

Combined Protocol: Watchmaker RNA Library Prep Kit with Polaris™ Depletion

Product Description

The Watchmaker RNA Library Prep Kit with Polaris Depletion enables the highly streamlined preparation of stranded, whole transcriptome sequencing (WTS) libraries from 1 ng to 1 µg total RNA with high library complexity and low coverage bias. The workflow is compatible with human, mouse, and rat species and both high- and low-quality samples, including FFPE material.

Kit Contents

Bundled kit	Kit	Kit code	Description	Component volume			
				24 rxn	96 rxn	384 rxn	
Watchmaker RNA Library Prep Kit with Polaris Depletion 7BK0002-024 (24 rxn) 7BK0002-096 (96 rxn) 7BK0002-384 (384 rxn)	Polaris Depletion Kit – rRNA/Globin (HMR)	7K0077-024 (24 rxn)	Depletion Master Mix	120 µL	540 µL	2.15 mL	
		7K0077-096 (96 rxn)	Depletion Probes – rRNA/Globin (HMR)	70 µL	300 µL	1.15 mL	
		7K0077-384 (384 rxn)	Probe Digestion Master Mix	925 µL	4.2 mL	16.15 mL	
	Watchmaker RNA Library Prep Kit		7K0078-024 (24 rxn)	FFPE Treatment Buffer	140 µL	600 µL	2.40 mL
			7K0078-024 (24 rxn)	Frag & Prime Buffer	290 µL	1.3 mL	5 mL
			7K0078-024 (24 rxn)	1st Strand Buffer	240 µL	1.08 mL	4.14 mL
			7K0078-024 (24 rxn)	1st Strand Enzyme	30 µL	120 µL	0.46 mL
			7K0078-096 (96 rxn)	2nd Strand Buffer	370 µL	1.68 mL	6.40 mL
			7K0078-096 (96 rxn)	2nd Strand Enzyme	30 µL	120 µL	0.46 mL
			7K0078-384 (384 rxn)	Ligation Buffer	1.06 mL	4.8 mL	18.40 mL
			7K0078-384 (384 rxn)	Ligation Enzyme	140 µL	600 µL	2.40 mL
			7K0078-384 (384 rxn)	Equinox Amplification Master Mix (2X)	690 µL	3 mL	12 mL
			7K0078-384 (384 rxn)	P5/P7 Primer Mix (10X)	144 µL	600 µL	2.40 mL

For custom formats, contact the **Sales Team** at sales@watchmakergenomics.com.

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Product Description (continued)

To improve the detection of RNAs of interest, the Polaris Depletion Kit – rRNA/Globin (HMR) specifically targets and efficiently depletes:

- 28S, 18S, 5.8S, and 5S cytoplasmic rRNAs
- 16S and 12S mitochondrial rRNAs
- 45S ETS and ITS rRNAs (probes designed for human only)
- HBA1/2, HBB, HBD, HBM, HBG1/2, HBE1, HBQ1, and HBZ globin RNAs (probes designed for human only)

The Watchmaker RNA Library Prep Kit converts enriched RNAs to adapter-ligated, double-stranded cDNA fragments through a small number of chemical and enzymatic manipulations:

- RNA fragmentation and priming for 1st strand cDNA synthesis
- 1st strand cDNA synthesis leveraging a specifically engineered reverse transcriptase
- Combined 2nd strand cDNA synthesis and A-tailing, during which dUTP is incorporated to maintain strand information
- Adapter ligation, where truncated, full length, or custom adapters utilizing a 3' T overhang are ligated to fragments
- Library amplification with Equinox® Amplification Master Mix (2X) for high-fidelity and high-efficiency PCR

Product Applications

This workflow was developed to address the highly specific needs of WTS and the associated areas of variant calling, isoform and gene fusion identification, and gene expression analysis. These applications require high library complexity, low bias, uniform coverage, and minimal experimental variability in order to support robust sensitivity, specificity, and reproducibility.

The workflow is ideally suited for:

- Whole transcriptome sequencing
- High-quality or degraded samples including FFPE
- Gene expression analysis
- Isoform/splice variant/gene fusion identification
- Single nucleotide variant detection
- Novel transcript discovery

- Targeted sequencing employing hybridization capture

Storage and Handling

The Polaris Depletion and Watchmaker RNA Library Prep Kits are shipped on cold packs. Upon receipt, store all components at $-20 \pm 5^\circ\text{C}$.

Keep all components and reaction mixes on ice or a cooled reagent block during routine use, unless otherwise stated. Some components are viscous; therefore take care to homogenize solutions thoroughly before use and during reaction setup. SPRI beads should be handled as per the manufacturer's guidelines.

All buffers should be vortexed for at least 5 sec before use. Enzymes and the Equinox Amplification Master Mix (2X) should be inverted ten times prior to use. The 1st Strand Buffer is photosensitive. Keep it in the kit box until thawing is required. Take care to protect it from direct light while thawing and in use.

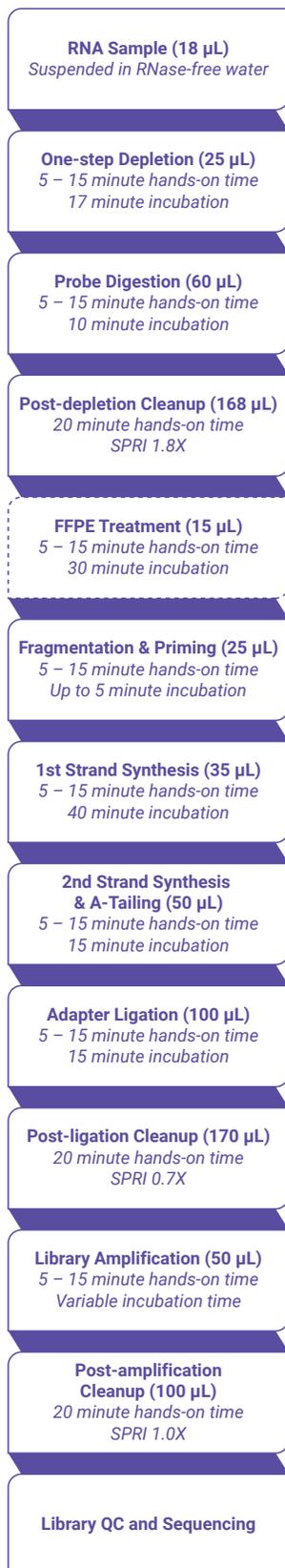
All master mixes (buffer and enzyme combined) prepared in the protocol should be stored at 4°C unless stated otherwise. The master mixes should be stable for up to 24 hours at 4°C .

Required Materials Not Included

- Adapters (see **Prior to Starting**)
- Adapter diluent (e.g., 10 mM Tris-HCl, pH 8.0, 10 mM NaCl)
- Magnetic rack compatible with 0.2 mL PCR tubes and/or 96-well plate
- Ampure® XP Beads (Beckman Coulter, Inc. #A63881) or equivalent
- 80% Ethanol
- 200 μL thin-wall PCR tubes compatible with thermocycler, or 96-well 0.2 mL PCR Plates and Plate-Seal
- 1 mL, 2 mL, 5 mL tubes (RNase-free)
- RNase-free water
- 10 mM Tris-HCl, pH 8.0
- Thermocycler
- Touch vortex
- Fragment Analyzer™ e.g., Bioanalyzer® or TapeStation (Agilent Technologies, Inc.), or similar instrument and consumables

These protocols are designed for use with the specified labware, consumables, and calibrated equipment.

