

Watchmaker DNA Library Prep Kit with TAPS+

Product Description

The Watchmaker DNA Library Prep Kit with TAPS+ is designed for the detection of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC). The highly streamlined workflow prepares direct methyl-sequencing libraries from 1 ng to 200 ng of total DNA input into library preparation. The Watchmaker DNA Library Prep Kit with TAPS+ has high 5mC and 5hmC conversion rates, minimal off-target conversion, a high degree of repeatability, low coverage bias resulting from the conversion, and is compatible with high-quality genomic DNA, cfDNA, and low-quality samples such as DNA derived from FFPE. TAPS+ is a next-generation, positive-readout methylation technology that delivers multimodal analysis of DNA methylation, SNVs, and CNVs from a single, streamlined assay.

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Kit Contents

Bundled kit	Kit	Kit code	Description	Component volume	
				24 rxn	96 rxn
Watchmaker DNA Library Prep Kit with TAPS+ 7BK0003-024 (24 rxn) 7BK0003-096 (96 rxn)	Watchmaker DNA Library Prep Kit (PCR-free)	7K0101-024 (24 rxn)	ER/AT Buffer	185 µL	840 µL
			ER/AT Enzyme Mix	80 µL	360 µL
		7K0101-096 (96 rxn)	Ligation Buffer	660 µL	3.0 mL
			Ligation Enzyme	135 µL	600 µL
	TAPS+ Kit Methyl-seq	7K0130-024 (24 rxn) 7K0130-096 (96 rxn)	Oxidation Buffer	265 µL	1.2 mL
			50 mM DTT	80 µL	360 µL
			Iron (II)	80 µL	360 µL
			Oxidation Cofactor	80 µL	360 µL
			Oxidation Enzyme	80 µL	360 µL
			Stop Solution	135 µL	600 µL
			Reduction Buffer	185 µL	840 µL
			Reduction Reagent	1 x 750 µL	4 x 750 µL
Equinox DHU Tolerant Amplification Master Mix (2X)	690 µL	3.0 mL			

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

Product Description (continued)

DNA fragments generated by biologically occurring DNA fragmentation, such as cell-free DNA (cfDNA), mechanical (e.g., Covaris®), or enzymatic fragmentation methods designed to retain methylation information, are suitable as input into the workflow. Watchmaker DNA Library Prep Kits generate A-tailed, 5' phosphorylated DNA fragments during the End Repair and A-tailing step, which are then ligated to T-tailed adapters in a rapid, single-tube workflow. The Watchmaker TAPS+ Kit – Methyl Seq then directly converts 5mC and 5hmC in double stranded DNA to T through an oxidation of 5mC/5hmC to 5caC (5-carboxylcytosine) followed by a reduction to DHU (dihydrouracil). During amplification, the DHU is recognized as 'T', resulting in final libraries containing 'T' in the place of 5mC and 5hmC bases. The Equinox DHU Tolerant Amplification Master Mix (2X) contains an optimized PCR buffer and enzyme formulation that enables library amplification with high fidelity and efficiency across a broad range of GC content and DHU.

This workflow enables a positive readout of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) as only the modified bases are converted. This is in contrast to EM-seq and bisulfite conversion methods, where the modified bases are “protected” and all unmodified bases are converted to T. The positive readout offers many advantages, including:

- Simultaneous detection of DNA methylation, SNVs, and CNVs
- Improved variant detection, including accurate calling of C>T mutations
- Broad sample compatibility, including cfDNA, FFPE, and other degraded or low-input samples
- No methylated adapters required
- High base complexity supports improved sequencing quality with minimal PhiX spike-in

Product Applications

This workflow was developed to address the highly specific needs of single-base resolution methylome analysis. TAPS+ results in the direct conversion of 5mC and 5hmC as a positive readout for methylation status – improving sequencing quality, base quality, and mapping. Enzymatic oxidation and gentle chemical reduction result in high conversion efficiency and produce high-

complexity libraries with improved sequencing efficiency, requiring fewer reads to achieve the same coverage. TAPS+ preserves genomic information, thus enabling multimodal analysis including methylation detection, variant detection (single nucleotide variants and indels), and copy number detection. These applications require high 5mC conversion rates, low off-target conversion (false positives), a high degree of reproducibility, and compatibility with low quantity and quality samples.

