xGen™ Broad-Range RNA Library Prep Kit

REVISION HISTORY

Version	Release date	Description of changes
4	January 2024	Correction to fragmentation off bead protocol
3	June 2022	Adjustment to product size offerings
2	April 2022	Adaptase adapter name update
1	December 2021	Initial release

Table of contents

OVERVIEW

The xGen Broad-Range RNA Library Prep Kit for stranded RNA-seq enables the preparation of comprehensive next generation sequencing (NGS) libraries. This workflow utilizes Adaptase™ technology to produce RNA-seq libraries following first-strand cDNA synthesis. This approach preserves the template, whether it is the coding or non-coding strand, in a process without conventional second-strand cDNA synthesis. This kit supports a broad range of RNA inputs and sample types, making it suited for applications utilizing low-input quantities or challenging samples.

The xGen Broad-Range RNA Library Prep Kit works with the following sample types:

- ribosomal RNA-depleted RNA
- poly(A)-selected RNA
- total RNA
- formalin-fixed, paraffin-embedded (FFPE) RNA*
- RNA with low RNA Integrity Number (RIN), i.e., RIN <7 and low DV_{200} scores†

This kit can accommodate an mRNA input range of 100 pg to 100 ng directly into library prep, a range of 10 ng to 1 µg of total RNA input into an appropriate Poly(A)-selection or Ribosomal RNA (rRNA) depletion module, and a range of 10–500 ng total RNA directly into a library prep to be used for xGen hybridization capture.

Note: The xGen Broad-Range RNA Library Prep Kit does not include the Poly(A)-selection or rRNA depletion modules. If using one of these upstream modules, see more detail in Appendix A. If using a downstream xGen Hybridization capture, see Appendix C for the required protocol adjustments; if using the xGen Normalase™ Module, refer to Appendix D.

Feature	Available options
Input RNA ranges	10 ng to 1 µg (total RNA into an upstream module*) 10–500 ng (total RNA into library prep for downstream xGen hybridization capture) 100 pg to 100 ng (purified Poly(A)-selected RNA or purified rRNA-depleted RNA into library prep)
Library insert size	200–250, 250–300, 300–350 bp

* Quantification is not required upon completion of rRNA depletion or Poly(A) enrichment modules. These upstream modules are not included with this kit. The xGen Broad-Range RNA Library Prep Kit is compatible with many upstream modules, see Appendix A for more details.

* For more information, see the application note Optimizing RNA-seq data quality and costs for FFPE samples with the xGen™ Broad-Range RNA Library Preparation Kit.

† DV_{200} = % of RNA fragments >200 bases in length

xGen Broad-Range RNA Library Prep Kit workflow

Reverse transcription
90 minutes

Adaptase technology
30 minutes

Extension
6 minutes

Ligation
30 minutes

Indexing PCR
Time varies

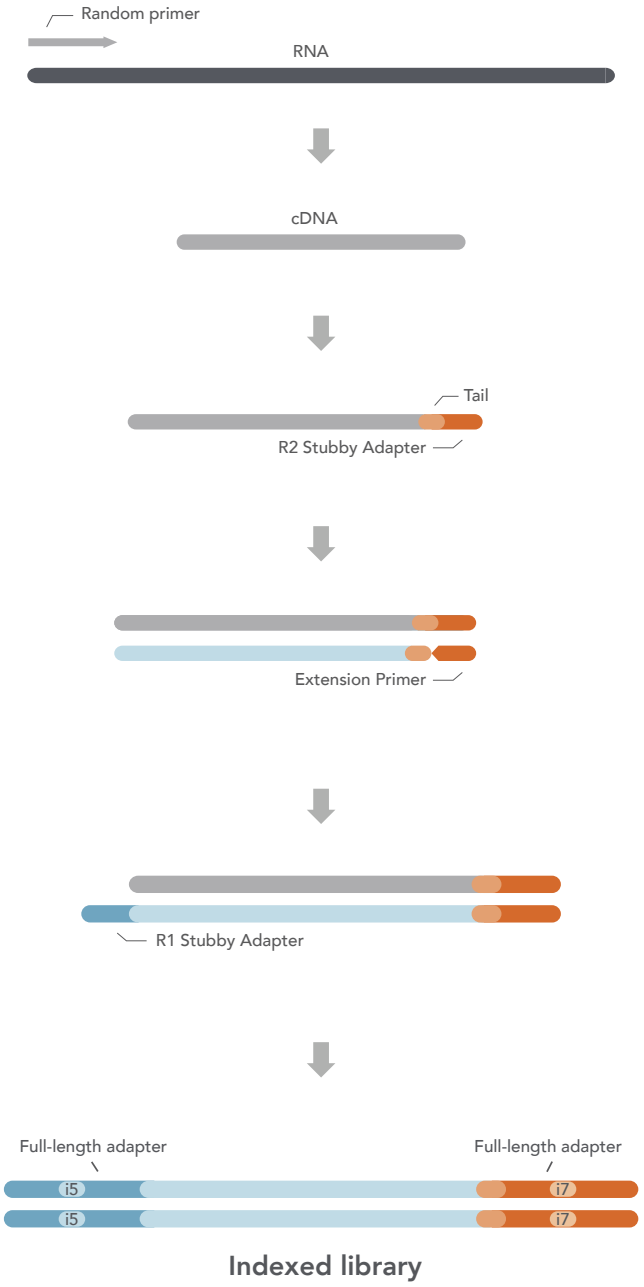

Figure 1. xGen Broad-Range Library Prep Kit workflow. After RNA fragmentation, the reverse transcriptase step uses random primers to generate the first strand cDNA. Next, Adaptase technology simultaneously performs tailing and ligation to incorporate the R2 Stubby Adapter to the 3′ ends of the cDNA molecules. The extension step produces a dsDNA duplex, while ligation add the R1 Stubby Adapter to the 3′ end of the primer extension products. Finally, indexing PCR increases library yield, incorporates single or dual indexes, and results in full-length adapters at the ends of each library molecule. In addition, bead cleanup steps are needed after extension, ligation, and final indexing PCR steps.

This workflow has the following steps:

- **Reverse transcription.** RNA fragmentation followed by random priming and reverse transcription generates first-strand cDNA.
- **Adaptase.** With the Adaptase technology, this step simultaneously performs tailing and ligation to incorporate R2 Stubby Adapter to the 3′ ends of the cDNA molecules.
- **Extension.** This step produces a dsDNA duplex for adapter ligation.
- **Ligation.** This step adds R1 Stubby Adapter to the 3′ ends of the primer-extended cDNA molecules.
- **Indexing PCR.** This step increases library yield, incorporates dual indexes, and results in full-length adapters at the ends of each molecule.

CONSUMABLES AND EQUIPMENT

This kit contains sufficient reagents for the preparation of 16 or 96 libraries (10% excess volume provided).

Consumables from IDT—Kit contents

Workflow stage	Component	Amount for 16 rxn (µL)	Amount for 96 rxn (µL)	Storage
Fragmentation	• Reagent F1	18	106	–20°C
	• Reagent F2	35	212	
	• Buffer F3	71	423	
	• Reagent F4	35	212	
Reverse transcription	• Enzyme R1	18	106	
	• Enzyme R2	18	106	
Adaptase reaction	• Buffer A1	35	212	
	• Reagent A2	35	212	
	• Reagent A3	22	132	
	• Enzyme A4	9	53	
	• Enzyme A5	9	53	
Extension and PCR	• Reagent E1	18	106	
	• PCR Master Mix	827	4964	
Ligation	• Buffer L1	53	317	
	• Reagent L2	176	1056	
	• Enzyme L3	35	212	
Additional reagents	Nuclease-Free Water	1000	1000	
	Low EDTA TE	6	24	Room temperature

Consumables from IDT—Reagents

Workflow component	Product name	Index #	Reaction size (rxn)	Catalog #
xGen Core Reagents	xGen Broad-Range RNA Library Prep Kit, 16 rxn	N/A	16	10009865
	xGen Broad-Range RNA Library Prep Kit, 96 rxn	N/A	96	10009813
xGen Normalase Module (optional)	xGen Normalase Module	N/A	96	10009793
xGen CDI Primers*	xGen CDI Primers	D501-D508/ D701-D712	96	10009815
xGen Normalase CDI Primers*	xGen Normalase CDI Primers	D501N-D508N/ D701N-D712N	96	10009794
xGen Normalase UDI Primer Plates*	xGen Normalase UDI Primer Plate 1	SU001-SU096	96	10009796
	xGen Normalase UDI Primer Plate 2	SU097-SU192	96	10009797
	xGen Normalase UDI Primer Plate 3	SU193-SU288	96	10009798
	xGen Normalase UDI Primer Plate 4	SU289-SU384	96	10009799
	xGen Normalase UDI Primer Set 1	SU001-SU384	4x96	10009795
	xGen Normalase UDI Primer Set 2	SU385-SU768	4x96	10009800
	xGen Normalase UDI Primer Set 3	SU769-SU1152	4x96	10009811
	xGen Normalase UDI Primer Set 4	SU1153-SU1536	4x96	10009812

* Each UDI primer set contains four single-use, 96-well plates. Each well in the plate contains one of the 96 premixed primer pairs.

