Watchmaker RNA Library Prep Reagents

Product Description

The Watchmaker RNA Library Prep Reagents are designed for the highly streamlined preparation of stranded RNA sequencing libraries from 0.25 ng to 100 ng total or previously enriched RNA with high library complexity and low coverage bias. The workflow is compatible with both high- and low-quality samples, including FFPE material.

The Watchmaker RNA Library Prep Reagents convert 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

Table of Contents

Kit Contents

Kit	Kit Code	Description	Component Volume	
			24 rxn	96 rxn
Watchmaker RNA Library Prep Reagents	**9K0078-024** (24 rxn) **9K0078-096** (96 rxn)	FFPE Treatment Buffer	140 µL	600 µL
		Frag & Prime Buffer	290 µL	1.3 mL
		1st Strand Buffer	240 µL	1.08 mL
		1st Strand Enzyme	30 µL	120 µL
		2nd Strand Buffer	370 µL	1.68 mL
		2nd Strand Enzyme	30 µL	120 µL
		Ligation Buffer	1.06 mL	4.8 mL
		Ligation Enzyme	140 µL	600 µL
		Equinox Amplification Master Mix (2X)	690 µL	3 mL
		P5/P7 Primer Mix (10X)	144 µL	600 µL

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

Product Applications

This workflow was developed to address the highly specific needs of RNA sequencing 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 Watchmaker RNA Library Prep Reagents are ideally suited for:

- Targeted sequencing protocols employing hybridization capture
- Whole transcriptome sequencing, where both protein-coding on noncoding transcripts are interrogated, from both high-quality and degraded samples
- mRNA sequencing, when using previously enriched poly(A)-tailed RNA
- Gene expression analysis
- Isoform/splice variant/gene fusion identification
- Single nucleotide variant detection
- Novel transcript discovery
- Workflows that employ unique molecular identifiers (UMIs) for improved sensitivity

Storage and Handling

The Watchmaker RNA Library Prep Reagents are shipped on cold packs. Upon receipt, store all components at -20 ± 5°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 sunlight while thawing and in use.

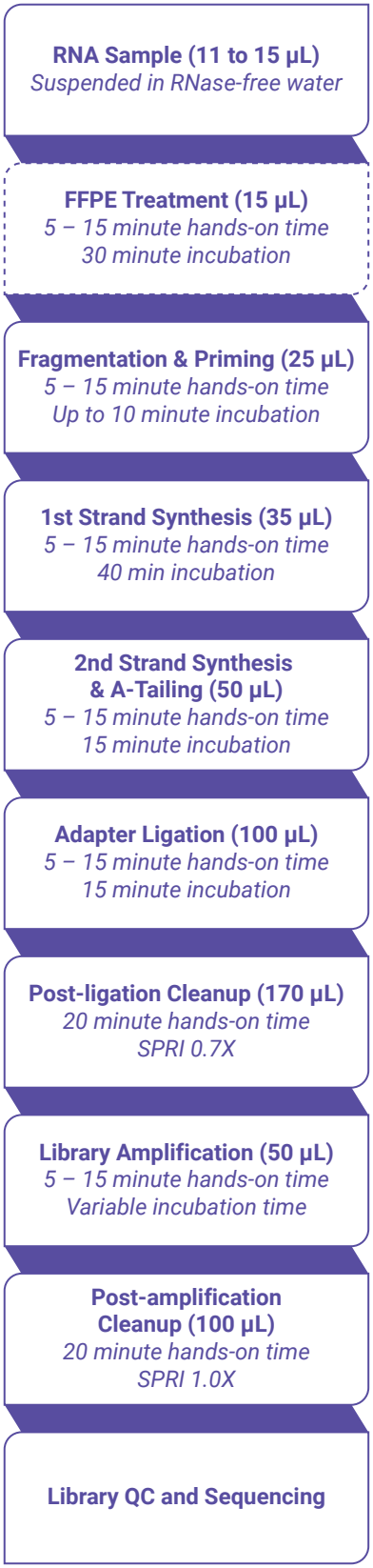
All master mixes (buffer and enzyme combined) prepared in the protocol should be stored at 4˚C unless stated otherwise. The master mixes should be stable for up to 24 hours at 4˚C.

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
- 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 those derived from FFPE.

