xGen™ RNA Library Prep Kit

REVISION HISTORY

Version	Release date	Description of changes
6	March 2024	Correction to protocol information
5	November 2022	Updated xGen Core Reagents catalog numbers
4	June 2022	Adjusted product size offerings
3	April 2022	Adaptase adapter name update
2	January 2022	Update volume for post exonuclease 1 cleanup
1	December 2021	Initial release

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OVERVIEW

The xGen™ RNA Library Prep Kit for stranded RNA-seq enables the preparation of high-complexity, next generation sequencing (NGS) libraries. This protocol utilizes Adaptase™ technology to produce high-quality RNA-seq libraries following first-strand cDNA synthesis.This approach preserves the original strand orientation without conventional second-strand cDNA synthesis. This workflow has fewer steps than comparable kits and takes less hands-on time, making it ideal for high-throughput applications.

This kit can accommodate an mRNA input range of 5–100 ng directly into library preparation, a range of 100 ng to 1 µg of total RNA input into an appropriate upstream poly(A)-selection or ribosomal RNA (rRNA) depletion module, and a range of 100–500 ng total RNA directly into library preparation to be used for xGen Hybridization Capture. For inputs outside of these ranges, we recommend the xGen™ Broad-Range RNA Library Prep Kit. If using Normalase™ Indexing Primers, read Appendix E before performing Normalase™ indexing PCR, as a protocol modification is required. If using a downstream xGen™ Hybridization Capture, see Appendix D for required protocol adjustments.

Supported applications and sample types

The xGen RNA Library Prep Kit works with following sample types:

- Ribosomal RNA-depleted RNA
- Poly(A)-selected mRNA
- Total RNA
- Formalin-fixed, paraffin-embedded (FFPE) RNA*
- RNA with a low RNA Integrity Number (i.e., RIN < 7) and DV_{200} scores > 50%

Input	Protocol options
RNA	100 ng to 1 µg (total RNA into an upstream module*) 100–500 ng (total RNA into library prep for hybridization capture) 5–100 ng (poly(A)-selected RNA, rRNA-depleted RNA into library prep)
Library insert size	200–250, 250–300, 300–350 bp

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

xGen RNA Library Prep workflow

Reverse transcription
- 90 minutes

Adaptase technology
- 30 minutes

Indexing PCR
- 40–70 minutes

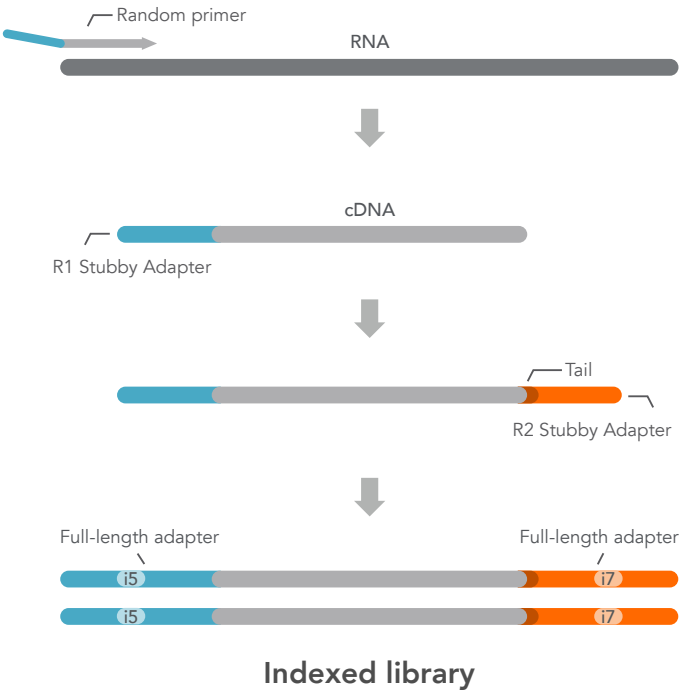

Figure 1. xGen RNA Library Prep workflow. RNA-Seq libraries are produced in three main steps: 1) Fragmentation and reverse transcription with a tailed random primer to incorporate an R1 Stubby Adapter; 2) addition of the R2 Stubby Adapter using Adaptase enzyme; and 3) indexing PCR.

The major steps to perform this protocol include:

- **Reverse transcription.** RNA fragmentation followed by random priming and reverse transcription generates first-strand cDNA. The random primer incorporates an R1 Stubby Adapter during cDNA synthesis (90 minutes). This is followed by an Exonuclease 1 treatment and SPRI cleanup (module sold separately), see Consumables–Other suppliers.
- **Adaptase treatment.** With the Adaptase technology this step simultaneously performs tailing and ligation to incorporate an R2 Stubby Adapter to the 3′ ends of the cDNA molecules (30 minutes).
- **Indexing PCR**. This step increases library yield, incorporates indexes, and results in full-length adapters at the ends of each molecule (40–70 minutes).

Note: The xGen Normalase primers can also be used for compatibility with the Normalase workflow (see Appendix E and the xGen Normalase Module protocol for a required workflow modification before starting the Indexing PCR setup).

CONSUMABLES AND EQUIPMENT

The xGen RNA Library Prep Kit contains enough reagents for the preparation of 16 or 96 libraries (10% excess volume provided). Indexing reagents are provided separately in the available indexing primer kits listed below.

Consumables from IDT—Kit contents

Workflow stage	Component	Volume (µL)		Storage
		16 rxns	96 rxns	
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	● Buffer A1	35	212	
	● Reagent A2	35	212	
	● Reagent A3	22	132	
	● Enzyme A4	9	53	
	● Enzyme A5	9	53	
PCR	● PCR Master Mix	440	2640	
Additional reagents	Nuclease-Free Water	1	1	
	Low EDTA TE	6	24	Room temperature

Consumables from IDT—Reagents

Workflow component	Product name	Index number	Reaction size (rxn)	Catalog #
xGen Core Reagents	xGen RNA Library Prep, 16 rxn	N/A	16	10009866
	xGen RNA Library Prep, 96 rxn	N/A	96	10009814
xGen Normalase™ Module (optional)	xGen Normalase Module	N/A	96	10009793
xGen Probes, Hyb, and Wash Kits (optional)	xGen Hybridization and Wash Kit	N/A	16	1080577
	xGen Hybridization and Wash Kit	N/A	96	1080584
xGen CDI Primers*	xGen CDI Primers	D501-D508/ D701-D712	96	10009815
xGen Normalse CDI Primers*	xGen Normalase CDI Primers	D501N-D508N/ D701N-D712N	96	10009794
xGen Normalse 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

* Normalase primers are compatible with both Normalase and non-Normalase workflows. Each UDI primer set contains four single-use, 96-well plates. Each plate contains 96 premixed primer pairs.