Note: Normalase primers are compatible with both Normalase and non-Normalase workflows.

Consumables—Other suppliers

Item	Supplier	Catalog number
Ribosomal RNA depletion kit	See Appendix A for more information	See Appendix A for more information
Poly(A)-selection kit		
RNaseZap™	Thermo Fisher Scientific	AM9780
KAPA HiFi HotStart ReadyMix (optional for pre-hybridization PCR)	KAPA Biosystems	KK2602
SPRIselect® beads or Agencourt® AMPure® XP	Beckman Coulter	B23317/B23318/B23319 A63880/A63881/A63882
RNA 6000 Pico™ kit	Agilent	5067-1513
0.2 mL PCR tubes or 96-well plates	Various suppliers	Varies
Aerosol resistant tips and pipette ranging from 1–1000 µL	Various suppliers	Varies
200-proof/absolute ethanol	Various suppliers	Varies
Nuclease-free water for preparation of 80% ethanol	Various suppliers	Varies
Fluorometric assay (Qubit™) for library quantification	Thermo Fisher Scientific	Varies
High-sensitivity Bioanalyzer® and Tapestation®	Agilent	Varies
qPCR library quantification kit	KAPA Biosystems	Varies

Equipment

Item	Supplier	Catalog number
0.2 mL magnets for individual tubes and plates	Permagen	MSR812, MSP750
Fluorometer (Qubit)	Thermo Fisher Scientific	Varies
Bioanalyzer or Tapestation for RNA and library size determination	Agilent	Varies
Quantitative PCR instrument	Various suppliers	Varies
Microcentrifuge	Various suppliers	Varies
Programmable thermal cycler	Various suppliers	Varies
Vortex machine	Various suppliers	Varies

GUIDELINES

RNA input considerations

- The xGen Broad-Range RNA Library Prep Kit can be used at input ranges of 100 pg to 100 ng of purified poly(A)-selected or purified ribo-depleted RNA, a range of 10 ng to 1 µg of total RNA into an upstream module, and a range of 10–500 ng of total RNA directly into library prep to be used for downstream xGen hybridization capture.
- Consider transcriptome complexity and sample quality when choosing input RNA quantity. Although libraries may be successfully prepared from low inputs, reduced representation of transcriptome complexity may occur.
- To improve sequencing data, RNA samples should be of high quality (RIN ≥ 7) before using this protocol. For FFPE, or other low-quality RNA (RIN <7), see Appendix B for more detail.
- As ribosomal RNA (rRNA) makes up ~80–90% of all RNA molecules, it is best to remove rRNA by depletion, perform poly(A)-selection, or enrich for specific target regions using hybridization capture to obtain sufficient coverage for RNA sequencing. See Appendix A and C for suggestions on pairing the xGen Broad-Range RNA Library Prep Kit with upstream or downstream modules.
- RNA samples used with the xGen Broad-Range RNA Library Kit must have a volume of 5 µL. See Appendix A for recommendations on achieving this volume with compatible upstream modules. If the volume is greater than 5 µL, contact applicationsupport@idtdna.com for options to concentrate the sample.

Avoid cross-contamination

To reduce the risk of RNase and library contamination, particularly at low inputs follow these guidelines:

- Apply DNase to RNA samples upon isolation
- Perform all handling of RNA in an RNA-only workstation
- Clean lab areas and equipment using 0.5% sodium hypochlorite (10% bleach) and then apply RNaseZap™ (Thermo Fisher Scientific) to all surfaces to reduce the possibility of RNA degradation
- After coming into contact with surfaces outside of the RNA-only workstation, change gloves or clean gloves with RNaseZap
- Use barrier pipette tips
- Physically separate the laboratory space, equipment, and supplies where pre-PCR and post-PCR processes are performed

Important:

- Keep RNA samples on ice and return them to –80°C as soon as possible to reduce sample degradation.
- Minimize the number of freeze-thaw cycles to avoid sample degradation.
- Do not vortex RNA samples. Instead, gently pipette up and down or gently flick to mix.

RNA-seq library preparation

- After thawing reagents, invert or briefly vortex to mix well, but do not vortex enzymes. Spin all tubes in a microcentrifuge to collect contents before opening.
- Assemble reagent master mixes for each protocol stage: fragmentation, reverse transcription, Adaptase, extension, ligation, and PCR. Scale master mix volumes as appropriate, using 5% excess volume to compensate for pipetting loss. A master mix calculator is available here.
- Plan to prepare a minimum of 4 reactions for a 16-reaction kit or 24 reactions for a 96-reaction kit to avoid excessive reagent loss from preparing >4 master mixes with 5% overage each.
- Always add enzymes last to master mixes and immediately before adding the master mix to samples.
- Before starting, prepare a fresh 80% ethanol solution for bead-based clean-up steps. Prepare enough for approximately 1 mL per library.
- Preheat each thermal cycler program with lid heating ON (~105°C) before preparing samples and master mixes.

Automation

- This protocol is automatable. A 10% overage volume of reagents is supplied to accommodate automation. Contact us at applicationsupport@idtdna.com if you require additional reagent overage volume or would like to learn about our custom packaging options.

Tip: While IDT does not supply automated liquid handling instruments or consumables, our automation team collaborates with automation solution providers and customers to develop automated scripts for use of our kits with liquid handling platforms routinely used in NGS library preparation. Contact us at applicationsupport@idtdna.com to discuss automation options for the xGen Broad-Range RNA Library Prep Kit.

PROTOCOL

Notes:

- Review this protocol before using an upstream RNA processing kit (e.g., ribosomal RNA depletion or poly(A)-selection).
- If using a ribosomal RNA depletion kit, elute the RNA in 7 µL of nuclease-free water and transfer 5 µL to a new PCR tube for input into the fragmentation off bead step below. See Appendix A for more details.
- If using a poly(A)-selection kit, resuspend the mRNA capture beads in RNA Fragmentation Master Mix, following the fragmentation on bead protocol below. See Appendix A for more details.
- If using hybridization capture, see Appendix C for protocol adjustments.

Perform RNA fragmentation

Follow the appropriate fragmentation protocol below depending on your selected upstream workflow:

- If using purified mRNA, total RNA, or depleted/selected RNA eluted in elution buffer, nuclease-free water, or another appropriate buffer, follow the fragmentation off bead protocol.
- If using a poly(A)-selection module, and the RNA will go directly into the xGen Broad-Range RNA Library Prep Kit following mRNA capture, follow the fragmentation on bead protocol.

Off bead

Note: This procedure is compatible with purified mRNA, total RNA, or depleted/selected RNA input following elution.

1. On ice, bring RNA sample to a total volume of 5 µL in a 0.2 mL PCR tube, adding nuclease-free water if necessary.
2. Assemble the Fragmentation Master Mix on ice. Thoroughly mix, then pulse-spin to collect contents. Add 9 µL of the mixture below to each sample tube, thoroughly mix, then pulse-spin to collect contents (14 µL total reaction volume):

Component	Volume per reaction (µL)
• Reagent F1	1
• Reagent F2	2
• Buffer F3	4
• Reagent F4	2
Total volume	9

3. Preheat thermal cycler to 94°C (lid heating ON). Once the thermal cycler has reached 94°C, add the sample tubes and run the program which is appropriate for each sample:

RIN score	Insert size (bp)	Temperature (°C)	Time (min)
≥7	300–350	94	10
≥7	250–300	94	12
≥7	200–250	94	15
2-7	300–350	94	5
FFPE	200–250	65	5

4. When the program has completed, **immediately** transfer samples to ice and incubate for 2 min. Proceed directly to Perform reverse transcription.

