Application Note

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SureSelect XT HS2 RNA Sequencing of Ribosomal RNA-Depleted Samples

Author

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Abstract

In this application note, we present the use of commercially available ribosomal RNA (rRNA) depletion kits in conjunction with SureSelect XT HS2 RNA reagents for Illumina sequencers. A panel of kits that employ different approaches for the depletion of rRNA sequences was examined, and data from two of the better-performing kits is presented. Finally, a detailed SureSelect XT HS2 rRNA protocol is provided.

Introduction

Next-generation sequencing (NGS) of RNA can provide useful information regarding expression profiles, splice variants, fusion transcripts, and post-translational modifications. To allow for efficient transcript analysis, it is highly beneficial to remove abundant RNA species from the test sample. Three common approaches to enriching RNA samples are isolation of mRNA using oligo(dT), enrichment with targeted probes, and depletion of ribosomal RNA. As ribosomal RNA is the most highly abundant component of total RNA (80 to 90%), its elimination is a requirement before any analysis of the transcriptome. Targeted RNA sequencing (RNA-Seq) is a focused evaluation of selected transcripts and is shown to generate quality libraries using formalin-fixed, paraffin-embedded (FFPE) samples. However, if a method to interrogate an unbiased population is required, then mRNA enrichment or rRNA depletion are more suitable methods to use. Oligo(dT) enrichment for mRNA is only advisable for use on high-quality samples as highly fragmented samples will result in large sample losses and a population of terminal ends. The use of rRNA depletion over mRNA enrichment enables researchers to prepare RNA libraries from both high-quality and highly fragmented samples (such as FFPE) and examine non-polyadenylated RNA species that may be of interest.

The Agilent SureSelect XT HS2 RNA portfolio does not currently provide a commercial ribosomal depletion module. Therefore, to support customers wishing to prepare rRNA-depleted XT HS2 RNA libraries, we optimized a procedure and evaluated it with many commercially available depletion kits. Table 1 describes the five rRNA depletion kits that were examined in an initial round of testing. All the kits target rRNA species from human, mouse, and rat (H/M/R); however, only human RNA was tested in these experiments. In addition to human rRNA, Kit J also targets bacterial rRNA and human beta-globin RNA. Four different modes of action for rRNA depletion were represented by the kits tested. Three of the modes of action depleted the RNA sample before library preparation, and one targeted DNA after the library was constructed (Kit K). Based on performance, ease of use, and compatibility with the existing RNA XT HS2 reagents and workflow, two kits were chosen for more extensive testing (Kit O and Kit M).

Kit O targets rRNA with sequence-specific DNA oligonucleotides followed by treatment with RNase H, which digests DNA/RNA hybrids. The procedure consists of three incubations followed by a SPRI purification of the depleted RNA. RNA-specific SPRI beads are purchased separately. Kit M targets rRNA sequences using tagged oligos that are then removed from the solution by tag-binding magnetic beads. Kit M takes less time to complete and includes all the reagents needed to perform the depletion. However, it requires more hands-on steps than Kit O. Data from running these two kits using both high-quality and FFPE RNA is presented.

Table 1. Commercially available rRNA depletion kits tested. The five kits detailed in Table 1 were all examined for compatibility with the Agilent SureSelect RNA XT HS2 chemistry and workflow. Four different modes of action are represented within the five kits tested. Four of the kits process the RNA sample before library construction, but one kit targets DNA after library construction (Kit K). The time to perform the depletion, manufacturer recommended input ranges, FFPE sample compatibility, and our assessment of each of the kits are also included.

