Automated Walk Away NGS Sample Preparation for Constructing In-Line Molecular Barcoded RNA Libraries from Fresh Frozen and FFPE Samples

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Introduction

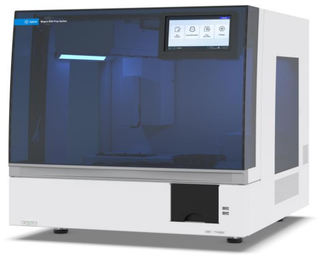
RNA sequencing (RNA-Seq) is a revolutionary technology for transcriptome analysis, which has been used to quantify transcript levels, confirm gene annotation, identify novel transcripts, splice variants, and fusion detection. Traditionally, the usefulness of RNA-Seq has been limited by pre-analytical (sample availability, low integrity, tumor heterogeneity, complex protocols) and analytical (small mRNA fraction, high read duplicate rate) factors, which can lead to higher costs, longer turnaround times, and false negative results. We have developed and automated a streamlined workflow for construction of highly complex RNA libraries from fresh frozen and FFPE samples that addresses these concerns. We use target enrichment by bait selection with FFPE-derived samples, where rRNA depletion is found to be inconsistent and poly(A) enrichment cannot be used due to degradation of the source material. A fully automated protocol using prealiquoted reagents has been optimized on the Agilent Magnis NGS Prep system for a wide range of RNA inputs (10 to 200 ng), various sample types (intact and FFPE total RNA) and equipped with 192 unique dual sample indices (UDIs) to minimize the effects of possible index hopping and contamination. Addition of in-line molecular barcodes incorporated at the ligation step helps differentiate fragmentation from PCR duplicates, which increases the total number of reads available for further analysis (gene expression and fusion detection). With minimal risk of contamination, this automated protocol delivers up to eight target-enriched NGS Illumina sequencing-ready cDNA libraries from total RNA input, per run, in about 12 hours, without any user intervention. Both catalog and custom probes can be used, and PCR cycles can easily be adjusted from the touchscreen to optimize the library yields.

Methods

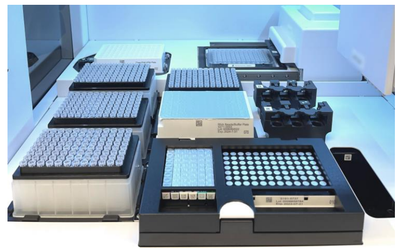
Automated targeted RNA-Seq libraries were generated with the Agilent Magnis SureSelect XT HS2 RNA reagent kit from over 80 runs (640 libraries) on 10 Magnis instruments from high-quality intact RNA at 10 to 200 ng sample inputs and FFPE RNA at 10 ng input, using small, medium, and large probe panels. RNA is thermally fragmented on deck for intact RNA samples (no fragmentation is required for FFPE RNA). Sequencing data shown in Table 1 is from SureSelect Cancer CGP RNA libraries generated by the automated Magnis NGS Prep system using intact Quantitative Human Reference RNA (QHRR) as sample input. Sequencing results shown in Tables 2 and 3 are libraries generated from intact QHRR and FFPE RNA (SeraCare V4 reference standard) using Magnis SureSelect XT HS2, RNA, Exome V8, ILM, 96 (Exome V8) probe set. Gene expression correlation between Magnis and manually-prepped libraries using 10 ng SeraCare V4 is shown in Figure 2.

Fusion detection results are displayed in Table 4. Intrarun (Table 5) and interrun (data not shown) contamination assays were performed using 10 ng of input RNA for all eight samples (15,000 copies). Every other sample was spiked in with 10 million copies of Alien-6 RNA fragment (sequence not present in human genome). Taqman qPCR assay specific for the Alien-6 RNA was used for the pre- and post-capture samples after the run to rule out intrarun contamination, and the next run to rule out interrun contamination. Quantification of copies per µL of sample was done using a standard curve of spiked-in samples.

Figures and Tables



A: Magnis exterior



B: Magnis interior and consumables

C: Magnis SureSelect XT HS2 RNA reagent kit

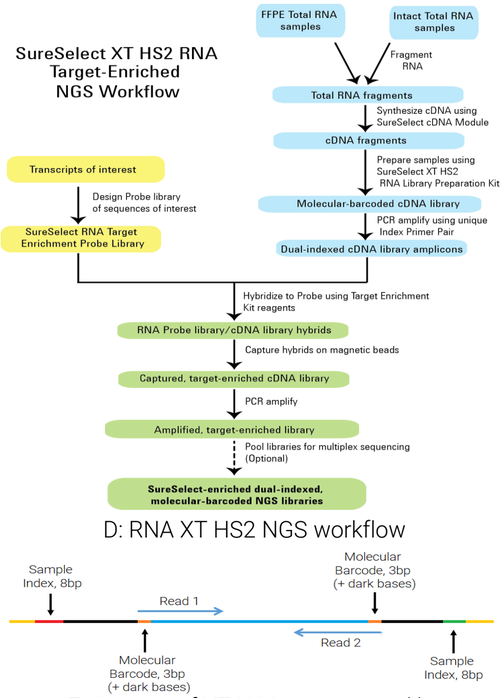
Magnis NGS Prep system modules:
(Initially patented as sample processing apparatus)

1. Gantry with an eight-channel pipette
2. Barcode reader – auto-teach and reagent verification
3. Heater Shaker Magnet (HSM) – patented
4. Thermal Cycler – multi-zone with self-opening/closing lid
5. Chiller block – holds six strips as well as one standard 96-well plate
6. Tube holder for reagents requiring special handling and storage
7. Four-tip box holding positions (96/box)
8. Beads and buffer position with a built-in plate for camera calibration
9. Touchscreen UI – robust and user-friendly software
10. UV decontamination system (UV bulb)