On bead

Note: This procedure is only compatible with poly(A)-selection modules.

1. Assemble the Fragmentation Master Mix on ice. Thoroughly mix, then pulse-spin to collect contents (15 µL total reaction volume):

Component	Volume per reaction (µL)
• Reagent F1	1
• Reagent F2	2
• Buffer F3	4
• Reagent F4	2
Nuclease-free water	6
Total volume	15

2. Follow the Poly(A)-selection module protocol according to the manufacturer's specifications (see Appendix A).
3. At the last step of the Poly(A)-selection module protocol, do not resuspend the mRNA capture beads in elution buffer. Instead, resuspend the beads in 15 µL of the above Fragmentation Master Mix. Pipette up and down to thoroughly mix.
4. Preheat thermal cycler to 94°C. Once the thermal cycler has reached 94°C, add the sample tubes and run the program most appropriate for each sample (lid heating ON):

RIN score	Insert size (bp)	Temperature (°C)	Time (min)
≥7	300–350	94	10
≥7	250–300	94	12
≥7	200–250	94	15
2–7	300–350	94	5
FFPE	200–250	65	5

5. When the thermal cycler program has completed, **immediately** place the tubes on a magnetic rack, wait for the solution to clear, then transfer 14 µL of the supernatant to a new tube. **Do not let the samples cool** prior to placing them on the magnetic rack. If cooled, mRNA fragments will re-hybridize to the mRNA capture beads.
6. **Immediately** transfer samples to ice and incubate for 2 min. Proceed directly to Perform reverse transcription.

Perform reverse transcription

1. Assemble the Reverse Transcription Master Mix on ice. Thoroughly mix, then pulse-spin to collect contents. Add 6 µL of the mix below to each sample tube, then thoroughly mix and pulse-spin to collect contents (20 µL total reaction volume):

Component	Volume per sample (µL)
• Enzyme R1	1
• Enzyme R2	1
Nuclease-free water	4
Total volume	6

2. Run the thermal cycler program (lid heating ON):

Temperature (°C)	Time (min)
25	10
42	30
70	15
4	Hold

Perform post-reverse transcription cleanup

1. Prepare a fresh 80% ethanol solution. Prepare enough volume for ~1 mL per sample.
2. Add 30 µL Low EDTA TE to each sample (50 µL total volume).
3. Add SPRISelect® beads (Thermo Fisher Scientific) according to the bead volumes listed here:

Insert size (bp)	SPRI ratio*	Bead volume (µL)
300–350	1.0X	50
250–300	1.4X	70
200–250	1.8X	90

* For alternate bead-based DNA clean-up products, the ratios will need to be adjusted specific to the characteristics of the products.

RNA sample	SPRI ratio	Bead volume (µL)
FFPE	1.2X	60

4. Mix each tube by pipetting 10 times or until the mixture is homogenous. Make sure there are no bead-sample suspension droplets left on the sides of the tube.
5. Incubate the samples for 5 minutes at room temperature.
6. Pulse-spin the samples in a microcentrifuge. Place the sample tubes on a magnetic rack until the solution clears and a pellet is formed (~2 minutes).
7. Remove and discard the supernatant without disturbing the pellet.
8. Add 200 µL of freshly prepared 80% ethanol solution to the pellet while it is still on the magnet. Be careful not to disturb the pellet. Incubate for 30 seconds, then carefully remove the ethanol solution using a pipette.
9. Repeat step 8 once for a second wash with the ethanol solution.
10. Pulse-spin the samples in a microcentrifuge, place them back onto the magnet, and remove any residual

ethanol solution from the bottom of the tube.

11. Without delay to avoid over-drying of beads, add 12 µL of Low EDTA TE to resuspend the pellet and mix well by pipetting up and down until homogenous. If droplets of the resuspension remain on the side of the tube, pulse spin in a microcentrifuge to collect contents. After at least 2 minutes, place the tube on the magnetic rack and wait until the solution clears and a pellet is formed (~2 minutes).
12. Transfer 10 µL elute to a new 0.2 mL PCR tube. Make sure that the eluate does not contain magnetic beads (indicated by brown coloration in eluate). If magnetic beads are present, place the tube back on the magnet and transfer the eluate again.

Safe stopping point: Samples can be stored at 4°C up to 24 hours, or at –20°C up to one month.

Perform Adaptase reaction

1. Pre-assemble the Adaptase Master Mix on ice. Mix thoroughly and pulse spin to collect contents. Do not add the Adaptase Master Mix to samples until after denaturation (step 2). Keep Master Mix on ice until step 4.

Component	Volume per sample (µL)
• Buffer A1	2
• Reagent A2	2
• Reagent A3	1.25
• Enzyme A4	0.5
• Enzyme A5	0.5
Low EDTA TE	4.25
Total volume	10.5

2. Preheat thermal cycler to 95°C. When the thermal cycler has reached 95°C, add the sample tubes (10 µL eluate), then run the following thermal cycler program (lid heating ON):

Temperature (°C)	Time (min)
95	2

3. When the program has completed, immediately transfer samples to ice and incubate for 2 min. Proceed directly to the next step.
4. Add 10.5 µL of the Adaptase Master Mix from step 2 to each sample tube, thoroughly mix, then pulse-spin to collect contents (20.5 µL total reaction volume).
5. Run the following thermal cycler program (lid heating ON):

Temperature (°C)	Time (min)
37	15
95	2
4	Hold

Perform extension reaction

1. Assemble the Extension Master Mix on ice. Thoroughly mix, then pulse-spin to collect contents. Add 23 µL of the mix to each sample tube, thoroughly mix, and pulse-spin to collect contents (43.5 µL total reaction volume):

Component	Volume per sample (µL)
• Reagent E1	1
• PCR Master Mix	22
Total volume	23

2. Run the following thermal cycler program (lid heating ON):

Temperature (°C)	Time (min)
98	1
63	2
72	5
4	Hold

Perform post-extension cleanups

Perform two 1.2X SPRI cleanups.

1. Add 52.2 µL SPRI beads to each sample.
2. Mix by pipetting 10 times or until homogenous. Make sure that there are no bead-sample suspension droplets left on the side of the tube.
3. Incubate the samples for 5 minutes at room temperature.
4. Pulse-spin the samples in a microcentrifuge. Place the sample tubes on a magnetic rack until the solution clears and a pellet is formed (~2 minutes).
5. Remove the supernatant, using a pipette, without disturbing the pellet, and discard the supernatant.
6. Add 200 µL of freshly prepared 80% ethanol solution to each pellet while tubes are still on the magnet. Be careful not to disturb the pellets. Incubate for 30 seconds, then carefully remove the ethanol solution.
7. Repeat step 6 once for a second wash with the ethanol solution.
8. Pulse-spin the samples in a microcentrifuge, place them back onto the magnet, and remove any residual ethanol solution from the bottom of each tube using a pipette.
9. Elute in 52 µL Low EDTA TE and transfer 50 µL eluate to a fresh 0.2 mL PCR tube.
10. Perform a second clean-up at a 1.2X ratio. Add 60 µL SPRI beads to each sample and repeat step 2–8.
11. Elute in 17 µL Low EDTA TE, then transfer 15 µL eluate to a fresh tube.

Safe stopping point: Samples can be stored at 4°C up to 24 hours, or at –20°C up to one month.

Perform ligation reaction

1. Assemble the Ligation Master Mix on ice. Mix thoroughly and pulse-spin to collect contents. Add 15 µL of the mix to each sample tube, mix thoroughly, and pulse-spin to collect (30 µL total reaction volume):

Component	Volume per reaction (µL)
• Buffer L1	3
• Reagent L2	10
• Enzyme L3	2
Total volume	15

2. Run the following thermal cycler program (lid heating ON):

Temperature (°C)	Time (min)
25	15
4	Hold

Perform post-ligation cleanup

Perform a 1.0X SPRI cleanup before PCR.