Workflow Overview



Prior to Starting

Input RNA quality and quantity

This kit is compatible with both high- and low-quality samples, including blood-derived and FFPE samples suspended in 18 µL of RNase-free water.

NOTE: If you are working with FFPE samples, use Protocol B on page 13.

RNA should be accurately quantified by Qubit® Fluorometer or similar prior to starting.

High-quality total RNA ranging from 1 – 1000 ng has been tested and shown to produce high-performing libraries. Every total RNA sample contains different levels of rRNA, which will affect library yields.

Note that libraries have been successfully generated with input amounts as low as 10 ng with FFPE-derived RNA. However, the utility of these libraries is ultimately dependent on the application.

FFPE RNA workflow efficiency will most likely be lower and more variable sample to sample due to the significant damage incurred during fixation. Performance with particularly degraded and challenging samples can often be salvaged by increasing the total RNA input amount, with a maximum of 1000 ng. Additionally, some extraction protocols fail to adequately de-crosslink FFPE-derived RNA. Our FFPE treatment step may enhance library quality in these instances. If you are working with FFPE samples, use **Protocol B** on page 13.

Assess total RNA quality via an electrophoretic method, such as Agilent BioAnalyzer or TapeStation.

Partially degraded samples, with RINs <7, may require a reduced adapter concentration in the ligation reaction to mitigate adapter-dimer carryover. Alternatively, a second post-ligation cleanup can be executed.

Input RNA Purity

RNA inputs should be free from contaminating DNA. If the total RNA contains DNA, remove the contamination by incubating with DNase I (not supplied with kit). Residual DNase I may interfere with library preparation, so it is important to ensure no residual enzyme remains in the sample.

RNA should be suspended in RNase-free water and be free of salts (e.g., Mg²⁺, or guanidinium salts), chelating agents (e.g., EDTA or EGTA), and organics (e.g., phenol or ethanol).

RNA Handling

To avoid RNase contamination, work in a laminar flow hood, if available, and keep all sample and reagent tubes closed unless in use. Wear gloves when handling reagents and preparing libraries. Change gloves and pipette tips if they come into contact with non-sterile surfaces.

To avoid RNA degradation, store RNA in an RNase-free diluent and limit the number of sample freeze-thaw cycles.

Fragmentation Optimization

Our **Fragmentation and Priming** step (A4.5 and B5.1) has been optimized to create inserts of 180 – 260 bp in length depending on RNA quality and condition (see Table A1). Inserts of this length are ideal for most RNA-seq applications including differential gene expression (DGE) analysis and targeted sequencing involving hybrid capture.

For some RNA library preparation workflows, it may be necessary or preferred to create libraries with longer inserts. Recommendations are provided in **Appendix B** for generating inserts >260 bp.

Adapters

This kit is compatible with adapters that have a 3' overhanging T to facilitate adapter orientation during double stranded cDNA ligation. Note that adapter quality impacts overall library preparation efficiency. Ensure that adapters are adequately duplexed and at the appropriate concentration prior to use. For more details, contact support@watchmakergenomics.com for our Adapter Recommendations Guide.

When using 'stubby', adapters where sample indexes are added during subsequent library amplification, user-supplied, uniquely indexed PCR primers will be required for the amplification of each library to be sequenced on the same flow cell.

Stubby adapters provide improved library prep efficiency due to the ability to include them at increased concentrations in the ligation reaction. We strongly recommend the use of stubby adapters for maximum performance.

This workflow is also compatible with full-length adapters where sample indexes are added during ligation. When using full-length adapters, a unique sample index is required for all samples to be sequenced on the same flow cell. Refer to the technical documentation provided by the adapter vendor for recommendations on optimal pooling.

P5/P7 Amplification Primers

The P5/P7 Primer Mix (10X) is supplied at a concentration of 20 µM of each primer and is appropriate for the amplification of full-length adapter-ligated libraries.

P5: AATGATACGGCGACCACCGA

P7: CAAGCAGAAGACGGCATAACGAGAT

User-supplied Amplification Primers

When using truncated, or 'stubby', adapters in multiplexed sequencing workflows, a uniquely barcoded primer mix will be required (and must be added individually) for each library to be sequenced on the same flow cell.

Primers should always use equimolar concentrations of the forward and reverse primers. A primer pre-mix containing 20 µM of each primer (resulting in a final concentration of 2 µM each in the amplification reaction) is recommended.

Primers may also incorporate chemical modifications (e.g., one or more 3'-phosphorothioate bonds) to improve specificity.

Use a buffered solution, such as 10 mM Tris-HCl, pH 8.0, to store and dilute primers. Limit the number of freeze-thaw cycles.

Library Amplification Optimization

Annealing Temperature

For the truncated adapter scheme detailed in Glenn et. al. 2019,¹ use an annealing temperature of 55°C. For other primers, an annealing temperature gradient (55°C to 70°C) may be performed to determine the optimal condition for amplification.

Extension Time

Longer extension times may be employed to ensure efficient amplification of longer-insert libraries. A 30-sec extension is sufficient for libraries with a mode fragment size up to 500 bp; a 45-sec extension time is recommended for libraries with mode fragment sizes >500 bp. The optimal condition for each application may have to be determined empirically.

Cycle Number

This protocol provides a starting point for PCR cycle number optimization based on RNA input into library preparation. For inserts >260 bp, an additional cycle of amplification is recommended (see **Appendix B**). FFPE and other degraded samples may require additional cycles. Adapter-ligated libraries may be quantified by qPCR or estimated by other means or methods to determine the optimal number of amplification cycles for the desired library yield.

SPRI Purification Beads

The protocol outlined below assumes the use of AMPure® XP (Beckman Coulter) reagent for bead purification steps.

Other SPRI bead brands may be used, assuming they are nuclease-free and deliver equivalent sizing performance at the given bead ratios. Otherwise, bead purification ratios will need to be optimized for the bead brand used.

Ensure beads are equilibrated to room temperature and thoroughly resuspended by vortexing prior to use.

¹Glenn TC, Nilsen RA, Kieran TJ, et al. Adapterama I: universal stubs and primers for 384 unique dual-indexed or 147,456 combinatorially-indexed Illumina libraries (iTru & iNext). *PeerJ*. 2019;7:e7755. Published 2019 Oct 11. doi:10.7717/peerj.7755

Library Construction Protocol A: High-quality and partially degraded samples

Recommendations

- Keep all sample tubes, buffers, and enzymes on ice unless stated otherwise.
- **NOTE:** This protocol is not intended for FFPE samples. Please see **Library Construction Protocol B: FFPE Samples** (page 13) for an FFPE-specific protocol.
- Vortex mixing is recommended for master mix generation and subsequent addition to sample. Pipette mixing is an acceptable alternative so long as care is taken to ensure a completely homogeneous reaction.
- The 1st Strand Buffer is photosensitive. Keep it in the kit box until thawing is required. Take care to protect it from direct light while thawing and in use.
- Ensure all the buffers are fully thawed on ice before use. Once thawed, invert the tubes several times, and vortex for at least 5 sec to ensure the reagent is fully mixed.
- Place enzymes and the Equinox Amplification Master Mix (2X) on ice before use. Invert the tubes 10 times to mix.
- Where possible, centrifuge briefly to remove any excess liquid from the tube and collect all liquid from the tube lid prior to opening.
- We recommend making master mixes with a 10% excess to account for loss during pipetting.
- Ensure SPRI purification beads are fully equilibrated to room temperature and thoroughly resuspended by vortexing prior to use.