Kit/Vendor (Species Targeted)	Mode of Action	Time	Input Range	FFPE	Notes
Kit J (human, mouse, rat, bacterial, human beta globin)	RNase H	1.5 to 2 hours	1 to 1,000 ng	Yes	Easy to use, works well, broad spectrum depletion, fits seamlessly into the SureSelect XT HS2 workflow. RNA-specific SPRI beads purchased separately.
Kit O (human, mouse, rat)	RNase H	1.5 to 2 hours	10 to 1,000 ng	Yes	Easy to use, works well, fits seamlessly into the SureSelect XT HS2 workflow. RNA-specific SPRI beads purchased separately.
Kit M (human, mouse, rat)	Magnetic bead-based adsorption	1.5 hours	1 to 1,000 ng	Yes	Enzyme-free, easy to use, works well, fits seamlessly into the SureSelect XT HS2 workflow, everything included.
Kit K (human, mouse, rat)	Cas 9 cleavage of rRNA sequences	2 to 2.5 hours	5 to 100 ng	No	A configuration is available to deplete pools of libraries which would impart ease and cost savings. Requires changes to the SureSelect XT HS2 workflow.
Kit R (human, mouse, rat)	Blocks cDNA synthesis	15 minutes	1 to 1,000 ng	Yes	Short protocol, requires optimization for use with SureSelect XT HS2 workflow.

Experimental

RNA sources

Universal Human Reference RNA (UHRR) for qPCR was obtained from Agilent Technologies (Santa Clara, CA p/n 750500-41). SeraSeq FFPE Fusion RNA Reference Material v4 was purchased from SeraCare (Gaithersburg, MD p/n 0710-0496). A melanoma FFPE tissue section was obtained from Cureline Human Biospecimen CRO (Brisbane, CA, p/n custom).

RNA isolation and qualification

RNA was isolated from FFPE tissue sections using the RNeasy FFPE Kit from Qiagen (Germantown, MD p/n 73504). The concentration of each extraction was determined on a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Samples were further analyzed on an Agilent Bioanalyzer 2100 system using the RNA 6000 Nano kit (Santa Clara, CA p/n 5067-1511). DV_{200} values (% of sample with >200 nt fragment length) were determined by performing a smear analysis using the Bioanalyzer software.

SureSelect XT HS2 RNA library preparation

RNA samples were depleted of ribosomal RNA using either Kit O or Kit M according to the manufacturer instructions except that final sample volumes were adjusted to accommodate the RNA XT HS2 start volume of 10 µL. Libraries were prepared from depleted RNA samples using the SureSelect XT HS2 RNA Library Prep kit for Illumina (Agilent Technologies p/n G9993A-D) and the procedure provided in the Appendix.

Sequencing and data analysis

Libraries were analyzed on an Illumina HiSeq 4000 by paired-end sequencing using a 2 × 150 read format. FASTQ files were aligned to human reference genome GRCh38 using STAR RNA-Seq aligner v2.7.2. Libraries were down sampled to 20 million reads for analysis. PCR duplicates were marked using a Picard duplicate marking tool (BROAD Institute) and the duplication rate and estimated library size were reported from the output. The duplicate-marked .bam files were then analyzed using RNA-SeQC v1.1.8 (BROAD Institute) and strand specificity and % mapped reads were reported from the output. The residual percent rRNA was analyzed by aligning FASTQ files to a rRNA reference file using BWA-MEM, the percentage of the sample aligned corresponds to the percent of rRNA in the library. Gene expression profiles, measured in transcripts per million (TPM), were generated using RSEM v0.8.1. Correlation coefficients were calculated from the TPM output using Microsoft Excel. Fusion transcripts were identified using STAR-Fusion v1.8.0.

Results and Discussion

High-quality RNA

To assess performance of the two rRNA depletion methods, libraries were prepared from UHRR using a wide range of inputs. Inputs of 1 to 1,000 ng for both depletion methods resulted in high-quality libraries. The strand specificities and mapping rates are high for both kits but the mapping rates were slightly better for Kit O. An input of 1 ng is not recommended by the manufacturer for either SureSelect XT HS2 RNA or the Kit O depletion kit, and the results in Figure 1 support that recommendation. As observed for both test kits, the low 1 ng input shows a steep increase in the duplication rate in addition to reduced library sizes. Both methods did well at removing rRNA (all had less than 1% residual rRNA). However, the highest input for Kit M showed an increase in residual rRNA indicating that 1,000 ng may be close to the high input capabilities of the kit. The column graph to the right of the metrics table shows more detail regarding the residual RNA content of the individual libraries.