The Watchmaker DNA Library Prep Kit with TAPS+ workflow is ideally suited for:

- Whole-genome or targeted methylation sequencing
- Detection of methylation, SNVs, and CNVs in a single workflow
- High- or low-input double stranded DNA samples, including cfDNA, FFPE, and tissue
- Assay development for multi-cancer early detection or monitoring
- Pre-fragmented double stranded DNA analysis
- Workflows that employ unique molecular identifiers (UMIs) for improved sensitivity

Storage and Handling

The Watchmaker DNA Library Prep Kit with TAPS+ is shipped on cold packs or dry ice. Upon receipt, store all components at $-20 \pm 5^{\circ}\text{C}$.

Keep all components, except the Reduction Reagent and Reduction Buffer, on ice or a cooled reagent block during routine use. The Reduction Reagent should be thawed and kept at room temperature while in use. After use, Reduction Reagent should be promptly and tightly closed to minimize exposure to atmospheric water and stored at a temperature of $-20 \pm 5^{\circ}\text{C}$.

Some components are viscous; therefore, take care to homogenize solutions thoroughly before use and during reaction setup. Enzymes should be inverted ten times prior to use. This includes the ER/AT, ligation, and oxidation enzymes. Equinox DHU Tolerant Amplification Master Mix (2X), 50 mM DTT, Iron (II), Oxidation Cofactor, Stop Solution, Reduction Reagent, and buffers should be vortexed for at least 5 seconds before use. AMPure XP reagent should be handled per the manufacturer's guidelines.

Storage and Handling (continued)

All master mixes (buffer and enzyme combined) prepared in the protocol should be stored at 4°C or on ice unless stated otherwise. During the **Reduction Reaction** and **Post-reduction Cleanup (Steps 7 and 8)**, the reaction mixes should be kept at room temperature to prevent precipitate formation.

For further details on storage and handling, our Safety Data Sheet (SDS) can be requested from support@watchmakergenomics.com.

Required Materials Not Included

- Unmethylated Adapters (see **Prior to Starting** for more detail)

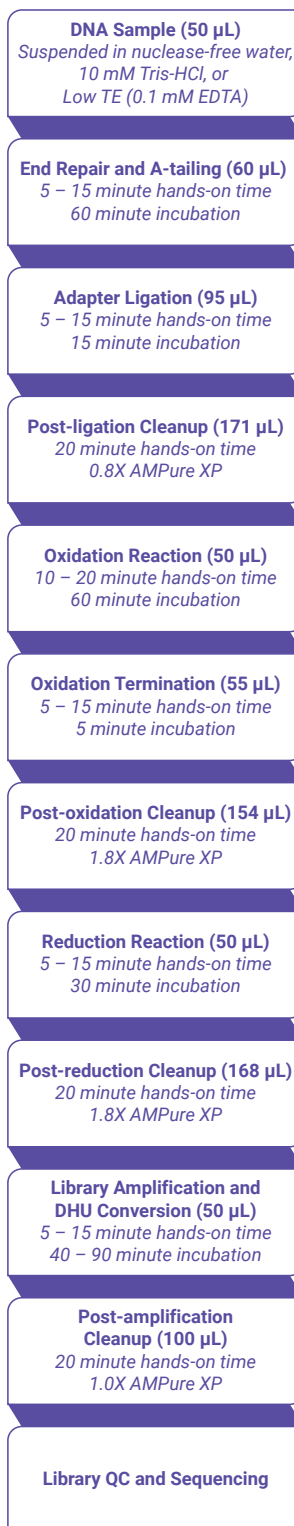
IMPORTANT NOTE: Unmethylated adapters are required. The IDT xGen™ Stubby adapter is methylated and thus not compatible with the TAPS+ chemistry.

- Amplification Primers (see **Prior to Starting** for more detail)
- Adapter diluent (e.g., 10 mM Tris-HCl, pH 8.0, 10 mM NaCl)
- AMPure® XP (Beckman Coulter, Inc. #A63881) Reagent

IMPORTANT NOTE: Conversion failure has been observed with other SPRI beads
- 80% ethanol
- Isopropanol, molecular biology grade
- 200 µL thin-wall PCR tubes compatible with thermocycler, or 96-well 0.2 mL PCR Plates and Plate-Seals
- 1 mL, 2 mL, 5 mL tubes (nuclease-free)
- Nuclease-free water
- 10 mM Tris-HCl, pH 8.0
- Magnetic rack compatible with 0.2 mL PCR tubes and/or 96-well plate
- Thermocycler
- Vortex mixer
- 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 DNA

This kit is effective with a broad range of fragmented input amounts (1 ng to 200 ng) from both high- and low-quality DNA, including FFPE. DNA fragments generated by biologically occurring DNA fragmentation, such as cell-free DNA (cfDNA), mechanical (e.g., Covaris®), or enzymatic fragmentation methods, which are designed to maintain methylation marks, are suitable as input into the workflow. DNA input refers to the input into the End Repair and A-tailing reaction.