Consumables—Other suppliers

Item	Supplier	Catalog #
Exonuclease 1 and Reaction Buffer	New England BioLabs	M0293S/M0293L
Ribosomal RNA depletion kit	See Appendix B for more information	See Appendix B for more information
Poly(A)-selection kit		
RNaseZap™	Invitrogen	AM9780
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 pipettes 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 assays for library quantification (e.g. Qubit™)	Thermo Fisher Scientific	Varies

Equipment

Item	Supplier	Catalog #
0.2 mL magnets for individual tubes and plates	Permagen	MSR812 MSP750
Fluorometer (Qubit™), spectrophotometer (Nanodrop®) or similar input RNA quantification assay	Various suppliers	Varies
Bioanalyzer® for RNA and library size determination	Agilent	Varies
Quantitative PCR	Various suppliers	Varies
Microcentrifuge	Various suppliers	Varies
Programmable thermal cycler	Various suppliers	Varies
Vortex	Various suppliers	Varies

GUIDELINES

Avoid cross-contamination

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

- Physically separate the laboratory space, equipment, and supplies where pre-PCR and post-PCR processes are performed.
- DNase-treat 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 treat all surfaces with RNaseZap™ or similar RNase removal product to reduce the possibility of RNA degradation.
- After touching surfaces outside of the RNA-only workstation, change gloves or treat gloves with RNaseZap™ or similar RNase removal product.
- Use barrier pipette tips to avoid exposure to potential contaminants.
- Always change tips between each sample.

To achieve best results, follow these guidelines:

- Keep RNA samples on ice and return 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 input considerations

The xGen RNA Library Prep Kit should be used with inputs of 5–100 ng of mRNA, a range of 100 ng to 1 µg of total RNA into an upstream module, and a range of 100–500 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 obtain the best sequence data, make sure that samples are of high quality (RIN ≥ 7) before proceeding. For FFPE or other low-quality RNA (RIN < 7), see Appendix C or contact us for further details.

Since ribosomal RNA (rRNA) makes up ~80–90% of all RNA molecules, it is recommended to remove rRNA by depletion, perform poly(A) selection of mRNA, or enrich for specific target regions using xGen Hybridization Capture to obtain sufficient RNA-seq coverage. See Appendix B and Appendix D for recommendations on pairing the xGen RNA Library Prep Kit with upstream or downstream modules.

Make sure that the RNA samples prepared using the RNA Library Kit 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 us for options to concentrate the sample.

RNA-seq library preparation

For best results, follow these suggestions:

- After thawing reagents, invert or briefly vortex (except enzymes) to mix well. Briefly centrifuge to collect contents. Place enzymes on ice for 10 minutes prior to pipetting. Attempting to pipette enzymes at –20°C may lead to reagent shortage.
- Assemble reagent master mixes for each protocol step: Fragmentation, Reverse Transcription, Adaptase reaction, and PCR. Scale master mix volumes as appropriate, using 5% excess volume to compensate for pipetting loss. A Master Mix calculator is available.
- When using the 16-reaction kit, prepare at least for 4 reactions; when using the 96-reaction kit, prepare for at least 24 reactions. In both cases prepare with an excess overage of 5% to avoid excessive reagent loss.
- 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 cleanup steps. Prepare ~1 mL of 80% ethanol for each library.
- Preheat each thermal cycler with lid heating set to 105°C before preparing samples and master mixes.

Automation

- This protocol is readily automatable. A 10% overage volume of reagents is supplied to accommodate automation. Contact us if you require additional reagent overage volume or would like to learn about our custom packaging options.
- While Integrated DNA Technologies does not supply automated liquid handling instruments or consumables, our automation team collaborates with automation solution providers and customers to develop and qualify optimized automated scripts for use of our kits with liquid handling platforms routinely used in NGS library preparation. Contact us to discuss automating the xGen RNA Library Prep Kit workflow with your automated liquid handling system.

PROTOCOL

- Review this protocol before beginning an upstream module (e.g., ribosomal RNA depletion or poly(A) selection).
- For best results, incorporate Exonuclease 1 treatment after reverse transcription. See the Consumables–Other suppliers table.
- If using a ribosomal RNA depletion kit, elute the final RNA in a volume of 5 µL for input during the Perform RNA Fragmentation: Off bead instructions.
- If using a Poly(A) Selection Kit, resuspend the mRNA capture beads in RNA fragmentation buffer, then follow the Perform RNA Fragmentation: On bead instructions. See Appendix B for more detailed recommendations.
- If using xGen Hybridization Capture, see Appendix D for specific protocol adjustments.

Perform RNA fragmentation

Follow one of these two fragmentation instructions, depending on the upstream module used:

- Use the Off bead instructions if purified mRNA, total RNA, or depleted/selected RNA has been eluted in elution buffer, nuclease free water, or another appropriate buffer.
- Use the On bead instructions if a Poly(A) selection module is used and the xGen RNA Library Prep Kit steps follow the mRNA capture.

Off bead

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. Mix thoroughly and pulse-spin to collect contents. Add 9 µL of the mix below to each sample tube, mix thoroughly, and 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. Once it has reached 94°C, add sample tubes to the thermal cycler and run the appropriate program for each sample, according to the table below (lid heating ON):

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

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

On bead

Compatible only 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 B).
3. At the last step, do not resuspend the mRNA capture beads in elution buffer. Instead, resuspend the beads in 15 µL of Fragmentation Master Mix. Pipette up and down to mix thoroughly.
4. Preheat thermal cycler to 94°C. Once it has reached 94°C, add sample tubes to the thermal cycler and run the appropriate program for each sample, according to the table below (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, and transfer 14 µL of the supernatant to a new tube.