High Quality RNA (UHRR)								
rRNA Depletion Kit	**Kit O**				**Kit M**			
Sample Input (ng)	1	10	100	1000	1	10	100	1000
Estimated Library Size	1.4E+07	1.6E+08	4.2E+08	6.0E+08	2.4E+07	1.5E+08	4.0E+08	3.3E+08
Duplication Rate	27%	5%	3%	3%	18%	6%	3%	4%
Strand Specificity	97%	98%	98%	98%	97%	97%	98%	98%
Mapping Rate	89%	93%	93%	92%	87%	90%	87%	88%
% Residual rRNA	0.2%	0.2%	0.1%	0.1%	0.5%	0.6%	0.6%	0.9%

average of duplicate reactions

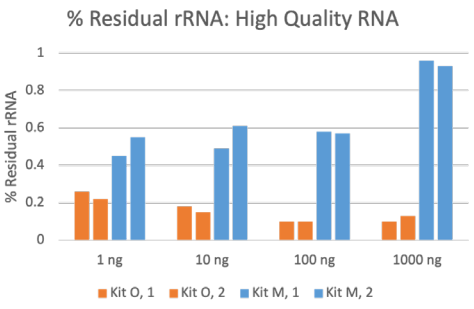

Figure 1. RNA sequencing metrics of rRNA-depleted libraries prepared from high-quality RNA. Duplicate rRNA depletion reactions were performed on 1, 10, 100, and 1,000 ng of Universal Human Reference RNA using both Kit O and Kit M rRNA depletion kits. SureSelect XT HS2 RNA libraries were prepared from the resulting RNA samples. Libraries were then sequenced and analyzed as described. The sequencing metrics table represents the average results from duplicate libraries. The bar graph shows the percent residual rRNA for each individual library. Kit O libraries are represented by orange bars and Kit M libraries are represented by blue bars.

FFPE RNA

Because of the poor condition of FFPE samples, not all the RNA molecules in a given sample are of sufficient quality to be manipulated in enzymatic reactions. In addition, very small fragments get washed away during SPRI purification, which also contributes to sample loss. Low inputs can also result in the undesirable formation of adaptor dimers during library construction. Because of these negative impacts, higher inputs of FFPE are strongly recommended to make up for the loss of "usable" template. With this in mind, the range of inputs tested for the melanoma FFPE sample started at 50 ng on the low end. The FFPE libraries depleted using Kit M have four to five times more residual rRNA than Kit O depleted samples. Due to the contribution from residual rRNA, the duplication rates of Kit M libraries are perceived to be lower, and the library sizes are perceived to be larger. The rRNA incorporated into these libraries contain adaptors with molecular bar codes (MBC), as such they are counted as unique molecules by the duplicate marking software. Their presence increases library complexity and there are fewer duplicates. However, these are uninformative library members that take away space in the library from more meaningful ones. This conclusion is supported by the lower mapping rates observed for Kit M as only a few species of rRNA map to the GRCh38 reference file. The mapping rates for the Kit O depletion method are higher, which demonstrates the benefit of minimizing the rRNA content. As anticipated, all library metrics improved with increased input for both depletion methods. If available, higher inputs of FFPE RNA are expected to be highly beneficial and is recommended (≥ 100 ng). The chart to the right of the metrics table shows the percent residual rRNA for the individual libraries. One of the 50 ng Kit O duplicates is unusually high compared to all the other Kit O libraries tested and is considered to be an outlier.

RNA Isolated from FFPE (Melanoma DV_{200} 40%)								
rRNA Depletion Kit	**Kit O**				**Kit M**			
Sample Input (ng)	50	100	250	1000	50	100	250	1000
Estimated Library Size	1.6E+07	3.1E+07	8.6E+07	2.7E+08	4.0E+07	9.3E+07	1.9E+08	3.4E+08
Duplication Rate	23%	14%	6%	3%	11%	6%	5%	4%
Strand Specificity	96%	96%	97%	97%	96%	96%	96%	96%
Mapping Rate	85%	89%	91%	92%	77%	77%	80%	80%
% Residual rRNA	2.6%	0.9%	0.5%	0.4%	5.2%	5.6%	4.8%	4.8%