If DNA was quantified prior to fragmentation, the actual input into library preparation may be lower. This should be considered when evaluating library preparation efficiency and during library amplification cycle number optimization.

NOTE: When starting with mechanically sheared input DNA, it is important to proceed directly into the End Repair and A-tailing reaction. Post-shearing DNA cleanups before End Repair and A-tailing have been shown to introduce significant AT and GC bias in the final libraries.

This kit is compatible with DNA extracted from formalin fixed paraffin-embedded tissues (FFPE). Due to the significant damage incurred by fixation and extraction, conversion efficiencies for FFPE DNA samples will most likely be lower and may be more variable sample to sample. The quality of FFPE DNA can vary greatly depending on factors such as fixation, storage, and extraction method. This variation can have a significant impact on the library preparation efficiency. Adding an additional 1 – 2 cycles of PCR is recommended.

Nuclease-free water, 10 mM Tris-HCl pH 8.0, and Low TE (0.1 mM EDTA) are the recommended buffers for input DNA suspension.

Fluorescence-based methods such as Qubit or PicoGreen™ are recommended for accurate input DNA quantification over spectrophotometric methods, such as NanoDrop™.

Adapters

This kit is compatible with adapters that have a 3' overhanging T to facilitate adapter orientation during double stranded DNA ligation. Unlike classical methyl-seq preparation techniques such as Bisulfite conversion, TAPS+ chemistry directly converts 5mC and 5hmC to T and thus **methylated adapters are not compatible**.

NOTE: TAPS+ requires the use of unmethylated adapters. The IDT xGen™ Stubby Adapter is methylated and **not compatible** with this chemistry. Contact support@watchmakergenomics.com if you have questions about adapter compatibility or need help selecting an alternative.

Adapter quality has an impact on the efficiency of the ligation reaction and library yield. High-quality adapters may be sourced from a variety of reliable vendors in convenient, ready-to-use formats. Adapter concentrations may need to be optimized based on input mass, quality, fragment size, and adapter type. Refer to **Step 2.4** of the protocol for specific recommendations for sample type, input mass, and adapter style.

'Stubby' adapters provide improved library prep efficiency as they can be included at increased concentrations in the ligation reaction. We strongly recommend the use of stubby adapters for maximum performance.

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.

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**.

User-supplied Amplification Primers

When using 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 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.

P5/P7 Amplification Primers at a concentration of 20 μM of each primer is appropriate for the amplification of full-length adapter-ligated libraries.

P5: AATGATACGGCGACCACCGA

P7: CAAGCAGAAGACGGCATACGAGAT

Library Amplification Optimization

Pre-extension

This protocol includes a 30-minute pre-extension step as the first PCR cycle. This step facilitates efficient read through of DHU bases generated by the TAPS+ chemistry. Inclusion of this step is necessary to ensure optimal amplification efficiency and downstream library performance.

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 DNA 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) reagents for bead purification steps.

IMPORTANT! Failures have been observed with other SPRI beads. Use of AMPure XP reagent is essential.

Ensure beads are equilibrated to room temperature and thoroughly resuspended via 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

Recommendations

- Keep all sample tubes, buffers, and enzymes on ice unless stated otherwise. This will ensure proper compatibility with TAPS+ conversion.

NOTE: Reduction Reagent and Reduction Buffer should be thawed and kept at room temperature prior to and during use. After use, the Reduction Reagent should be promptly closed to minimize exposure to atmospheric water and stored at a temperature of $-20 \pm 5^{\circ}\text{C}$. Each tube of Reduction Reagent can be used a maximum of 4 times.

- 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.
- Besides Reduction Reagent and Buffer, ensure all the components are fully thawed on ice before use. Once thawed, invert enzyme tubes 10 times to mix. For all other components, invert the tubes several times, and vortex for at least 5 sec to ensure the reagent is fully mixed.
- Where possible, centrifuge briefly to remove any excess liquid from the tube lid prior to opening.
- Prepare master mixes for each reaction step with a 10% excess to account for loss during pipetting.
- Ensure AMPure XP Beads are fully equilibrated to room temperature and thoroughly resuspended by vortexing prior to use.