1. Add 30 µL SPRI beads to each sample.
2. Mix each sample by pipetting 10 times or until homogenous. Make sure that no bead-sample suspension droplets are left on the side of the tube.
3. Incubate the samples for 5 minutes at room temperature.
4. Pulse-spin the samples in a microcentrifuge. Place the sample tubes on a magnetic rack until the solution clears and a pellet is formed (~2 minutes).
5. Remove and discard the supernatant, using a pipette, without disturbing the pellet.
6. Add 200 µL of freshly prepared 80% ethanol solution to the pellet while it is still on the magnet. Be careful not to disturb the pellet. Incubate for 30 seconds, then carefully remove the ethanol solution.
7. Repeat step 6 once for a second wash with the ethanol solution.
8. Pulse-spin the samples in a microcentrifuge, place them back onto the magnet, and remove any residual ethanol solution from the bottom of the tube using a pipette.
9. Elute in 22 µL Low EDTA TE and transfer 20 µL eluate to a fresh tube.

Safe stopping point: Samples can be stored at 4°C up to 24 hours or, at –20°C up to one month.

Perform indexing PCR

1. Add 2.5 µL (of each combinatorial dual index CDI primer) or 5 µL (single or unique dual index UDI primer) directly to each sample following the volumes listed in the table below (25 µL total reaction volume).

   Note: If using xGen Normalase primers, see Appendix D: xGen Normalase Module instructions.

Indexing Kit	Primer	Volume added to each sample (µL)
xGen CDI primer pairs	i5	2.5
	i7	2.5
xGen UDI primer pairs	Premixed i5 and i7	5

2. Assemble the PCR Master Mix on ice. Mix thoroughly and pulse-spin to collect contents. Add 25 µL of the mix to each tube, mix thoroughly, and pulse-spin to collect contents (50 µL total reaction volume):

Component	Volume per reaction (µL)
• PCR Master Mix	25
Total volume	25

3. Run the following thermal cycler program, adjusting the number of cycles depending on the input amount and sample quality (see table below) (lid heating ON):

Step	Cycle number	Temperature (°C)	Time
Activate enzyme		98	2 min
Denature	Perform X cycles*	98	20 sec
Anneal		60	30 sec
Extend		72	30 sec
Final extension		72	1 min
Hold		4	∞

* The recommended minimum number of cycles, based on various input amounts, to provide sufficient yields suitable for direct sequencing is as follows:

Input amount (depleted/selected)	Minimum number of cycles	Input amount (total RNA)	Minimum number of cycles
100 pg	18	10 ng	17
1 ng	14	50 ng	14
10 ng	11	100 ng	13
100 ng	8	1 µg	8

Tip: The number of cycles required may vary based on the input amount, as detailed above, but also on the quality of the sample. Recommendations above are for high-quality input RNA (RIN ≥ 7). Low-quality and FFPE RNA may require additional cycles to reach a minimum yield of 4 nM.

Perform post-indexing PCR cleanup

Perform a 0.85X SPRI cleanup after completing the indexing PCR step.

1. Add 42.5 µL SPRI beads and mix by pipetting 10 times or until homogenous. Make sure there are no bead-sample suspension droplets left on the side of the tube.
2. Incubate the samples for 5 minutes at room temperature.
3. Pulse-spin the samples in a microcentrifuge. Place the sample tubes on a magnetic rack until the solution clears and a pellet is formed (~2 minutes).
4. Remove and discard the supernatant, using a pipette, without disturbing the pellet.
5. Add 200 µL of freshly prepared 80% ethanol solution to the pellet while it is still on the magnet. Be careful not to disturb the pellet. Incubate for 30 seconds, then carefully remove the ethanol solution using a pipette.
6. Repeat step 5 once for a second wash with the ethanol solution.
7. Pulse-spin the samples in a microcentrifuge, place them back onto the magnet, and remove any residual ethanol solution from the bottom of the tube using a pipette.
8. Elute the pellet in 22 µL Low EDTA TE and transfer 20 µL eluate to a fresh tube.

Notes: When performing direct sequencing with low-input RNA, adapter dimers may be present. These dimers are evident by a peak at ~135 bp by Bioanalyzer® instrument (Agilent) to check the size and purity of nucleotide polymers. If adapter dimers are present in the sample, perform a second 0.85X SPRI cleanup prior to library quantification. An additional cleanup is not needed when performing hybridization capture.

1. If necessary, add Low EDTA TE to sample to bring it to a total volume of 20 µL.
2. Add 17 µL SPRI beads and elute in 22 µL Low EDTA TE, then transfer 20 µL eluate to a fresh tube.

Optionally, if you are direct sequencing on patterned flow cells, perform a second 0.85X SPRI cleanup to remove the unincorporated primers which can increase index hopping. This second purification is not necessary if using xGen Unique Dual Indexing primer pairs, xGen Normalase Module, or xGen Hybridization Capture.

Safe stopping point: Samples can be stored at 4°C up to 24 hours, or at –20°C for a longer time. The library is now ready for quantification and sequencing.

Results analysis

Yields for varying input quantities using this protocol with human brain mRNA (Clontech™) are shown below. Mean library size includes the adapter sequences, which add ~135 bp to the final library insert size.

Input amount (mRNA)	PCR cycles
100 pg	18
1 ng	14
100 ng	8

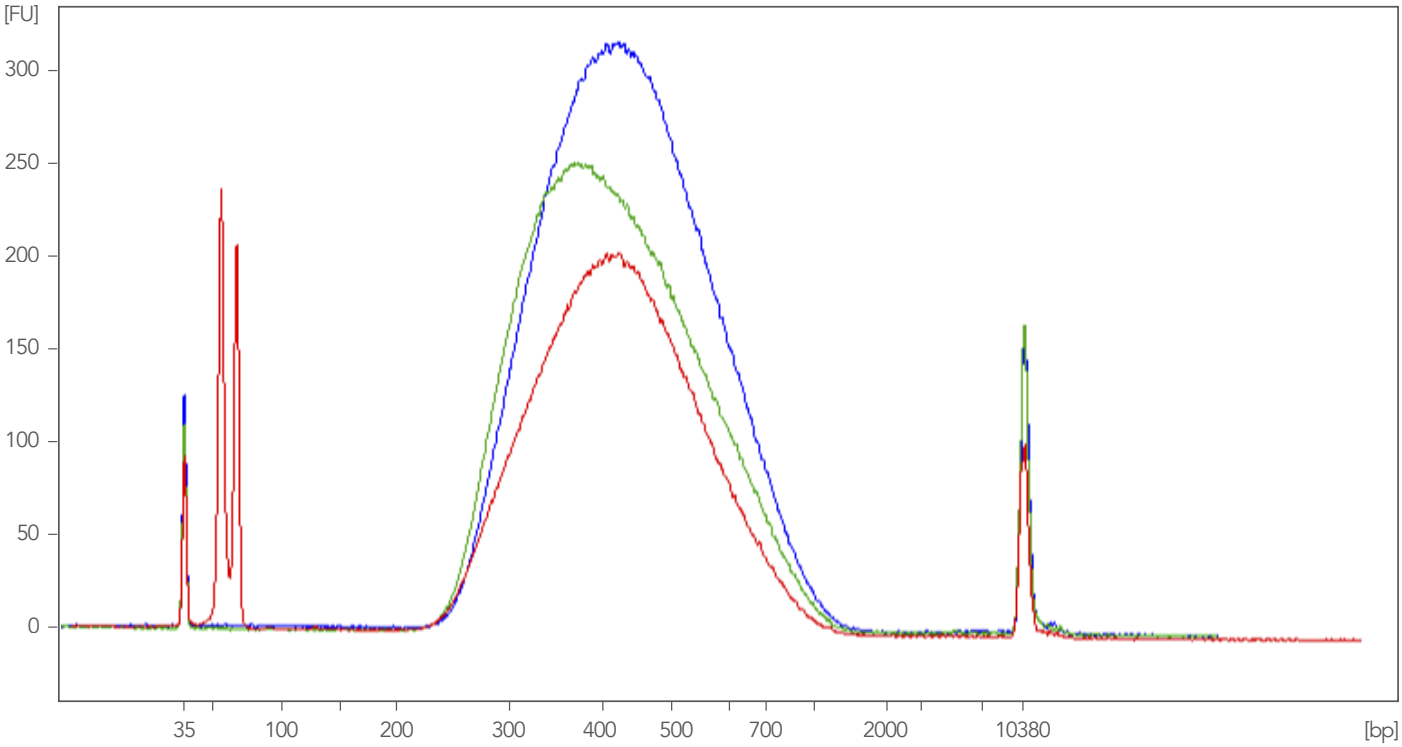

Figure 2. RNA Bioanalyzer results. Fragment sizes and quantities for RNA libraries produced from starting inputs (*n* = 1) of 100 pg, 1 ng, or 100 ng human brain mRNA (Clontech™ 636102). Libraries were run on the Bioanalyzer using a HS DNA chip.