- Intact and partially degraded samples should be suspended in 15 µL of RNase-free water.
- FFPE samples should be suspended in 11 µL of RNase-free water.

  NOTE: If you are working with FFPE samples, use Protocol B on page 12.
- RNA should be accurately quantified by Qubit® Fluorometer or similar prior to starting.

High-quality total RNA ranging from 0.25 – 100 ng has been tested and shown to produce high-performing libraries.

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 100 ng. Additionally, some extraction protocols fail to adequately de-crosslink FFPE-derived RNA. Our de-crosslinking step may enhance library quality in these instances. If you are working with FFPE samples, use **Protocol B** on page 12.

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 that may be carried over from extraction. If the total RNA contains DNA, remove the contamination by incubating with DNase I (not supplied with the 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., Mg^{2+}, 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.

Adapters

This kit is compatible with adapters that have a 3' overhanging T to facilitate adapter orientation during dscDNA ligation. Note that adapter quality impacts overall library preparation efficiency. Ensure that adapters are adequately duplexed and at the appropriate concentration prior to use.

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: CAAGCAGAAGACGGCATACGAGAT

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. 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 via vortexing prior to use.

[1]Glenn 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 12) 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.
- 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 in the tube lids prior to opening a tube.
- We recommend making master mixes for each reaction step 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. Fragmentation and Priming

A1.1 On ice, prepare input RNA in a total volume of 15 µL using RNase-free water and add to labeled 0.2 mL PCR tubes or PCR plate.

A1.2 To each tube, add the Frag & Prime Buffer as specified below on ice:

Component	Volume (uL)
RNA	15
Frag & Prime Buffer	10

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 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]	Fragmentation Condition
Intact	>7	230 – 250 bp	80°C for 10 min
		220 – 240 bp	85°C for 10 min
		210 – 230 bp	90°C for 10 min
		175 – 185 bp	95°C for 10 min
Partially degraded[3]	2 – 7	100 – 300 bp	85°C for 2 – 5 min
Degraded, non-FFPE	1 – 2	Dictated by RNA quality	65ºC for 1 min

[1]As assessed by NGS – Picard mean insert size.

[2]Assumes a 0.7X Post-ligation Cleanup ratio.

[3]Lower fragmentation times and temperatures may facilitate longer inserts for partially degraded samples.

A1.5 Place the tubes into the chilled thermocycler. Advance the thermocycler from the initial 4°C hold to start the RNA Fragmentation incubation.

A1.6 After the program has finished and the samples have returned to 12°C, proceed immediately to **1st Strand Synthesis.**

A2. 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 sunlight while thawing and in use.

A2.1 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

A2.2 For each reaction, prepare the 1st Strand Master Mix as follows on ice:

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

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 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

A2.5 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

A2.6 Place reactions in the chilled thermocycler (programmed and initiated in **Step A2.1**). Advance the thermocycler from the initial 4°C hold to start the 25°C incubation.

A2.7 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**.

A3. 2nd Strand Synthesis and A-Tailing

A3.1 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

A3.2 For each reaction, prepare the 2nd Strand Master Mix as follows on ice:

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

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 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

A3.5 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

A3.6 Place reactions in the chilled thermocycler (programmed and initiated in **Step A3.1**). Advance the thermocycler from the initial 4°C hold to start the 42°C incubation.

A3.7 Proceed to adapter ligation after the program has finished and the samples have returned to 4°C. Proceed immediately to **Adapter Ligation**.

A4. Adapter Ligation

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

A4.1 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]

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

A4.2 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 and placing it on ice.

A4.3 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)
<0.25[2]	0.1
0.25 – 42	0.25
5 – 29	1
30 – 74	2.5
≥75	7.5

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

[2]A second post-ligation cleanup (**Step A6**) is recommended for inputs under 1 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)
<0.25	0.25
0.25 – 1	0.5
1 – 4	2
5 – 29	4
30 – 74	10
≥75	15

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

A4.4 Add 5 µL of appropriately diluted adapter to each tube.

A4.5 Prepare the Ligation Master Mix as follows:

Component	Volume (µL)
Ligation Buffer	40
Ligation Enzyme	5

A4.6 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

A4.7 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

A4.8 The Ligation Master Mix is viscous. Carefully pipette a minimum volume of 80 µL up and down a minimum of ten times. Briefly centrifuge if needed to collect all liquid in the bottom of the tube.

