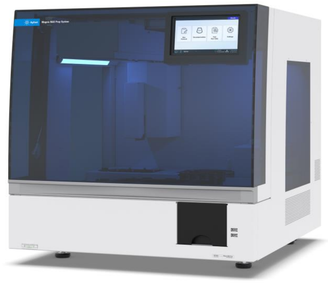
Liquid biopsy has transformed oncology by high sensitivity detection of genomic alterations from circulating cell-free DNA (cfDNA). Issues such as sensitivity, PCR bias, contamination, reproducibility, and long and laborious workflows are yet to be resolved. We have fully automated the workflow for a novel library preparation and target enrichment/probe technology, Agilent Avida, that tackles some of the existing challenges by being highly optimized to work with circulating tumor DNA (ctDNA). Our automated protocol using pre-aliquoted reagents, was developed on the Agilent Magnis NGS Prep System for a wide range of DNA inputs (1–100 ng) and various sample types (intact, FFPE DNA, and cfDNA). Using this platform, we construct libraries with a high sample recovery that scales linearly with the input amount and is capable of high sensitivity variant detection without a need for pre-enrichment PCR, minimizing GC bias. Our automated Avida DNA protocol generates up to eight target-enriched Illumina sequencing-ready libraries in about 8 hours without the need of any user intervention, thus minimizing potential contamination. We have generated data using both Avida catalog and custom panels covering a broad range of target sizes to determine assay sensitivity. Using these probes, we were able to reliably detect cancer-associated genomic alterations, including SNVs/indels, CNVs, and translocations across key oncogenes using well characterized cfDNA and formalin compromised reference standards as well as real ctDNA samples. For example, using 15 ng of SeraCare V4 ctDNA sample enriched with Avida DNA Onco LB panel (1.17 Mb covering 164 pan-cancer-associated genes) and sequenced with a budget of 100 M read pairs, we were able to detect SNP frequencies down to 0.25%. Furthermore, in our automated runs, we obtained high reproducibility across eight technical replicates. For example, for five independent Magnis runs (40 samples) of various sample types and inputs per run using the Avida DNA Onco LB panel, the % Coefficient of Variation (CV) ranged from 0.8 to 2.3% for on-target rate, 1.9 to 8.2% for UMI recovery (library complexity), 1 to 3.2% for base coverage (raw medium coverage), and 0.03 to 0.09% for uniformity (20% of mean coverage per region). With minimal risk of contamination, this automated protocol delivers up to eight target-enriched NGS Illumina sequencing-ready cDNA libraries.

Methods

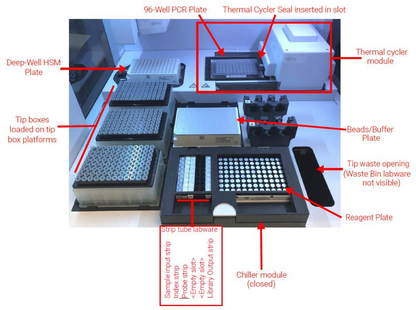
Over 800 Avida DNA libraries were generated across 100 automated runs on ten Magnis instruments, using Magnis Avida DNA reagent kits. Sample inputs were gDNA and FFPE DNA (10-100 ng) and cfDNA (1-100 ng). Targets were enriched using oncology focused panels of various sizes (27 kb to 2.5 Mb). The Avida-DNA-ILM automated protocol on Magnis NGS Prep System supports two Avida DNA workflows within the same package. One workflow covers the pre-sheared DNA (covaris as well as cfDNA), while the second workflow supports non-sheared DNA that is fragmented on-deck using the enzymatic fragmentation option. Fragment sizes and sequencing data shown in Table 1. are libraries generated by the Magnis using pre-sheared as well as non-sheared (sheared on-deck) FFPE samples of various qualities at 10 ng input. The 2 workflows are compared side-by-side using the Avida DNA Expanded Cancer panel run on separate instruments. These FFPE samples are from various qualities to show performance as well as providing a side-by-side comparison between the two workflows. Table 2. includes representative Coefficient of Variation (CV) for sequencing outputs of gDNA samples ran with the Avida Onco LB panel to show reproducibility and tightness of the replicates within 40 samples (5 runs) using both workflows, various sample types, and input amounts. Table 3. shows variant detection data for 15 ng SeraCare V4 ctDNA using the Avida DNA Onco LB panel. Figure 1. shows different aspects of the Magnis NGS prep system including the instrument, the Avida DNA workflow, and the Avida-DNA-ILM automated workflow. Figure 2. is a visual representation of sequencing outputs from 3 ng cfDNA generated by various panels. Libraries are generated from Biochain cfDNA samples using 6 Avida DNA panels and utilizing the sheared workflow.