average of duplicate reactions

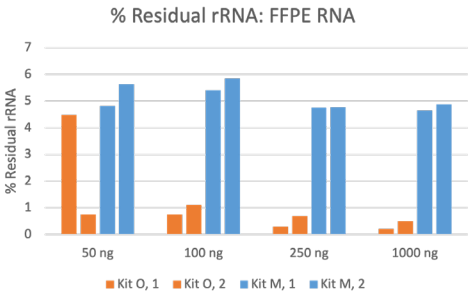

Figure 2. RNA sequencing metrics of rRNA-depleted libraries prepared from fragmented FFPE RNA. Duplicate rRNA depletion reactions were performed on 50, 100, 250, and 1,000 ng of FFPE RNA using both the Kit O and Kit M rRNA depletion kits. SureSelect XT HS2 RNA libraries were prepared from the resulting RNA samples. Libraries were then sequenced and analyzed as described. The sequencing metrics table represents the average results from duplicate libraries. The bar graph shows the percent residual rRNA for each individual library. Kit O libraries are represented by orange bars and Kit M libraries are represented by blue bars.

Gene expression

To determine if the gene expression profiles of rRNA-depleted RNA XT HS2 libraries were significantly altered by the ribodepletion process, a correlation analysis comparing an unbiased control data set to libraries prepared from rRNA-depleted samples was performed. To minimize as much bias as possible, a ground truth library was prepared from 10 ng unenriched UHRR. This library was deep sequenced and all rRNA sequences were manually removed from the resulting data set. The test data set was an RNA XT HS2 library prepared from 10 ng UHRR that had been enriched using Kit O and Kit M rRNA depletion kits. RNA-Seq by expectation-maximization (RSEM) analysis was performed on all the data sets and the transcripts per million (TPM) outputs were used as input for the correlation study. Correlation coefficients for pairings of all five libraries are presented in Table 2. A high degree of correlation was observed for all the libraries (≥ 94%) and differences observed between the two depletion kits were negligible.

Table 2. Gene expression correlation analysis of rRNA-depleted SureSelect XT HS2 RNA libraries to a ground truth library. Duplicate reactions of 10 ng UHRR were ribodepleted using Kit O and Kit M according to manufacturer instructions. SureSelect XT HS2 RNA libraries were constructed from the resulting depleted RNA samples and sequenced on the Illumina HiSeq 4000 platform. As a reference, a library from an unenriched 10 ng UHRR sample was constructed then deep sequenced. The rRNA sequences were then manually removed from the resulting data to provide a ground truth data set for comparison. A TPM gene expression analysis was performed on each library. Correlation coefficients (r) were calculated from the TPM using Microsoft Excel and are presented below.

GEx Correlation	Kit O Rep A	Kit O Rep B	Kit M Rep A	Kit M Rep B	Unenriched
Kit O Rep A	1.00				
Kit O Rep B	0.96	1.00			
Kit M Rep A	0.95	0.95	1.00		
Kit M Rep B	0.95	0.95	0.96	1.00	
Unenriched	0.94	0.94	0.94	0.94	1.00

Fusion detection

To investigate rRNA depletion kit performance for the detection of gene fusion transcripts, RNA XT HS2 libraries were prepared from duplicate 50 ng input reactions of SeraSeq FFPE Fusion RNA v4 (DV_{200} 60%). Sixteen fusions are reported for this reference sample, as listed in Table 3. STAR-Fusion analysis software was used to report the fusions detected in each of the libraries. Junction and supporting reads were combined and those resulting values were averaged between replicate libraries and reported in Table 3. Samples depleted using Kit M showed higher fusion counts overall and higher counts for 13 of the 16 fusions on the list. In addition, the Kit M libraries detected all 16 fusions, but Kit O missed one (EGFR-SEPT14). When considered in the context of the other sequencing metrics, the level of fusion detection observed here for Kit M-depleted libraries improves further. As observed in the melanoma FFPE libraries example (Figure 2), the SeraSeq FFPE Kit M-depleted libraries had higher amounts of residual rRNA and lower mapping rates than those prepared with the Kit O depletion kit (data not shown). Taking this into consideration, the fusion counts for Kit M libraries would be expected to be even higher if an equal number of mapped reads had been analyzed.