A4.9 Place the sample tubes in the thermocycler and initiate the program (programmed in **Step A4.1**).

A4.10 Once the program has completed, proceed immediately to **Post-ligation Cleanup**.

A5. Post-ligation Cleanup

A5.1 Freshly prepare at least 0.4 mL of an 80% ethanol solution for each reaction.

A5.2 Vortex **room temperature** SPRI beads to thoroughly mix. Add 70 µL (0.7X) of room temperature resuspended beads to each ligation reaction.

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

A5.5 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.

A5.6 Carefully remove and discard the supernatant from each tube.

A5.7 Add 200 µL of freshly prepared 80% ethanol to each tube, taking care to not disturb the bead pellet on the tube wall.

A5.8 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.

A5.9 Repeat **Steps A5.7 – A5.8**, for a total of two washes.

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

A5.10 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.

A5.11 Remove sample tubes from the magnet and carefully resuspend each bead pellet in 22 µL of 10 mM Tris-Cl pH 8.0.

A5.12 Incubate tubes at room temperature for at least 2 min before placing back on the magnet.

A5.13 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.

A5.14 Carefully transfer 20 µL of each library-containing supernatant to a new, labeled tube.

Safe stopping point. Samples can be frozen at -20˚C for up to 4 weeks.

A6. 2nd Post-ligation Cleanup (Optional)

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

- Full-length adapters and less than 1 ng of RNA
- Full-length adapters and partially degraded samples as an alternative to titrating adapter concentration in the ligation reaction

A6.1 Freshly prepare at least 0.4 mL of an 80% ethanol solution for each reaction.

A6.2 Vortex room temperature SPRI beads to thoroughly mix. Add 20 µL (1X) of room temperature resuspended beads to each eluted library from **Step A5.14**.

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

A6.5 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.

A6.6 Carefully remove and discard the supernatant from each tube.

A6.7 Add 200 µL of freshly prepared 80% ethanol to each tube, taking care to not disturb the bead pellet on the tube wall.

A6.8 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.

A6.9 Repeat **Steps A6.7 – A6.8**, for a total of two washes.

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

A6.10 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.

A6.11 Remove sample tubes from the magnet and carefully resuspend each bead pellet in 22 µL of 10 mM Tris-Cl pH 8.0.

A6.12 Incubate tubes at room temperature for at least 2 min before placing back on the magnet.

A6.13 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.

A6.14 Carefully transfer 20 µL of each library-containing supernatant to a new, labeled tube.

Safe stopping point. Samples can be frozen at -20˚C for up to 4 weeks.

A7. 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.
- If your application requires high amplified library yields (e.g., hybridization capture workflows), refer to **Appendix B** for PCR cycle number recommendations.

A7.1 Thaw and equilibrate the Equinox Amplification Master Mix (2X) on ice. Once thawed, invert several times or swirl vigorously to mix (**DO NOT VORTEX**).

A7.2 Program a thermocycler as indicated below:

Step[1]	Temperature	Time	Cycles
Initial denaturation	98ºC	45 sec	1
Denaturation	98ºC	15 sec	See Table A4
Annealing	P5/P7 primers: 60ºC[2] Indexed primers: 55ºC[3]	30 sec	
Extension	72ºC	30 – 45 sec	
Final extension	72ºC	60 sec	1
–	12ºC	Hold	–

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

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

[3]For the truncated adapter scheme detailed in Glenn et. al. 2019, use 55˚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
100	8	7[1]
50	8 – 9	
25	9 – 10	8
10	10 – 11	8 – 9
5	11 – 12	9 – 10
1	14 – 15	10 – 11
<1	18 – 19	14 – 15

[1]A minimum of 6 amplification cycles is recommended to ensure transcript strand origin information is maintained.

A7.3 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

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

A7.4 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

A7.5 Place tubes in the thermocycler (programmed in **Step A7.2**) and initiate the program.

A7.6 Once the program has completed, proceed immediately to **Post-amplification Cleanup**.

A8. Post-amplification 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 50 µL (1X) of beads to each amplification 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 all beads have collected on the tube wall and until the solution is clear.

A8.6 Carefully remove and discard the supernatant from each tube.

A8.7 Add 200 µL 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.

A8.11 Remove sample tubes from the magnet and carefully resuspend each bead pellet in 22 µL of 10 mM Tris pH 8.0.

