XCeloSeq® cfDNA Library Preparation Kit

SEQ001

FOR RESEARCH USE ONLY

Store at -20°C or 4°C - Component Dependant

Instructions for Use – English

Version 1.0 – March 2020

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1 Copyright and Trademarks

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2 Notices

For **Research Use Only**. Not for use in diagnostic procedures. Not intended to be used for treatment of human or animal diseases.

Safety data sheets pertaining to this product are available upon request.

The use of **caution** symbols identifies steps in the procedure where there is risk of assay failure if the protocol is not fully understood and followed.

The use of **stop** symbols indicates points in the protocol where it is safe to stop.

3 Upon Delivery

Immediately upon delivery remove the ATO Purification Beads (transparent tube, red cap) from the box and store at 4°C.

4 Intended Use

The XCeloSeq cfDNA Library Prep Kit is intended for use with the targeted enrichment of nucleic acids to generate high quality, high-complexity next-generation sequencing libraries suitable for use with Illumina next-generation sequencing (NGS) instruments.

This is a Research Use Only product.

5 XCeloSeq Technological Principle

All XCeloSeq products are built on the strongest technical foundation, **A**daptor **T**emplate **O**ligo **M**ediated **Seq**uencing – **ATOM-Seq**.

ATOM-Seq is a patented technology which uses a unique, advanced capture chemistry designed for compatibility with targeted enrichment of nucleic acid fragments to generate a sequencing ready NGS library. The chemistry underpinning ATOM-Seq has been developed to be specifically capable of capturing nucleic acid molecules from ultra-low input, highly fragmented, single and double strand, or highly damaged templates. These include both cfDNA and fragmented FFPE samples.

ATOM-Seq is entirely ligation independent and as such can avoid inefficiencies associated with, and has advantages over, ligation-capture based methods, ligation-amplicon based methods, and purely amplicon-based chemistries.

6 Kit Contents

6.1 Materials supplied with the kit

Reagent	Tube Colour	Tube Cap Colour	Storage Conditions
ATO - 1	Transparent	Blue	-20°C
ATO Reagents	Transparent	Amber	-20°C
ATO Enzyme	Transparent	Green	-20°C
ATO Treatment	Transparent	White	-20°C
Amplification Primers	Transparent	Yellow	-20°C
Universal Enzyme Mix	Transparent	Lilac	-20°C
ATO Purification Beads	Transparent	Red	4°C
ATO - 2	Transparent	Orange	-20°C

Reagents between different XCeloSeq kits are not interchangeable.
Only use “XCeloSeq cfDNA Library Prep Kit” reagents with the following protocol.

6.2 Indexes supplied with the kit

Reagent	Tube Colour	Tube Cap Colour	Storage Conditions	Index	Machine Compatibility
i5-L-001 (i5 Index Primer)	Amber	Blue	-20°C	ATCCGTAC	MiSeq, NovaSeq, HiSeq2500, HiSeq2000
				GTACGGAT	MiniSeq, NextSeq, HiSeq4000, HiSeq3000
i7-001 (i7 Index Primer)	Amber	Green	-20°C	CATAGCCG	All

