Overview

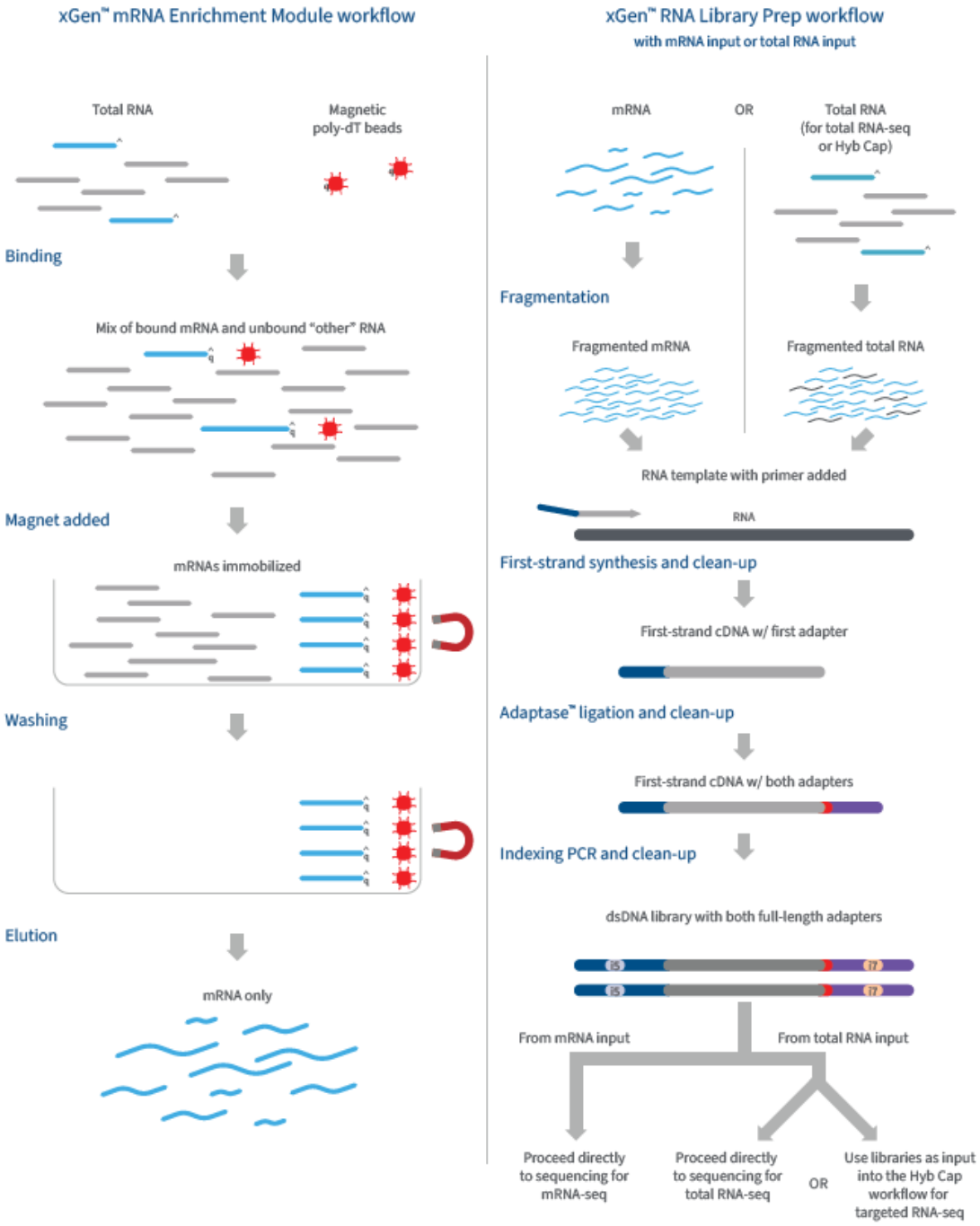
Product Description

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.

The xGen™ mRNA Enrichment Module offers an upstream module for poly(A) selection of total RNA and can be bundled with the xGen RNA Library Kit as a full solution for mRNA sequencing. This mRNA enrichment module provides an oligo dT-based mRNA isolation option that efficiently removes ribosomal RNA (rRNA) and other non-polyadenylated transcripts from total RNA samples and is ideal for use with eukaryotic samples containing intact poly(A) tails. This enrichment module is not recommended for use with FFPE or other damaged samples.