1. End Repair and A-tailing

NOTE: Input DNA must be fragmented prior to End Repair and A-tailing reaction.

- 1.1 Program a thermocycler as indicated below and initiate the program:

Step	Temperature	Time
Lid temperature	85°C	N/A
Pre-heating	20°C	HOLD
End repair	20°C	30:00
A-tailing	65°C	30:00
HOLD	4°C	HOLD

- 1.2 On ice, prepare fragmented input DNA in a total volume of 50 μL . Dilute fragmented DNA in nuclease free water, 10 mM Tris-HCl, pH 8.0, or Low TE (0.1 mM EDTA).

- 1.3 Prepare the End Repair and A-tailing (ER/AT) master mix as specified below on ice:

Component	Volume (μL)
ER/AT Buffer	7
ER/AT Enzyme Mix	3

- 1.4 Mix on a touch vortexer (or 2,000 rpm) for 5 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.

- 1.5 To each tube, add the following on ice:

Component	Volume (μL)
Input DNA	50
End repair and A-tailing master mix	10

- 1.6 Mix on a touch vortexer (or 2,000 rpm) for 5 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.
- 1.7 Place the tube in the initiated thermocycler (programmed and initiated in **Step 1.1**). Advance the thermocycler from the initial 20°C hold.
- 1.8 Proceed immediately to **Adapter Ligation** after the program has completed and the samples have returned to 4°C. Hold samples at 4°C until **Step 2.5**.

2. Adapter Ligation

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

- 2.1 Program a thermocycler as indicated below:

Step	Temperature	Time
Lid temperature	OFF	N/A
Ligation	20°C	15:00 ¹

¹Ligation time may be extended to a maximum of 16 hours. Library quality decreases with overnight ligation.

- 2.2 Prepare the Ligation master mix as specified below on ice:

Component	Volume (µL)
Ligation Buffer	25
Ligation Enzyme	5

- 2.3 Mix on a touch vortexer (or 2,000 rpm) for 5 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.
- 2.4 Using an appropriate diluent (e.g., 10 mM Tris-HCl, pH 8.0, 10 mM NaCl), prepare the required volume of each adapter (stubby OR full-length) at the concentration specified below. 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.

Stubby adapters:

For most stubby adapters and DNA of any source (including FFPE and cfDNA), an adapter concentration of 15 µM is recommended, irrespective of DNA input amount.

UMI stubby adapters dimerize more readily and are removed less efficiently. In these instances, use the recommendations below:

- For inputs <10 ng, use 3 µM adapter
- For inputs ≥10 ng, use 10 µM adapter

Full-length adapters:

For cfDNA, an adapter concentration of 15 μ M is recommended.

For non-cfDNA (including FFPE), the following adapter concentrations are recommended (Table 1):

Table 1. Recommended full-length adapter concentrations into library prep for non-cfDNA¹

DNA input (ng)	Adapter concentration (μ M)
>50	15.0
25 – 50	7.5
10 – 24	3.0
2 – 9	1.5
<2	0.3

¹Adapter concentrations may need to be optimized based on input mass, quality (FFPE), fragment size, and adapter source. Use the maximum concentration of adapter while minimizing adapter dimer product.

- 2.5 Remove the reaction tubes (from **Step 1.8**) from the thermocycler.
- 2.6 To each tube, add the following in order, on ice:

Component	Volume (μ L)
End repair and A-tailed DNA	60
Adapter	5
Ligation master mix	30

- 2.7 The Ligation master mix is viscous. Mix the ligation reaction on a touch vortexer (or 2,000 rpm) for 5 sec or carefully pipette a minimum of 50 μ L up and down ten times to ensure proper mixing. Briefly centrifuge if needed to collect all liquid in the bottom of the tube.

NOTE: Ensure the reaction is homogeneous prior to proceeding.

- 2.8 Place the tubes in the thermocycler and initiate the Ligation incubation program (programmed in **Step 2.1**).
- 2.9 Proceed to **Post-ligation Cleanup** after the program has completed.