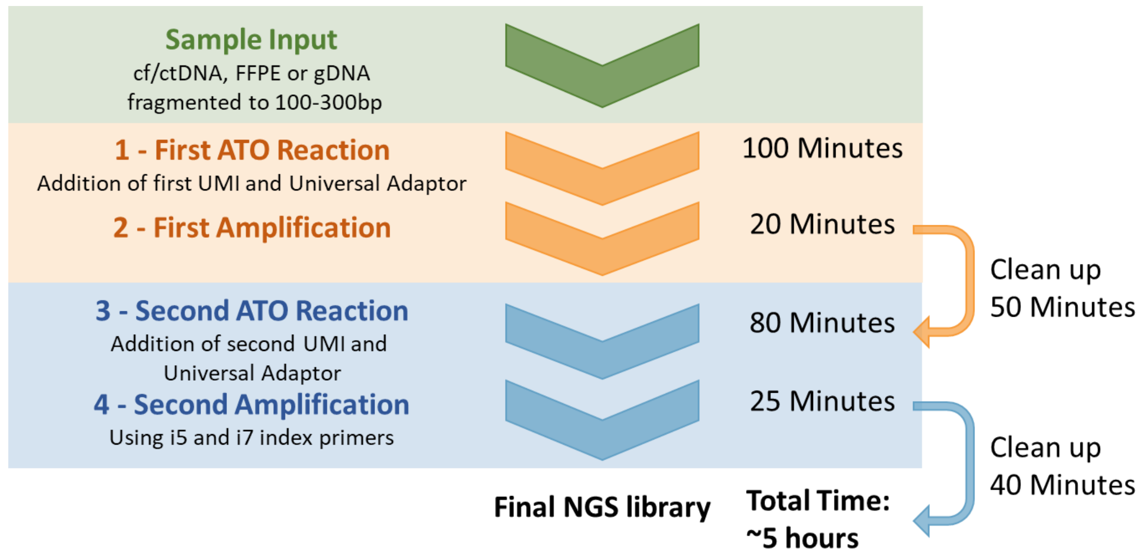
All SEQ005-i5-XN primers supplied in XCeloSeq Indexing Kits can directly replace the i5-L-001 primer supplied with this kit.

All SEQ005-i7-XN primers supplied in XCeloSeq Indexing Kits can directly replace the i7-001 primer supplied with this kit.

6.3 Additional equipment and reagents required (not provided in the kit)

- Reagents and equipment for specimen collection, filtration, and nucleic acid extraction
- Distilled water (molecular biology grade)
- 100% Ethanol (molecular biology grade)
- DNase and RNase-free pipette tips with aerosol barriers
- DNase and RNase-free tubes for preparing Reaction Mix
- Agencourt AMPure XP magnetic beads
- Suitable magnet (Thermofisher, Magnetic Stand-96, AM10027, or any suitable alternative)
- Pipettes, adjustable (P10, P20, P200 and P1000, or similar)
- Vortex mixer
- Microcentrifuge
- Standard PCR Thermal Cycler
- PCR tubes, plates, and accessories compatible with the PCR system used
- Ice

7 Protocol Overview



Times shown represent protocol duration for 10 ng of starting material.

Before Starting

8 Before Starting

- Read this protocol in its entirety before beginning the library preparation, ensuring that everything is prepared and that the process is clear before proceeding
- Ensure good laboratory practice is used at all times to prevent contamination of the samples or kit by any double or single stranded DNA (such as PCR products)
- Workstations and equipment should be clean, calibrated and in good working order. Cleaning products such as DNA AWAY (Thermofisher) may be used.
- All kit components should be fully thawed, mixed by vortexing, and briefly spun down before use
- All reagents should be kept on ice and steps should be performed on ice, unless stated otherwise
- Adding consistent and precise amounts of reagents is critically important for accurate results

Starting material: cfDNA, total cell free nucleic acids or enzymatically fragmented gDNA/FFPE are the recommended starting materials. The use of gDNA/FFPE which has been fragmented by either using sonication or other similar physical sheering methods is supported but **-is not recommended-** and will result in greatly reduced sensitivities.

The optimal starting template is DNA in EDTA-free buffer or Ultra-Pure Water. If EDTA buffers are used TE is suitable (10mM Tris-Hcl pH 8.0, 1mM EDTA).

*The recommended range of starting material is **5-50 ng** of cfDNA. Larger quantities of DNA should be used where possible to improve maximum sensitivity. Starting quantities of DNA should not exceed 50 ng. When using greater than or less than 5 ng of starting material Amplification Two cycle number can be adjusted depending on the desired library yields.*

Before starting it is necessary to ensure the following are prepared and ready for use.

