Watchmaker DNA Library Prep Kit

with Fragmentation

Product Description

The Watchmaker DNA Library Prep Kit with Fragmentation is designed for the highly efficient conversion of DNA of variable quality into Illumina®-compatible sequencing-ready libraries. The chemistry and streamlined, single-tube protocol have been optimized to produce libraries from a broad range of inputs and sample types while minimizing bias and artifacts. The integrated enzymatic fragmentation, end-repair and A-tailing module produces fragmented, A-tailed DNA in a single reaction with a high degree of consistency. The ligation module adds full-length or truncated Illumina adapters (not included) to DNA fragments with industry-leading efficiency and without the need for a prior cleanup step.

The kit supports PCR-free workflows for input DNA of sufficient mass and quality. For workflows where library amplification is desirable or required, the kit includes the Equinox Amplification Master Mix (previously named Library Amplification Hot Start Master Mix (2X)). This ready-to-use mix contains an optimized PCR buffer and hot start enzyme formulation that enables library amplification with high fidelity and efficiency across a broad range of GC content and in the presence of a variety of paramagnetic beads. The hot start functionality inhibits both the polymerase and exonuclease activities of the enzyme, ensuring no loss of performance when amplification reactions are set up at room temperature.

Table of Contents

Kit Contents

Kit	Kit Code	Description	Component Volume	
			24 rxn kit	96 rxn kit
Watchmaker DNA Library Prep Kit with Fragmentation (PCR-free)[1]	**7K0013-024** (24 rxns) **7K0013-096** (96 rxns)	Frag/AT Buffer	110 µL	480 µL
		Frag/AT Enzyme Mix[2]	160 µL	720 µL
		Ligation Master Mix	530 µL	2.4 mL
Watchmaker DNA Library Prep Kit with Fragmentation (w/o primers)[3]	**7K0022-024** (24 rxns) **7K0022-096** (96 rxns)	Includes all components listed for **7K0013**, and:		
		Equinox Amplification Master Mix (2X)[4]	690 µL	3.0 mL
Watchmaker DNA Library Prep Kit with Fragmentation[5]	**7K0019-024** (24 rxns) **7K0019-096** (96 rxns)	Includes all components listed for **7K0022**, and:		
		P5/P7 Primer Mix (10X)	144 µL	600 µL

[1]Previously named **Library Preparation Kit – Enzymatic Fragmentation**
[2]Previously named **Frag/AT Enzymes**
[3]Previously named **Library Preparation and Amplification Kit – Enzymatic Fragmentation (w/o primers)**
[4]Previously named **Library Amplification Hot Start Master Mix (2X)**
[5]Previously named **Library Preparation and Amplification Kit – Enzymatic Fragmentation**

Product Applications

This workflow was developed to address unmet performance needs in next-generation sequencing (NGS) applications such as rare variant detection, single-cell analysis, and hybrid capture. These applications require high conversion efficiency, high library complexity, low bias, uniform coverage, and minimal experimental artifacts in order to support robust sensitivity and specificity. This library preparation kit is designed for high-performance use in germline and somatic whole-genome and targeted sequencing, is compatible with ultra-low inputs, and enables high conversion rates with low-quality DNA samples.

Watchmaker DNA Library Prep Kits with Fragmentation are ideally suited for:

- High-quality genomic DNA, DNA extracted from FFPE tissue, plasmid DNA and long PCR products
- A broad range of DNA inputs (1 ng – 500 ng)
- High- and low-complexity genomes, and genomes with extreme (15 – 85%) GC content
- -Whole-genome sequencing (including PCR-free workflows)
- Targeted sequencing protocols employing hybridization capture
- Workflows that employ unique molecular identifiers (UMIs) for improved sensitivity
- Amplicon sequencing

Storage and Handling

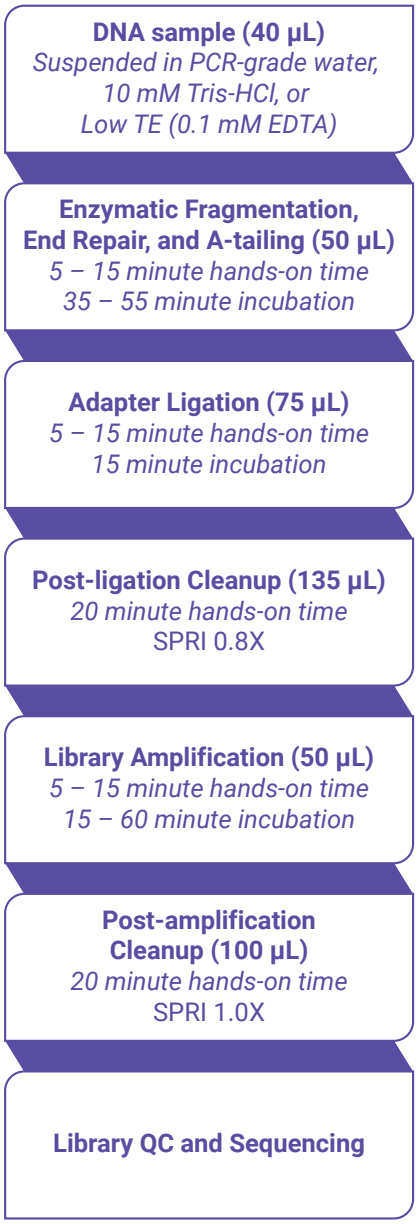
The Watchmaker DNA Library Prep Kit with Fragmentation is shipped on cold packs. Upon receipt, store all components at -20 ± 5°C.