A1. rRNA and Globin Depletion

- A1.1 Program a thermocycler as indicated below and initiate the run to heat the block:

Step	Temperature	Time
Lid temperature	80°C	N/A
HOLD	77°C	HOLD
Denaturation	77°C	2 min
rRNA digestion	65°C	15 min
HOLD	4°C	HOLD

- A1.2 For each reaction, prepare the Depletion Reaction Mix as specified below on ice:

Component	Volume (µL)
Depletion Master Mix	4.5
Depletion Probes – rRNA/Globin (HMR)	2.5

- A1.3 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

- A1.4 On ice, prepare input RNA in a total volume of 18 µL using RNase-free water and add to labeled 0.2 mL PCR tubes or PCR plate.

- A1.5 To each sample, add the following on ice:

Component	Volume (µL)
RNA sample	18
Depletion Reaction Mix	7

- A1.6 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

- A1.7 Place the tube on ice until ready for thermocycling.

NOTE: Samples should remain on ice for no longer than 10 min.

- A1.8 Place the tube into the heated thermocycler (programmed and initiated in **Step A1.1**). Advance the thermocycler from the initial 77°C hold.

- A1.9 Upon completion of thermocycling, proceed immediately to **Probe Digestion**.

A2. Probe Digestion

- A2.1 Program a thermocycler as indicated below and initiate the run to cool the block:

Step	Temperature	Time
Lid temperature	40°C	N/A
Pre-cooling	4°C	HOLD
Probe digestion	37°C	10 min
HOLD	4°C	HOLD

- A2.2 To each sample, add the Probe Digestion Master Mix as specified below on ice:

Component	Volume (µL)
Depleted RNA	25
Probe Digestion Master Mix	35

- A2.3 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.
- A2.4 Place the tube in the chilled thermocycler (programmed and initiated in **Step A2.1**). Advance the thermocycler from the initial 4°C hold to start the 37°C incubation.
- A2.5 Upon completion of the Probe Digestion incubation, proceed immediately to **Post-depletion Cleanup**.

A3. Post-depletion Cleanup

- A3.1 Freshly prepare at least 0.4 mL of an 80% ethanol solution for each reaction.
- A3.2 Vortex **room temperature** SPRI beads to thoroughly mix. Add 108 µL (1.8X) of beads to each reaction.
- A3.3 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.
- A3.4 Incubate the reaction-bead mixtures at room temperature for 5 min.
- A3.5 Place sample tubes on a magnet for at least 5 min, or until all beads have collected on the tube wall and the solution is clear.
- A3.6 Carefully remove and discard the supernatant from each tube.
- A3.7 Add 200 µL of freshly prepared 80% ethanol to each tube, taking care to not disturb the bead pellet on the tube wall.
- A3.8 Incubate tubes at room temperature for at least 30 sec without removing the tube from the magnet or disturbing the bead pellet. Carefully remove and discard the ethanol.
- A3.9 Repeat **Steps A3.7 – A3.8**, for a total of two washes.

OPTIONAL: Tubes can be briefly spun to pull down excess ethanol prior to removing with a p10 pipette.

- A3.10 Allow remaining ethanol to evaporate by allowing the pellets to air dry for 3 – 5 min.

NOTE: Beads have been sufficiently dried when cracks appear in the surface of the bead pellet and no liquid is visible in the tube.

A4. Fragmentation and Priming

- A4.1 Prepare the Frag & Prime Master Mix as follows for each reaction:

Component	Volume (µL)
Frag & Prime Buffer	10.8
RNase-free water	16.2

- A4.2 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.
- A4.3 Add 27 µL of the Frag & Prime Master Mix to the dry beads from **Step A3.10**.
- A4.4 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.



Safe stopping point. Fully resuspended samples can be stored at 4°C for <24 hours. Ensure samples are mixed on a touch vortexer (or 3,000 rpm) for 4 sec after storing at 4°C

- A4.5 Program a thermocycler as indicated below, and initiate the run to cool the block.

NOTE: Insert sizes can be adjusted through modulating the Fragmentation and Priming incubation time and temperature. Optimal conditions vary depending on the RNA input quality. See **Table A1** for more details.

Step	Temperature	Time
Lid temperature	105°C	N/A
Pre-cooling	4°C	HOLD
RNA fragmentation	See Table A1	
HOLD (Priming)	12°C	HOLD

Table A1. Recommended RNA fragmentation conditions based on RNA quality and desired insert size

RNA Quality	RIN	Desired Insert Size ^{1,2,3}	Fragmentation Condition
Intact	>7	180 – 260 bp	85°C for 5 min
Partially degraded	2 – 7	100 – 300 bp	85°C for 1 – 5 min
Degraded, non-FFPE	1 – 2	Dictated by RNA quality	65°C for 1 min

¹As assessed by NGS – Picard mean insert size.

²Assumes a 0.7X Post-ligation Cleanup ratio.

³For inserts >260 bp, see recommendations provided in **Appendix B**.

- A4.6 Place the tubes into the chilled thermocycler. Advance the thermocycler from the initial 4°C hold to start the RNA Fragmentation incubation.
- A4.7 After the program has finished and the samples have returned to 12°C, place tubes on a magnet for at least 5 min, or until all beads have been collected on the tube wall and the solution is clear.
- A4.8 Proceed immediately to **1st Strand Synthesis**.

A5. 1st Strand Synthesis

NOTE: The 1st Strand Buffer is photosensitive. Keep it in the kit box until thawing is required. Take care to protect it from direct light while thawing and in use.

- A5.1 Program a thermocycler as indicated below and initiate the run to cool the block:

Step	Temperature	Time
Lid temperature	105°C	N/A
Pre-cooling	4°C	HOLD
cDNA Synthesis	25°C	10 min
	42°C	15 min
RT Inactivation	70°C	15 min
HOLD	4°C	HOLD

- A5.2 For each reaction, prepare the 1st Strand Master Mix as follows on ice:

Component	Volume (µL)
1st Strand Buffer	9
1st Strand Enzyme	1

- A5.3 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

- A5.4 Carefully remove 25 µL of the supernatant (from **Step 4.7**) and add to a fresh tube on ice. To each tube, add the 1st Strand Master Mix as specified below:

Component	Volume (µL)
Fragmented RNA (from Step A4.7)	25
1st Strand Master Mix	10

- A5.5 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

- A5.6 Place reactions in the chilled thermocycler (programmed and initiated in **Step A5.1**). Advance the thermocycler from the initial 4°C hold to start the 25°C incubation.

- A5.7 Once the 1st Strand Synthesis reaction has completed, place the samples on ice or leave in the thermocycler at 4°C. Proceed immediately to **2nd Strand Synthesis and A-Tailing**.