- Freshly prepared 70% and 80% ethanol. Ensure the vessel is tightly closed when not in use to prevent unwanted evaporation

9 Operating Procedure

9.1 ATO 1 Reaction – Step 1: ATO 1 Reaction Mix and Incubation

- In a PCR vessel, add **1 µl 'ATO – 1' (transparent tube, blue cap)** to your DNA sample. The total volume of this mixture must not exceed 7.5 µl. If required add molecular biology grade water to a final volume of 7.5 µl
- Seal the PCR vessel using PCR caps, mix by vortexing, and centrifuge briefly. Samples should be sealed tightly to avoid evaporation and sample loss
- Incubate the mixture in a thermocycler as detailed in the table below:

Stage	Temperature	Duration
1	65 °C	2.5 minutes
2	10 °C	1 minute
3	4 °C	Hold

9.2 ATO 1 Reaction – Step 2: Enzyme and Reagent Addition

- Prepare the following mixture:

Order	Name	Tube colour	Tube Cap colour	Volume per single reaction (µl)
1	**ATO Reagents**	**Transparent**	**Amber**	**1.65**
2	**ATO Enzyme**	**Transparent**	**Green**	**1.1**
	Total Volume			**2.75**

- Enough reagents for a 10% excess of this mix are supplied
- Mix by vortexing, and centrifuge briefly
- Remove the sample from the thermocycler, carefully open the PCR vessel, and add **2.5 µl of the ATO Enzyme** and **ATO Reagents** mixture**.** The total volume of each sample will now be 10 µl
- Seal the PCR vessel, mix by vortexing, and centrifuge briefly
- Ensure that the thermocycler is precooled to 4 °C. Place your PCR vessel into the machine and continue to thermocycle as detailed at the top of the following page

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ATO 1 Reaction

Stage	Cycles	Temperature	Duration
1	-	4 °C	Hold/Pause
2	1x	10 °C	1 minute
2	1x	26 °C	6 minutes
3		30 °C	**10 minutes**
4		65 °C	1 minute
5		10 °C	1 minute
6		26 °C	6 minutes
7		30 °C	**10 minutes**
8	2x	65 °C	1 minute
9		10 °C	1 minute
10		26 °C	6 minutes
11		30 °C	**5 minutes**
12	-	4 °C	Hold

9.3 ATO 1 Reaction – Step 3: ATO 1 Treatment

- Remove the samples from the thermocycler and briefly spin down. Add **1 µl ATO Treatment (transparent tube, white cap)** to each sample. The total volume of each sample is now 11 µl
- Vortex, centrifuge and incubate the mixture in a thermocycler as detailed in the table below:

Stage	Temperature	Duration
1	37 °C	20 minutes
2	25 °C	10 minutes
3	4 °C	Hold

Safe Stopping Point

After the incubation step has completed, samples can be stored at 4°C overnight. Samples must proceed to Amplification One within 24 hours.

Amplification One

9.4 Amplification One

- Remove the samples from the thermocycler and briefly spin down. Add **12.5 µl Universal Enzyme Mix (transparent tube, lilac cap)** and **1.5 µl Amplification Primers (transparent tube, yellow cap)** to each sample. The total volume per sample is now 25 µl
- Seal the PCR vessel, mix by vortexing, and centrifuge briefly
- Incubate the mixture in a thermocycler as detailed in the table at the top of the following page

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Stage	Cycles	Temperature	Duration
1	1x	98 °C	30 seconds
2	6x	98 °C	10 seconds
		65 °C	75 seconds
3	1x	65 °C	2 minutes
4	-	4 °C	Hold

9.5 Bead Purification

Before proceeding, 25 µl of **molecular biology grade water** must be added into the **Amplification 1** product from **step 9.4**. The volume of the sample will now be 50 µl.