The xGen RNA Library Prep Kit can accommodate a range of 100 ng to 1 µg of total RNA input into the xGen mRNA Enrichment module. For inputs outside of these ranges, we recommend the xGen™ Broad-Range RNA Library Prep Kit. This workflow is compatible with CDI Indexing Primers, UDI Indexing Primers or Normalase™ Indexing Primers.

mRNA Extraction and Library Preparation Workflow



Reagents

xGen mRNA Enrichment Module

- 16 rxn part number: 10017545
- 96 rxn part number: 10017546

Kit Contents

Kit	Kit Components	16 rxn (uL)	96 rxn (uL)	Storage
xGen mRNA Enrichment Module	PolyA Beads	352	2,112	Store at 4°C.
	Binding Buffer	3,520	21,120	
	Wash Buffer	7,040	42,240	
	Elution Buffer	1,179	7,075	
	Nuclease-Free Water	880	5,280	

xGen RNA Library Prep Kit

- 16 rxn part number: 10009866
- 96 rxn part number: 10009814

Kit Contents

Kit	Workflow Stage	Component	16 rxn (uL)	96 rxn (uL)	Storage
xGen RNA Library Prep	Fragmentation	Reagent F1	18	106	Store at -20°C
		Reagent F2	35	212	
		Buffer F3	71	423	
		Reagent F4	35	212	
		Enzyme R1	18	106	

	Reverse transcription	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	
	PCR	PCR Master Mix	440	2,640	
	Additional reagents	Nuclease-Free Water	1,000	1,000	Room Temperature
		Low EDTA TE	6,000	24,000	Room Temperature

Reagents from IDT (required but not included in the kit)

Component	Product Name	Index Number	Reaction Size	Part Number
xGen Normalase™ Module (optional)	xGen Normalase Module	N/A	96	10009793
xGen CDI Primers*	xGen CDI Primers	D501-D508/ D701-D712	96	10009815
xGen UDI Primers	xGen UDI Index Primers	1-96	96	10005922 10009816
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-US384	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.

Reagents - Other Suppliers (required but not included in the kit)

Description	Supplier	Part Number
Nuclease-free water for preparation of 80% ethanol	General laboratory supplier	Varies
200-proof/absolute ethanol	General laboratory supplier	Varies

RNase Decontamination Solution: RNase Away or RNaseZap™	Fisher Scientific Invitrogen	21-402-178 AM9780
Exonuclease 1 and Reaction Buffer (optional)	New England BioLabs	M0293S/M0293L
SPRIselect® beads or Agencourt® AMPure® XP	Beckman Coulter	B23317/B23318/B23319 A63880/A63881/A63882
RNA 6000 Pico Kit for RNA qualification	Agilent	5067-1513
Fluorometic assays for RNA quantification (e.g. Qubit)	Thermo Fisher	Varies

Materials and Equipment

Consumables

Description	Supplier	Part Number
RNase-free Low bind 0.2 mL PCR tubes or 96-well plates	Various suppliers	Varies
RNase-free Low Bind 1.5mL microcentrifuge tubes	Various suppliers	Varies
RNase-free aerosol resistant tips and pipettes ranging from 1-1000 µL	Various suppliers	Varies
Adhesive PCR Plate Seals	Various supplies	Varies

Equipment

Item	Supplier	Part Number

0.2 mL magnets for individual tubes and plates	Alpaqua Permagen	A000380 MSR812, MSP750
Fluorometer (Qubit), spectrophotometer (Nanodrop) or similar input RNA quantification assay	Various suppliers	Varies
Pipettes ranging from 1-1000 µL	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 100 ng to 1 µg of total RNA into the upstream xGen mRNA Enrichment Module.
- 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.
- Since ribosomal RNA (rRNA) makes up ~80 – 90% of all RNA molecules, it is recommended to remove rRNA by poly(A) selection of mRNA to obtain sufficient RNA-seq coverage.

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.

- When using the 16-reaction kit, prepare at least four 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 to master mixes last 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 ON (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.