Table 3. Fusion detection. RNA was isolated from SeraSeq FFPE Fusion RNA Reference v4 curls using the Qiagen RNeasy FFPE Kit. Duplicate 50 ng input reactions were ribodepleted using both Kit O and Kit M according to the manufacturer instructions. SureSelect XT HS2 RNA libraries were constructed from the rRNA-depleted samples and sequenced on the Illumina HiSeq 4000 platform. Samples were normalized to 20 million reads and fusions were determined using the STAR-Fusion (by BWA) analysis tool. Junction and supporting read numbers for the fusions detected were combined and results from replicate samples were averaged.

Read Counts of Fusions in rRNA-Depleted SeraSeq FFPE RNA (DV_{200} 60%)		
Expected Fusions	**Kit O**	**Kit M**
CCDC6-RET	2.5	6.5
CD74-ROS1	5	9
EGFR-SEPT14	0	1.5
EML4-ALK	1	2.5
ETV6-NTRK3	1.5	4
FGFR3-BAIAP2L1	9	5
FGFR3-TACC3	6.5	10
KIF5B-RET	3	3.5
LMNA-NTRK1	3	2.5
NCOA4-RET	1.5	3.5
PAX8-PPARG1	1.5	2
SLC34A2-ROS1	1.5	2
SLC45A3-BRAF	2	3
TFG-NTRK1	3	2.5
TMPRSS2-ERG	1	10
TPM3-NTRK1	2	6.5

junction + supporting reads (average of duplicate reactions)

Conclusion

Five rRNA depletion kits were initially screened for use in conjunction with the SureSelect XT HS2 RNA reagents and workflow. Three of those kits fit seamlessly into the workflow and performed well. Two of those kits, Kit O and Kit M, were selected for extensive testing with both high-quality and FFPE RNA samples. Both kits performed well on high-quality RNA, but Kit O was significantly better at removing rRNA from FFPE samples. An input range of 10 to 1,000 ng of high-quality RNA resulted in quality libraries for both kits tested. Higher inputs for FFPE RNA improved library quality for both kits. At least 100 ng input of FFPE RNA is recommended if available. Both kits produced libraries with gene expression profiles that had a high correlation to that of a ground truth control library and each other. Kit M was superior to Kit O in detecting gene fusions. Overall, both rRNA depletion kits performed well in addition to being reliable and easy to use. Either kit is recommended for use with the SureSelect XT HS2 RNA chemistry and the protocol is provided in the Appendix.

Appendix

Reagents and Procedure for the Construction of rRNA-Depleted XT HS2 RNA Libraries

Reagents required

Reagent	Vendor	Part Number
Ribosomal RNA Depletion kit (human)	*User preference	
SureSelect XT HS2 RNA Library Prep kit for Illumina	Agilent Technologies	G9993A-D (96 rxn) G9990A (16 rxn)**
AMPure XP Beads	Beckman Coulter	A63882 (4 L), A63881 (60 ml), A63880 (5 ml)
High Sensitivity DNA1000 ScreenTape assay	Agilent Technologies	5067-5584
High Sensitivity DNA1000 Sample buffer	Agilent Technologies	5067-5603

**Kit O and/or Kit M are recommended, but kits from other vendors may be acceptable. If using an alternative vendor, it is recommended to perform the depletion on the RNA before library construction and to adhere to the sample guidelines provided in Table 4.*
***G9990A is the 16 reaction kit and includes AMPure beads. It also includes target enrichment reagents, which are not required for this protocol.*

Sample guidelines

rRNA-depleted samples are prepared using a method selected by the user following the manufacturer guidelines with the following exceptions:

- The RNA XT HS2 library preparation protocol input volume must be 10 µL to begin (in water). The output volume of the depletion kit protocol should be adjusted accordingly.
- For library preparation, adhere to recommendations for the quality-based sample input amount instead of the depletion kit input range (see Table 4 below). Samples with DV_{200} <20% are not recommended for use.

Table 4. Recommended RNA inputs based on sample quality.