   Important: Do not let the samples cool before placing 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. Mix thoroughly and pulse-spin to collect contents. Add 6 µL of the mix below to each sample tube. Mix each sample tube thoroughly and pulse-spin to collect contents (20 µL total reaction volume):

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

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

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

For best results, proceed Perform Exonuclease 1 treatment next, then post-SPRI cleanup. Alternatively, a double SPRI cleanup can be performed (see Appendix A).

Perform Exonuclease 1 treatment

1. Assemble the Exonuclease 1 Master Mix on ice. Mix thoroughly and pulse spin to collect contents. Add 5 µL of the master mix to each sample tube. Mix each sample tube thoroughly and pulse spin to collect contents (25 µL total reaction volume):

Component	Volume per reaction (µL)
Exonuclease 1	2.5
Exonuclease 1 buffer	2.5

2. Run the following thermal cycler program. Once it has reached 37°C, add sample tubes to the thermal cycler and run the program (lid heating ON):

Temperature (°C)	Time
37	10 min
4	Hold

Perform post-exonuclease 1 cleanup

Add 25 µL Low EDTA TE to each sample before performing the cleanup.

1. Prepare ~1 mL of fresh, 80% ethanol solution per sample.
2. Invert or briefly vortex beads to homogenize the suspension before use.
3. Add the specified bead volume to each sample according to the table below.

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

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

4. Mix by pipetting 10 times or until 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, using a clean pipette tip, 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.
9. Repeat step 8 once for a second wash with the ethanol solution.
10. Pulse-spin the samples in a microcentrifuge, place 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, mixing well by pipetting up and down until homogenous. If droplets of the resuspension are on the side of the tube, pulse-spin the tube 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 eluate 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 back on magnet, and transfer eluate again.

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

Perform Adaptase Treatment

1. Preassemble the Adaptase Master Mix on ice. Mix thoroughly and pulse-spin to collect contents. Do NOT add to samples until after denaturation (step 3). Keep Master Mix on ice until step 4 below.

Component	Volume per reaction (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 the thermal cycler to 95°C for denaturation of the samples. When the thermal cycler has reached 95°C, add sample tubes (10 µL eluate) and run the following thermal cycler program (lid heating ON):

Temperature (°C)	Time
95	2 min

3. When the program has completed, **immediately** transfer samples to ice and incubate on ice 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, mix thoroughly and pulse-spin to collect contents (20.5 µL total reaction volume).
5. Run the following thermal cycler program (lid heating ON):

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

6. If using Combinatorial Dual, or Unique Dual Indexing primers, proceed to Perform indexing PCR. If using Normalase Indexing Primers, perform a 2.0X SPRI bead cleanup before proceeding to Perform Normalase Indexing PCR in Appendix E.

Perform indexing PCR

1. Add 2.5 µL (of each Combinatorial Dual) or 5 µL (Unique Dual) of the appropriate indexing primers directly to each sample, according to the table below (25.5 µL total reaction volume).

   Note: If using Normalase Indexing Primers, see Appendix E: Normalase instructions for a specific protocol modification to make before PCR.

Indexing kit	Reagent contents	Volume added to each sample (µL)
xGen CDI Primer Pairs	i5 primer	2.5
	i7 primer	2.5
xGen UDI Primer Pairs	Premixed i5 and i7 primers	5

2. Assemble the PCR Master Mix on ice. Thoroughly mix, then pulse-spin to collect contents. Add 25 µL of the mix to each tube, then mix thoroughly and pulse-spin to collect contents (50.5 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):

Cycle no.	Temperature (°C)	Time
	98	2 min
Perform X cycles*	98	20 sec
	60	30 sec
	72	30 sec
	72	1 min
	4	Hold

   * The recommended minimum number of cycles for each input for direct sequencing is as follows:

Input amount (depleted/selected, ng)	Minimum number of cycles	Input amount (total RNA)	Minimum number of cycles
5	13	100 ng	14
10	12	500 ng	11
100	7	1 µg	8

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

4. Proceed to Perform post-indexing PCR cleanup.

Perform post-indexing PCR cleanup

Perform a 0.8X cleanup after completing the indexing PCR step.

1. Add 40.4 µL beads to each sample.
2. Mix by pipetting 10 times or until homogenous. Make sure there are no bead-sample suspension droplets left on the sides 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 clean pipette tip, 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, then place samples back onto the magnet, and remove any residual ethanol solution from the bottom of the tube.
9. Elute in 22 µL Low EDTA TE and transfer 20 µL eluate to a fresh tube.

Note: When direct sequencing with low inputs, a "random primer library" artifact may be present, evident by a peak at ~150 bp by Bioanalyzer®. For these samples, perform a second 0.8X bead cleanup on the samples prior to library quantification. If performing hybridization capture, this additional cleanup is not needed.

Safe Stop: Samples can be stored at 4°C for up to 24 hours or at –20°C long-term. The library is now ready for quantification and sequencing.

Expected results

Figure 2 shows expected yields for varying input quantities using the xGen RNA Library Prep protocol with a starting input of human brain mRNA (Clontech). Mean library size includes the adapter sequences which adds ~130 bp to the library insert size.