Before getting started

Important precautions

- **Read through the entire protocol before starting your mRNA enrichment.**
- Never freeze the beads.
- Thoroughly clean gloves, bench surfaces, pipettes, tube racks, centrifuges, etc with an RNase Decontamination Solution before handling RNA.
- All thermal cycler programs are to have the lid set to “On” or 105°C.
- Prepare the Fragmentation Master Mix from xGen RNA Library Prep Kit prior to starting this mRNA enrichment protocol.
- For best results, incorporate Exonuclease 1 treatment after reverse transcription. See the Reagents-Other Suppliers table.

Working with liquid reagents

- Thoroughly resuspend the beads and all reagents by vortexing prior to starting protocol to ensure reagents are homogenous.

Input nucleic acid

- xGen RNA: 100 – 1000 ng Total RNA

- Before starting, working on ice, bring volume of total RNA sample to 50 µL using Nuclease-free water for each reaction.

Reagents to prepare before starting

- Volumes of reagents needed for each reaction:
  - 20 µL Beads
  - 220 µL Binding Buffer
  - 440 µL Wash Buffer
  - 50 µL Elution Buffer

mRNA Enrichment Protocol

Prepare Fragmentation Master Mix

1. Assemble the Fragmentation Master Mix using reagents from the xGen RNA Library Prep Kit. Thoroughly mix, then pulse-spin to collect contents (15 µL total reaction volume):

Component	Volume per reaction
• Reagent F1	1.0 µL
• Reagent F2	2.0 µL
• Buffer F3	4.0 µL
• Reagent F4	2.0 µL
Nuclease-free water	6.0 µL
Total volume	**15.0 µL**

2. Store On-Bead Fragmentation Master Mix on ice while performing enrichment.

Perform Enrichment

Bead Preparation Procedure

1. Thoroughly resuspend beads by vortexing. Ensure no beads remain settled at the bottom of the tube.
2. Transfer 100 µL of binding buffer to fresh wells of a new 96-well PCR plate. Each sample requires its own well.
3. Add 20 µL of beads to each well and mix until homogenous. Each well must contain 100 µL of binding buffer and 20 µL of beads.
4. Place the 96-well PCR plate on the magnet for 30 seconds or until the solution becomes clear.
5. Discard the clear supernatant while the 96-well PCR plate remains on the magnet.
6. Remove 96-well PCR plate from magnet.
7. Resuspend pellet in 50 µL of binding buffer. Proceed to First Bead Binding.

First Bead Binding

Bring volume of total RNA sample to 50 µL using nuclease-free water.

1. Add 50 µL total RNA to the prepared beads. Mix thoroughly until homogenized.

2. Each well must contain 50 µL of the total RNA sample and 50 µL of the prepared beads.
3. Apply adhesive PCR plate seal and place in thermal cycler that is programmed as follows:

Step	Temperature (°C)	Time (minutes)
1	65	5
2	4	Hold

4. Remove the 96-well PCR plate from the thermal cycler as soon as the plate is at 4°C.
5. Mix thoroughly until homogenized.
6. Incubate at room temperature for 5 minutes.

First Bead Wash

1. Place the 96-well PCR plate on the magnetic stand at room temperature for 30 seconds or until the supernatant appears completely clear.
2. Remove and discard the clear supernatant taking care not to disturb beads.
3. Remove the 96-well PCR plate from the magnetic stand.
4. Resuspend pellet with 200 µL of wash buffer. Mix thoroughly until homogenized.
5. Place the 96-well PCR plate on the magnetic stand for 30 seconds or until the supernatant appears completely clear.
6. Remove and discard clear supernatant taking care not to disturb beads.
7. Remove the 96-well PCR plate from the magnetic stand.
8. Resuspend pellet with 50 µL of elution buffer.

Second Bead Binding

1. Apply adhesive PCR plate seal and place in thermal cycler that is programed as follows:

Step	Temperature (°C)	Time (minutes)
1	70	5
2	25	Hold

2. Remove the 96-well plate from the thermal cycler once the plate is at 25°C.
3. Add 50 µL of binding buffer to each sample. Mix thoroughly until homogenized. Do not magnetize.
4. Incubate at room temperature for 5 minutes.