3. Post-ligation Cleanup

- 3.1 Freshly prepare at least 0.4 mL of an 80% ethanol solution for each reaction.
- 3.2 Vortex room temperature AMPure XP beads to thoroughly mix. Add 76 μ L (0.8X) of beads to each ligation reaction and mix thoroughly by pipetting.

NOTE: SPRI-to-sample bead ratios may be optimized for different applications or adapter configurations. A ratio of 0.8X is recommended as a starting point when using high-quality DNA and full-length adapters. When stubby adapters are used, the bead ratio may be increased to 1X (95 μ L) to improve performance.

- 3.3 Mix on a touch vortexer (or 2,000 rpm) for 5 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.
- 3.4 Incubate the library-bead mixtures at room temperature for at least 5 min to maximize library recovery.
- 3.5 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.
- 3.6 Carefully remove and discard the supernatant from each tube.
- 3.7 Add 200 μ L of freshly prepared 80% ethanol to each tube, taking care not to disturb the bead pellet on the tube wall.

3.8 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.

3.9 Repeat **Steps 3.7 – 3.8** for a total of two washes.

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

3.10 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.

3.11 Remove sample tubes from the magnet and carefully resuspend each bead pellet in 22 μ L nuclease-free water. Pipetting carefully will minimize bubbling and allow for greater library recovery.

NOTE: It is critical that elution be performed with nuclease-free water to prevent interference with downstream chemistry.

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

3.13 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.

3.14 Carefully transfer 20 μ L of each adapter-ligated supernatant to a new, labeled tube.

NOTE: Take care not to transfer any beads across as bead carryover can potentially impact library yield and methyl conversion.



Safe stopping point. Samples can be stored at 4°C for up to 1 week and at -20°C for up to 1 month.

4. Oxidation Reaction (5mC and 5hmC oxidation to 5caC)

4.1 Program a thermocycler as indicated below and initiate the program:

Step	Temperature	Time
Lid temperature	40°C	N/A
Pre-heating	37°C	HOLD
Oxidation	37°C	60:00

4.2 Prepare the Oxidation master mix as specified below on ice. Combine reagents in the order listed.

NOTE: The presence of iron in the master mix may cause a pink coloration in both the master mix and the final reaction. This is expected and does not affect reaction efficiency.

Component	Volume (μ L)
Oxidation buffer	10
Nuclease free water	9
50 mM DTT	3
Iron (II)	3
Oxidation Cofactor	3
Oxidation Enzyme	3

4.3 Mix on a touch vortexer (or 2,000 rpm) for 5 seconds. Briefly centrifuge to collect all liquid in the bottom of the tube.

- 4.4 To each adapter-ligated DNA tube, add the Oxidation master mix as specified below on ice:

Component	Volume (μL)
DNA (adapter-ligated)	20
Oxidation master mix	30

- 4.5 Mix on a touch vortexer (or 2,000 rpm) for 5 seconds. Briefly centrifuge to collect all liquid in the bottom of the tube.
- 4.6 Immediately place the tube into the heated thermocycler (programmed and initiated in **Step 4.1**). Advance the thermocycler from the initial 37°C hold.
- 4.7 Proceed directly to **Oxidation Termination** after the program has completed.

5. Oxidation Termination

- 5.1 Program a thermocycler as indicated below and initiate the program:

Step	Temperature	Time
Lid temperature	40°C	N/A
Pre-heating	37°C	HOLD
Oxidation termination	37°C	5:00
HOLD	4°C	HOLD

- 5.2 Add 5 μL of Stop Solution to each oxidation reaction on ice.
- 5.3 Mix on a touch vortexer (or 2,000 rpm) for 5 seconds. Briefly centrifuge to collect all liquid in the bottom of the tube.
- 5.4 Place the tube into the heated thermocycler for the oxidation termination step (programmed and initiated in **Step 5.1**). Advance the thermocycler from the 37°C hold.
- 5.5 Once the program has completed, proceed to **Post-oxidation Cleanup**.

6. Post-oxidation Cleanup

- 6.1 Freshly prepare at least 0.4 mL of an 80% ethanol solution for each reaction.
- 6.2 Vortex room temperature AMPure XP beads to thoroughly mix. Add 99 μL (1.8X) of beads to each oxidation reaction and mix thoroughly by pipetting.
- 6.3 Mix on a touch vortexer (or 2,000 rpm) for 5 seconds. Briefly centrifuge to collect all liquid in the bottom of the tube.
- 6.4 Incubate the reaction-bead mixtures at room temperature for at least 5 min to maximize library recovery.
- 6.5 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.
- 6.6 Carefully remove and discard the supernatant from each tube.
- 6.7 Add 200 μL of freshly prepared 80% ethanol to each tube, taking care not to disturb the bead pellet on the tube wall.
- 6.8 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.
- 6.9 Repeat **Steps 6.7 – 6.8**, for a total of two washes.
- 6.10 Briefly spin down the tubes to pull down excess ethanol prior to removing with a p10 pipette.
- 6.11 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.