Keep all components and reaction mixes on ice or a cooled reagent block during routine use. Many components are viscous; therefore, take care to homogenize solutions thoroughly before use and during reaction setup. Frag/AT buffer and P5/P7 Primer Mix (10X) should be vortexed for 5 sec before use. The Frag/AT Enzyme Mix, Ligation Master Mix and Equinox Amplification Master Mix (2X) should be inverted ten times prior to reaction setup. The combined fragmentation/end-repair and A-tailing reaction must be set up on ice. The accompanying ligation and library amplification reactions may be set up at room temperature.

The Frag/AT Buffer, P5/P7 Primer Mix (10X), and Equinox Amplification Master Mix (2X) can withstand >20 freeze-thaw cycles. The Frag/AT Enzyme Mix and ligation master mix should not freeze during storage, and these components do not tolerate multiple freeze thaw cycles.

When stored and handled as indicated, the product will retain full performance until the expiry date printed on the kit box.

Workflow Overview



Required Materials not Included

- Adapters (see **Prior to Starting** section for more detail)
- SPRI Purification Beads (see **Prior to Starting** section for more detail)
- 100% ethanol
- PCR-grade water or Low TE (0.1 mM EDTA); optional
- 10 mM Tris-HCl
- Magnetic rack compatible with 0.2 mL PCR tubes and/or 96 well plate
- Thermocycler
- Vortex mixer

Prior to Starting

Input DNA

This kit is compatible with a broad range of input amounts (1 ng – 500 ng). Conversion efficiencies should be >80% across this range for high-quality DNA inputs. 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.

> **NOTE:** Libraries have been successfully constructed using less than 1 ng of input DNA with the protocol outlined below.

The protocol outlined below applies to high-quality genomic DNA, PCR products, and plasmids.

This kit is compatible with DNA extracted from formalin-fixed paraffin-embedded tissues (FFPE). The quality of FFPE DNA can vary greatly depending on factors such as fixation, storage, and extraction method and this variation can have a significant impact on the fragmentation reaction. As a starting point, decrease fragmentation times by half to achieve the same insert size as for high-quality DNA. Fragmentation rates for FFPE DNA samples may vary depending on the quality of the DNA sample, and fragmentation time may have to be optimized for different batches of samples.

PCR-grade water, 10 mM Tris-HCl pH 8.0, and Low TE (0.1 mM EDTA) are the recommended buffers for DNA suspension. Higher EDTA concentrations will significantly slow the enzymatic fragmentation reaction.

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

Adapters

Watchmaker DNA Library Prep Kit with Fragmentation are compatible with adapters that have a 3' overhanging T to facilitate adapter orientation during dsDNA ligation. Note that adapter quality impacts overall library preparation efficiency. Ensure that adapters are adequately duplexed and at the appropriate concentration prior to use.

This workflow has been verified using full-length Illumina®-compatible adapters, where the P5 and P7 cluster generation sequences are added at the ligation step.

When using full-length adapters, a unique sample (sequencing) barcode is required for all samples to be sequenced on the same flow cell.

The workflow is compatible with truncated or 'stubby' adapters, though purification bead ratios may need to be tuned to account for molecular length differences, and user-supplied, uniquely indexed PCR primers will be required for the amplification of each library to be sequenced on the same flow cell.

Library Amplification Primers

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

- P5: AATGATACGGCGACCACCGA
- P7: CAAGCAGAAGACGGCATACGAGAT

For other primers, an annealing temperature gradient (55°C to 70°C) may be performed to determine the optimal condition for amplification.

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.

User-supplied 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.

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

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 (0.8X and 1X). 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.

Library Construction Protocol

Note: Ensure SPRI purification beads are fully equilibrated to room temperature and thoroughly resuspended via vortexing prior to use.

