Watchmaker mRNA Library Prep Kit

Table of Contents

Product Description

The Watchmaker mRNA Library Prep Kit is designed for the highly streamlined preparation of stranded mRNA sequencing libraries from 2.5 ng to 1000 ng intact total eukaryotic RNA with high library complexity and low coverage bias.

The Watchmaker mRNA Library Prep Kit enriches for mature, polyadenylated mRNAs and converts those mRNAs to adapter-ligated, double-stranded cDNA fragments through a small number of chemical and enzymatic manipulations:

- Highly specific capture of full-length polyadenylated (poly(A)) RNA transcripts such as eukaryotic mRNA and some non-coding RNAs
- 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

Kit Contents

Bundled kit	Kit	Kit code	Description	Component volume		
				24 rxn	96 rxn	384 rxn
Watchmaker mRNA Library Prep Kit **7BK0001-024** (24 rxn) **7BK0001-096** (96 rxn) **7BK0001-384** (384 rxn)	Watchmaker mRNA Capture Kit	**7K0105-024** (24 rxn) **7K0105-096** (96 rxn) **7K0105-384** (384 rxn)	mRNA Capture Beads	270 µL	1.2 mL	4.80 mL
			Bead Prep Buffer	2.8 mL	12 mL	46 mL
			Capture Buffer	9.5 mL	37 mL	161 mL
			Final Wash Buffer	6.8 mL	27 mL	115 mL
	Watchmaker RNA Library Prep Kit	**7K0078-024** (24 rxn) **7K0078-096** (96 rxn) **7K0078-384** (384 rxn)	Frag & Prime Buffer	290 µL	1.3 mL	5 mL
			1st Strand Buffer	240 µL	1.08 mL	4.14 mL
			1st Strand Enzyme	30 µL	120 µL	0.46 mL
			2nd Strand Buffer	370 µL	1.68 mL	6.40 mL
			2nd Strand Enzyme	30 µL	120 µL	0.46 mL
			Ligation Buffer	1.06 mL	4.8 mL	18.40 mL
			Ligation Enzyme	140 µL	600 µL	2.40 mL
			Equinox Amplification Master Mix (2X)	690 µL	3 mL	12 mL
			P5/P7 Primer Mix (10X)	144 µL	600 µL	2.40 mL
			FFPE Treatment Buffer[1]	*140 µL*	*600 µL*	*2.40 mL*

[1]The FFPE Treatment Buffer is not required or recommended for mRNA library preparation. When used on its own or in conjunction with the Polaris™ Depletion Kit, the Watchmaker RNA Library Prep Kit is compatible with the FFPE Treatment Buffer.

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

Product Applications

This workflow was developed to address the highly specific needs of mRNA 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.

This library preparation kit is ideally suited for:

- 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 mRNA Library Prep Kits are shipped on cold packs. **Upon receipt, store the Watchmaker mRNA Capture Kit (K0105) at 4°C and the Watchmaker RNA Library Prep Kit (K0078) at -20 ± 5°C.**

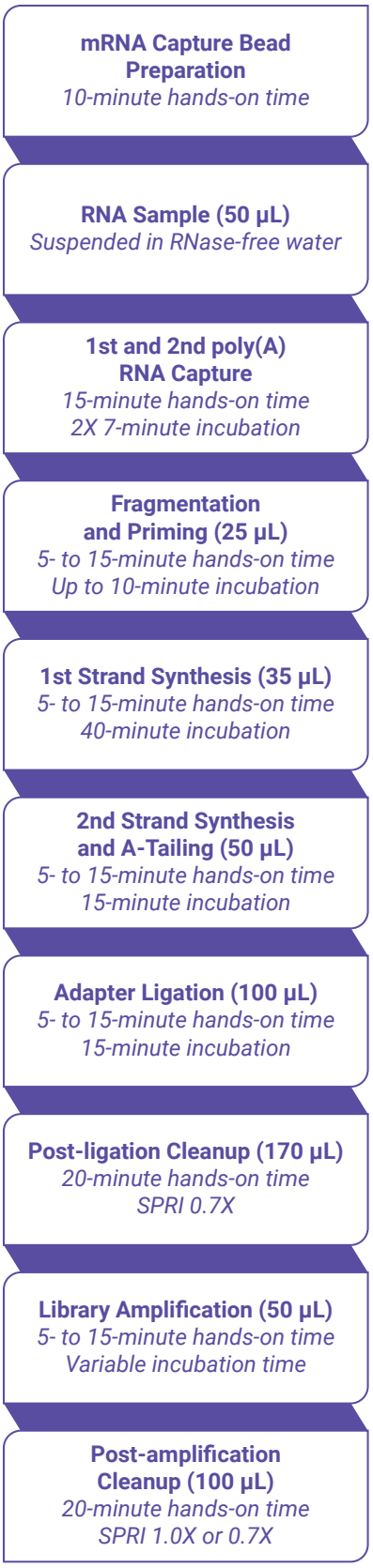
Some components are viscous; therefore, take care to homogenize solutions thoroughly before use and during reaction setup. SPRI beads should be handled as per the manufacturer's guidelines.