- 6.12 Remove sample tubes from the magnet and carefully resuspend each bead pellet in 20 μL of nuclease-free water.
NOTE: It is critical that elution be performed with nuclease-free water to prevent interference with downstream chemistry.
- 6.13 Incubate tubes at room temperature for at least 2 min before placing back on the magnet.
- 6.14 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.
- 6.15 Carefully transfer 18 μL of each library-containing supernatant to a new, labeled tube.
NOTE: Take care not to transfer any beads across as bead carryover can significantly impact library yield and methyl conversion.



Safe stopping point. Samples can be stored at 4°C overnight or at -20°C for up to 1 week.

7. Reduction Reaction (5caC reduction to DHU)

IMPORTANT! During the **Reduction Reaction** and **Post-Reduction Cleanup (Steps 7 and 8)**, the reaction mixes should be kept at room temperature.

- 7.1 Program a thermocycler as indicated below and initiate the program:

Step	Temperature	Time
Lid temperature	65°C	N/A
Pre-heating	50°C	HOLD
Reduction	50°C	30:00
HOLD	25°C	HOLD

- 7.2 Prepare the Reduction master mix as specified below, at room temperature:

NOTE: After use, Reduction Reagent should be promptly and tightly closed to minimize exposure to atmospheric water and stored at a temperature of $-20 \pm 5^\circ\text{C}$. Each tube of Reduction Reagent can be used a maximum of 4 times.

NOTE: Prepare master mix immediately prior to use. Bubble formation may occur if prepared in advance. Bubbles do not impair the reaction provided the full volume of master mix is transferred to the reaction.

Component	Volume (μL)
Reduction Buffer	7
Reduction Reagent	25

- 7.3 Mix on a touch vortexer (or 2,000 rpm) for 5 seconds. Briefly centrifuge to collect all liquid in the bottom of the tube.
- 7.4 To each tube, add the Reduction master mix as specified below at room temperature:

Component	Volume (μL)
Oxidized DNA	18
Reduction master mix	32

- 7.5 Mix on a touch vortexer (or 2,000 rpm) for 5 seconds. Briefly centrifuge to collect all liquid in the bottom of the tube.
NOTE: Reduction reactions should be assembled at room temperature. Place tubes in thermocycler immediately after assembly.
- 7.6 Place the tube into the heated thermocycler (programmed and initiated in **Step 7.1**). Advance the thermocycler from the initial 50°C hold.

7.7 Directly upon completion of the reduction reaction, remove the tubes from the thermocycler and place at room temperature.

IMPORTANT! The reaction should not be placed on ice after its completion.

7.8 Proceed to **Post-reduction Cleanup** after the program has completed and the samples have returned to 25°C.

8. Post-reduction Cleanup

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

8.2 Vortex room temperature AMPure XP Beads to thoroughly mix. Add 90 µL (1.8X) of beads to each reaction.

8.3 Add 28 µL of isopropanol to each tube.

8.4 Mix on a touch vortexer (or 2,000 rpm) for 5 seconds. Briefly centrifuge to collect all liquid in the bottom of the tube.

8.5 Incubate the reaction-bead mixtures at room temperature for 5 minutes.

8.6 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.

NOTE: Supernatant may form visible precipitate; this will not impact performance.

8.7 Carefully remove and discard the supernatant from each tube.

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

8.9 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.

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

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

8.11 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.

8.12 Remove sample tubes from the magnet and carefully resuspend each bead pellet in 22 µL of nuclease-free water.

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

8.14 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.

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



Safe stopping point. Samples can be stored at -20°C for up to 2 weeks.

9. Library Amplification and DHU Conversion

NOTE: Library amplification is required for the final conversion step of 5mC and 5hmC to T.