Second Bead Wash

1. Place the 96-well PCR plate on the magnetic stand at room temperature for 30 seconds or until the supernatant appears completely clear.
2. Remove and discard clear supernatant taking care not to disturb beads.
3. Remove the 96-well PCR plate from the magnetic stand.

4. Resuspend pellet with 200 µL of Wash Buffer. Mix thoroughly until homogenized.
5. Place the 96-well PCR plate on the magnetic stand for 30 seconds or until supernatant appears completely clear.
6. Remove and discard clear supernatant taking care not to disturb beads.
7. Using a P20, remove all remaining supernatant from the wells.
8. Remove 96-well PCR plate from the magnetic stand.
9. Proceed directly to RNA Fragmentation.

xGen RNA Library Prep Protocol

Perform RNA Fragmentation

1. Resuspend beads in each well with 15 µL Fragmentation Master Mix from the first step of the enrichment protocol.
2. Pipette up and down to mix, apply adhesive PCR plate seal.
3. Preheat thermal cycler to 94 °C (Lid heating set to 105 °C).
4. Ensure the thermal cycler has reached 94 °C, add samples to the thermal cycler and run the program for each sample:

RIN score	Insert Size (bp)	Temperature (lid 105°C)	Time (minutes)
≥7	300-350	94 °C	10
	250-300		12
	200-250		15

3. When the program is complete, **immediately** transfer the hot plate to a magnet. Do not allow samples in the 96-well PCR plate to cool to room temperature.
4. Wait until solution clears then transfer 14µL eluate to a new 96-well PCR plate.
5. Immediately transfer plate to ice and incubate for 2 min. Proceed directly to 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 reaction well, mix thoroughly and pulse spin to collect contents (20 µL total reaction volume):

Component	Volume per reaction
• Enzyme R1	1 µL
• Enzyme R2	1 µL
Nuclease-free water	4 µL

Total volume	6 µL

2. Run the thermal cycler program (Lid heating set to 105 °C):

Step	Temperature (lid 105°C)	Time (minutes)
1	25 °C	10
2	42 °C	30
3	70 °C	15
4	4 °C	Hold

Safe Stopping point: Samples can be stored at 4 °C for up to 24 hours.

Perform Exonuclease I Treatment (optional)

Exonuclease I Treatment with post-SPRI cleanup may be replaced with a Double SPRI cleanup, proceed to next section for Double SPRI cleanup instructions

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

Component	Volume per reaction
Exonuclease 1	2.5 µL
Exonuclease 1 buffer	2.5 µL
Total volume	**5.0 µL**

2. Run the following thermal cycler program. Once thermal cycler has reached 37 °C, add sample(s) to the thermal cycler and run the program (lid heating set to 105 °C):

Step	Temperature (lid 105°C)	Time (minutes)
1	37 °C	10
2	4 °C	Hold*

*Proceed directly to Post-Exonuclease I Cleanup.

Perform Post-Exonuclease I Cleanup

1. Prepare a fresh 80% ethanol solution; prepare enough for ~ 1 mL per sample.
2. Add 25 uL Low EDTA TE to each sample before performing the cleanup.
3. Briefly vortex beads to homogenize the suspension before use.
4. Add the specified bead volume to each sample according to the table below:

Insert Size (bp)	SPRI ratio	Bead Volume
300-350	1.0X	50 µL
250-300	1.4X	70 µL
200-250	1.8X	90 µL

5. Mix by pipetting 10 times or until homogenous. Ensure no bead-sample suspension droplets are left on the sides of the tube/well.
6. Incubate the samples for 5 minutes at room temperature.
7. Pulse-spin the samples in a microfuge. Place the samples on a magnetic rack until the solution clears and a pellet is formed (~ 2 minutes).
8. Remove and discard the supernatant without disturbing the pellet.
9. Add 200 µL of freshly prepared 80% ethanol solution to the pellet while it is still on the magnet. Use care not to disturb the pellet. Incubate for 30 seconds, then carefully remove the ethanol solution.
10. Repeat step 7 once for a second wash with the ethanol solution.
11. Keep the samples on the magnet for 1 min and remove any residual ethanol solution from the bottom of the tube/well.
12. Without delay to avoid over-drying of beads, remove the plate from the magnet and 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/well, pulse-spin the samples in a microfuge to collect contents. After at least 2 minutes, place the samples on the magnetic rack and wait until the solution clears and a pellet is formed (~2 minutes).
13. Transfer 10 µL eluate to a new tube/well. Ensure that eluate does not contain magnetic beads (indicated by brown coloration in eluate). If magnetic beads are present, place back on the magnet, and transfer eluate again.
14. Proceed to Perform Adaptase.

