Maximizing the output RNA-Seq for low input, challenging samples using the xGen™ Broad Range Library Prep Kit

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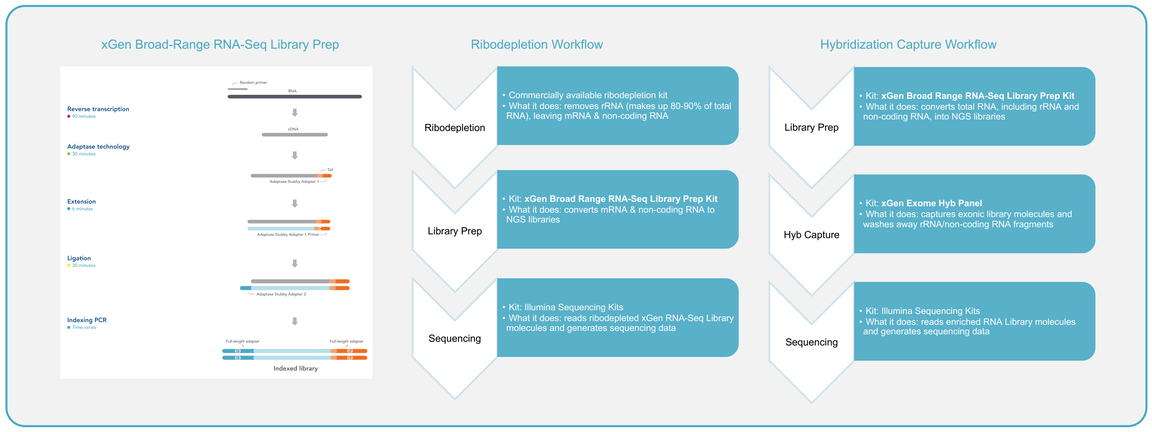
Introduction

Most labs consider the depletion of ribosomal RNA (rRNA) as the default approach for processing formalin fixation and paraffin-embedding (FFPE) RNA samples—despite high costs for reagents and sequencing. By using hybridization capture for enriching desired targets can produce quality data from FFPE RNA-Seq libraries. As a solution for FFPE samples in research, we present the IDT xGen™ Broad Range RNA Library Prep Kit. This workflow leverages patented Adaptase™ technology, a method for adapter ligation to single-stranded substrates, to directly convert first-strand cDNA into NGS libraries in four hours. This approach has been shown to save time for preparing stranded libraries without requiring conventional second-strand cDNA synthesis or degradation and increases yield and complexity while reducing bias and undesired library. Here, we present workflow options and summarize the resulting tradeoffs to data and costs. Due to the high variability in quality and quantity of RNA derived from FFPE, we present data to support the use of the IDT xGen Broad-Range RNA Library Kit with challenging and low input RNA samples.

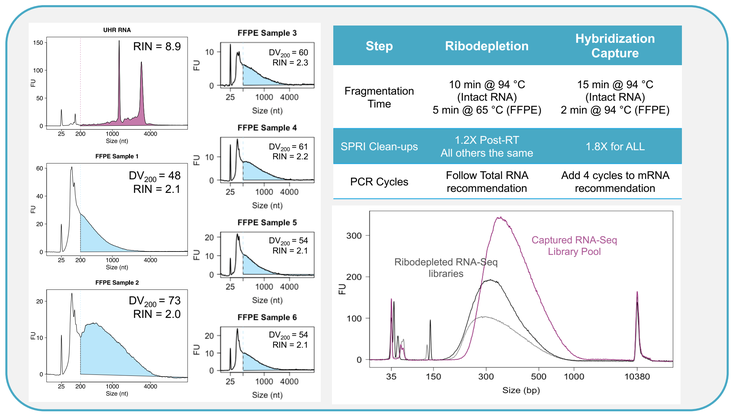
RNA-Seq library- Ribodepletion vs. downstream enrichment



The choice between ribodepletion and hybridization capture should be informed by the RNA types and sequencing data of interest. Below, we discuss each workflow and provide representative data from Universal Human Reference (UHR) RNA and multiple breast cancer tumor FFPE samples for research.

RNA characterization and protocol considerations

RIN scores are useful for determining whether a poly(A)-selection module can be used, which is typically only recommended for samples with RIN ≥7. High-quality samples typically have RIN scores ≥7 whereas FFPE samples often have RIN scores <7. For samples with RIN <7, ribodepletion and hybridization capture are the preferred workflows. More information about sample quality can be obtained through the DV_{200} score which can help inform the fragmentation time, SPRI ratio, and PCR cycling. In general, it is not recommended to make libraries using samples with a RIN < 2 or a DV_{200} < 30.