Input amount (mRNA)	PCR cycles
5 ng	13
10 ng	12
100 ng	7

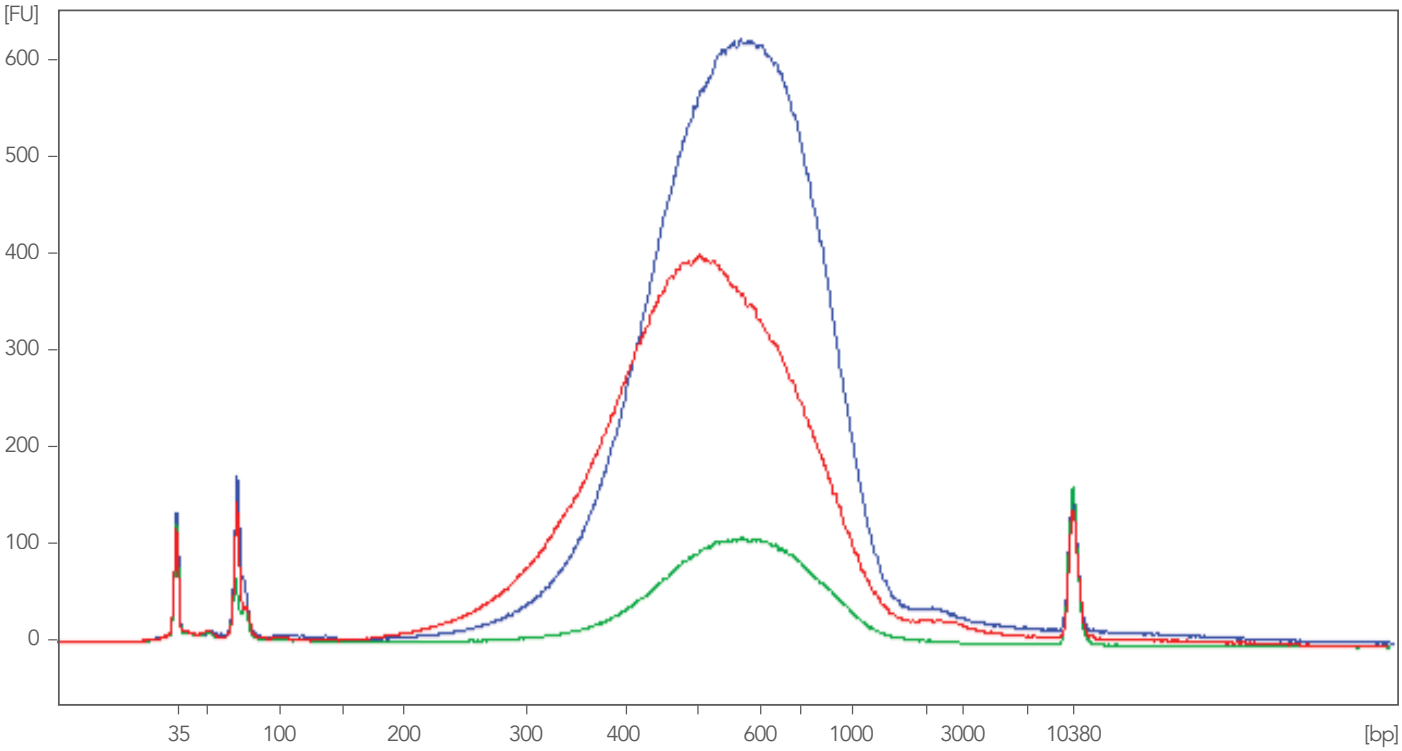

Figure 2. xGen RNA Library Prep Bioanalyzer® results. Expected results for xGen RNA Library Prep Kit produced from starting inputs of 5 ng, 10 ng, or 100 ng human brain mRNA (Clontech 636102). Libraries were run on the Bioanalyzer® using a HS DNA chip.

A peak at ~150 bp indicates a "random primer library" artifact—tailed random primers that survive the post-reverse transcription SPRI can serve as templates during the Adaptase reaction (not shown above). These artifacts can be removed with a second post-PCR SPRI (see Perform post-Exonuclease 1 cleanup). Note that a peak around ~60 bp represents carryover of indexing primers from PCR and is expected. These fragments cannot cluster and will not impact quantification or sequencing but can be removed by a second post-PCR cleanup, if necessary.

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

xGen RNA libraries can be sequenced using single-end or paired-end sequencing on Illumina® instruments. We recommend using a minimum of 2 x 50 paired-end reads. The 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™ instruments (Illumina). The NextSeq™ 550 (Illumina) may be sensitive to the low complexity Adaptase tail present at the beginning of Read 2, so PhiX or another suitable high-complexity library spike-in may be required. Contact Illumina technical support for further information regarding sequencing instrument compatibility with low-complexity sequences. If sequencing low-plex, pooled, single-indexed libraries, follow the recommendations outlined in the Illumina Index Adapters Pooling Guide. Use additional index combinations as needed for color-balanced index reads if required for the instrument.

Important: To ensure optimal mapping efficiency, we recommend using the STAR aligner [1]. If using an alternate software for alignment, bioinformatic trimming of the low complexity Adaptase tail from these libraries may be necessary (see Appendix G).

APPENDIX A: POST-REVERSE TRANSCRIPTION CLEANUPS

If an Exonuclease 1 treatment was not performed, perform two (2) cleanups following the ratio selected from the table below.

1. Add 30 µL Low EDTA TE to each sample (50 µL total volume).
2. Add SPRI beads according to the bead volume (1st SPRI) in the table below and follow the clean-up protocol.

Insert size (bp)	Bead ratio	Bead volume (1st SPRI) (µL)	Bead volume (2nd SPRI) (µL)
300–350	1.0X	50	20
250–300	1.4X	70	28
200–250	1.8X	90	36

RNA sample	Bead ratio	Bead volume (1st SPRI) (µL)	Bead volume (2nd SPRI) (µL)
FFPE	1.2X	60	24

3. Mix by pipetting 10 times or until homogenous. Make sure there are no bead-sample suspension droplets left on the sides of the tube.
4. Incubate the samples for 5 minutes at room temperature.
5. 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).
6. Remove and discard the supernatant, using a clean pipette tip, without disturbing the pellet.
7. 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, and then carefully remove the ethanol solution.
8. Repeat step 7 once for a second wash with the ethanol solution.
9. Pulse-spin the samples in a microcentrifuge, place back onto the magnet, and remove any residual ethanol solution from the bottom of the tube.
10. Without delay to avoid over-drying of beads, elute in 22 µL Low EDTA TE to resuspend the pellet, mix well by pipetting up and down until homogenous. If droplets of the resuspension are on the side of the tube, pulse-spin the tube 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).
11. Transfer 20 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 back on magnet, and transfer eluate again.
12. Perform a second SPRI cleanup. Add SPRI beads according to the bead volume (2nd SPRI) in the table above.
13. Mix by pipetting 10 times or until homogenous. Make sure there are no bead-sample suspension droplets are left on the sides of the tube.
14. Incubate the samples for 5 minutes at room temperature.