Buffers in the Watchmaker mRNA Capture Kit should be mixed by inverting the tubes and NOT by vortexing to prevent foaming. All buffers in the Watchmaker RNA Library Prep Kit 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.

Workflow Overview



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
- Magnetic rack compatible with 1.5 – 2 mL microtubes
- 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 microtubes (RNase-free)
- RNase-free water
- 10 mM Tris-HCl, pH 8.0
- Thermocycler
- Vortex mixer and/or plate vortexer
- Plate centrifuge and/or benchtop picofuge
- Fragment Analyzer™, Bioanalyzer®, TapeStation (Agilent Technologies, Inc.), or similar instrument and consumables

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

Prior to Starting

Input RNA Quality and Quantity

This kit is compatible with intact (RIN >7) total RNA samples suspended in 50 µL of RNase-free water.

High-quality total RNA ranging from 2.5 – 1000 ng has been tested and shown to produce high-performing libraries.

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

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

ERCC RNA Spike-In Controls are not recommended for use with the Watchmaker mRNA Library Prep Kit due to their short poly(A) tails (20 – 26 nt long), which do not imitate typical polyadenylated RNA transcripts. If ERCC RNA Spike-In Controls are included, a lower number of reads mapping to ERCCs is expected due to lower efficiency hybridization to the oligo dT.

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., 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.

Insert Size

The standard protocol targets ~200 bp inserts.[1] When targeting ~300 bp inserts, add 2 extra cycles of PCR amplification (**Step 10**) and make use of a 0.7X post-amplification SPRI cleanup (**Step 11.2**).

Mixing Considerations

The Watchmaker mRNA Library Prep Kit has been developed to provide robust and reproducible data when employing either vortex or pipette mixing.

[1]As assessed by Illumina NovaSeq (Picard mean insert size)

It is recommended to make use of vortexing (2,000 rpm on an appropriate vortex mixer) where possible as it provides uniform and reproducible mixing of samples. Tubes and/or plates should be sealed prior to vortexing and briefly centrifuged after vortexing. Extreme care should be taken to ensure that no residual droplets remain on the side or at the top of tubes and/or plates after centrifuging.

For automation, or if access to an appropriate vortex mixer is not available, use pipette mixing. Samples should be pipetted carefully to minimize foaming and tubes/plates briefly centrifuged after mixing.

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.

Contact **support@watchmakergenomics.com** for more details and our Adapter Recommendations Guide.

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,[2] 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 and insert size. 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.

[2]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

Recommendations

- All mRNA Capture components should be equilibrated to room temperature for 20 minutes and inverted several times before use.

  NOTE: Do NOT vortex Watchmaker mRNA Capture Kit buffers.

- Ensure all Watchmaker RNA Library Prep Kit 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.
- The Fragmentation & Priming Master mix (**Step 4.1**) may be prepared ahead of time and stored on ice before use.
- Place enzymes and the Equinox Amplification Master Mix (2X) on ice before use. Invert the tubes 10 times to mix.
- Vortexing is recommended for reaction mixing throughout. Where possible, centrifuge briefly to remove any excess liquid from the tubes and collect all liquid in the tube lids prior to opening them—see **Prior to Starting: Mixing Considerations**.
- We recommend making master mixes for each enzymatic 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.

1. RNA and mRNA Capture Bead Preparation

1.1 After thawing on ice, dilute the total RNA sample to a final volume of 50 µL with RNase-free water.

1.2 Vortex mRNA Capture Beads until all beads are well resuspended (5 – 10 secs).

1.3 Prepare the mRNA Capture Beads in bulk by aliquoting 10.5 µL of beads (10 µL + 5% excess) per reaction into a microtube.