A peak at ~150 bp would indicate adapter dimer carry-over (not shown). These can be removed with a second post-PCR SPRI (repeat Perform post-indexing PCR cleanup). Be aware that a peak at ~60 bp represents carryover of indexing primers from PCR and is expected. These fragments cannot cluster and will not impact quantification or sequencing; however, they can still be removed by a second post-PCR SPRI.

Library quantification

Accurate library quantification is essential to properly load the sequencing instrument. Libraries can be quantified using fluorometric-, electrophoretic-, or qPCR-based methods. Electrophoresis-based methods also enable examination of library insert size distribution. There are many commercially available kits suitable for library quantification.

Sequence the RNA libraries

RNA libraries can be sequenced with single-end or paired-end sequencing on Illumina® instruments. We recommend using a minimum of 2 x 50 paired-end reads. The read length and depth of coverage required will depend on the application.

Due to the complexity of the transcriptome, no PhiX spike-in is required on MiSeq® or MiniSeq® (Illumina) instruments. The NextSeq550® may be sensitive to the low complexity Adaptase tail, which is present at the beginning of Read 2 during the sequencing run. PhiX or another suitable library spike-in may be required. Contact Illumina® technical support for further information regarding the compatibility of the sequencing instrument with low-complexity sequences.

Important: To ensure optimal mapping efficiency, use the STAR aligner [1]. If using a different software for alignment, be aware that you may have to use bioinformatic trimming of the low complexity Adaptase tail from these libraries (see Appendix F).

APPENDIXES

Appendix A: Kit compatibility with upstream modules

The xGen Broad-Range RNA Library Prep Kit is compatible with many poly(A)-selection and ribosomal RNA depletion modules. See the details below for specific modules and recommendations on combining them with the xGen Broad-Range RNA Library Prep Kit workflow. Contact us for additional questions or recommendations for modules not listed below.

Poly(A)-selection modules

Poly(A)-selection enables the selection of mRNA by capturing the poly(A)-tail of transcripts using oligo-dT beads. We suggest using the following modules/kits. Internal testing confirms their compatibility with the xGen Broad-Range RNA Library Prep Kit.

- NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England Biolabs) – recommended
- Poly(A) RNA Selection Kit (Lexogen®) – alternate

If using the NEBNext Poly(A) mRNA Magnetic Isolation Module, follow the manufacturer's specifications until the final elution in the NEB Instruction Manual or the NEB Online Protocol. Instead of resuspending the mRNA capture beads in 17 µL of Tris Buffer, resuspend the mRNA capture beads in 15 µL of the Fragmentation Master Mix, then proceed to Perform RNA fragmentation: On bead.

If using the Lexogen Poly(A) RNA Selection Kit, follow the manufacturer's specifications until the final elution step. Completely remove the supernatant from the last wash. Instead of resuspending the beads in 25 µL of molecular biology grade water, resuspend the beads in 15 µL of the Fragmentation Master Mix, then proceed to Perform RNA fragmentation: On bead.

Ribosomal RNA-depletion modules

Ribosomal RNAs comprise 80–90% of total RNA and should be removed before sequencing to avoid repetive sequencing of these transcripts over the rest of the transcriptome. Our internal testing found that one of the following kits to remove ribosomal RNA is compatible with the xGen Broad-Range RNA Library Prep Kit:

- RiboCop® rRNA Depletion Kit V2 (Lexogen) – recommended
- NEBNext® rRNA Depletion Kit (New England Biolabs) – alternate

If using the Lexogen RiboCop rRNA Depletion Kit V2, follow the manufacturer's specifications until the final elution step. Instead of adding 12 µL of Elution Buffer to the tube, add 7 µL of Elution Buffer to the tube. Remove the tube from magnet and resuspend the beads by pipetting up and down. Incubate for 2 minutes at room temperature. Place the tube on a magnetic rack. When the solution clears, transfer 5 µL of the supernatant into a new PCR tube then proceed to Perform RNA fragmentation: Off bead.

If using the NEBNext rRNA Depletion Kit, follow the manufacturer's specifications until the final elution step. Remove the tube/plate from the magnetic stand. Instead of eluting the RNA in 8 µL of nuclease-free water, elute the RNA in 7 µL of nuclease-free water. Mix well by pipetting up and down 10 times. Incubate for at least 2 minutes. Place the tube/plate on the magnetic stand. When the solution is clear, transfer 5 µL to a new PCR tube then proceed to Perform RNA fragmentation: Off bead.

Appendix B: Low-quality and FFPE RNA

The quality of input RNA into an RNA-Seq workflow can impact the library yield and data quality. RNA quality and integrity can be evaluated using two different metrics: RIN score (RNA Integrity Number, which is the ratio of the 28S to 18S rRNA peaks) and DV_{200} (percentage of RNA fragments that are greater than 200 nucleotides). Both metrics can be obtained using an electrophoretic instrument, such as an Agilent Bioanalyzer® or TapeStation®.

Samples that have RIN scores ≥ 7 are considered high-quality and are suitable for poly(A)-selection. Samples with RIN scores between 2 and 7 are suitable for ribodepletion or hybridization capture. For these samples, more information about sample quality can be obtained through the DV_{200} score, which can help inform the fragmentation time, SPRISelect® bead ratio, and PCR cycling. In general, it is not recommended to make libraries using samples with a RIN <2 or a DV_{200} <30.

Experimental results with low-quality RNA (RIN <7)

Universal Human Reference (UHR) RNA (Agilent) was heat-damaged according to the table below to produce variable RIN scores. RIN scores were evaluated using an RNA Pico 6000 Bioanalyzer Kit (Figure 3).

RIN score	Heat-treatment temperature	Heat-treatment time
9.2	80°C	NA
3.2	80°C	5 min
2.3	80°C	8 min
1.7	80°C	15 min