RNA Sample Type	Input Range	Comment
High quality (non-FFPE)	10 to 1,000 ng	Low RIN OK (>4)
FFPE DV_{200} >40%	50 to 1,000 ng	Higher input recommended for lower quality
FFPE DV_{200} 20 to 40%	100 to 1,000 ng	

SureSelect RNA XT HS2 Library Construction

Before starting:

- Thaw required reagents on ice and mix well by vortexing. Briefly spin to seat the solution in the tube.
- Bring AMPure beads to room temperature.
- Prepare 70% ethanol (400 µL/SPRI purification/sample) before use.

cDNA synthesis

1. Add **10 µL** of SureSelect RNA 2X Priming buffer to each 10 µL rRNA-depleted sample. Store FFPE samples on ice until ready to proceed with cDNA synthesis.
   FFPE
   FFPE samples are already highly degraded and do not require any additional fragmentation. Skip step 2 below (94 °C heat fragmentation).
   Non-FFPE
   RNA samples isloated from cell lines, tissue, or fresh frozen tissue, regardless of quality, need to go through the fragmentation process. Proceed to step 2.

2. Fragment **non-FFPE** samples according to the table below using a thermocycler. Return to ice after completion.

Cycles	Temp	Time	Step
1X	94 °C	4:00	fragmentation
1X	4 °C	HOLD	cold storage

Set volume to **20 µL** if prompted.

3. Program and prewarm a thermocylcer for the first strand synthesis as follows:

Cycles	Temp	Time	Step
1X	25 °C	10:00	hexamer extension
1X	37 °C	40:00	cDNA synthesis
1X	4 °C	HOLD	cold storage

Set volume to **24 µL** if prompted.

4. Vortex First Strand Master Mix and briefly spin.

5. Dispense **4 µL** to each sample on ice. Cap, vortex to mix, and briefly spin.

6. Transfer samples to the thermocycler and run the program. Remove samples and transfer to ice when complete.

7. Program and prewarm a thermocycler for the second strand synthesis as follows:

Cycles	Temp	Time	Step
1X	16 °C	60:00	second strand synthesis
1X	4 °C	HOLD	cold storage

Set volume to **54 µL** if prompted.

8. Vortex the thawed vials of Second Strand Enzyme Mix, and Second Strand Oligo Mix and briefly spin.

9. To each reaction on ice, add **25 µL** of Second Strand Enzyme Mix and **5 µL** of Second Strand Oligo Mix. Cap, vortex to mix, and transfer the reactions to the prewarmed thermocyler and run the program.

10. Following second strand synthesis, SPRI purify the cDNA using the following parameters:

Post cDNA Synthesis SPRI Purification				
54 µL Volume at 1.9X				
Cycles	Action		Volume	Incubation Time
1X	Bind	AMPure XP Beads	105 µL	5:00
2X	Wash	70% EtOH	200 µL	1:00
1X	Dry	RT or 37 °C	Watch	
1X	Elute	Water	52 µL	2:00
1X	Transfer		50 µL	

11. Transfer 50 µL of the purified cDNA to freshly labeled tubes.

This is a stopping point in the procedure. If not continuing to library construction, freeze samples at -20 °C until ready to use.

End repair and adaptor ligation

12. Prepare the appropriate volume of ligation master mix by combining the reagents in table below. Mix reagents well before use and scale up or down as required. Pipette slowly as the buffer is viscous. Mix well. **Keep the master mix at room temperature while performing the end repair reaction.**

Ligation Master Mix		
Reagent	1X	8X+
Ligation Buffer	23 µL	207 µL
T4 DNA Ligase	2 µL	18 µL
	25 µL	

13. Program and prewarm a thermocycler for the end repair/dA tailing reaction using the parameters outlined in the table below. Make sure that the hot top is on.

Cycles	Temp	Time	Step
1X	20 °C	15:00	end repair
1X	72 °C	15:00	A tailing
1X	4 °C	HOLD	cold storage

Set volume to **70 µL** if prompted.

14. Prepare the appropriate volume of ER/A master mix by combining the reagents in the table below. Mix reagents well before use and scale up or down as required. Mix well, spin briefly, then dispense **20 µL** to each 50 µL purified cDNA sample.