1. Enzymatic Fragmentation, End Repair and A-tailing (Frag/AT)

1.1 Remove all kit components from the freezer and thaw/equilibrate them on ice.

1.2 Vortex the Frag/AT Buffer for 5 seconds. Briefly centrifuge to collect all liquid in the bottom of the tube.

1.3 Invert the Frag/AT Enzyme Mix 10 times to homogenize, or briefly vortex the enzyme mix to ensure complete mixing. Briefly centrifuge to collect all liquid in the bottom of the tube.

1.4 Prepare input DNA in a total volume of 40 µL. Dilute DNA in PCR-grade water, 10 mM Tris-HCl pH 8.0, or Low TE (0.1 mM EDTA). Higher concentrations of EDTA will significantly slow the enzymatic fragmentation reaction.

1.5 Fragmentation may be performed at **either** 37˚C (Option 1) **OR** at 30˚C (Option 2) according to the tables below. Fragmentation temperature does not impact data quality. One temperature may be preferred over the other based on application or for operational reasons.

NOTE: As a starting point for sample DNA derived from FFPE sources, use half of the recommended fragmentation time for the desired insert size as a starting point. Fragmentation rates for FFPE DNA samples may vary depending on the quality of the DNA sample, and the fragmentation time may need to be optimized for different batches of samples.

Option 1: 37°C Incubation*

Time (min)	5	10	20	30
Mode insert size (bp)	300	250	200	150

OR

Option 2: 30°C Incubation*

Time (min)	3	4	5	10	15
Mode insert size (bp)	550	400	350	300	250

*Recommended fragmentation times/sizes are a starting point and may require optimization for different sample types/applications. Fragmentation time is independent of DNA input amount when working within the recommended input range of the kit.

1.6 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
Fragmentation, end repair and A-tailing	Refer to **Step 1.5**: either 37˚C or 30˚C may be used.	
	65°C	30 min
HOLD	4°C	HOLD

1.7 For each reaction, combine 4 µL of chilled Frag/AT Buffer and 6 µL of chilled Frag/AT Enzyme Mix to create a 5X Frag/AT master mix. Homogenize by vortexing at a moderate intensity (avoid bubbling) for 5 seconds. Prepare at least one extra reaction to ensure an adequate volume of 5X master mix for all reactions. This mixture is stable on ice for up to 4 hrs.

NOTE: When preparing a small number of reactions, it is acceptable to add Frag/AT Buffer and Frag/AT Enzyme Mix directly to each reaction without preparing a master mix.

1.8 Label a 0.2 mL PCR tube for each library to be prepared and place tubes on ice. To each tube, add the following in the order specified below.

Component	Volume (µL)
DNA sample	40
5X Frag/AT master mix	10

1.9 Vortex each reaction to homogenize the reaction. The consistency of this mixing step from tube to tube and experiment to experiment is important for consistent fragmentation results between samples. Briefly centrifuge to collect the sample at the bottom of the tube, then place back on ice.

NOTE: Complete mixing is critical to achieve desired fragment lengths. Mixing by pipetting is also acceptable. On ice, pipette 25 µL of the reaction up and down 10 times to mix completely.

1.10 Place reactions in the chilled thermocycler (programmed and initiated in **Step 1.6**). Advance the thermocycler from the initial 4°C hold to start the 30°C or 37°C incubation.

1.11 If required, prepare adapter stock solutions (**Step 2.3**) during the Frag/AT incubation.

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

2. Adapter Ligation

2.1 Invert the Ligation Master Mix ten times to homogenize (**DO NOT VORTEX**), and place on ice.

2.2 Remove the reaction tubes (from **Step 1.10**) from the thermocycler.

2.3 Prepare an appropriate volume of each adapter at the concentration specified in Table 1 using 10 mM Tris-HCl pH 8.0. 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. Adapter concentration by DNA input amount into library prep

DNA input (ng)	Adapter concentration*	Adapter:insert molar ratio
>10	15 µM	Up to 1500:1
1 – 10	3 µM	300 – 3000:1
<1	0.6 µM	>300:1

*Adapter-to-insert ratio estimated based on 300 bp fragment sizes.

2.4 Add 5 µL of appropriately diluted adapter to each reaction and mix thoroughly by brief vortexing or pipetting. Briefly centrifuge to collect all liquid in the bottom of the tube.

2.5 Add 20 µL of Ligation Master Mix to each reaction. The Ligation Master Mix is viscous. Carefully pipette a minimum of 40 µL up and down 10 times to ensure proper mixing.

2.6 Place the sample tubes in a thermocycler programmed as follows:

Step	Temperature	Time
Lid temperature	OFF	N/A
Ligation	20°C	15 min*

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

2.7 Proceed immediately to **Post-ligation Cleanup**.

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** SPRI beads to thoroughly mix. Add 60 µL (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. This ratio is recommended as a starting point when using high-quality DNA and full-length adapters.