NOTE: A recommended maximum of 28 or 36 reactions can be prepared in a 1.5 mL and 2 mL tube, respectively. Multiple tubes, or larger microtubes, may be required depending on the number of reactions.

1.4 Place the tube on the magnetic rack at room temperature for 2 min or until the solution is clear.

1.5 Remove and discard all of the storage buffer from the tube. Take care not to disturb the beads.

1.6 Remove the tube from the magnetic rack and keep at room temperature.

1.7 Wash the beads by adding 52.5 µL of Bead Prep Buffer per reaction to the bulk beads from **Step 1.6**. Pipette mix at the highest feasible volume ≥10 times to fully resuspend the beads. Take care to avoid foaming.

1.8 Place the tube on the magnetic rack at room temperature for 2 min or until the solution is clear.

1.9 Remove and discard all of the Bead Prep Buffer from the tube. Take care not to disturb the beads.

1.10 Remove the tube from the magnetic rack and keep at room temperature.

1.11 Add 52.5 µL of Bead Prep Buffer per reaction to the bulk beads from **Step 1.10**. Pipette at the highest feasible volume ≥10 times to fully resuspend the beads. Take care to avoid foaming.

1.12 Store the washed mRNA Capture Beads at room temperature and proceed to **1st Poly(A) RNA Capture**.

NOTE: Washed beads from **Step 1.11** can be stored in the Bead Prep Buffer for up to 1 week at 4°C. Ensure beads are fully resuspended after storage before continuing.

2. 1st Poly(A) RNA Capture

2.1 Program a thermocycler as indicated below and initiate the run to heat the block:

NOTE: This thermocycler program is used again in **Step 3.1**.

Step	Temperature	Time
Lid temperature	80°C	N/A
HOLD	25°C	HOLD
Denaturation	75°C	2 min
Hybridization	25°C	5 min
HOLD	25°C	HOLD

2.2 To each tube, add the following:

Component	Volume (µL)
RNA sample	50
Washed mRNA Capture Beads (from **Step 1.12**)	50

2.3 Mix on an appropriate vortex mixer (2,000 rpm) for 5 sec. Briefly centrifuge to collect all liquid in the bottom of the tubes.

WORKFLOW: If pipette mixing, mix the reaction 10 times with a pipette set to 80 µL. Briefly centrifuge if possible. See **Prior to Starting: Mixing Considerations**.

2.4 Place the tubes into the preheated thermocycler (programmed and initiated in **Step 2.1**). Advance the thermocycler from the initial 25°C hold.

2.5 Upon completion of thermocycling, place the tubes on the magnetic rack at room temperature for 2 min, or until the beads have collected on the side of the tubes.

2.6 While on the magnet, remove and discard all of the supernatant from each tube. Avoid disturbing the beads.

2.7 While on the magnet, and without disturbing the beads, gently add 180 µL Capture Buffer to the tubes. If using a 0.3 mL tube, then use 250 µL to fill tube capacity. **This is to remove any remaining uncaptured RNA adsorbed to the tubes.**

2.8 While still on the magnet, remove and discard all of the supernatant from each tube. Avoid disturbing the beads.

2.9 Remove the tubes from the magnetic rack and add 100 µL of Capture Buffer to each tube.

2.10 Mix on an appropriate vortex mixer (2,000 rpm) for 5 sec to resuspend the beads. Briefly centrifuge to collect all liquid in the bottom of the tubes.

WORKFLOW: If pipette mixing, mix the reaction 10 times with a pipette set to 80 µL to fully resuspend the beads in the Capture Buffer and to avoid excessive foaming. Briefly centrifuge if possible. See **Prior to Starting: Mixing Considerations**.

2.11 Proceed immediately to **2nd Poly(A) RNA Capture**.

3. 2nd Poly(A) RNA Capture

3.1 Program a thermocycler as indicated below and initiate the run to heat the block:

Step	Temperature	Time
Lid temperature	80°C	N/A
HOLD	25°C	HOLD
Denaturation	75°C	2 min
Hybridization	25°C	5 min
HOLD	25°C	HOLD

3.2 Place the tubes into the preheated thermocycler (programmed and initiated in **Step 3.1**). Advance the thermocycler from the initial 25°C hold.