End Repair/A Tailing Master Mix		
Reagent	**1X**	**8X+**
ER/A Buffer	16 µL	144 µL
ER/A Enzyme Mix	4 µL	36 µL
	20 µL	

15. Mix well and briefly spin. Transfer the reactions to the prewarmed thermocylcer and resume the run.

16. Once the run has completed, transfer the samples to ice and program a thermocycler for the next reaction using the parameters in the table below. Start the run then pause it to prewarm the instrument.

Cycles	Temp	Time	Step
1X	20 °C	30:00	Adaptor ligation
1X	4 °C	HOLD	Cold storage

Set volume to **100 µL** if prompted.

17. To each ER/A reaction on ice, add **25 µL** of the room temperature ligation master mix. Pipette slowly since the reagent is viscous. Cap and vortex the samples well. Briefly spin to seat the samples.

18. Remove the caps and add **5 µL** of SureSelect XT HS2 RNA Adaptor Oligo Mix to each sample. Cap the samples and vortex the samples well. Briefly spin to seat the samples. Transfer the reactions to the preprogramed cycler and resume the run.

19. Remove the samples from the cycler and transfer to a rack at room temperature.

20. Perform back-to-back SPRI purifications on the ligation reactions. The second SPRI purification greatly helps reduce the formation of adaptor dimers. The purification parameters are detailed in the two tables below.

Note: The bead and elution volumes are different for each purification.

Post-Ligation SPRI Purification #1				
100 µL Volume at 0.8X				
Cycles	**Action**		**Volume**	**Incubation Time**
1X	Bind	AMPure XP Beads	80 µL	5:00
2X	Wash	70% EtOH	200 µL	1:00
1X	Dry	RT or 37 °C	Watch	
1X	Elute	Water	50 µL	2:00
1X	Transfer		50 µL	

Post-Ligation SPRI Purification #2				
50 µL Volume at 1.2X				
Cycles	**Action**		**Volume**	**Incubation Time**
1X	Bind	AMPure XP Beads	60 µL	5:00
2X	Wash	70% EtOH	200 µL	1:00
1X	Dry	RT or 37 °C	Watch	
1X	Elute	Water	35 µL	2:00
1X	Transfer		34 µL	

21. Transfer the eluate from the second SPRI purification to fresh tubes and keep on ice.

Amplification of rRNA-depleted libraries

22. Remove the SureSelect Index Primers from frozen storage and allow to thaw at room temperature. Once thawed, vortex to mix, then spin to seat the sample in the bottom of the well.

23. Program and prewarm a thermocycler for PCR amplification of the adaptor-ligated library using the parameters outlined in the table below. Use the appropriate cycle number indicated in Table 5.

PCR Cycling Parameters			
Segment	**Number of Cycles**	**Temperature**	**Time (Minutes:Seconds)**
1	1	98 °C	2:00
2	See Table 5.	98 °C	0:30
		60 °C	0:30
		72 °C	1:00
3	1	72 °C	5:00
4	1	4 °C	Hold

Set volume to **50 µL** if prompted.

Table 5. Recommended PCR cycle numbers for SureSelect XT HS2 rRNA-depleted libraries.*

Input (ng)	Non-FFPE	Good FFPE (DV_{200} ≥50%)	Average FFPE (DV_{200} 30-50%)	Poor FFPE (DV_{200} 20-30%)
10	12			
50	10	13	15	
100	9	12	14	16
250	8	11	13	15
500	7	10	12	14
1,000	7	9	11	13

**The recommended PCR cycle numbers are an integral difference for this Agilent SureSelect ribosomal depletion application.*

24. Prepare the appropriate volume of PCR reaction mix using the table below. Mix well.

PCR Amplification Master Mix		
Reagent	**1X**	**8X+**
5X Herculase II Buffer with dNTPs	10 µL	90 µL
Herculase II DNA Polymerase	1 µL	9 µL
	11 µL	

25. Dispense **11 µL** of PCR master mix to each purified adaptor-ligated library.

26. Add **5 µL** of a SureSelect XT HS2 index primer pair to each reaction. Cap, mix, and spin briefly.

27. Place the reactions in the preprogrammed thermocycler and resume the run.

28. Following the PCR reactions, transfer the reactions to a rack at room temperature and SPRI purify using the parameters described in the table below.