A6. 2nd Strand Synthesis and A-Tailing

- A6.1 Program a thermocycler as indicated below and initiate the run to cool the block:

Step	Temperature	Time
Lid temperature	80°C	N/A
Pre-cooling	4°C	HOLD
2nd Strand Synthesis	42°C	5 min
A-Tailing	62°C	10 min
HOLD	4°C	HOLD

- A6.2 For each reaction, prepare the 2nd Strand Master Mix as follows on ice:

Component	Volume (µL)
2nd Strand Buffer	14
2nd Strand Enzyme	1

- A6.3 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

- A6.4 To each tube, add the 2nd Strand Master Mix as specified below on ice:

Component	Volume (µL)
1st Strand Synthesis product	35
2nd Strand Master Mix	15

- A6.5 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

- A6.6 Place reactions in the chilled thermocycler (programmed and initiated in **Step A6.1**). Advance the thermocycler from the initial 4°C hold to start the 42°C incubation.

- A6.7 Proceed to adapter ligation after the program has finished and the samples have returned to 4°C. Proceed immediately to **Adapter Ligation**.

A7. Adapter Ligation

NOTE: See **Prior to Starting** for considerations in adapter selection and design.

- A7.1 Program a thermocycler as indicated below and initiate the run to cool the block:

Step	Temperature	Time
Lid temperature	OFF	N/A
Pre-cooling	4°C	HOLD
Ligation	20°C	15 min
HOLD	4°C	HOLD ¹

¹Maintain samples on ice following ligation to reduce adapter-dimer formation prior to **Post-ligation Cleanup**.

- A7.2 Vortex the thawed Ligation Buffer for 20 sec to fully homogenize the solution before centrifuging to collect all of the solution at the bottom of the tube. Then place it on ice.
- A7.3 Using an appropriate diluent (e.g., 10 mM Tris-HCl, pH 8.0, 10 mM NaCl), prepare the required volume of each adapter at the concentration specified in either **Table A2** or **Table A3** based on adapter design. 5 µL of adapter at the appropriate concentration is required per ligation reaction.

NOTE: Storing adapter solutions at concentrations <10 µM for extended periods of time is not recommended.

Table A2. Full-length adapter concentration by RNA input amount into library prep

RNA input (ng) ¹	Adapter concentration (µM)
<10	Not recommended
10 – 49 ²	0.25
50 – 199	1
200 – 499	2
500 – 749	2.5
≥750	5

¹When using partially degraded RNA samples, either reducing the adapter concentration or executing a second post-ligation cleanup may help to mitigate adapter-dimer carryover.

²A second post-ligation cleanup (**Step A8**) is recommended for inputs under 20 ng when using full-length adapters.

Table A3. Truncated ('stubby') adapter concentration by RNA input amount into library prep

RNA input (ng) ¹	Adapter concentration (µM)
<10	0.2
10 – 99	2
100 – 249	4
≥250	6

¹When using partially degraded RNA samples, either reducing the adapter concentration or executing a second post-ligation cleanup may help to mitigate adapter-dimer carryover.

- A7.4 Add 5 µL of appropriately diluted adapter to each tube.
- A7.5 Prepare the Ligation Master Mix as follows:

Component	Volume (µL)
Ligation Buffer	40
Ligation Enzyme	5

- A7.6 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.
- A7.7 To each tube, add the Ligation Master Mix as specified below on ice:

Component	Volume (µL)
2nd Strand Synthesis product and adapter	55
Ligation Master Mix	45

- A7.8 The Ligation Master Mix is viscous. Mix the ligation reaction on a touch vortexer (or 3,000 rpm) for 4 sec or carefully pipette a minimum of 80 µL up and down ≥10 times to ensure proper mixing. Briefly centrifuge if needed to collect all liquid in the bottom of the tube.
- A7.9 Place the sample tubes in the thermocycler and initiate the program (programmed in **Step A7.1**).
- A7.10 Once the program has completed, proceed immediately to **Post-ligation Cleanup**.

A8. Post-ligation Cleanup

- A8.1 Freshly prepare at least 0.4 mL of an 80% ethanol solution for each reaction.
- A8.2 Vortex **room temperature** SPRI beads to thoroughly mix. Add 70 µL (0.7X) of resuspended beads to each ligation reaction.
- A8.3 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

- A8.4 Incubate the library-bead mixtures at room temperature for at least 5 min to maximize library recovery.
- A8.5 Place sample tubes on a magnet for at least 5 min, or until all beads have been collected on the tube wall and the solution is clear.
- A8.6 Carefully remove and discard the supernatant from each tube.
- A8.7 Add 200 μ L of freshly prepared 80% ethanol to each tube, taking care to not disturb the bead pellet on the tube wall.
- A8.8 Incubate tubes at room temperature for at least 30 sec without removing the tube from the magnet or disturbing the bead pellet. Carefully remove and discard the ethanol.
- A8.9 Repeat **Steps A8.7 – A8.8**, for a total of two washes.
OPTIONAL: Tubes can be briefly spun to pull down excess ethanol prior to removing with a p10 pipette.
- A8.10 Allow remaining ethanol to evaporate by allowing the pellets to air dry for 3 – 5 min.
NOTE: Beads have been sufficiently dried when cracks appear in the surface of the bead pellet and no liquid is visible in the tube. Residual trace ethanol in the library amplification reaction may decrease performance.
- A8.11 Remove sample tubes from the magnet and carefully resuspend each bead pellet in 22 μ L of 10 mM Tris-HCl, pH 8.0.
- A8.12 Incubate tubes at room temperature for at least 2 min before placing back on the magnet.
- A8.13 Leave tubes on the magnet for at least 2 min, or until all beads have collected on the tube wall and the solution is clear.
- A8.14 Carefully transfer 20 μ L of each library-containing supernatant to a new, labeled tube.
-  **Safe stopping point.** Samples can be stored at 4°C for <24 hours or frozen at -20°C for up to 4 weeks.
- A9. 2nd Post-ligation Cleanup (Optional)**
- NOTE:** This is an optional step and only recommended when working with:
- Full-length adapters and less than 20 ng of RNA
 - Partially degraded samples as an alternative to titrating adapter concentration in the ligation reaction
- A9.1 Freshly prepare at least 0.4 mL of an 80% ethanol solution for each reaction.
- A9.2 Vortex **room temperature** SPRI beads to thoroughly mix. Add 20 μ L (1X) of room temperature resuspended beads to each eluted library from **Step A8.14**.
- A9.3 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.
- A9.4 Incubate the library-bead mixtures at room temperature for at least 5 min to maximize library recovery.
- A9.5 Place sample tubes on a magnet for at least 5 min, or until all beads have been collected on the tube wall and the solution is clear.
- A9.6 Carefully remove and discard the supernatant from each tube.
- A9.7 Add 200 μ L of freshly prepared 80% ethanol to each tube, taking care to not disturb the bead pellet on the tube wall.
- A9.8 Incubate tubes at room temperature for at least 30 sec without removing the tube from the magnet or disturbing the bead pellet. Carefully remove and discard the ethanol.
- A9.9 Repeat **Steps A9.7 – A9.8**, for a total of two washes.
OPTIONAL: Tubes can be briefly spun to pull down excess ethanol prior to removing with a p10 pipette.
- A9.10 Allow remaining ethanol to evaporate by allowing the pellets to air dry for 3 – 5 min.
NOTE: Beads have been sufficiently dried when cracks appear in the surface of the bead pellet and no liquid is visible in the tube. Residual trace ethanol in the library amplification reaction may decrease performance.
- A9.11 Remove sample tubes from the magnet and carefully resuspend each bead pellet in 22 μ L of 10 mM Tris-HCl, pH 8.0.
- A9.12 Incubate tubes at room temperature for at least 2 min before placing back on the magnet.
- A9.13 Leave tubes on the magnet for at least 2 min, or until all beads have collected on the tube wall and the solution is clear.
- A9.14 Carefully transfer 20 μ L of each library-containing supernatant to a new, labeled tube.
-  **Safe stopping point.** Samples can be stored at 4°C for <24 hours or frozen at -20°C for up to 4 weeks.