- 9.1 Thaw and equilibrate the Equinox DHU Tolerant Amplification Master Mix (2X) on ice. Vortex 5 seconds prior to use.
- 9.2 Program a thermocycler as indicated below:

Step ¹	Temperature	Time	Cycles
Lid temperature	105°C	N/A	N/A
Initial denaturation	98°C	1:00	1
Annealing	25°C	0:30	1
Pre-extension	72°C	30:00	1
Denaturation	98°C	0:15	
Annealing	P5/P7 primers: 60°C ² Indexed primers: 55°C ³	0:30	See Table 2
Extension	72°C	0:30 – 0:45 ¹	
Final extension	72°C	1:00	1
–	12°C	HOLD	–

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

²Appropriate temperature for Illumina P5/P7 Primers

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

Table 2. Recommended PCR cycle numbers by DNA input amount into library prep

DNA input into library preparation (ng)	PCR cycles to generate 10 – 50 nM ¹ library		
	cfDNA	gDNA (HQ)	FFPE DNA
≥75	3	3 – 4	4 – 5
20	3 – 4	4 – 5	5 – 6
5	5 – 6	7 – 8	8 – 9
1	7 – 8	9 – 11	10 – 12

¹For downstream target enrichment, a higher library yield may be required. Add 4 cycles (cfDNA) or 3 cycles (gDNA and FFPE) to generate 1 µg of library.

- 9.3 Assemble each amplification reaction in the order specified below.

Component	Volume (µL)
Reduced library	20
Primer Mix ¹	5
Equinox DHU Tolerant Amplification Master Mix (2X)	25

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

- 9.4 Mix on a touch vortexer (or 2,000 rpm) for 5 seconds. Briefly centrifuge to collect all liquid in the bottom of the tube.
- 9.5 Place tubes in the thermocycler (programmed in **Step 9.2**) and initiate the program.
- 9.6 Once the program has completed, proceed immediately to **Post-amplification Cleanup**.

10. Post-amplification Cleanup

- 10.1 Freshly prepare at least 0.4 mL of an 80% ethanol solution for each reaction.
- 10.2 Vortex room temperature AMPure XP beads to thoroughly mix. Add 50 μ L (1X) of beads to each amplification reaction.
- 10.3 Mix on a touch vortexer (or 2,000 rpm) for 5 sec. Briefly centrifuge to collect all liquid in the bottom of the tube.
- 10.4 Incubate the library-bead mixtures at room temperature for at least 5 min to maximize library recovery.
- 10.5 Place sample tubes on a magnet for at least 5 min, or until beads have collected on the tube wall and the solution is clear.
- 10.6 Carefully remove and discard the supernatant from each tube.
- 10.7 Add 200 μ L of freshly prepared 80% ethanol to each tube, taking care not to disturb the bead pellet on the tube wall.
- 10.8 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.
- 10.9 Repeat **Steps 10.7 – 10.8** for a total of two washes.
OPTIONAL: Tubes can be briefly spun to pull down excess ethanol prior to removing with a p10 pipette.
- 10.10 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.
- 10.11 Remove sample tubes from the magnet and carefully resuspend each bead pellet in 22 μ L of 10 mM Tris-HCl, pH 8.0.
- 10.12 Incubate tubes at room temperature for at least 2 min before placing back on the magnet.
- 10.13 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.
- 10.14 Carefully transfer 20 μ L of each library-containing supernatant to a new, labeled tube.
- 10.15 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: Methylation Controls

Libraries prepared with the Watchmaker DNA Library Prep Kit with TAPS+ undergo standard NGS library QC procedures (quantification and electrophoretic analysis) and look indistinguishable from normal NGS DNA libraries. As such, these QC procedures should not be used to indicate success or failure to convert 5mC and 5hmC. To best assess conversion, the use of suitable positive and negative controls is recommended.

A positive control is used to assess the global conversion rate of 5mC to T. An effective positive control should contain known 5mC locations. In contrast, an effective negative control is used to assess the false positive rate and should contain known unmodified cytosine locations that can be used for this analysis.

Process controls

For evaluation, we recommend an in-process control (parallel control processed as its own sample) as the best way to assess 5mC conversion performance. One strategy for assessing both conversion of modified cytosines and false positive rates is to use a mixture of a fully methylated lambda phage DNA mixed with NA12878 human gDNA. A mixture of 95% NA12878 human gDNA with 5% fully methylated lambda DNA is recommended. The lambda serves as the positive control, while CH sites (i.e., CpA, CpC, CpT) in NA12878 human gDNA are used as the negative control. NA12878 is compatible with this approach as CH methylation is not present. However, some cell lines maintain CH methylation and thus may be unsuitable to serve as a negative control in this context. Additional benefits of this type of process control are:

- Sequencing shallowly (0.5 – 1M read pairs) is sufficient to assess 5mC conversion from the reads mapping to the lambda genome and the false positive rate from the reads mapping to the human genome.
- It can be sequenced separately and in advance, allowing a 5mC conversion as a batch QC, ahead of the high output sequence run required for whole genome sequencing on test samples.