A8.12 Incubate tubes at room temperature for at least 2 min before placing back on the magnet.

A8.13 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.

A8.14 Carefully transfer 20 µL of each library-containing supernatant to a new tube.

A8.15 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.
- 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 in the tube lids prior to opening a tube.
- We recommend making master mixes for each reaction step 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. FFPE Treatment

B1.1 On ice, dilute input FFPE-derived RNA to 11 µL using RNase-free water and add 4 µL of FFPE Treatment Buffer.

B1.2 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

B1.3 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

B1.4 Place tubes in the thermocycler and initiate the run.

B1.5 After the program completes, remove tubes from the thermocycler, and add the Frag & Prime Buffer as specified below on ice:

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

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 Proceed immediately to **Fragmentation and Priming**.

B2. Fragmentation and Priming

B2.1 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	65°C	1 min
HOLD (Priming)	12°C	HOLD

B2.2 Place the tubes into the chilled thermocycler. Advance the thermocycler from the initial 4°C hold to start the RNA Fragmentation incubation.

B2.3 Proceed immediately to **1st Strand Synthesis**.

B3. 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 sunlight while thawing and in use.

B3.1 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

B3.2 For each reaction, prepare the 1st Strand Master Mix as follows on ice:

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

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 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

B3.5 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

B3.6 Place reactions in the chilled thermocycler (programmed and initiated in **Step B3.1**). Advance the thermocycler from the initial 4°C hold to start the 25°C incubation.

B3.7 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**.

B4. 2nd Strand Synthesis & A-Tailing

B4.1 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

B4.2 For each reaction, prepare the 2nd Strand Master Mix as follows on ice:

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

B4.3 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

B4.4 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

B4.5 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

B4.6 Place reactions in the chilled thermocycler (programmed and initiated in **Step B4.1**). Advance the thermocycler from the initial 4°C hold to start the 42°C incubation.

B4.7 Proceed to adapter ligation after the program has finished and the samples have returned to 4°C. Proceed immediately to **Adapter Ligation**.

B5. Adapter Ligation

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

B5.1 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]

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

B5.2 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 and placing it on ice.

B5.3 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)	Adapter concentration (µM)
<1[1]	0.05
1 – 4[1]	0.1
5 – 9[1]	0.25
≥10	0.5

[1]A second post-ligation cleanup (**Step B7**) is recommended for inputs 5 ng and less when using full-length adapters.

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

RNA input (ng)	Adapter concentration (µM)
<2[2]	0.35
2 – 4	0.5
5 – 9	1
≥10	2

[2]A second post-ligation cleanup (**Step B7**) is recommended for inputs 2 ng and less when using stubby adapters.

B5.4 Add 5 µL of appropriately diluted adapter to each tube.

B5.5 Prepare the Ligation Master Mix as follows:

Component	Volume (µL)
Ligation Buffer	40
Ligation Enzyme	5

B5.6 Mix on a touch vortexer (or 3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

B5.7 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

B5.8 The Ligation Master Mix is viscous. Carefully pipette a minimum volume of 80 µL up and down a minimum of ten times. Briefly centrifuge if needed to collect all liquid in the bottom of the tube.

B5.9 Place the sample tubes in the thermocycler and initiate the program (programmed in **Step B5.1**).

B5.10 Once the program has completed, proceed immediately to **Post-ligation Cleanup**.

B6. Post-ligation Cleanup

B6.1 Freshly prepare at least 0.4 mL of an 80% ethanol solution for each reaction.

B6.2 Vortex **room temperature** SPRI beads to thoroughly mix. Add 70 µL (0.7X) of room temperature resuspended beads to each ligation reaction.

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

B6.5 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.

B6.6 Carefully remove and discard the supernatant from each tube.

B6.7 Add 200 µL of freshly prepared 80% ethanol to each tube, taking care to not disturb the bead pellet on the tube wall.

B6.8 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.

B6.9 Repeat **Steps B6.7 – B6.8**, for a total of two washes.

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

B6.10 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.

B6.11 Remove sample tubes from the magnet and carefully resuspend each bead pellet in 22 µL of 10 mM Tris-Cl pH 8.0.

B6.12 Incubate tubes at room temperature for at least 2 min before placing back on the magnet.

B6.13 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.

B6.14 Carefully transfer 20 µL of each library-containing supernatant to a new, labeled tube.