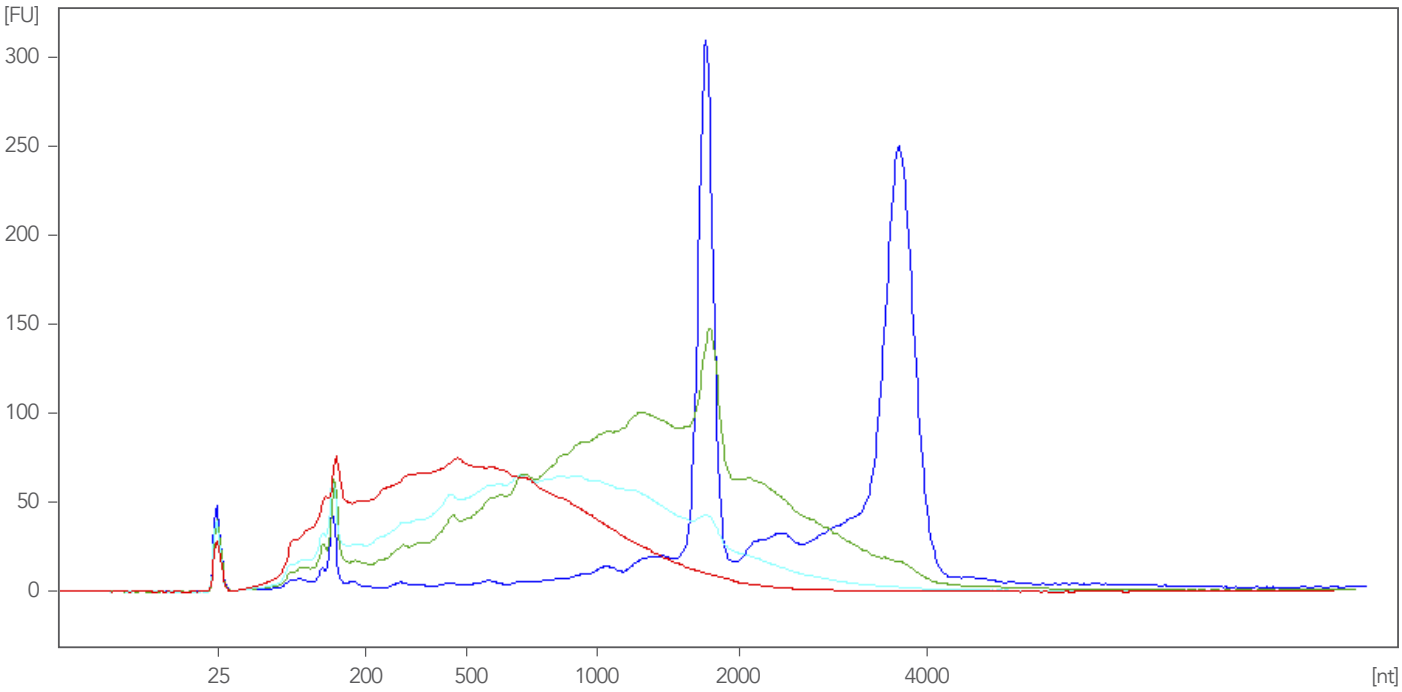

Figure 3. Bioanalyzer results for heat damaged UHR. RNA Bioanalyzer results for heat damaged UHR to obtain various RIN scores. UHR RNA samples, one sample (*n* = 1) per condition, were run on the Bioanalyzer using an RNA Pico 6000 chip. The Total Eukaryote RNA program was run to obtain RIN score estimates.

Expected library results using 100 ng UHR with RIN scores of 9.2, 3.2, 2.3, and 1.7 as input into the xGen Broad-Range RNA Library Prep Kit. Fragmentation times were adjusted, as recommended when 8 PCR cycles are used for each library.

Experimental results with FFPE RNA (RIN ~2, DV_{200} <80)

FFPE curls were obtained from two breast cancer tumor samples (Spectrum Health, Grand Rapids, MI). FFPE RNA was extracted using the RNeasy® FFPE Kit (QIAGEN). RIN and DV_{200} scores were evaluated using an RNA Pico 6000 Bioanalyzer Kit. Trace analyses (Figure 4) indicate that the samples have RIN scores of ~2 and DV_{200} scores of 48 and 73.

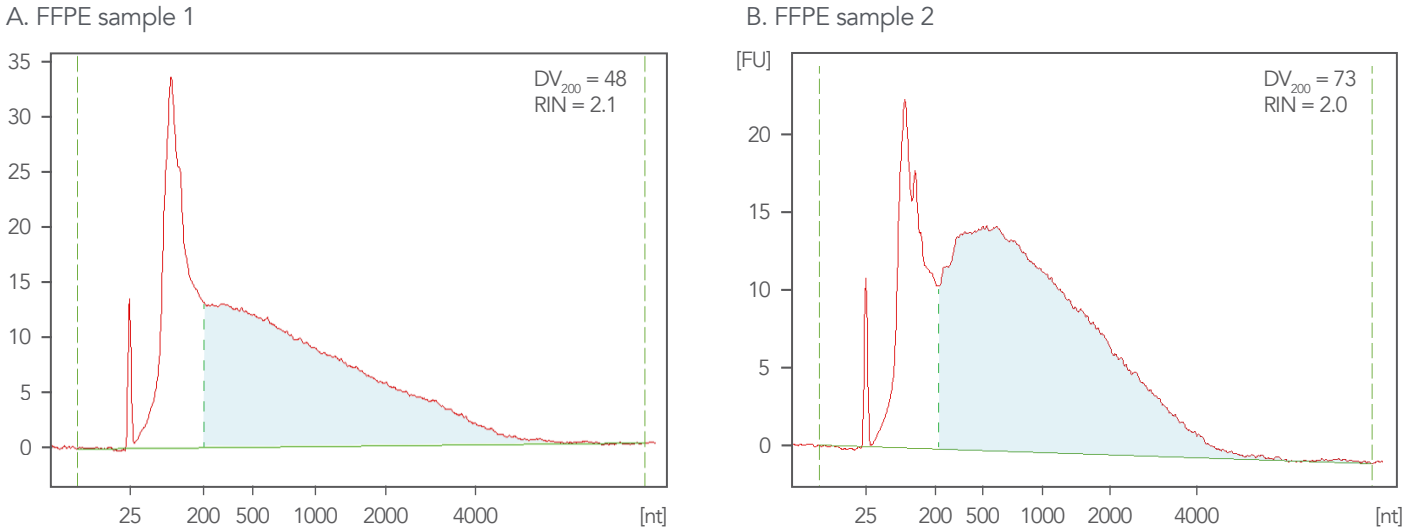

Figure 4. Bioanalyzer results for FFPE RNA. RNA was isolated from two breast cancer tumor samples. RNA samples were run on the Bioanalyzer using an RNA Pico 6000 chip. The Total Eukaryote RNA program was used to obtain RIN scores. The DV_{200} RNA Pico program was used to obtain DV_{200} scores. The xGen Broad-Range RNA Library Prep Kit is for research purposes only.

FFPE RNA samples typically have low integrity (RIN < 7), so the recommended NGS workflows are ribodepletion or hybridization capture. For ribodepletion, see Appendix A for instructions on pairing this kit with compatible ribodepletion modules. For hybridization capture, see Appendix C for recommended protocol adjustments.

Following ribodepletion, FFPE-specific adjustments to this protocol are as follows:

- **Fragmentation.** Instead of fragmenting samples at 94°C, heat samples at 65°C for 5 minutes (see Perform RNA fragmentation). This step may require sample-specific testing to determine the best conditions for fragmentation. To test different conditions, reduce fragmentation time by 1-2 min for low DV_{200} scores (DV_{200} <40) or add 1-2 min for high DV_{200} scores (DV_{200} >70).
- **Post-RT SPRI bead cleanup ratio.** Instead of using a 1.0X SPRI ratio in the cleanup after reverse transcription, use a 1.2X SPRI ratio. To achieve a 1.2X ratio, add 60 µL of SPRI beads to the 50 µL sample volume. This step may require sample-specific testing to determine the best ratio. To test different conditions, increase the SPRI ratio up to 1.8X to retain smaller fragments for low DV_{200} scores (DV_{200} <40).

Library results using 100 ng FFPE RNA and the Lexogen RiboCop v1.3 ribodepletion module. In an in-house test, ribodepleted RNA was used as input into the xGen Broad-Range RNA Library Prep Kit with the following adjustments:

- Fragmentation at 65°C for 5 min
- 1.2X post-RT SPRI cleanups
- Libraries amplified with 12 PCR cycles

Sample	RIN score	DV_{200} score	Fragmentation time	Post-RT SPRI	Mean library size (bp)	Mean insert size (bp)	Library yield
FFPE sample 1	2.1	48	5 min at 65°C	1.2X	400	270	20 nM
FFPE sample 2	2.0	73	5 min at 65°C	1.2X	470	340	24 nM