Results and Discussion

Figure 1. Magnis NGS Prep System



A: Magnis Exterior



B: Magnis Interior & Consumables

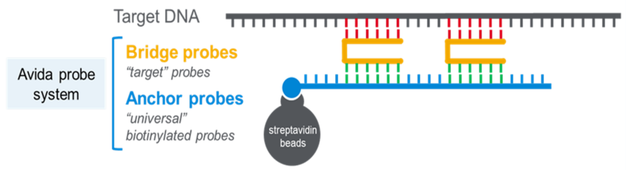
C: Magnis Avida DNA Reagent Kit

Magnis Modules:

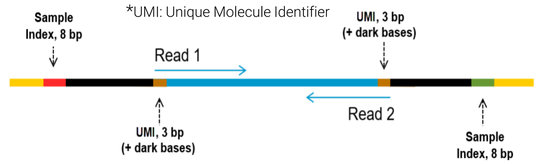
1. Gantry with an 8-channel pipette
2. Barcode reader – Auto-teach & reagent verification
3. Heater Shaker Magnet (HSM) – Patented
4. Thermal Cycler – Multi-zone with self-opening/closing lid
5. Chiller block – Holds 6 strips as well as one 96-well plate
6. Tube holder - for reagents requiring special handling & storage
7. Four tip box holding positions (96/box)
8. Built-in plate for camera calibration
9. Touchscreen UI – Robust & user-friendly Software
10. UV decontamination system (UV Bulb)

Special Features:

1. All module position auto-teach system using the barcode reader's camera
   - A built-in calibration plate with special marks
   - An algorithm to calculate the teaching position for each module
2. Fully validated Avida DNA and previously launched SureSelect NGS protocols developed by Agilent coupled with pre-filled & barcoded labware for traceability & confirmation of user setup
   - Full automation of laborious, time-consuming NGS chemistries with multiple pipetting & incubation steps & lengthy hands-on times, enables labs with no prior NGS background to easily generate reliable and reproducible NGS libraries.
   - Avida DNA and its tested probes enable reliable detection of cancer-associated genomic alterations without pre-enrichment
3. Disposable labware, PCR lid, and liners to prevent cross-contamination



D: Avida DNA NGS Target Enrichment Principle



E: Content of Avida DNA sequencing libraries

Each fragment contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), unique dual sample indexes (red and green), duplex UMIs (brown), and the library PCR primers (yellow).

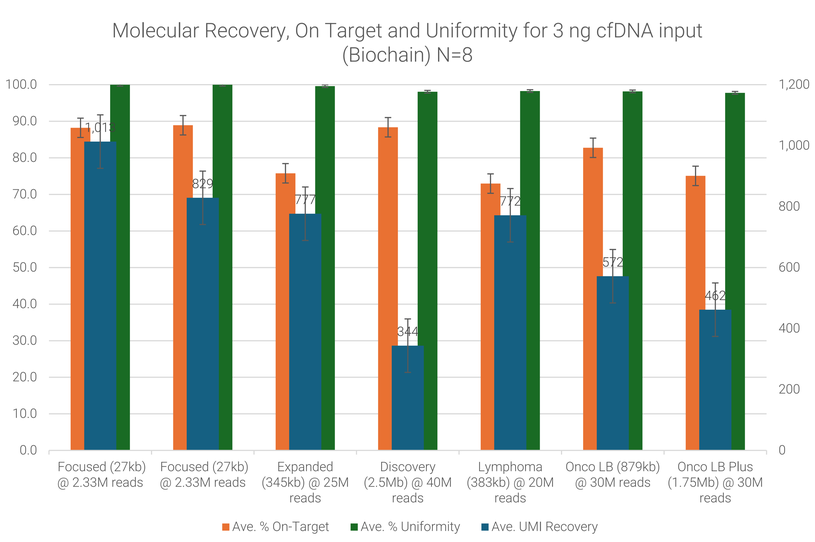


Figure 2. Avida DNA on Magnis Sequencing Metrics – 3 ng cfDNA– 27 kb to 2.5 Mb Panels