A10. Library Amplification and Strand Selection

- Library amplification is required for strand-specific sequencing regardless of adapter configuration used.
- If your workflow requires library amplification in the presence of paramagnetic beads, refer to **Appendix A** for bead compatibility.

A10.1 Thaw and equilibrate the Equinox Amplification Master Mix (2X) on ice. Once thawed, vortex for 5 sec to mix.

A10.2 Program a thermocycler as indicated below:

Step ¹	Temperature	Time	Cycles
Lid temperature	105°C	N/A	N/A
Initial denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	
Annealing	P5/P7 primers: 60°C ² Indexed primers: 55°C ³	30 sec	See Table A4
Extension	72°C	30 – 45 sec	
Final extension	72°C	60 sec	1
HOLD	12°C	HOLD	–

¹For additional details on optimizing amplification, see **Prior to Starting**.

²Appropriate temperature for P5/P7 Primer Mix (10X).

³For indexes used with the truncated adapter scheme detailed in Glenn, et. al. 2019, use 55°C unless using IDT™ xGen Stubby-Adapter UDI primers which should be annealed at 64°C. Optimization may be required for other adapter/primer configurations (see **Prior to Starting**).

Table A4. Recommended PCR cycle numbers by RNA input amount into library prep

RNA input into Library Preparation (ng)	PCR cycles to generate 10 – 50 nM library	
	Full-length adapters	Stubby adapters
1000	7 – 8	6 – 7
500	8 – 9	7 – 8
250	9 – 10	8 – 9
100	11 – 12	10 – 11
50	12 – 13	11 – 12
10	15 – 16	13 – 14
1	Not recommended	17 – 18

A10.3 Assemble each amplification reaction in the order specified below:

Component	Volume (µL)
Adapter-ligated library	20
P5/P7 Primer Mix (10X) or User-supplied primers ¹	5
Equinox Amplification Master Mix (2X)	25

¹See **Prior to Starting** for more information on user-supplied primers.

A10.4 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

A10.5 Place tubes in the thermocycler (programmed in **Step A10.2**) and initiate the program.

A10.6 Once the program has completed, proceed immediately to **Post-amplification Cleanup**.

A11. Post-amplification Cleanup

A11.1 Freshly prepare at least 0.4 mL of an 80% ethanol solution for each reaction.

A11.2 Vortex **room temperature** SPRI beads to thoroughly mix. Add 50 µL (1X) of beads to each amplification reaction.

A11.3 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

A11.4 Incubate the library-bead mixtures at room temperature for at least 5 min to maximize library recovery.

A11.5 Place sample tubes on a magnet for at least 5 min, or until all beads have collected on the tube wall and the solution is clear.

A11.6 Carefully remove and discard the supernatant from each tube.

A11.7 Add 200 µL of freshly prepared 80% ethanol to each tube, taking care to not disturb the bead pellet on the tube wall.

A11.8 Incubate tubes at room temperature for at least 30 sec without removing the tube from the magnet or disturbing the bead pellet. Carefully remove and discard the ethanol.

A11.9 Repeat **Steps A11.7 – A11.8** for a total of two washes.

OPTIONAL: Tubes can be briefly spun to pull down excess ethanol prior to removing with a p10 pipette.

A11.10 Allow remaining ethanol to evaporate by allowing the pellets to air dry for 3 – 5 min.

NOTE: Beads have been sufficiently dried when cracks appear in the surface of the bead pellet and no liquid is visible in the tube.

A11.11 Remove sample tubes from the magnet and carefully resuspend each bead pellet in 22 μ L of 10 mM Tris-HCl, pH 8.0.

A11.12 Incubate tubes at room temperature for at least 2 min before placing back on the magnet.

A11.13 Leave sample tubes on the magnet for at least 2 min, or until all beads have collected on the tube wall and the solution is clear.

A11.14 Carefully transfer 20 μ L of each library-containing supernatant to a new tube.

A11.15 At this point, libraries are ready for quantification, normalization, pooling, hybrid capture, and/or sequencing.

NOTE: We recommend quantifying libraries using qPCR and analyzing quality and sizing using capillary electrophoresis prior to preparing the libraries for sequencing.

Library Construction Protocol B: FFPE Samples

Recommendations

- Keep all sample tubes, buffers, and enzymes on ice unless stated otherwise.
- Vortex mixing is recommended for master mix generation and subsequent addition to sample. Pipette mixing is an acceptable alternative so long as care is taken to ensure a completely homogeneous reaction.
- The 1st Strand Buffer is photosensitive. Keep it in the kit box until thawing is required. Take care to protect it from direct light while thawing and in use.
- Ensure all the buffers are fully thawed on ice before use. Once thawed, invert the tubes several times, and vortex for at least 5 sec to ensure the reagent is fully mixed.
- Place enzymes and the Equinox Amplification Master Mix (2X) on ice before use. Invert the tubes 10 times to mix.
- Where possible, centrifuge briefly to remove any excess liquid from the tube and collect all liquid from the tube lid prior to opening.
- We recommend making master mixes with a 10% excess to account for loss during pipetting.
- Ensure SPRI purification beads are fully equilibrated to room temperature and thoroughly resuspended by vortexing prior to use.



Indicates a deviation from Protocol A specifically tailored for FFPE samples

B1. rRNA and Globin Depletion

B1.1 Program a thermocycler as indicated below and initiate the run to heat the block:

Step	Temperature	Time
Lid temperature	80°C	N/A
HOLD	77°C	HOLD
Denaturation	77°C	2 min
rRNA digestion	65°C	15 min
HOLD	4°C	HOLD

B1.2 For each reaction, prepare the Depletion Reaction Mix as specified below on ice:

Component	Volume (µL)
Depletion Master Mix	4.5
Depletion Probes - rRNA/Globin (HMR)	2.5

B1.3 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

B1.4 On ice, prepare input RNA in a total volume of 18 µL using RNase-free water and add to labeled 0.2 mL PCR tubes or PCR plate.

B1.5 To each sample, add the following on ice:

Component	Volume (µL)
RNA sample	18
Depletion Reaction Mix	7

B1.6 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

B1.7 Place the tube on ice until ready for thermocycling.

NOTE: Samples should remain on ice for no longer than 10 min.

B1.8 Place the tube into the heated thermocycler (programmed and initiated in **Step B1.1**). Advance the thermocycler from the initial 77°C hold.

B1.9 Upon completion of thermocycling, proceed immediately to **Probe Digestion**.