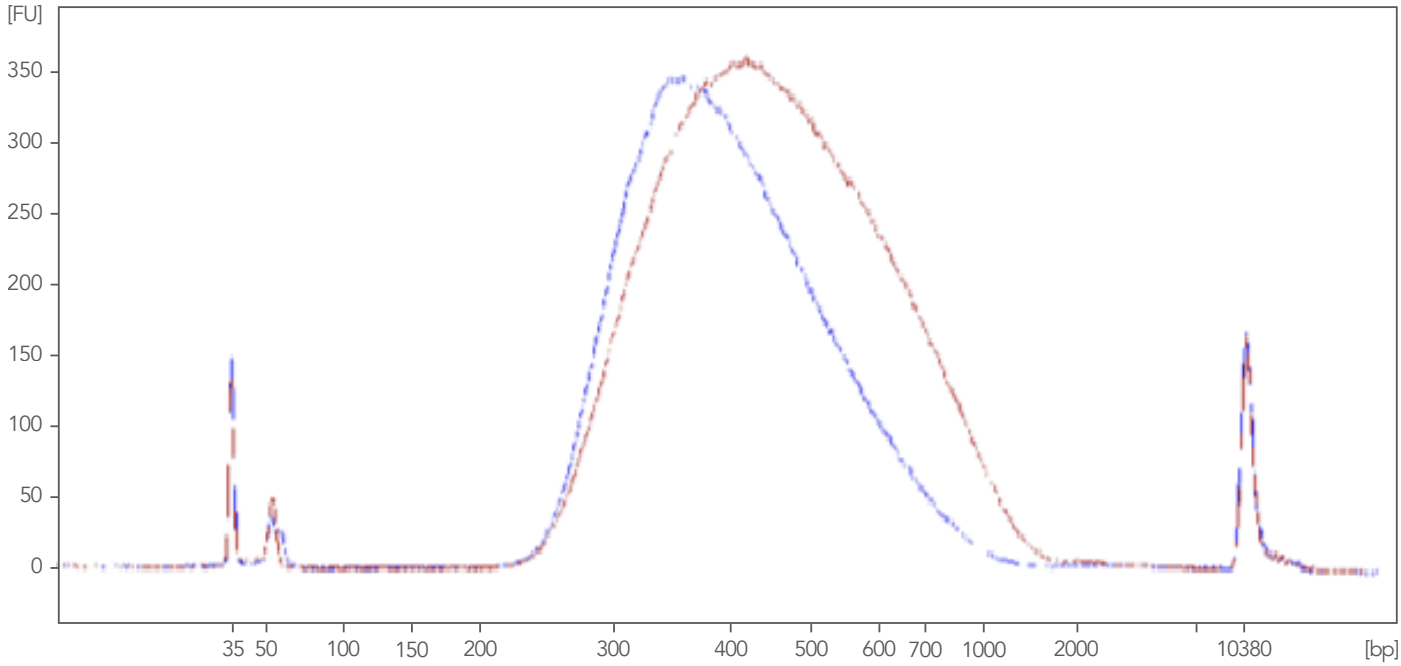

Figure 5. Bioanalyzer results for libraries from ribodepleted FFPE RNA. Expected results for RNA libraries produced from 100 ng FFPE RNA following ribodepletion using Lexogen RiboCop v1.3. Libraries were run on the Bioanalyzer using a HS DNA chip.

For more information, see our application note **Optimizing RNA-seq data quality and costs for FFPE samples with the xGen™ Broad-Range RNA Library Preparation Kit**.

Appendix C: Adjustments for xGen Hybridization Capture Panels

Hybridization capture enables the capture and sequencing of specific regions of the transcriptome. First, libraries are made using total RNA isolated from tissue or an organism. Next, a probe set designed to a target region (such as the exome) is used to hybridize and capture complementary fragments. The captured library is then sequenced.

Hybridization capture is compatible with intact as well as damaged or degraded samples, such as FFPE RNA. For more information, see our application note **Optimizing RNA-seq data quality and costs for FFPE samples with the xGen™ Broad-RangeRNA Library Preparation Kit.** For Indexing PCR, use the PCR Master Mix included in the kit.

For each hybridization capture library, 500 ng of each library is recommended. For more information, see the **xGen Hybridization Capture** protocol.

During library prep, follow these protocol adjustments:

- **SPRI bead-based cleanup ratio**. Follow the standard SPRI ratios if working with moderate- to high-integrity RNA samples. To accommodate smaller insert sizes while working with damaged FFPE, use the 200 bp SPRI recommendations found in the Beckman Coulter product instructions to retain short fragments.
- **PCR cycles.** To obtain library yields >500 ng, follow the PCR cycling recommendations below. No additional PCR cycles are required if substituting the included PCR Master Mix with KAPA HiFi HotStart ReadyMix, or similar PCR mix. Comparable yields can be obtained for both polymerases.

Note: This step may require sample-specific testing to determine the number of PCR cycles. To test this parameter, increase the number of PCR cycles if yields are lower than the required amount for hybridization capture.

Total RNA into library prep (ng)	PCR cycle recommendations
10	15
50	14
100	12
500	10

These PCR cycle numbers were determined using Universal Human Reference (UHR) RNA fragmented to a 250–300 bp insert size. Independent determination of PCR cycle number may be required with samples of varying quality and fragment size.

Appendix D: xGen Normalase™ Module instructions

Review this section and the xGen Normalase Module protocol before setting up Normalase indexing PCR. To make the library fragments compatible with the xGen Normalase Module, amplify each library using xGen Normalase primer pairs (options can be found in the Consumables from IDT—Reagents). Choose the appropriate number of cycles and thermal cycler conditions as shown below, to obtain a library yield of 12 nM, or greater, in a 20 µL eluate.

1. Add the appropriate volume of Normalase indexing primers to each sample:

Indexing options	Primer	Volume per sample (µL)
xGen Normalase UDI primer pairs	Premixed primer pair	4
xGen Normalase CDI primer pairs	i5	2
	i7	2

2. Assemble the PCR Master Mix on ice. Mix the Master Mix thoroughly, using a pipette, and pulse-spin to collect contents. Add 26 µL of the mix to each sample tube, thoroughly mix, then pulse-spin to collect contents (50 µL total reaction volume):

xGen Normalase UDI primer pairs

Component	Volume per reaction (µL)
• PCR Master Mix	25
• Reagent R7	1
Total Master Mix	**26**
Eluted sample + primers	24
Total volume	**50**

xGen Normalase CDI primer pairs

Component	Volume per reaction (µL)
• PCR Master Mix	25
• Reagent R6	1
Total Master Mix	**26**
Eluted sample + primers	24
Total volume	**50**

3. Run the following thermal cycler program, adjusting the number of cycles depending on the input amount and sample quality (see table below) (lid heating ON):

Step	Cycle number	Temperature (°C)	Time
Activate enzyme		98	2 min
Denature	Perform X cycles*	98	20 sec
Anneal		60	30 sec
Extend		72	30 sec
Final extension		72	1 min
Hold		4	∞

* The recommended minimum number of cycles for each input to provide ≥ 12 nM yields suitable for the xGen Normalase workflow is as follows.

Input amount (depleted/selected)	Minimum number of cycles for ≥12 nM*	Input amount (total RNA)	Minimum number of cycles for ≥12 nM
100 pg	20	10 ng	20
1 ng	17	50 ng	17
100 ng	9	1 mg	9

* The number of cycles required may vary based on the input amount, as detailed above, but also on the quality of the sample. Recommendations above are for high-quality input RNA (RIN ≥ 7).

4. Proceed to Perform post-ligation cleanup.
5. Proceed to Normalase I, pooling, and Normalase II in the xGen Normalase Module protocol.

Appendix E: Expected results for alternate insert sizes

To adjust the final, mean library size, adjust both the fragmentation time and SPRI ratio following reverse transcription. Increase both the fragmentation time and post-RT SPRI ratio to achieve a smaller insert size.

Expected library results using 100 ng human brain mRNA into the xGen Broad-Range RNA Library Prep Kit with fragmentation time and post-RT SPRI ratio adjustments are detailed in the table below. Libraries were amplified with 8 PCR cycles.