Table 1. Avida DNA on Magnis Sequencing Metrics – 10 ng Various Quality FFPE Samples – Covaris Vs. Enzymatic Fragmentation – Avida DNA Expanded Panel

Panel: Expanded (345 kb) @ 25 M read pairs	FFPE # 1 - DIN 2.7		FFPE # 2 - DIN 3.0		FFPE # 3 - DIN 3.0		FFPE # 4 - DIN 4.9	
10 ng FFPE averaged over duplicates	Covaris Sheared	Enz. Frag. (27 min)	Covaris Sheared	Enz. Frag. (27 min)	Covaris Sheared	Enz. Frag. (27 min)	Covaris Sheared	Enz. Frag. (27 min)
Run	1	2	1	2	1	2	1	2
Ave. Fragment Size (bp)	331	347	352	373	328	334	417	410
Ave. Base Coverage	4,145	4,644	4,463	3,939	4,020	4,658	5,821	5,083
Ave. UMI Recovery	41	46	39	32	41	40	205	184
Ave. % On-Target	51.7	61.4	61.4	59.7	50.7	60.7	70.3	65.7
Ave. % Uniformity	99.4	99.4	98.6	97.8	99.4	99.1	99.8	99.7

Table 2. Intra-Run Sample to Sample Reproducibility – Various Sample Types

Panel	Avida DNA Onco LB panel (879 kb) @ 30 M reads				
Specifications: %CV over 8 samples per run	10 ng gDNA Covaris	10 ng gDNA Covaris	10 ng gDNA Covaris	10 ng gDNA Covaris	3 ng cfDNA
Run	1	2	3	4	5
% CV Base Coverage	2.17	3.17	1.50	2.78	1.00
% CV UMI Recovery	1.83	5.98	5.53	8.19	3.33
% CV % On-Target	2.04	2.27	0.78	2.66	0.81
% CV % Uniformity	0.03	0.03	0.07	0.04	0.09

Table 3. Variant Detection – 15 ng SeraCare V4 ctDNA – Avida DNA Onco LB Panel

Panel		Avida DNA Onco LB panel (879 kb)				
Specifications: 15 ng ctDNA SeraCare V4 Mix (93 unique multiplexed variants in 71 genes covering 43 SNVs, 19 deletions, 5 insertions, 4 INDELs, 12 CNVs, and 10 translocations) Specifications: 15 ng ctDNA (3 replicate runs /8 samples per run)		% Expected Allele Frequency				
		5	2	1	0.5	0.25
SNV/InDel	% Sensitivity	97.8	98.3	95.8	93.3	66.7
	% Specificity	100.0	100.0	100.0	100.0	100.0
CNV	% Sensitivity	100.0	100.0	100.0	100.0	0.0
	% Specificity	98.9	99.4	99.6	99.6	98.9
Fusion (Analysis Method: QCI 2)	% Sensitivity	100.0	100.0	81.0	74.0	48.0

Conclusions

- The Magnis NGS Prep system generates highly reproducible libraries using a variety of panel sizes (27 kb to 2.5 Mb) including oncology specific panels.
- gDNA (data not shown), cfDNA, and FFPE DNA samples can be processed using pre-sheared workflow (Covaris/mechanically sheared or cfDNA), as well as non-sheared workflow (on-deck using enzymatic fragmentation) through a simple setup with minimal hands-on time.
- There is good correlation between libraries (fragment size, and sequencing results) generated by the non-sheared & pre-sheared workflows with fragmentation time set to 27 min. as default.

Acknowledgments

Agilent Technologies La Jolla, Santa Clara, Cedar Creek, and Penang. Mohd-Aizuddin Kamaruddin, Chain-Siong Siow, Jia-Wei Lee, Sim-Siang Teh, Teik-Chuan Goo, Rajesh Bagga, John Halladay, Kelly Flesher, Sarah Jandle, Dawn Lesperance, Alesa Jacob, and Clay Smith