B2. Probe Digestion

B2.1 Program a thermocycler as indicated below and initiate the run to cool the block:

Step	Temperature	Time
Lid temperature	40°C	N/A
Pre-cooling	4°C	HOLD
Probe digestion	37°C	10 min
HOLD	4°C	HOLD

B2.2 To each sample, add the Probe Digestion Master Mix as specified below on ice:

Component	Volume (µL)
Depleted RNA	25
Probe Digestion Master Mix	35

- B2.3 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.
- B2.4 Place the tube in the chilled thermocycler (programmed and initiated in **Step B2.1**). Advance the thermocycler from the initial 4°C hold to start the 37°C incubation.
- B2.5 Upon completion of the Probe Digestion incubation, proceed immediately to **Post-depletion Cleanup**.

B3. Post-depletion Cleanup

- B3.1 Freshly prepare at least 0.4 mL of an 80% ethanol solution for each reaction.
- B3.2 Vortex **room temperature** SPRI beads to thoroughly mix. Add 108 µL (1.8X) of beads to each reaction.
- B3.3 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.
- B3.4 Incubate the reaction-bead mixtures at room temperature for 5 min.
- B3.5 Place sample tubes on a magnet for at least 5 min, or until all beads have collected on the tube wall and the solution is clear.
- B3.6 Carefully remove and discard the supernatant from each tube.
- B3.7 Add 200 µL of freshly prepared 80% ethanol to each tube, taking care to not disturb the bead pellet on the tube wall.
- B3.8 Incubate tubes at room temperature for at least 30 sec without removing the tube from the magnet or disturbing the bead pellet. Carefully remove and discard the ethanol.
- B3.9 Repeat **Steps B3.7 – B3.8**, for a total of two washes.
- OPTIONAL:** Tubes can be briefly spun to pull down excess ethanol prior to removing with a p10 pipette.
- B3.10 Allow remaining ethanol to evaporate by allowing the pellets to air dry for 3 – 5 min.

NOTE: Beads have been sufficiently dried when cracks appear in the surface of the bead pellet and no liquid is visible in the tube.

B4. FFPE Treatment

- B4.1 Prepare the FFPE Treatment Master Mix as follows for each reaction:

Component	Volume (µL)
FFPE Treatment Buffer	4.5
RNase-free water	12.5

- B4.2 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.
- B4.3 Add 17 µL of FFPE Treatment Master Mix to the dry beads from **Step 3.10**.
- B4.4 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.



Safe stopping point. Fully resuspended samples can be stored at 4°C for <24 hours. Ensure samples are mixed on a touch vortexer (or 3,000 rpm) for 4 sec after storing at 4°C.

- B4.5 Place tubes on a magnet for at least 5 min, or until all beads have been collected on the tube wall and the solution is clear.
- B4.6 Carefully remove 15 µL of the supernatant and transfer to new tubes.
- B4.7 Program a thermocycler as indicated below:

Step	Temperature	Time
Lid temperature	105°C	N/A
FFPE heat treatment	70°C	30 min
HOLD	4°C	HOLD

- B4.8 Place tubes in the thermocycler and initiate the run.
- B4.9 After the program completes, proceed immediately to **Fragmentation and Priming**.

B5. Fragmentation and Priming

B5.1 Program a thermocycler as indicated below, and initiate the run to cool the block:

Step	Temperature	Time
Lid temperature	105°C	N/A
Pre-cooling	4°C	HOLD
RNA fragmentation ¹	65°C	1 min
HOLD (Priming)	12°C	HOLD

¹Longer insert lengths may be achieved with different SPRI cleanup parameters. For fragments >260 bp, see **Appendix B**.

B5.2 To each reaction, add the Frag & Prime Buffer as specified below on ice:

Component	Volume (µL)
Treated RNA	15
Frag & Prime Buffer	10

B5.3 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

B5.4 Place the tubes into the chilled thermocycler. Advance the thermocycler from the initial 4°C hold to start the RNA Fragmentation incubation.

B5.5 Proceed immediately to **1st Strand Synthesis**.

B6. 1st Strand Synthesis

NOTE: The 1st Strand Buffer is photosensitive. Keep it in the kit box until thawing is required. Take care to protect it from direct light while thawing and in use.

B6.1 Program a thermocycler as indicated below and initiate the run to cool the block:

Step	Temperature	Time
Lid temperature	105°C	N/A
Pre-cooling	4°C	HOLD
cDNA synthesis	25°C 42°C	10 min 15 min
RT inactivation	70°C	15 min
HOLD	4°C	HOLD

B6.2 For each reaction, prepare the 1st Strand Master Mix as follows on ice:

Component	Volume (µL)
1st Strand Buffer	9
1st Strand Enzyme	1

B6.3 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

B6.4 To each tube, add the 1st Strand Master Mix as specified below on ice:

Component	Volume (µL)
Fragmented RNA	25
1st Strand Synthesis Master Mix	10

B6.5 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

B6.6 Place reactions in the chilled thermocycler (programmed and initiated in **Step B6.1**). Advance the thermocycler from the initial 4°C hold to start the 25°C incubation.

B6.7 Once the 1st Strand Synthesis reaction has completed, place the samples on ice or leave in the thermocycler at 4°C. Proceed immediately to **2nd Strand Synthesis & A-Tailing**.

B7. 2nd Strand Synthesis & A-Tailing

B7.1 Program a thermocycler as indicated below and initiate the run to cool the block:

Step	Temperature	Time
Lid temperature	80°C	N/A
Pre-cooling	4°C	HOLD
2nd Strand Synthesis	42°C	5 min
A-Tailing	62°C	10 min
HOLD	4°C	HOLD

B7.2 For each reaction, prepare the 2nd Strand Master Mix as follows on ice:

Component	Volume (μL)
2nd Strand Buffer	14
2nd Strand Enzyme	1

B7.3 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

B7.4 To each tube, add the 2nd Strand Master Mix as specified below on ice:

Component	Volume (μL)
1st Strand Synthesis product	35
2nd Strand Master Mix	15

B7.5 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

B7.6 Place reactions in the chilled thermocycler (programmed and initiated in **Step B7.1**). Advance the thermocycler from the initial 4°C hold to start the 42°C incubation.

B7.7 Proceed to adapter ligation after the program has finished and the samples have returned to 4°C. Proceed immediately to **Adapter Ligation**.

B8. Adapter Ligation

NOTE: See **Prior to Starting** for considerations in adapter selection and design.

B8.1 Program a thermocycler as indicated below:

Step	Temperature	Time
Lid temperature	OFF	N/A
Pre-cooling	4°C	HOLD
Ligation	20°C	15 min
HOLD	4°C	HOLD ¹

¹Maintain samples on ice following ligation to reduce adapter-dimer formation prior to **Post-ligation Cleanup**.

B8.2 Vortex the thawed Ligation Buffer for 20 sec to fully homogenize the solution before centrifuging to collect all of the solution at the bottom of the tube. Then place it on ice.

B8.3 Using an appropriate diluent (e.g., 10 mM Tris-HCl, pH 8.0, 10 mM NaCl), prepare the required volume of each adapter at the concentration specified in either **Table B1** or **Table B2** based on adapter design. 5 μL of adapter at the appropriate concentration is required per ligation reaction.