15. 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).
16. With a clean pipette tip, remove and discard the supernatant without disturbing the pellet.
17. 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, and then carefully remove the ethanol solution.
18. Repeat step 17 once for a second wash with the ethanol solution.
19. Pulse-spin the samples in a microcentrifuge, place back onto the magnet, and remove any residual ethanol solution from the bottom of the tube.
20. Elute in 12 µL Low EDTA TE and transfer 10 µL eluate to a fresh tube.

APPENDIX B: KIT COMPATIBILITY WITH OTHER MODULES

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

Poly(A) selection modules

Poly(A) selection allows for the enrichment of mRNA by capturing the poly(A) tail of transcripts using oligo-dT beads. We recommend using the following, or an equivalent poly(A) magnetic selection module:

- NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England Biolabs)—recommended
- Lexogen 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 step. 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 and follow the Perform RNA Fragmentation: On bead instructions.

If using the Lexogen Poly(A) RNA Selection Kit, follow the manufacturer's specifications until the final elution step. Completely remove the supernatant after completing 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 and follow the Perform RNA Fragmentation: On bead instructions.

Ribosomal RNA depletion modules

Ribosomal RNA depletion allows for the depletion of ribosomal RNAs, which make up 80–90% of total RNA. We recommend using the following, or an equivalent ribosomal RNA depletion kit:

- Lexogen RiboCop rRNA Depletion Kit V2 (Lexogen, for Human/Mouse/Rat)—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. 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. Proceed to Perform RNA fragmentation: Off bead.

APPENDIX C: LOW-QUALITY AND FFPE RNA

The quality of input RNA into an RNA-seq workflow can substantially impact the library yield and resulting data quality. RNA quality and integrity can be evaluated using two different metrics: RIN score (RNA Integrity Number; 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™.

High-quality samples typically have RIN scores ≥ 7 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, SPRI ratio, and number of PCR cycles. In general, it is not recommended to make libraries using samples with a RIN < 2 or a DV_{200} < 30.

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

Universal Human Reference (UHR) RNA (Agilent 740000) was heat-damaged according to the table below to produce variable RIN scores. RIN scores were evaluated using an RNA Pico 6000 Bioanalyzer Kit.

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

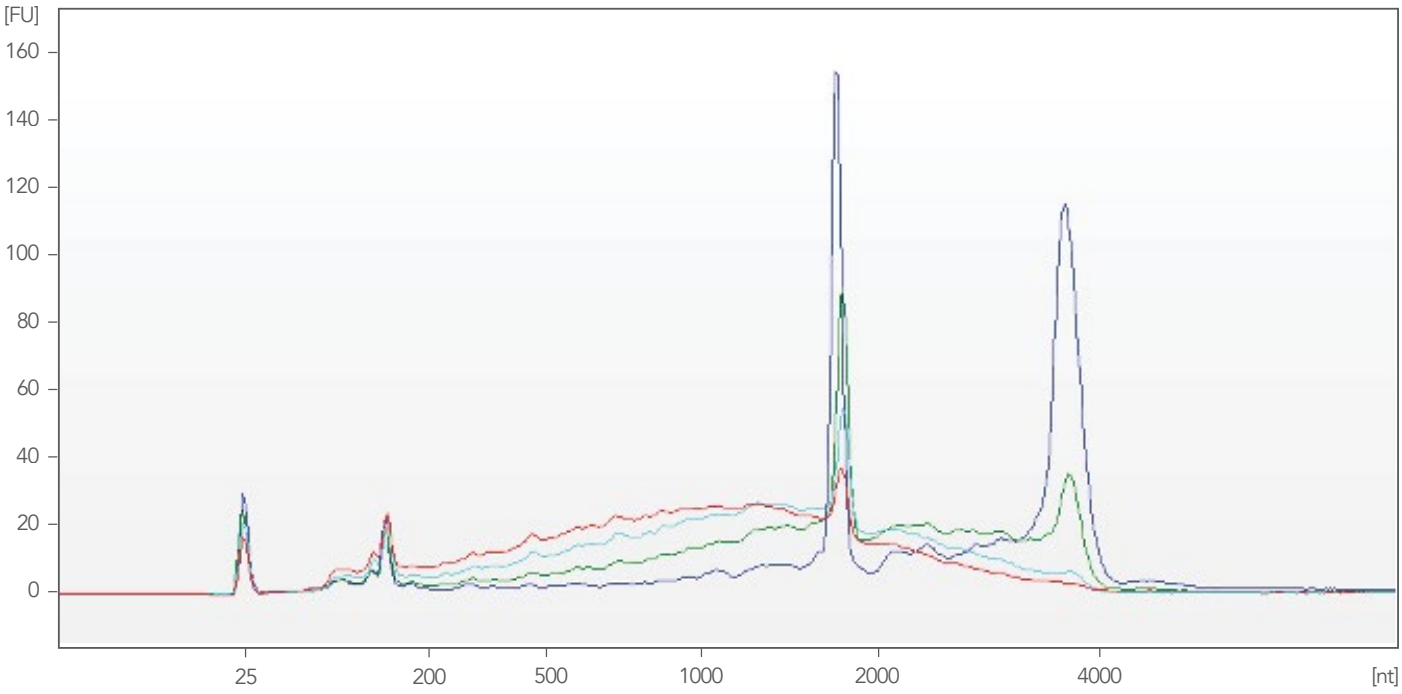

Figure 3. Bioanalyzer® results for heat-damaged UHR. Four UHR RNA samples were run on the Bioanalyzer® using an RNA Pico 6000 chip. The Total Eukaryote RNA program was used to obtain RIN score estimates.