Safe Stopping point: samples can be stored at 4 °C for up to 24 hours.

Perform Double SPRI Cleanup

- ***Perform if ExoI treatment was not completed.***
- ***If ExoI treatment with Post-ExoI Cleanup was performed, proceed directly to Perform Adaptase section of this protocol.***

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

1. Prepare a fresh 80% ethanol solution; prepare enough for ~ 1 mL per sample.
2. Add 30 µL Low EDTA TE to each sample (50 µL total volume) before performing the cleanup.
3. Briefly vortex beads to homogenize the suspension before use.
4. Add SPRI beads according to the Bead volume (1st SPRI) in the table below:

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

5. Mix by pipetting 10 times or until homogenous. Ensure no bead-sample suspension droplets are left on the sides of the tube/well.
6. Incubate the samples for 5 minutes at room temperature.
7. Pulse-spin the samples in a microfuge. Place the samples on a magnetic rack until the solution clears and a pellet is formed (~ 2 minutes).
8. Remove and discard the supernatant without disturbing the pellet.
9. Add 200 µL of freshly prepared 80% ethanol solution to the pellet while it is still on the magnet. Use care not to disturb the pellet. Incubate for 30 seconds, then carefully remove the ethanol solution.
10. Repeat step 7 once for a second wash with the ethanol solution.
11. Keep the samples on the magnet for 1 min and remove any residual ethanol solution from the bottom of the tube/well.
12. Without delay to avoid over-drying of beads, remove the sample tubes from the magnet and add 22 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/well, pulse-spin the samples in a microfuge to collect contents. After at least 2 minutes, place the samples on the magnetic rack and wait until the solution clears and a pellet is formed (~2 minutes).

13. Transfer 20 µL elute to a new tube/well. Ensure that eluate does not contain magnetic beads (indicated by brown coloration in eluate). If magnetic beads are present, place back on the magnet, and transfer eluate again.
14. Perform a second SPRI cleanup. Add SPRI beads according to the bead volume (2nd SPRI) in the table above.
15. Mix by pipetting 10 times or until homogenous. Ensure no bead-sample suspension droplets are left on the sides of the tube/well.
16. Incubate the samples for 5 minutes at room temperature.
17. Pulse-spin the samples in a microfuge. Place the samples on a magnetic rack until the solution clears and a pellet is formed (~ 2 minutes).
18. Remove and discard the supernatant without disturbing the pellet.
19. Add 200 µL of freshly prepared 80% ethanol solution to the pellet while it is still on the magnet. Use care not to disturb the pellet. Incubate for 30 seconds, then carefully remove the ethanol solution.
20. Repeat step 7 once for a second wash with the ethanol solution.
21. Keep the samples on the magnet for 1 min and remove any residual ethanol solution from the bottom of the tube/well.
22. Without delay to avoid over-drying of beads, remove the sample tubes from the magnet and 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/well, pulse-spin the samples in a microfuge to collect contents. After at least 2 minutes, place the samples on the magnetic rack and wait until the solution clears and a pellet is formed (~2 minutes).
23. Transfer 10 µL elute to a new tube/well. Ensure that eluate does not contain magnetic beads (indicated by brown coloration in eluate). If magnetic beads are present, place back on the magnet, and transfer eluate again.

Safe Stopping point: samples can be stored at 4 °C for up to 24 hours.

Perform Adaptase

1. Preheat thermal cycler to 95°C for denaturation of the samples.
2. Pre-assemble the Adaptase master mix on ice. Mix thoroughly and pulse spin to collect contents. Do NOT add to samples until after denaturation (Step 5). Keep master mix on ice until instructed to use below.