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

3.4 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.

3.5 Carefully remove and discard the supernatant from each tube.

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

3.7 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.

3.8 Repeat **Steps 3.6 – 3.7** for a total of two washes.

3.9 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.

3.10 Remove sample tubes from the magnet and carefully resuspend each bead pellet in 22 µL of 10 mM Tris-HCl pH 8.0. Pipetting carefully will minimize bubbling and allow for greater library recovery.

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

3.12 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.

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

NOTE: If desired, library amplification may be carried out in the same tube in the presence of SPRI beads. See **Step 4** for reaction setup and **Appendix A** for details regarding PCR in the presence of beads.

3.14 Libraries not requiring amplification can proceed into QC and sequencing.

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

3.15 Libraries generated from low inputs and/or with truncated ("stubby") adapters will require amplification (**Step 4**) prior to qPCR-based library quantification and/or sequencing.

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. Library Amplification

4.1 Adapter-ligated libraries (recovered in **Step 3.13**) can be used as template DNA in the library amplification reaction described below.

NOTE: If your workflow requires library amplification in the presence of paramagnetic beads, refer to **Appendix A** for bead compatibility.

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

4.3 Program a thermocycler according to the table below.

Step	Temperature (°C)	Time (sec)	Cycles
Initial denaturation	98	45	1
Denaturation	98	15	See **Table 2**
Annealing	60[1]	30	
Extension	72	30 – 45[2]	
Final extension	72	60	1
-	12	Hold	-

[1]An annealing temperature of 60°C is recommended for standard Illumina® P5 and P7 primers. P5: AATGATACGGCGACCACCGA; P7: CAAGCAGAAGACGGCATACGAGAT. For other primers, an annealing temperature gradient (55°C to 70°C) may be performed to determine the optimal condition.

[2]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 need to be determined empirically.

Table 2: Recommended PCR cycle numbers based on DNA input into library prep

DNA input into library preparation (ng)	PCR cycles[1,2] to generate:	
	10 – 40 nM library	1 µg library
500	0	0 – 2
100	0 – 1	3 – 4
50	0 – 2	4 – 5
10	2 – 4	6 – 7
5	3 – 5	7 – 8
1	7 – 9	10 – 12
0.5[3]	7 – 9	11 – 12
0.1[3]	12	13

[1]Estimated final yields are based on a mode library insert size of ~300 bp (mode library fragment size of ~450 bp).

[2]To minimize amplification bias and other experimental artifacts, always use the fewest number of cycles required to produce enough library for QC, downstream processing, and archiving. This table is based on the empirical yield from library preparation using high-quality input DNA, including any reasonable losses or variability in sample preparation. Low-quality (FFPE) DNA may require more PCR cycles. The amount of adapter-ligated library may be determined by qPCR to more accurately inform the desired number of amplification cycles.

[3]Depending on the sample type and application, it is possible to reliably produce high-quality sequencing libraries from input amounts <1 ng. However, the quality and precise quantification of low-input samples greatly influences cycling parameters, and as a result may need to be optimized for specific applications.

4.4 Assemble each amplification reaction in the order specified below:

Component	Volume (50 µL reaction)
Adapter-ligated library	20 µL
P5/P7 Primer Mix (10X) or user-supplied primers[1]	5 µL
Equinox Amplification Master Mix (2X)	25 µL

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

4.5 Mix thoroughly and centrifuge briefly.

4.6 Place reactions in the thermocycler (programmed in **Step 4.3**) and run the program.

4.7 Proceed immediately to **Post-amplification Clean-up**.

5. Post-amplification Clean-up

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

5.2 Vortex **room temperature** SPRI beads to thoroughly mix. Add 50 µL (1X) of beads to each amplification reaction and mix thoroughly by pipetting.

NOTE: SPRI-to-sample bead ratios may be optimized for different applications or adapter configurations. This ratio is recommended as a starting point when using standard P5/P7 amplification primers.

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

5.4 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.

5.5 Carefully remove and discard the supernatant from each tube.

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

5.7 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.

5.8 Repeat **Steps 5.6 – 5.7** for a total of two washes.

5.9 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.

5.10 Remove sample tubes from the magnet and carefully resuspend each bead pellet in 22 µL of 10 mM Tris-HCl pH 8.0. Pipetting carefully will minimize bubbling and allow for greater library recovery.

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

5.12 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.

5.13 Carefully transfer each library-containing supernatant to a new tube.

5.14 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 3 details the various paramagnetic bead types evaluated.

Table 3. 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).

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

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