Expected library results using 100 ng UHR total RNA with RIN scores of 1.7, 2.3, 3.2, and 9.2 as input into the xGen RNA Library Prep Kit. Fragmentation times were adjusted as recommended (See Perform RNA fragmentation) with 7 PCR cycles used for each library.

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

FFPE curls were obtained from 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 (Figure 4). Trace analyses revealed that the samples have RIN scores of ~2 and DV_{200} scores of 48 and 73.

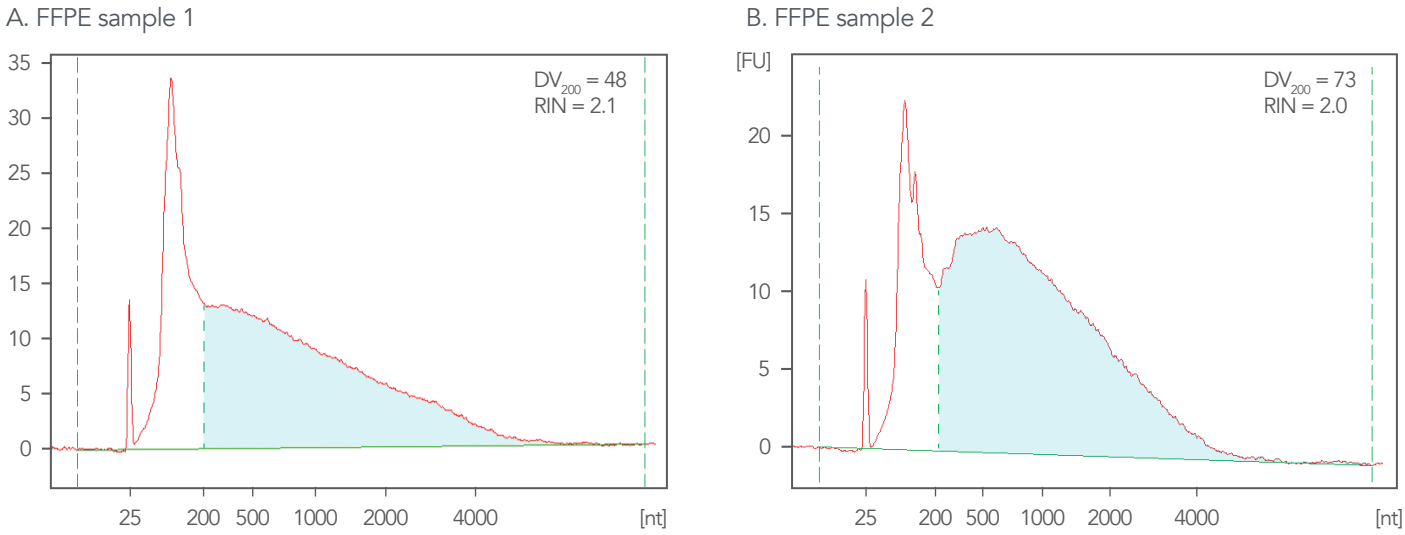

Figure 4. Bioanalyzer® results for FFPE RNA. RNA was isolated from breast cancer tumor samples. RNA samples were run on the Bioanalyzer® using an RNA Pico 6000 chip. The Total Eukaryote RNA program was used to obtain RIN scores. The DV_{200} RNA Pico program was used to obtain DV_{200} scores.

Because FFPE RNA samples typically have low integrity (RIN < 7), the recommended NGS workflows are ribodepletion or hybridization capture. For ribodepletion, see Appendix B for instructions on pairing the xGen RNA Library Prep Kit with compatible ribodepletion modules. For hybridization capture, see Appendix D for recommended protocol adjustments.

Following ribodepletion, FFPE-specific adjustments to the xGen RNA Library Prep Kit 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 optimization; 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 ratio.** Use the Exonuclease treatment followed by a SPRI cleanup. Follow the instructions in Perform Exonuclease 1 treatment. Add 25 µL Low EDTA TE to each sample before performing the cleanup. Achieve a 1.2X ratio by adding 60 µL SPRI beads.

  Tip: This step may require sample-specific optimization; increase the SPRI ratio up to 1.8X to retain smaller fragments for low DV_{200} scores (DV_{200} < 40) (Figure 5).

Figure 5 shows expected library results using 100 ng FFPE RNA into the Lexogen Ribocop V1.3 ribodepletion module. Ribodepleted RNA was used as input into the xGen RNA Library Prep Kit with the following adjustments: Fragmentation at 65°C for 5 min and 1.2X post-RT SPRI clean-ups. Libraries were amplified with 13 PCR cycles.

Sample	RIN score	DV_{200} score	Fragmentation time	Post-RT SPRI	Mean library size (bp)	Mean insert size (bp)	Library yield nM
FFPE Sample 1	2.1	48	5 min @ 65°C	1.2X	400	270	14
FFPE Sample 2	2.0	73	5 min @ 65°C	1.2X	470	340	15