Component	Volume per reaction
• Buffer A1	2.0 µL
• Reagent A2	2.0 µL
• Reagent A3	1.25 µL
• Enzyme A4	0.5 µL

• Enzyme A5	0.5 µL
Low EDTA TE	4.25 µL
Total volume	**10.5 µL**

3. When the thermal cycler has reached 95 °C, add samples (10 µL eluate) and run the following thermal cycler program (Lid heating set to 105 °C):

Step	Temperature	Time (minutes)
1	95 °C	2

4. When the program has completed, immediately transfer samples to ice and incubate on ice for 2 min. Proceed directly to the next step.
5. Add 10.5 µL of the Adaptase master mix from Step 1 to each sample, mix thoroughly and pulse spin to collect contents (20.5 µL total reaction volume).
6. Run the following thermal cycler program (Lid heating set to 105 °C):

Step	Temperature	Time (minutes)
1	37 °C	15
2	95 °C	2
3	4 °C	Hold*

* Proceed immediately to next step.

7. When using xGen CDI or UDI Primers, proceed directly to Perform Indexing PCR. If using Normalase Primers, proceed to Perform 2.0X Post-Adaptase Cleanup.

Perform 2.0X Post-Adaptase Cleanup

Only required when amplifying with Normalase Primers

1. Add 41 µL SPRI beads and follow cleanup protocol.
2. Mix by pipetting 10 times or until homogenous. Ensure no bead-sample suspension droplets are left on the sides of the tube/well.
3. Incubate the samples for 5 minutes at room temperature.
4. Pulse-spin the samples in a microfuge. Place the samples on a magnetic rack until the solution clears and a pellet is formed (~ 2 minutes).
5. Remove and discard the supernatant without disturbing the pellet.
6. Add 200 µL of freshly prepared 80% ethanol solution to the pellet while it is still on the magnet. Use care 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. Keep the samples on the magnet and remove any residual ethanol solution from the bottom of the tube/well.

9. Elute in 22.5 µL Low EDTA TE, mix samples until beads are homogenous. After 2 minutes, place the samples on the magnetic rack and wait until the solution clears and a pellet is formed (~2 minutes). Transfer 20.5 µL eluate to a fresh tube/well. Proceed immediately to indexing PCR.

Perform Indexing PCR

1. Add the appropriate indexing primer(s) directly to each sample following the table below:

Indexing Kit	Reagent Contents	Volume added per sample
xGen CDI or xGen UDI Primer Pairs	i5 and i7 primers: Individual or Pre-mixed	2.5uL + 2.5uL or 5 µL
xGen Normalase UDI Primer Pairs	Pre-mixed i5 and i7 primers	4 µL

2. Follow instructions for desired indexing primers pairs: xGen UDI Primers or xGen Normalase UDI Primers

For xGen CDI or xGen UDI Primers: Add PCR Master Mix to each sample, mix thoroughly and pulse spin to collect contents (50 µL total reaction volume):

Component	Volume per reaction
PCR Master Mix	25 µL
Eluted Sample	20.5 µL
xGen CDI Individual Primers or xGen UDI Primer Pairs	2.5uL + 2.5uL or 5 µL
Volume to add	**50.5 µL**

For xGen Normalase UDI Primer Pairs: prepare the following master mix and add 26 µL to each sample, mix thoroughly and pulse spin to collect contents (50 µL total reaction volume):

xGen Normalase UDI Primer Pairs	
Component	**Volume per reaction**
• **PCR Master Mix**	25 µl

• **Reagent R7**	1 µl
Total Master Mix	**26 µl**
Eluted Sample + Primers	24.5 µl
Total Volume	**50.5 µl**

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 set to 105 °C):

Step	Temperature	xGen CDI or xGen UDI Time	xGen Normalase Time	Number of Cycles
1	98 °C	2 min		1
2	98 °C	20 sec		*See table below for recommended cycles
3	60 °C	30 sec		
4	72 °C	30 sec		
5	72 °C	1 min	5 min	1
6	4 °C	Hold		1

* The recommended minimum number of cycles for each input to provide sufficient yields suitable for direct sequencing is as follows:

Starting Total RNA Input Amount	Number of Cycles xGen CDI or xGen UDI	Number of Cycles xGen Normalase
100 ng	14	16
500 ng	11	14
1000 ng	8	9

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

Perform Post-Indexing PCR Cleanup

Perform a 0.8X SPRI cleanup following the indexing PCR step.