Post-PCR SPRI Purification				
50 µL Volume at 1X				
Cycles	**Action**		**Volume**	**Incubation Time**
1X	Bind	AMPure XP Beads	50 µL	5:00
2X	Wash	70% EtOH	200 µL	1:00
1X	Dry	RT or 37 °C	Watch	
1X	Elute	Low TE Buffer	15 µL	2:00
1X	Transfer		15 µL	

29. Assess the quality and determine the yield of the libraries on an Agilent HS DNA1000 ScreenTape assay. See Figure 3 below for two examples.

30. Store completed libraries at -20 °C until ready to pool and submit for sequencing.

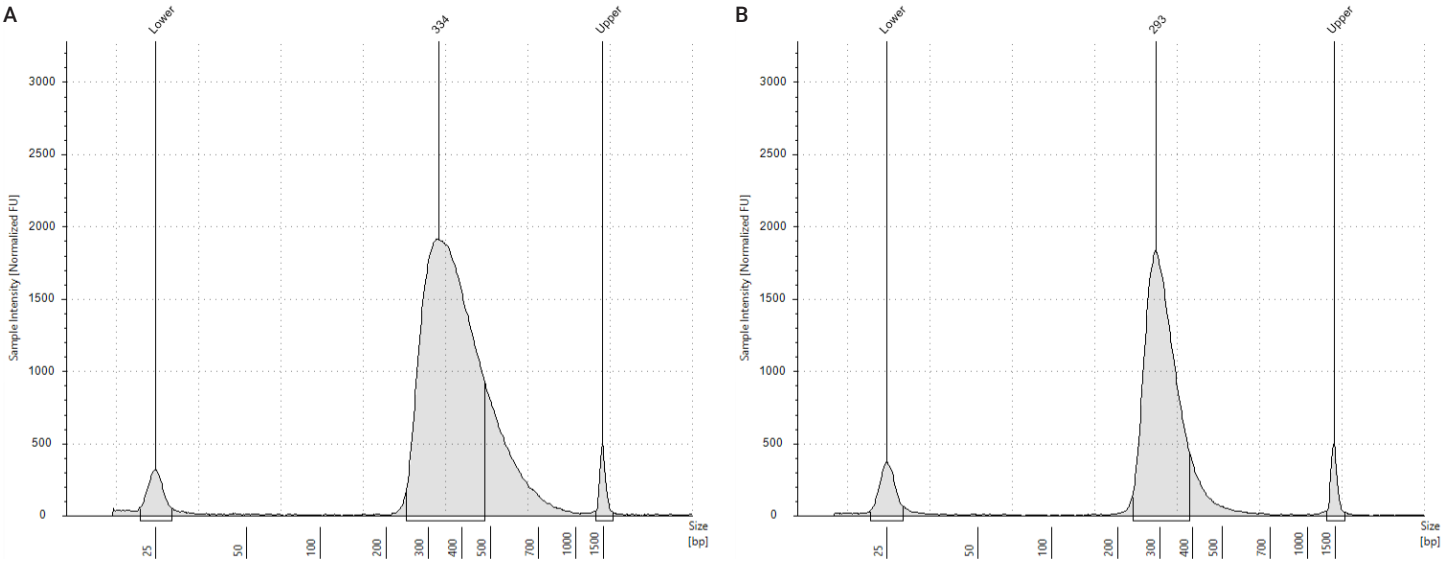

Figure 3. High Sensitivity D1000 ScreenTape electropherograms of rRNA-depleted SureSelect XT HS2 RNA libraries. Two examples of rRNA-depleted libraries prepared using the Kit O rRNA depletion kit and the procedure provided are shown. A) A library prepared from a 10 ng input of high-quality RNA (UHRR). B) A library prepared from a 100 ng input of RNA extracted from a melanoma FFPE tissue section (DV_{200} 43%).

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