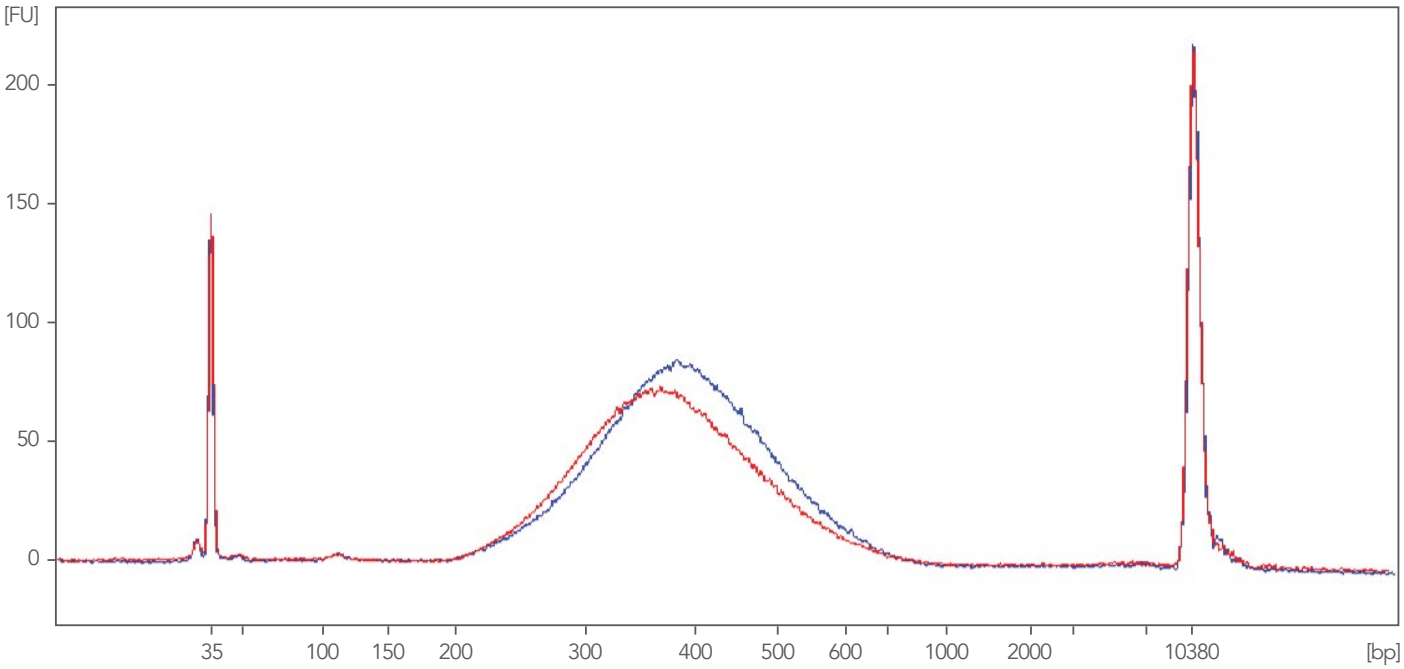

Figure 5. Bioanalyzer® results for libraries from ribodepleted FFPE RNA. Expected results for xGen RNA Library Prep Kit libraries produced from 100 ng FFPE RNA following ribodepletion using Lexogen RiboCop v1.3. Libraries were diluted 1:2 and run on the Bioanalyzer using a HS DNA chip.

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.**

APPENDIX D: PROTOCOL ADJUSTMENTS FOR xGEN HYBRIDIZATION CAPTURE

Hybridization capture enables the capture and sequencing of specific regions of the transcriptome. First, libraries are made using total RNA. 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.

Note: For each hybridization capture library, 500 ng of each library is recommended. For more information, refer to the xGen Hybridization Capture protocol.

During library prep, follow the protocol adjustments below:

- **Cleanup:** Follow the standard bead ratios if working with moderate to high integrity RNA samples. If working with damaged FFPE, use the 200 bp SPRI recommendations to retain short fragments and to accommodate smaller insert sizes.
- **PCR cycles:** To achieve library yields >500 ng, follow the PCR cycling recommendations below. This step may require sample-specific optimization; increase the number of PCR cycles if yields are lower than the required amount for hybridization capture.

Total RNA into library prep (ng)	PCR cycles
100	14–15
300	12–13
500	11

APPENDIX E: NORMALASE INSTRUCTIONS

Review this section and the xGen Normalase Module protocol before setting up your indexing PCR. To achieve expected results, amplify each library using xGen Normalase Indexing Primer Pairs with the appropriate number of cycles and thermocycling conditions (below) to obtain a library yield of 12 nM or greater in a 20 µL eluate.

SPRI cleanup

Important: A 2.0X SPRI cleanup is required before Indexing PCR with Normalase indexing primers.

1. Add 41 µL SPRI beads to each sample and follow cleanup protocol.
2. Mix by pipetting 10 times with a clean pipette tip or until homogenous. Make sure there are no bead-sample suspension droplets left on the sides 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 clean pipette tip, 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, and 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 back onto the magnet, and remove any residual ethanol solution from the bottom of the tube.
9. Elute in 22.5 µL Low EDTA TE and transfer 20.5 µL eluate to a fresh tube.

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

Perform Normalase indexing PCR

1. Add the specified amount of Normalase indexing primers to each sample as shown below:

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

2. Assemble the PCR Master Mix on ice. Mix thoroughly and pulse-spin to collect contents. Add 26 µL of the mix to each sample tube, mix thoroughly, and 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):

Number of cycles	Temperature (°C)	Time
	98	2 min
Perform X cycles*	98	20 sec
	60	30 sec
	72	30 sec
	72	5 min
	4	Hold

* 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, ng)	Minimum number of cycles for ≥12 nM*	Input amount (Total RNA)	Minimum number of cycles for ≥12 nM
5	16	100 ng	16
10	14	500 ng	14
100	9	1 µg	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 Post-indexing PCR clean-up.
5. Proceed to Normalase I, Pooling, and Normalase II steps in the xGen Normalase Module Protocol.

APPENDIX F: EXPECTED RESULTS FOR ALTERNATE FRAGMENTATION TIMES

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

Figure 6 shows expected library results using 100 ng human brain mRNA into the xGen RNA Library Prep Kit with fragmentation time and post-RT SPRI ratio adjustments as detailed in the table below. Libraries were amplified with 7 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