Special features:

1. All module position auto-teach system using the barcode reader's camera – invention disclosure submitted
   - A built-in calibration plate with special marks
   - An algorithm to calculate the accurate teaching position for each module
2. Fully-validated SureSelect NGS protocols developed by Agilent coupled with prefilled and barcoded labware for traceability and confirmation of user setup
   - Full automation of laborious, time-consuming NGS chemistries with multiple pipetting and incubation steps and lengthy hands-on times enables even labs with no prior NGS background to easily generate reliable and reproducible NGS libraries
   - DNA-XTHS, DNA-XTHS-EPIS, DNA-XTHS2, DNA-XTHS2-EPIS, RNA-XTHS2
3. Disposable labware, PCR lid, and liners to prevent cross-contamination
4. Custom protocols for key customers
5. Available to clinical and diagnostic customers CE-IVD, ISO 13485

Figure 1. Magnis NGS Prep system



D: RNA XT HS2 NGS workflow

E: Content of XT HS2 sequencing library

Table 1. RNA-XTHS2 Magnis sequencing metrics – 50 ng CGP probe

Specifications: 50 ng QHRR with Cancer CGP at 4,000,000 Reads Averaged for Four Samples per Run	Cancer CGP		
Run	1	2	3
Instrument	A	B	C
Percentage Reads in Targeted Regions	73.73%	72.94%	73.20%
Duplication Rate of Mapped Reads	18.65%	18.08%	19.35%
% Strand Specificity	96.99%	97.41%	97.55%
Average Estimated Library Size	3,670,767	3,864,780	3,657,386

Table 2. RNA-XTHS2 Magnis sequencing metrics – 10 ng QHRR – Exome V8 probe

Specifications: 10 ng QHRR with V8 at 20,000,000 Reads Averaged for Four Samples per Run	Exome V8		
Run	1	2	3
Instrument	A	B	C
Percentage reads in Targeted Regions	66.57%	66.52%	66.60%
Duplicate Rate of Mapped Reads	10.60%	9.53%	9.35%
% Strand Specificity	97.91%	98.21%	97.90%
Average Estimated Library Size	41,929,281	47,374,235	61,220,177

Table 3. RNA-XTHS2 Magnis sequencing metrics – 10 ng SeraCare V4 – Exome V8 probe

Specifications: 10 ng SeraCare V4 with V8 at 20,000,000 Reads Averaged for Eight Samples per Run	Exome V8		
Run	1	2	3
Instrument	A	B	C
Percentage reads in Targeted Regions	65.21%	65.06%	65.32%
Duplicate Rate of Mapped Reads	9.56%	8.25%	9.64%
% Strand Specificity	98.07%	97.83%	98.00%
Average Estimated Library Size	47,390,862	55,021,074	46,225,531
Fusions Detected out of 8	7 samples: 8/8 1 sample: 7/8	All samples: 8/8	All samples: 8/8

Table 4. RNA-XTHS2 fusion detection – 10 ng SeraCare V4 – Exome V8 probe

		Fusion in SeraCare V4 FFPE Sample with RNA Strip, Beads, and Buffer plates; Lot 1								
Run	Sample	EML4—ALK	ETV6--NTRK3	FGFR3-BAIAP2L1	FGFR3--TACC3	KIF5B--RET	LMNA--NTRK1	NCOA4—RET	SLC34A2--ROS1	Fusions Detected out of the 8 Tested
1	1	7	8	15	45	7	13	5	9	8
	2	3	7	27	33	12	15	11	10	8
	3	2	5	26	27	7	15	12	12	8
	4	3	7	18	56	10	12	12	12	8
	5	6	12	15	40	7	15	11	11	8
	6	ND	11	23	31	8	10	12	10	7
	7	4	7	14	23	13	8	7	15	8
	8	3	11	18	47	11	15	4	10	8

Table 5. Magnis intrarun contamination assay – every other well received 10 million copies of Alien6 RNA-SpikeIn

Copy Number per µL Library – Ran in Two Replicates			Run 1		Run 2		Run 3	
	Standards		A		B		C	
A	10,000,000	NTC	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq
B	1,000,000	NTC	15,800,884	17,335,437	15,032,314	16,119,650	19,183,797	17,072,560
C	100,000	NTC	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq
D	10,000	NTC	16,669,728	17,107,532	13,972,941	17,209,534	15,134,827	14,740,000
E	1,000	blank	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq
F	100	blank	12,807,028	12,132,320	11,947,389	11,363,026	8,445,820	10,669,528
G	10	blank	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq
H	1	blank	13,168,255	11,579,823	11,002,759	13,512,117	13,706,785	12,283,055
		Average	14,575,126		13,769,966		13,904,547	

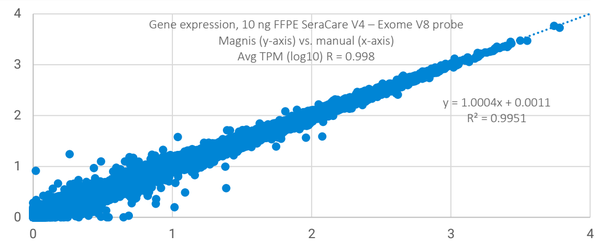


Figure 2. RNA-XTHS2 gene expression comparison Magnis vs. manual

Conclusions

The Magnis NGS Prep system generates highly reproducible, strand-specific libraries using a variety of bait sizes (CGP or Exome V8) from both intact and FFPE RNA samples, with a simple setup and minimal hands-on time.
Correlation of gene expression between Magnis and manual prep is extremely high (R=0.998).
Magnis detects SeraCare V4 fusions at 10 ng total RNA input level.
No inter- and intrarun contamination is observed.

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