Step	Ribodepletion	Hybridization Capture
Fragmentation Time	10 min @ 94 °C (Intact RNA) 5 min @ 65 °C (FFPE)	15 min @ 94 °C (Intact RNA) 2 min @ 94 °C (FFPE)
SPRI Clean-ups	1.2X Post-RT All others the same	1.8X for ALL
PCR Cycles	Follow Total RNA recommendation	Add 4 cycles to mRNA recommendation

The FFPE curls were obtained from breast cancer tumors (Spectrum Health, Grand Rapids, MI). The RNA samples were extracted using the RNeasy FFPE Kit (Qiagen 73504). Isolated RNA was analyzed using an RNA 6000 Pico Kit (Agilent 5067-1513) on the Agilent Bioanalyzer. Trace analyses showed all samples had a RIN score near two whereas the DV_{200} scores ranged from 48 to 73, providing a more accurate depiction of RNA integrity.

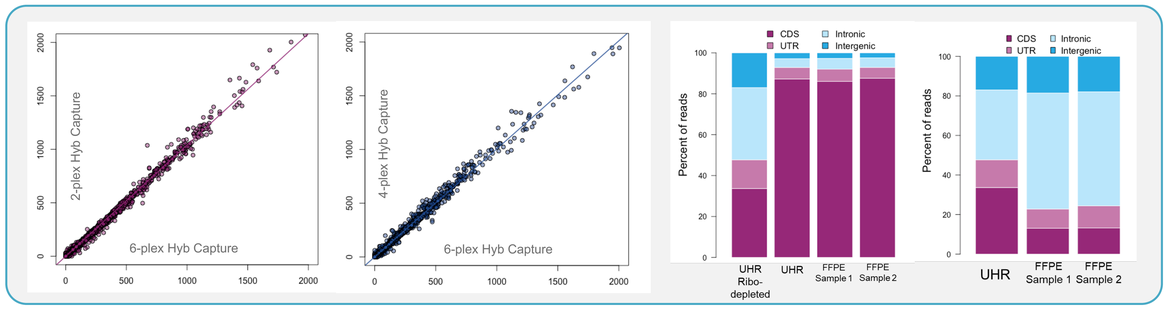
Workflow Tradeoffs

Workflow	Ribodepletion-based	Hyb-capture based
RNA types recovered	mRNA transcripts and fusion partners; non-coding RNA	mRNA transcripts and fusion partners
Kits used	1. Lexogen RiboCop V1.3 HMR (037.96) 2. xGen Broad-Range RNA-Seq Library Prep Kit (10009813) 3. xGen UDI Primer Pairs (10005922)	1. xGen Broad-Range RNA-Seq Library Prep Kit (10009813) 2. xGen UDI Primer Pairs (10005922) 3. xGen Exome Hyb Panel (10005152) 4. xGen Hybridization and Wash Kit (1080577) 5. xGen Universal Blockers (1075474) 6. xGen Library Amplification Primer Mix (1077675)
Steps	20	28
Library prep time (RNA-to-library)	6 hours	12 hours
Library price per sample (RNA-to-library)	$60	$92 (6-plex libraries per capture)
Sequencing price per sample (Library-to-fastq, 30M exonic reads)	$1488	$290
Total price per sample (FFPE RNA-to-data, $100/hr FTE)	$1548	$382

A compelling price per sample savings advantage is presented through the hybridization capture workflow relative to ribodepletion when exonic transcript information is the desired data output.

Robust data across FFPE samples of low quality

Sample	Workflow	Library Yield (nM)	STAR mapping rate (%)	Genes detected	Exonic rate (%)	rRNA rate (%)	ERCC (R^2) or selected bases (%)
UHR RNA	Ribo	44.2	86.2	17,132	46	3.4	0.94
	Hyb	82	89.6	17,758	91.2	1.3	96.7
FFPE Sample 1	Ribo	19.8	85.7	15,112	21.2	6.0	0.92
	Hyb	93	88.8	17,009	89.2	0.9	96.5
FFPE Sample 2	Ribo	24.9	84.2	15,274	24.6	4.8	0.92
	Hyb	73	87.9	17,024	88.1	1.0	95.8
FFPE Sample 3	Hyb	23.0	89.4	16,089	87.4	1.5	95.1
FFPE Sample 4	Hyb	54	88.0	16,065	88.7	1.3	95.6
FFPE Sample 5	Hyb	33.4	87.5	15,653	87.6	1.3	94.6
FFPE Sample 6	Hyb	56	87.6	16,246	88.4	1.2	95.4



xGen Broad-Range libraries followed by hybridization capture (xGen Exome Panel) resulted in sequencing data with high yields, mapping rates, and exonic rates across a range of FFPE samples. Further, libraries also produced high-quality data despite the lower input of 10 ng. RNA libraries can be combined prior to hybridization capture to maximize the value of each reaction. For DNA libraries, samples can often be multiplexed with up to 16 samples. However, because transcripts are expressed at variable levels, RNA libraries run the risk of depleting probes for highly-expressed genes, thus limiting the number of samples that can be multiplexed. Here, we showed FPKM (Fragments Per Kilobase of transcript per Million mapped reads) plots for UHR and FFPE RNA libraries that have been multiplexed with 2, 4, or 6 samples. Linear regression of the same library with different multiplexing shows extremely high correlation. Multiplexing can reduce the overall cost of each sample.