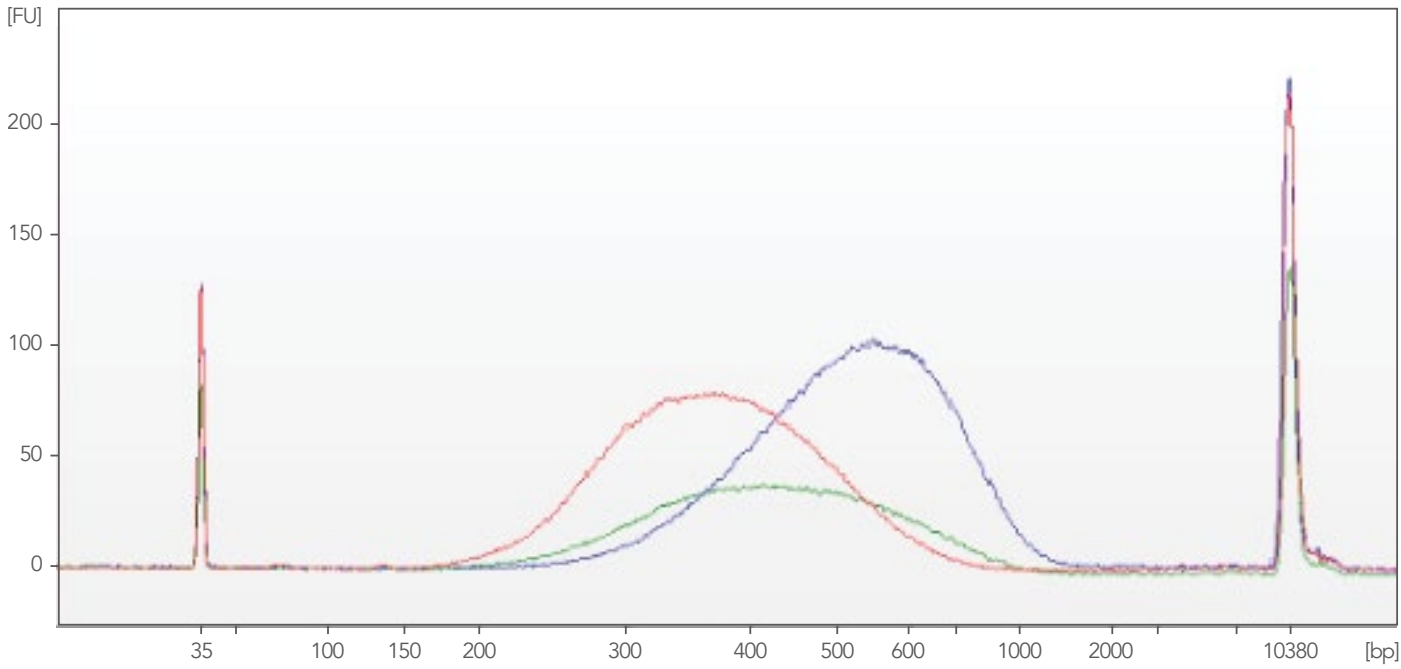

Figure 6. xGen RNA library preparation Bioanalyzer® results for alternate insert sizes. Libraries were produced from starting human brain mRNA (Clontech 636102) inputs of 100 ng using a fragmentation time of 10, 12, or 15 minutes with post-RT SPRI ratios of 1.0X, 1.4X, and 1.8X, respectively. Libraries were run on the Bioanalyzer® using a HS DNA chip.

APPENDIX G: DATA ANALYSIS AND BIOINFORMATICS

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

We recommend using the STAR aligner [1] for data analysis, as it is typically able 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 analysis, we recommend implementing STAR with the following argument: --clip5pNbases 10.

Other aligners may be unable to soft clip the synthetic tail sequence, which can interfere with alignment. In most cases, a reciprocal trim is preferred. We recommend trimming 15 bases from the beginning of both R1 and R2 if insert size is significantly larger than read length (i.e., 2 x 75 bp for a 250 bp insert library). If insert size is similar to the read length, you may encounter tails at the end of R1 (i.e., 2 x 125 bp for a 250 bp library). We recommend that 15 bases be trimmed 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 Cutadapt [2,3].

Note: Make sure that tail trimming is performed AFTER adapter trimming.

For additional tail trimming recommendations, refer to our technical note Tail Trimming for Better Data or contact us.

APPENDIX H: 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 8 bp or 10 bp for UDI. These sequences represent the adapter sequences following completion of the indexing PCR step.

Index 1 (i7) Adapters

5′–GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**XXXXXXXX(XX)**ATCTCGTATGCCGTCTTCTGCTTG–3′

Index 2 (i5) Adapters

5′–AATGATACGGCGACCACCGAGATCTACAC**YYYYYYYY(YY)**ACACTCTTTCCCTACACGACGCTCTTCCGATCT–3′

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

APPENDIX I: PRIMER SEQUENCES

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

i7 primer: Replace 8 bases for CDI and 8 or 10 bases for UDI **X's** with the **reverse complement** of the specified i7 index sequence in the Index Sequences Master List:

5´–CAAGCAGAAGACGGCATACGAGAT**XXXXXXXX(XX)**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT–3´

i5 primer: Replace 8 bases for CDI and 8 or 10 bases for UDI **Y's** with the specified Forward Strand Workflow i5 index sequence in the Index Sequences Master List:

5´–AATGATACGGCGACCACCGAGATCTACAC**YYYYYYYY(YY)**ACACTCTTTCCCTACACGACGCTCTTCCGATCT–3´

For assistance confirming compatibility of primers with the xGen Amplicon workflow, contact us. To purchase custom Normalase Indexing Primers, contact your local sales representative or distributor.

APPENDIX J: TROUBLESHOOTING

Issue	Possible cause	Suggested remedy
Input is less than 5 ng of RNA in 5 µL volume	Input RNA is too diluted	Concentrate RNA with column purification kit.
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	Random primers were carried over into the Adaptase reaction, resulting in library artifacts	Perform an additional 0.8X SPRI on samples to remove residual adapter dimers prior to quantifying or sequencing. Note that a peak around ~60 bp represents carryover 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 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 F for more details.
Yields are lower than expected (<4 nM)	Inaccurate RNA input quantification, low-quality RNA, or incorrect SPRI ratios	Ensure RNA input is accurately quantified with a Nanodrop®, Qubit™, or Bioanalyzer® prior to using the xGen RNA Library Prep Kit. If using low-quality RNA (RIN < 7), see our recommendations in Appendix C. 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.

REFERENCES

1. Dobin A, Davis CA, Schlesinger F, *et al*. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013;29(1):15-21.
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 RNA Library Prep Kit

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