Spike-in controls

Spike-in controls can be effectively employed to establish consistency across samples and as relative indicators of performance from one run to another. Watchmaker has evaluated several commercially available controls and assessed them to determine their overall methylation level and compatibility with the TAPS+ library. Please contact support@watchmakergenomics.com for information on specific spike-in controls.

Please note that the global methyl conversion rate of the spike-in controls may vary depending on the control and testing paradigm used. As such, it is important to determine the conversion rate for your sample type, input mass, and spike-in controls, and that the observed rate is consistent across different lots of the spike-in controls, as well as between batches and between samples within a batch.

The amount of spike-in control to add to your sample depends on the depth of sequencing and the genome size of the spike-in control. A good starting point is to target ~300X coverage of your spike-in controls. This amounts to 50,000 reads for the lambda genome (50 kb) and 2,500 reads for pUC19 plasmid. These numbers are not absolute but are recommendations as a starting point to determine the amount of each spike-in control to add.

Many spike-in controls are unsheared and thus need to be fragmented before the ER/AT reaction.

- For gDNA and FFPE DNA, add the spike-in control to the sample before shearing. Shear in the same tube, then proceed to **End Repair and A-tailing (Step 1)**.
- cfDNA samples do not require additional shearing. When using a spike-in control with these samples, shear the spike-in control, then add it to the cfDNA sample and proceed to **End Repair and A-tailing (Step 1)**.

Table 3. Summary of process controls vs. spike-in controls

	Process control	Spike-in control
Description	Positive and/or negative control processed as its own sample alongside test samples	Known quantities of orthogonal positive and/or negative control DNA are added directly to each test sample as an internal standard
Characteristics	Absolute reference to assess performance between batches	Reference point to adjust for variability in sample processing, library preparation, and measurement
What it controls for	Reagent performance, protocol execution, batch effects: <ul style="list-style-type: none"> • Establish baseline performance • Ensure consistency between runs • Validate chemistry and protocol execution 	Sample-to-sample variation: <ul style="list-style-type: none"> • Normalize data across samples • Monitor technical biases within a run • Calibrate measurements and sensitivity
Benefits	<ul style="list-style-type: none"> • No foreign material added to the test sample • Establish performance for the entire batch by sequencing one sample • Sequence ahead of test samples • Low data output requirements 	<ul style="list-style-type: none"> • Assess sample-to-sample performance • No need to run a separate sample • Supports quantitative comparisons within and across runs
Limitations	<ul style="list-style-type: none"> • Does not capture sample-to-sample variability • Consumes a reaction for the control sample 	<ul style="list-style-type: none"> • Foreign material added to each sample • Consumes sequencing reads in every library • Requires careful selection of orthogonal sequences
Additional considerations	<ul style="list-style-type: none"> • Should be representative of expected sample performance and workflow • Doesn't track sample-to-sample variability within a run 	<ul style="list-style-type: none"> • Wasted reads in each sample • Orthogonal genomes required for positive and negative controls • Spike-ins must not cross-map with actual sample genome or target regions
Recommended controls	95:5 mix of human gDNA (CH methylation negative) and fully methylated lambda DNA <ul style="list-style-type: none"> • CpG sites in lambda as true positives • CH (CC, CT, CA) sites in NA12878 as true negatives 	<ul style="list-style-type: none"> • Fully methylated lambda • Unmethylated pUC19

Contact support@watchmakergneomics.com for additional information for recommendations of commercially available methylation controls

Revision History

Version	Description	Date
1.0	• First release	10/2025
1.1	<ul style="list-style-type: none"> • Update of shipping conditions • Clarification that input DNA be fragmented • Caution about effects of bead carryover into libraries generated in Steps 3.14 and 6.15 • Reduction in number of recommended PCR amplification cycles to generate sufficient library for target enrichment in Step 9.2 • Rewrite of Appendix A: Methylation Controls 	05/2026



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