3.3 Upon completion of thermocycling, place the tubes on the magnetic rack at room temperature for 2 min.

3.4 Remove and discard all of the supernatant from each tube. Avoid disturbing the beads.

3.5 While on the magnet, and without disturbing the beads, gently add 180 µL Final Wash Buffer to the tubes. If using a 0.3 mL tube, then use 250 µL to fill tube capacity. **This is to remove any remaining uncaptured RNA adsorbed to the tubes.**

3.6 While on the magnet, remove and discard all of the supernatant from the tubes. Avoid disturbing the beads.

NOTE: Any remaining Final Wash Buffer in the tubes will dilute the poly(A) RNA proceeding into Library Preparation, but will not negatively impact the Library Preparation chemistry.

3.7 Remove the tubes from the magnetic rack and proceed immediately with **Fragmentation and Priming**.

4. Fragmentation and Priming

4.1 Prepare the Frag & Prime Master Mix as follows for each reaction:

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

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

4.3 Add 27 µL of the Frag & Prime Master Mix to the beads from **Step 3.7**.

4.4 Mix on an appropriate vortex mixer (3,000 rpm) for 4 sec to resuspend the beads. Briefly centrifuge to collect all liquid in the bottom of the tubes.

WORKFLOW: If pipette mixing, mix the reaction 10 times with a pipette set to 25 µL to resuspend the beads in the Frag & Prime Master Mix. Briefly centrifuge if possible. See **Prior to Starting: Mixing Considerations**.

4.5 Program a thermocycler as indicated below, and initiate the run to cool the block:

NOTE: Insert size is adjusted through modulating the Post-amplification SPRI ratio (**Step 11.2**) and amplification cycles (**Step 10**). Irrespective of insert size required, the RNA Fragmentation conditions remain the same. See **Prior to Starting: Insert Size** for more details.

Step	Temperature	Time
Lid temperature	105°C	N/A
Pre-cooling	4°C	HOLD
RNA Fragmentation	85°C	10 min
HOLD (Priming)	12°C	HOLD

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

4.7 After the program has finished and the samples have returned to 12°C, place the tubes on a magnet for at least 2 min, or until all beads have been collected on the tube walls and the solution is clear.

4.8 Carefully remove 25 uL of the supernatant and transfer to fresh, labeled tubes.

NOTE: Take care not to disturb the beads, as bead carryover can interfere with 1st Strand Synthesis.

Either proceed immediately to **1st Strand Synthesis** or store at -20°C for not more than 24 hours.

Safe stopping point. Samples can be frozen at -20°C for <24 hours.

5. 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.

5.1 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

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

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

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

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

Component	Volume (µL)
Fragmented RNA (from **Step 4.8**)	25
1st Strand Master Mix	10

5.5 Mix on an appropriate vortex mixer (3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tubes.

5.6 Place reactions in the chilled thermocycler (programmed and initiated in **Step 5.1**). Advance the thermocycler from the initial 4°C hold to start the 25°C incubation.

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

6. 2nd Strand Synthesis and A-Tailing

6.1 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

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

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

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

6.4 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

6.5 Mix on an appropriate vortex mixer (3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tubes.

6.6 Place reactions in the chilled thermocycler (programmed and initiated in **Step 6.1**). Advance the thermocycler from the initial 4°C hold to start the 42°C incubation.

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

7. Adapter Ligation

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

7.1 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**.

7.2 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 tubes and placing it on ice.

7.3 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 1** or **Table 2** 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 1. Full-length adapter concentration by RNA input into library prep

RNA input (ng)	Adapter concentration (µM)
≤50[1]	0.3
51 – 150	
151 – 1000	1

[1]A second post-ligation cleanup (**Step 9**) is recommended for inputs of 50 ng and lower when using full-length adapters.

Table 2. Truncated ('stubby') adapter concentration by RNA input into library prep

RNA input (ng)	Adapter concentration (µM)
≤10[1]	1
11 – 250	
251 – 1000	4

[1]A second post-ligation cleanup (**Step 9**) is recommended for inputs of 10 ng and lower when using 'stubby' adapters.

7.4 Add 5 µL of appropriately diluted adapter to each tube (**Step 6.7**).

7.5 Prepare the Ligation Master Mix as follows:

Component	Volume (µL)
Ligation Buffer	40
Ligation Enzyme	5

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

7.7 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

7.8 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 tubes.

7.9 Place the tubes in the thermocycler and initiate the program (programmed in **Step 7.1**).

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

8. Post-ligation Cleanup

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

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

8.3 Mix on an appropriate vortex mixer (3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tubes.

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

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

8.6 Carefully remove and discard the supernatant from each tube.

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

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

8.9 Repeat **Steps 8.7 – 8.8**, for a total of two washes.

OPTIONAL: Tubes can be briefly centrifuged to pull down excess ethanol prior to removal with a P10 pipette.