Safe stopping point. Samples can be frozen at -20˚C for up to 4 weeks.

B7. 2nd Post-ligation Cleanup (Optional)

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

- Full-length adapters and 5 ng or less of RNA
- Stubby adapters and less than 2 ng of RNA

B7.1 Freshly prepare at least 0.4 mL of an 80% ethanol solution for each reaction.

B7.2 Vortex **room temperature** SPRI beads to thoroughly mix. Add 20 µL (1X) of room temperature resuspended beads to each eluted library from **Step B6.14**.

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

B7.5 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.

B7.6 Carefully remove and discard the supernatant from each tube.

B7.7 Add 200 µL of freshly prepared 80% ethanol to each tube, taking care to not disturb the bead pellet on the tube wall.

B7.8 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.

B7.9 Repeat **Steps B7.7 – B7.8**, for a total of two washes.

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

B7.10 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.

B7.11 Remove sample tubes from the magnet and carefully resuspend each bead pellet in 22 µL of 10 mM Tris-Cl pH 8.0.

B7.12 Incubate tubes at room temperature for at least 2 min before placing back on the magnet.

B7.13 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.

B7.14 Carefully transfer 20 µL of each library-containing supernatant to a new, labeled tube.

Safe stopping point. Samples can be frozen at -20˚C for up to 4 weeks.

B8. 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.
- If your application requires high amplified library yields (e.g., hybridization capture workflows), refer to **Appendix B** for PCR cycle number recommendations.

B8.1 Thaw and equilibrate the Equinox Amplification Master Mix (2X) on ice. Once thawed, invert several times or swirl vigorously to mix (**DO NOT VORTEX**).

B8.2 Program a thermocycler as indicated below:

Step[1]	Temperature	Time	Cycles
Initial denaturation	98ºC	45 sec	1
Denaturation	98ºC	15 sec	See **Table B3**
Annealing	P5/P7 primers: 60ºC[2] Indexed primers: 55ºC[3]	30 sec	
Extension	72ºC	30 – 45 sec	
Final extension	72ºC	60 sec	1
–	12ºC	Hold	–

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

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

[3]For the truncated adapter scheme detailed in Glenn et. al. 2019, use 55˚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	
	Full-length adapters	Stubby adapters
100	9 – 10	8 – 9
50	11 – 12	9 – 10
25	13 – 14	11 – 12
10	14 – 15	12 – 13
5	20 – 21	16 – 17
1	25 – 26	20 – 21
<1	27 – 28	22 – 23

B8.3 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

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

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

B8.5 Place tubes in the thermocycler (programmed in **Step B8.2**) and initiate the program.

B8.6 Once the program has completed, proceed immediately to **Post-amplification Cleanup**.

B9. Post-amplification 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 50 µL (1X) of beads to each amplification 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 all beads have collected on the tube wall and until 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.

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

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

B9.13 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.

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

B9.15 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 A details the various paramagnetic bead types evaluated.

Table A. 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

[1]Volume of slurry or mass of beads per 50 µL amplification reaction.
[2]Used in xGen Hybridization and Wash Kit (Integrated DNA Technologies).

Appendix B: Amplifying Libraries for High-yield Applications

Some applications require high amplified library yields, such as those leveraging hybridization capture downstream of library preparation. The Equinox Library Amplification Master Mix (2X) is optimized for ultra-high-fidelity and efficient amplification for such applications. Tables B and C provide PCR cycle number recommendations based on adapter design and RNA input into library preparation for both intact and FFPE-derived RNA.

Table B. Intact RNA: Recommended PCR cycle numbers by RNA input amount into library prep

RNA input into Library Preparation (ng)	PCR cycles to generate 1 µg library	
	Full-length adapters	Stubby adapters
100	10	9
50	10 – 11	9
25	11 – 12	10
10	12 – 13	10 – 11

Table C. FFPE-derived RNA: Recommended PCR cycle numbers by RNA input amount into library prep

RNA input into Library Preparation (ng)	PCR cycles to generate 1 µg library	
	Full-length adapters	Stubby adapters
100	11 – 12	10 – 11
50	13 – 14	11 – 12
25	15 – 16	13 – 14
10	16 – 17	14 – 15

For Technical Support, please contact the **Scientific Support Team** at support@watchmakergenomics.com.

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