1. Add 40.4 µL SPRI beads and follow clean-up protocol.
2. Mix by pipetting 10 times or until homogenous. Ensure no bead-sample suspension droplets are left on the sides of the tube/well.
3. Incubate the samples for 5 minutes at room temperature.
4. Pulse-spin the samples in a microfuge. Place the samples on a magnetic rack until the solution clears and a pellet is formed (~ 2 minutes).
5. Remove and discard the supernatant without disturbing the pellet.
6. Add 200 µL of freshly prepared 80% ethanol solution to the pellet while it is still on the magnet. Use care 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. Keep the samples on the magnet and remove any residual ethanol solution from the bottom of the tube/well.
9. Elute in 22 µL Low EDTA TE, mix samples until beads are homogenous. After at least 2 minutes, place the samples on the magnetic rack and wait until the solution clears and a pellet is formed (~2 minutes). Transfer 20 µL eluate to a fresh tube/well.

NOTE: When direct sequencing with low inputs, a random primer library artifact my be present, evident by a peak at ~150bp by Bioanalyzer® or Tapestation®. For these samples, perform a second 0.8X bead cleanup on the samples prior to library quantification.

Safe Stopping point: samples can be stored at 4 °C for up to 24 hours or at -20 °C.

Expected Results

Yields for varying input quantities using this protocol with human universal reference total RNA (ThermoFisher) are shown below. Mean library size includes the adapter sequences which add ~130 bp to the library insert size.

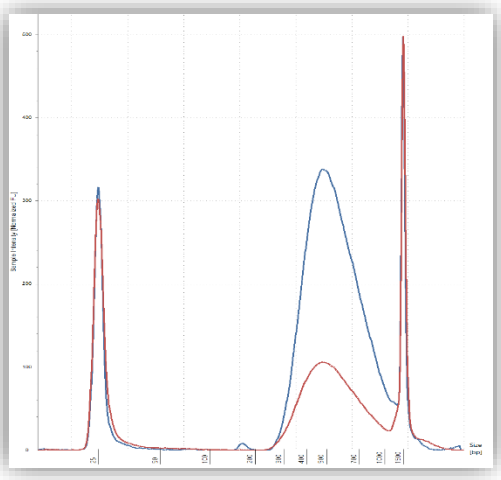


Figure 1. xGen RNA Library TapeStation results: 100 ng library with 14 cycles of amplification is represented by the blue line, and 1 ug library with 8 cycles of amplification is represented by the red line. Fragment sizes and quantities for RNA libraries produced from starting inputs (n=1) of 100 ng or 1 µg Universal Human Reference RNA (ThermoFisher). Libraries were run on the TapeStation 4200 using the High Sensitivity DNA 1000 reagents and screentape.

A peak at ~150-170 bp indicates a random primer library artifact – tailed random primers that survive the post-reverse transcription SPRI can serve as template during the Adaptase reaction. These artifacts can be removed with a second post-PCR SPRI. Note that a peak around ~60 bp (not shown) 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.

Limitations of use

For research use only. Not for use in diagnostic procedures. Unless otherwise agreed to in writing, IDT does not intend these products to be used in clinical applications and does not warrant their fitness or suitability for any clinical diagnostic use. Purchaser is solely responsible for all decisions regarding the use of these products and any associated regulatory or legal obligations.

Product in development. This protocol may serve as an initial guide for evaluation but may not be fully optimized. IDT does not guarantee the performance of this protocol, as development is in progress and verification testing has not been completed.

WARNING

Binding Buffer



Signal Word
Warning

Hazard Statements
H315 – Causes skin irritation
H319 – Causes serious eye irritation

Precautionary Statements
P264 – Wash thoroughly after handling.
P280 – Wear protective gloves/ eye protection/ face protection.
P302 – If on skin: Wash with plenty of water.
P305 – If in eyes: Rinse cautiously with water for several minutes. Remove contacts. Continue rinsing.
P332 – If skin irritation occurs: Get medical advice/ attention.
P337 – If eye irritation persists: Get medical advice/ attention.