8.10 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 tubes. Residual trace ethanol in the library amplification reaction may decrease performance.

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

8.12 Incubate tubes at room temperature for at least 2 mins before placing back on the magnet.

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

8.14 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.

9. 2nd Post-ligation Cleanup (Optional)

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

- Full-length adapters and equal to or lower than 50 ng of RNA.
- Truncated ('stubby') adapters and equal to or lower than 10 ng of RNA.

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

9.2 Vortex room temperature SPRI beads to thoroughly mix. Add 20 µL (1X) of room temperature resuspended beads to each eluted library from **Step 8.14**.

9.3 Mix on an appropriate vortex mixer (3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tubes.

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

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

9.6 Carefully remove and discard the supernatant from each tube.

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

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

9.9 Repeat **Steps 9.7 – 9.8** for a total of two washes.

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

9.10 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 tubes. Residual trace ethanol in the library amplification reaction may decrease performance.

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

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

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

9.14 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.

10. 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.

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

10.2 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	See **Table 3**
Annealing	P5/P7 primers: 60°C[2] Indexed primers: 55°C[3]	30 sec	
Extension	72°C	30 – 45 sec	
Final extension	72°C	60 sec	1
–	12°C	HOLD	1

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

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

[3]For indexes used with the truncated adapter scheme detailed in Glenn, et. al. 2019, use 55°C. For the IDT xGen™ Stubby Adapter-UDI Primers, an annealing temperature of 64°C is recommended. For other adapter/primer configurations, optimization may be required (see **Prior to Starting**).

Table 3. Recommended PCR cycle numbers by RNA amount input into library prep

RNA input into Library Preparation (ng)	PCR cycles to generate 10 – 50 nM library[1]
251 – 1000	8 – 9
101 – 250	10 – 12
51 – 100	12 – 13
11 – 50	13 – 15
2.5 – 10	16 – 18

[1]**INSERT SIZE NOTE:** When targeting 290 to 310 bp inserts using a 0.7X post-amplification cleanup (Step 11.2), an extra 2 PCR cycles are required.

NOTE: When targeting 290 to 310 bp inserts using a 0.7X post-amplification cleanup (**Step 11.2**), an extra 2 PCR cycles are required.

10.3 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.

10.4 Mix on an appropriate vortex mixer (3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tubes.

10.5 Place tubes in the thermocycler (programmed in **Step 10.2**) and initiate the program.

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

11. Post-amplification Cleanup

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

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

INSERT SIZE NOTE: When targeting 290 – 310 bp inserts, add 35 µL (a 0.7X ratio) of room temperature resuspended beads to each amplification reaction.

11.3 Mix on an appropriate vortex mixer (3,000 rpm) for 4 sec. Briefly centrifuge to collect all liquid in the bottom of the tubes.

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

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

11.6 Carefully remove and discard the supernatant from each tube.

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

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

11.9 Repeat **Steps 11.7 – 11.8** for a total of two washes.

OPTIONAL: Tubes can be briefly centrifuged to pull down excess ethanol prior to removing with a P10 pipette.

11.10 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 tubes.

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

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

11.13 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.

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

11.15 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, 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*
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**	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

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

**Used in xGen Hybridization and Wash Kit (Integrated DNA Technologies).

Revision History

Version	Description	Date
2.0	• Footnote 3 of amplification program table (10.2) modified to include specific annealing temperature recommendations for IDT xGen™ Stubby Adapter-UDI primers.	06/2024
2.1	• Temperatures in Steps 4.5, 4.6, and 4.7 corrected to align with temperatures in Table in Step 4.5 • Steps 4.8 and 7.8 rewritten for clarification purposes • Pg 10: Lid temperature in thermocycler post-ligation amplification program added in Step 10.2. • Trademark updated	10/2024
2.2	• Addition of 384 rxn pack sizes and volumes in Kit Contents • Step 10.1: Correction to vortex Equinox Amplification Master Mix (2X) • Step 11.2: Correction of “ligation” to “amplification”	12/2024

WATCHMAKER GENOMICS

For Technical Support, please contact support@watchmakergenomics.com.

5744 Central Avenue, Suite 100
Boulder, CO 80301
www.watchmakergenomics.com

PTD-32 WMTG214