NOTE: Storing adapter solutions at concentrations <10 μM for extended periods of time is not recommended.



Table B1. Full-length adapter concentration by RNA input amount into library prep

RNA input (ng) ¹	Adapter concentration (μM)
<20	0.05
20 – 49	0.1
50 – 199	0.25
200 – 499	0.5
≥500	1

¹A second post-ligation cleanup (**Step B10**) is recommended when using full-length adapters.



Table B2. Truncated ('stubby') adapter concentration by RNA input amount into library prep

RNA input (ng)	Adapter concentration (μM)
10 – 49 ¹	0.2
50 – 199	0.4
200 – 499	0.75
≥500	1

¹A second post-ligation cleanup (**Step B10**) is recommended for inputs under 50 ng when using stubby adapters.

B8.4 Add 5 μL of appropriately diluted adapter to each tube.

B8.5 Prepare the Ligation Master Mix as follows:

Component	Volume (μL)
Ligation Buffer	40
Ligation Enzyme	5

B8.6 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

B8.7 To each tube, add the Ligation Master Mix as specified below on ice:

Component	Volume (μL)
2nd Strand Synthesis product and adapter	55
Ligation Master Mix	45

B8.8 The Ligation Master Mix is viscous. Mix the ligation reaction on a touch vortexer (or 3,000 rpm) for 4 sec or carefully pipette a minimum of 80 μL up and down ≥10 times to ensure proper mixing. Briefly centrifuge if needed to collect all liquid in the bottom of the tube.

B8.9 Place the sample tubes in the thermocycler and initiate the program (programmed in **Step B8.1**).

B8.10 Once the program has completed, proceed immediately to **Post-ligation Cleanup**.

B9. Post-ligation Cleanup

B9.1 Freshly prepare at least 0.4 mL of an 80% ethanol solution for each reaction.

B9.2 Vortex **room temperature** SPRI beads to thoroughly mix. Add 70 μL (0.7X) of room temperature resuspended beads to each ligation reaction.

B9.3 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

B9.4 Incubate the library-bead mixtures at room temperature for at least 5 min to maximize library recovery.

B9.5 Place sample tubes on a magnet for at least 5 min, or until all beads have been collected on the tube wall and the solution is clear.

B9.6 Carefully remove and discard the supernatant from each tube.

B9.7 Add 200 μL of freshly prepared 80% ethanol to each tube, taking care to not disturb the bead pellet on the tube wall.

B9.8 Incubate tubes at room temperature for at least 30 sec without removing the tube from the magnet or disturbing the bead pellet. Carefully remove and discard the ethanol.

B9.9 Repeat **Steps B9.7 – B9.8**, for a total of two washes.

OPTIONAL: Tubes can be briefly spun to pull down excess ethanol prior to removing with a p10 pipette.

B9.10 Allow remaining ethanol to evaporate by allowing the pellets to air dry for 3 – 5 min.

NOTE: Beads have been sufficiently dried when cracks appear in the surface of the bead pellet and no liquid is visible in the tube. Residual trace ethanol in the library amplification reaction may decrease performance.

B9.11 Remove sample tubes from the magnet and carefully resuspend each bead pellet in 22 μL of 10 mM Tris-HCl, pH 8.0.

B9.12 Incubate tubes at room temperature for at least 2 min before placing back on the magnet.

B9.13 Leave tubes on the magnet for at least 2 min, or until all beads have collected on the tube wall and the solution is clear.

B9.14 Carefully transfer 20 μL of each library-containing supernatant to a new, labeled tube.



Safe stopping point. Samples can be stored at 4°C for <24 hours or frozen at -20°C for up to 4 weeks.

B10. 2nd Post-ligation Cleanup (Optional)

NOTE: This is an optional step and only recommended when working with:

- Full-length adapters
- Stubby adapters and <50 ng of RNA

B10.1 Freshly prepare at least 0.4 mL of an 80% ethanol solution for each reaction.

B10.2 Vortex **room temperature** SPRI beads to thoroughly mix. Add 20 µL (1X) of room temperature resuspended beads to each eluted library from **Step B9.14**.

B10.3 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

B10.4 Incubate the library-bead mixtures at room temperature for at least 5 min to maximize library recovery.

B10.5 Place sample tubes on a magnet for at least 5 min, or until all beads have been collected on the tube wall and the solution is clear.

B10.6 Carefully remove and discard the supernatant from each tube.

B10.7 Add 200 µL of freshly prepared 80% ethanol to each tube, taking care to not disturb the bead pellet on the tube wall.

B10.8 Incubate tubes at room temperature for at least 30 sec without removing the tube from the magnet or disturbing the bead pellet. Carefully remove and discard the ethanol.

B10.9 Repeat **Steps B10.7 – B10.8**, for a total of two washes.

OPTIONAL: Tubes can be briefly spun to pull down excess ethanol prior to removing with a p10 pipette.

B10.10 Allow remaining ethanol to evaporate by allowing the pellets to air dry for 3 – 5 min.

NOTE: Beads have been sufficiently dried when cracks appear in the surface of the bead pellet and no liquid is visible in the tube. Residual trace ethanol in the library amplification reaction may decrease performance.

B10.11 Remove sample tubes from the magnet and carefully resuspend each bead pellet in 22 µL of 10 mM Tris-HCl, pH 8.0.

B10.12 Incubate tubes at room temperature for at least 2 min before placing back on the magnet.

B10.13 Leave tubes on the magnet for at least 2 min, or until all beads have collected on the tube wall and the solution is clear.

B10.14 Carefully transfer 20 µL of each library-containing supernatant to a new, labeled tube.



Safe stopping point. Samples can be stored at 4°C for <24 hours or frozen at -20°C for up to 4 weeks.

B11. Library Amplification and Strand Selection

- Library amplification is required for strand-specific sequencing regardless of adapter configuration used.
- If your workflow requires library amplification in the presence of paramagnetic beads, refer to **Appendix A** for bead compatibility.

B11.1 Thaw and equilibrate the Equinox Amplification Master Mix (2X) on ice. Once thawed, vortex for 5 sec to mix.

B11.2 Program a thermocycler as indicated below:

Step ¹	Temperature	Time	Cycles
Lid temperature	105°C	N/A	N/A
Initial denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	
Annealing	P5/P7 primers: 60°C ² Indexed primers: 55°C ³	30 sec	See Table B3
Extension	72°C	30 – 45 sec	
Final extension	72°C	60 sec	1
–	12°C	Hold	–

¹For additional details on optimizing amplification, see **Prior to Starting**.

²Appropriate temperature for P5/P7 Primer Mix (10X).

³For indexes used with the truncated adapter scheme detailed in Glenn, et. al. 2019, use 55°C unless using IDT™ xGen Stubby-Adapter UDI primers which should be annealed at 64°C. Optimization may be required for other adapter/primer configurations (see **Prior to Starting**).

 Table B3. Recommended PCR cycle numbers by RNA input amount into library prep.

RNA input into Library Preparation (ng)	PCR cycles to generate 10 – 50 nM library ¹	
	Full-length adapters	Stubby adapters
≥500	16 – 17	11 – 14
250	17 – 18	13 – 16
100	18 – 19	15 – 18
25	20 – 21	18 – 20
≤10	21 – 23	19 – 22

¹PCR cycle numbers may vary depending on the quality of FFPE-derived RNA.