Fragmentation time	Post-RT SPRI ratio	Mean libary size (bp)	Mean insert size (bp)
10 min (default)	1.0X	480	350
12 min	1.4X	430	300
15 min	1.8X	380	250

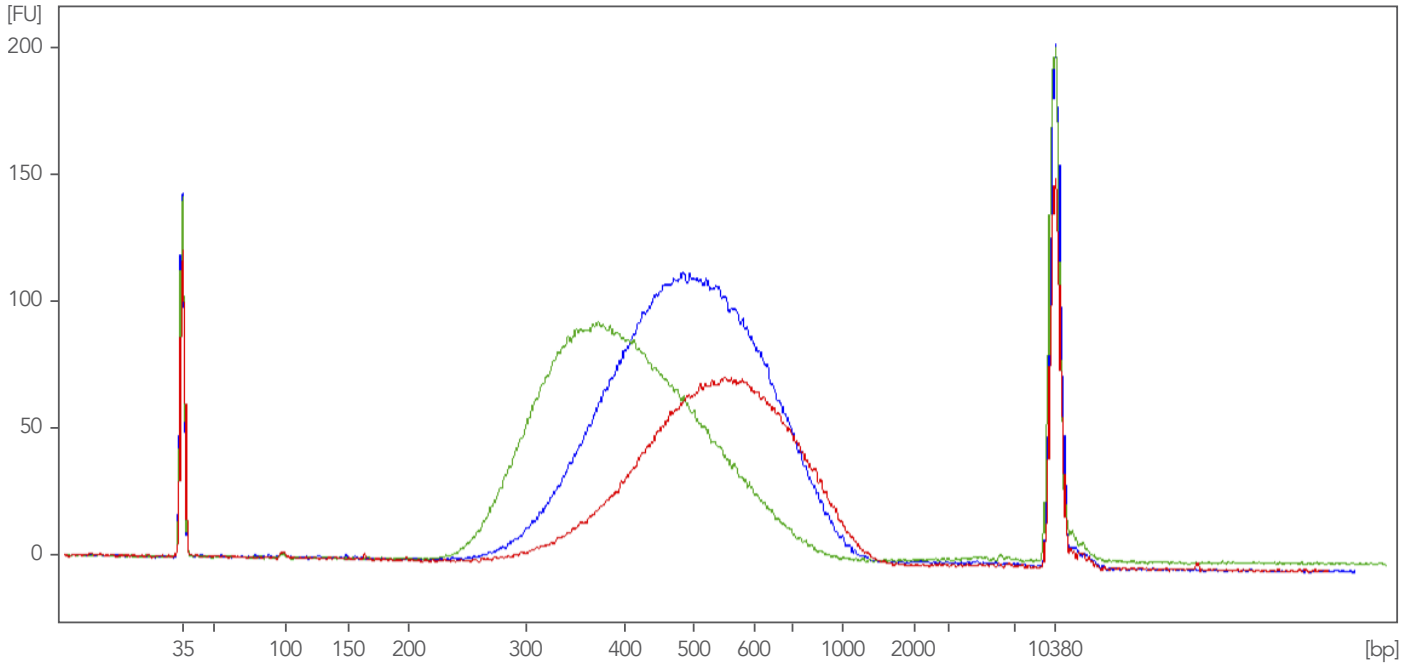

Figure 6: RNA Bioanalyzer results for alternate insert sizes. Expected results for RNA libraries produced from human brain mRNA (Clontech™ 636102) inputs of 100 ng using a fragmentation time of 10, 12, or 15 minutes with post-RT SPRI ratios of 1.0X, 1.4X, and 1.8X, respectively. Libraries were run on the Bioanalyzer using a HS DNA chip. The xGen Broad-Range RNA Library Prep Kit is for research purposes only.

Appendix F: Data analysis and informatics

The IDT xGen Adaptase technology, used in the xGen Broad-Range RNA Library Prep Kit, adds a low-complexity dinucleotide tail with a median length of 8 bases to the 3′ end of each fragment during the addition of the first NGS adapter molecule. Therefore, it is normal to observe this tail at the beginning of Read 2 (R2) on an Illumina® sequencer data output. When read length is close to fragment size, the tail may also be observed toward the end of Read 1 (R1) data.

The STAR aligner [1] has the bioinformatic capability to soft clip the synthetic Adaptase tail sequence as well as the synthetic random primer sequence at the beginning of Read 1 if any mismatches were introduced during the priming step. STAR provides efficient mapping without additional processing of the sequencing data. However, if you find that soft-clipping is not sufficient for your specific analysis, we recommend implementing STAR with the following argument: --clip5pNbases 10

Other alignment tools may be unable to soft clip the synthetic tail sequence, which can interfere with alignment. In most cases, a reciprocal trim is preferred. Trim 15 bases from the beginning of both R1 and R2 if the insert size is significantly larger than read length (i.e., 2 x 75 bp for a 250 bp insert library). If the insert size is like the read length, you may encounter tails at the end of R1 (i.e., 2 x 125 bp for a 250 bp library). In that case, trim 15 bases from the end of R1 and the beginning of R2. Tail and random primer trimming can be performed using publicly available tools like Trimmomatic, or another primer trimming informatics tool [2,3].

Important: Make sure that tail trimming is performed after adapter trimming. For additional tail trimming recommendations, see our technical note Tail Trimming for Better Data, or contact us at applicationsupport@idtdna.com.

Appendix G: Indexed adapter sequences

The full-length adapter sequences are below. The underlined text indicates the location of the index sequences, which are 8 bp for CDI and 10 bp for UDI. These sequences represent the adapter sequences following completion of the indexing PCR step.

Index 1 (i7) Adapters

5′–GATCGGAAGAGCACACGTCTGAACTCCAGTCACXXXXXXXX(XX)ATCTCGTATGCCGTCTTCTGCTTG–3′

Index 2 (i5) Adapters

5′–AATGATACGGCGACCACCGAGATCTACACYYYYYYYY(YY)ACACTCTTTCCCTACACGACGCTCTTCCGATCT–3′

Refer to the accompanying Index Sequences Master List for index sequences while preparing your Illumina® sequencing sample sheet on your instrument of choice.

Appendix H: Primer sequences

For reference, the primer sequences are below. These primers include full-length Illumina adapter and index sequences.

i7 primer: Replace 8 (CDI) or 10 (UDI) X's with the reverse complement of the specified i7 index sequence listed in the Index Sequences Master List.

5′–CAAGCAGAAGACGGCATACGAGATXXXXXXXX(XX)GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT–3′

i5 primer: Replace 8 (CDI) or 10 (UDI) Y's with the specified Forward Strand Workflow i5 index sequence in the Index Sequences Master List.

5′–AATGATACGGCGACCACCGAGATCTACACYYYYYYYY(YY)ACACTCTTTCCCTACACGACGCTCTTCCGATCT–3′

Appendix I: Troubleshooting

Issue	Possible cause	Suggested remedy
Low RNA input concentrations	Input RNA is too diluted	Contact applicationsupport@idtdna.com for options to concentrate the sample.
Difficulty resuspending beads after ethanol wash during SPRI steps	Over-drying of beads	Add Low EDTA TE immediately after removing the final ethanol wash. Continue pipetting the liquid over the beads for complete resuspension.
Lower than expected cluster density	Error in library quantification	Re-quantify library and confirm correct library insert size for calculating molarity.
Additional peak around ~150 bp visible on Bioanalyzer trace	Stubby adapters were carried over into the Ligation reaction, resulting in adapter dimers	Perform an additional 0.85X SPRI on samples to remove residual adapter dimers prior to quantification or sequencing. Note that a peak at ~60 bp represents carry-over of indexing primers from PCR and is expected. These fragments cannot cluster and will not impact quantification or sequencing.
Library size (insert size plus ~130 bp to account for adapters) is much smaller or larger than the expected	Too much or too little fragmentation, or sample had different RIN score than anticipated	Adjust the fragmentation time to optimize the library size to fit your experimental requirements. Increase fragmentation by ~2 minutes for a smaller library size or decrease by ~2 minutes for a larger library size. See Appendix E for more details.
Yields are lower than expected (<4 nM)	Inaccurate RNA input quantification, low-quality RNA, or incorrect SPRI ratios	Make sure you are accurately quantifying your RNA input with Nanodrop, Qubit™, or Bioanalyzer® before starting the xGen Broad-Range RNA Library Prep Kit. If using low-quality RNA (RIN <7), see our recommendations in Appendix B. The number of PCR cycles may need to be increased to accommodate your specific RNA input if yields are consistently lower than expected.
A second, large peak is visible in the Bioanalyzer trace (>2000 bp)	Libraries were overamplified, resulting in the formation of a heteroduplex	Reduce the number of PCR cycles to prevent the overamplification of your libraries.

Note: If you need further technical assistance, contact IDT at applicationsupport@idtdna.com.

REFERENCES

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2. Bolger AM, Lohse M, Usadel B. **Trimmomatic: a flexible trimmer for Illumina sequence data**. *Bioinformatics.* 2014;30(15):2114-2120.
3. Martin M. **Cutadapt removes adapter sequences from high-throughput sequencing reads**. *EMBnet.journal.* 2011;17(1):3.

xGen™ Broad-Range RNA Library Prep Kit

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