B11.3 Assemble each amplification reaction in the order specified below:

Component	Volume (μL)
Adapter-ligated library	20
P5/P7 Primer Mix (10X) or User-supplied primers ¹	5
Equinox Amplification Master Mix (2X)	25

¹See **Prior to Starting** for more information on user-supplied primers.

B11.4 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

B11.5 Place tubes in the thermocycler (programmed in **Step B11.2**) and initiate the program.

B11.6 Once the program has completed, proceed immediately to **Post-amplification Cleanup**.

B12. Post-amplification Cleanup

B12.1 Freshly prepare at least 0.4 mL of an 80% ethanol solution for each reaction.

B12.2 Vortex **room temperature** SPRI beads to thoroughly mix. Add 50 μL (1X) of beads to each amplification reaction.

B12.3 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

B12.4 Incubate the library-bead mixtures at room temperature for at least 5 min to maximize library recovery.

B12.5 Place sample tubes on a magnet for at least 5 min, or all beads have collected on the tube wall and until the solution is clear.

B12.6 Carefully remove and discard the supernatant from each tube.

B12.7 Add 200 μL of freshly prepared 80% ethanol to each tube, taking care to not disturb the bead pellet on the tube wall.

B12.8 Incubate tubes at room temperature for at least 30 sec without removing the tube from the magnet or disturbing the bead pellet. Carefully remove and discard the ethanol.

B12.9 Repeat **Steps B12.7 – B12.8** for a total of two washes.

OPTIONAL: Tubes can be briefly spun to pull down excess ethanol prior to removing with a p10 pipette.

B12.10 Allow remaining ethanol to evaporate by allowing the pellets to air dry for 3 – 5 min.

NOTE: Beads have been sufficiently dried when cracks appear in the surface of the bead pellet and no liquid is visible in the tube.

B12.11 Remove sample tubes from the magnet and carefully resuspend each bead pellet in 22 μL of 10 mM Tris-HCl, pH 8.0.

B12.12 Incubate tubes at room temperature for at least 2 min before placing back on the magnet.

B12.13 Leave sample tubes on the magnet for at least 2 min, or until all beads have collected on the tube wall and the solution is clear.

B12.14 Carefully transfer 20 μL of each library-containing supernatant to a new tube.

B12.15 At this point, libraries are ready for quantification, normalization, pooling, hybrid capture and/or sequencing.

NOTE: We recommend quantifying libraries using qPCR and analyzing quality and sizing using capillary electrophoresis prior to preparing the libraries for sequencing.

Appendix A: Amplification with Paramagnetic Purification Beads

For some library preparation workflows, it may be preferred or advantageous to perform PCR in the presence of paramagnetic purification beads. For example, in hybrid capture workflows, post-capture amplification may be performed by adding PCR mix directly to the capture beads with bound library, eliminating an elution step. Alternatively, libraries may be eluted directly from SPRI beads in the PCR reaction mixture.

We have found paramagnetic purification beads to fall into three categories of compatibility with PCR and sequencing based on their surface chemistry: Group I—fully compatible, Group II—potentially inhibitory, and Group III—incompatible. While Group II beads are inhibitory to non-optimized PCR systems, the Equinox Amplification Master Mix (2X) has been optimized to allow amplification in the presence of both Group I and Group II beads, with no observable loss in performance (e.g., efficiency, uniformity, or fidelity). Equinox Amplification Master Mix (2X) and other library amplification systems are not compatible with the extreme inhibition characterized by Group III beads. Table 5 details the various paramagnetic bead types evaluated.

Table 5. Paramagnetic purification bead types

Bead Type	Vendor	Catalog Number	Compatibility/amount tested ¹
Group I (Tosyl-activated beads)			
Dynabeads™ M280 Streptavidin	Thermo Fisher	11205D	500 µg
Dynabeads MyOne™ Streptavidin T1	Thermo Fisher	65601	500 µg
Group II (Carboxylic acid-activated beads)			
Dynabeads M270 Streptavidin ²	Thermo Fisher	65305	500 µg
SPRI	Various, incl. Beckman Coulter	A63882	100 µL
Dynabeads MyOne Streptavidin C1	Thermo Fisher	65001	500 µg
Group III (Not compatible with PCR)			
Dynabeads M270 Carboxylic Acid	Thermo Fisher	14305D	500 µg

¹Volume of slurry or mass of beads per 50 µL amplification reaction.

²Used in xGen Hybridization and Wash Kit (Integrated DNA Technologies). Appendix A: Amplification with Paramagnetic Purification Beads

Appendix B: Recommendations for Generating Insert Lengths >260 bp

While there are different strategies for preparing RNA libraries, longer insert sizes may be preferred for sequencing economy, better detection of structural variants, and more accurate representation of complex regions of the genome.

Here, we describe optimized conditions for generation of longer insert sizes as assessed by capillary electrophoresis (mode library sizes: 400 – 450 bp), for both high-quality RNA and FFPE RNA. For any further support required on fragment length optimization, please contact support@watchmakergenomics.com.

High-quality RNA

For high-quality RNA we recommend a shorter fragmentation time coupled with a more stringent post-amplification SPRI cleanup ratio. An additional amplification cycle is utilized to account for a potential loss in yield due to the adjusted SPRI cleanup.

Follow **Library Construction Protocol A** (pg. 6), with the following modifications:

- A4.5 Refer to Table 6 below for optimized fragmentation conditions.

Table 6: Optimized fragmentation conditions

Step	Temperature	Time
Lid temperature	105°C	N/A
Pre-cooling	4°C	HOLD
RNA fragmentation	80°C	1 min
HOLD (Priming)	12°C	HOLD

- A10.2 (Table A4): For library amplification, increase the number of cycles by one additional cycle.
- A11.2 Adjust the post-amplification SPRI cleanup ratio to 0.7X (standard is 1X).

FFPE RNA

For FFPE RNA we recommend maintaining the fragmentation conditions as described in **Step B5.1**, with an additional amplification cycle to account for a potential loss in yield with a more stringent post-amplification SPRI cleanup ratio.

Follow **Library Construction Protocol B** (pg. 13), with the following modifications:

- B11.2 (Table B3): For library amplification, increase the number of cycles by one additional cycle.
- B12.2 Adjust the post-amplification SPRI cleanup ratio to 0.8X (standard is 1X).

Revision History

Version	Description	Date
4.0	<ul style="list-style-type: none"> Included ordering information under Kit Contents for Bundled Kit option Workflow clarification to Step A5.4 Footnote 3 of amplification program tables (A7.2 and B8.2) modified to include specific annealing temperature recommendations for IDT xGen™ Stubby Adapter-UDI primers. 	06/2024
4.1	<ul style="list-style-type: none"> Pg 9 and Pg 17: Steps A7.8 and B8.8 re-written for clarification purposes, respectively Pg 11 and Pg 18: Lid temperature in thermocycler post-ligation amplification program added in Steps A10.2 and B11.2. Trademark updated 	10/2024
4.2	<ul style="list-style-type: none"> Addition of 384 rxn pack sizes and volumes in Kit Contents Steps A10.1 and B11.1: Correction to vortex Equinox Amplification Master Mix (2X) 	12/2024



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