1) Allow the **ATO Purification Beads (transparent tube, red cap)** to come to room temperature, then completely resuspend the beads by vortexing. **Note:** All bead purification steps must be performed at room temperature, and reagents should not be kept on ice.
2) Add 1.8X volumes (**90 µl**) of **ATO Purification Beads** to each reaction.
3) Vortex, or mix by pipetting each sample 15 times, to ensure a homogenous mixture of beads and sample.
4) Leave samples at room temperature for 20 minutes. If required, spin down briefly to collect sample in the bottom of the vial.
5) Place the samples on the magnet for 3 minutes, or until all beads have been collected.
6) Without disturbing the collected beads, use a pipette to collect and dispose of the supernatant. If the beads are disturbed, return the solution back into the vial and repeat the incubation to allow them to collect on the side of the tube again.
7) Whilst leaving the vial on the magnet, add **180 µl** of freshly prepared **70% ethanol**.
8) Incubate for 30 seconds at room temperature.
9) Carefully discard the supernatant, without disturbing the beads.
10) Repeat steps 7 – 9 two additional times.
11) After the third wash carefully remove as much of the residual ethanol as possible.
12) Allow the beads to air dry for 3 minutes. Take care not to over-dry the beads as this will have a significant effect on the overall yield of the purification.
13) Elute the DNA by resuspending the beads in **14 µl** of distilled water. Incubate the resuspended beads for 5 minutes at room temperature.
14) Place the samples on the magnet for 3 minutes, or until all the beads have been collected.
15) Carefully transfer **13 µl** eluted amplification product into a clean tube.

9.6 ATO 2 Reaction – Step 1: ATO 2 Reaction Mix and Incubation

- Add **2 µl 'ATO – 2' (transparent tube, orange cap)** to the purified Amplification 1 product. Mix by vortexing, and centrifuge briefly, ensuring lids remain tightly sealed. The total volume of each sample is now 15 µl
- Incubate the mixture in a thermocycler as detailed in the table below:

Stage	Temperature	Duration
1	65 °C	2.5 minutes
2	10 °C	1 minute
3	10 °C	Hold

9.7 ATO 2 Reaction – Step 2: Enzyme and Reagent Addition

- Prepare the following mixture

Order	Name	Tube colour	Tube Cap colour	Volume per single reaction (µl)
1	**ATO Reagents**	**Transparent**	**Amber**	**3.3**
2	**ATO Enzyme**	**Transparent**	**Green**	**2.2**
	Total Volume			**5.5**

- Enough reagents for a 10% excess of this mix are supplied
- Mix by vortexing, and centrifuge briefly
- Remove the sample from the thermocycler, carefully open the PCR vessel, and add **5.0 µl** of the **ATO Enzyme** and **ATO Reagents** mixture. The total volume of each sample will now be 20 µl
- Seal the PCR vessel, mix by vortexing, and centrifuge briefly
- Ensure that the thermocycler is precooled to 4 °C. Place the PCR vessel into the machine and continue to thermocycle as detailed below:

Stage	Temperature	Duration
1	4 °C	Hold/Pause
2	10 °C	1 minute
3	26 °C	6 minutes
4	30 °C	10 minutes
5	65 °C	1 minute
6	10 °C	1 minute
7	26 °C	6 minutes
8	30 °C	10 minutes
9	-	Hold

9.8 ATO 2 Reaction – Step 3: ATO 2 Treatment

- Remove the samples from the thermocycler and briefly spin down. Add **2 µl ATO Treatment (transparent tube, white cap)** to each sample. The total volume of each sample is now 22 µl
- Seal the PCR vessel, mix by vortexing, and centrifuge briefly
- Incubate the mixture in a thermocycler as detailed in the table at the top of the following page

Stage	Temperature	Duration
1	37 °C	20 minutes
2	25 °C	10 minutes
3	4 °C	Hold

9.9 Amplification Two

When preparing the sample mixes, different combinations of i7 and i5 primers can be used to allow for sample multiplexing. When using XCeloSeq i5 or i7 Indexing Kits the supplied i5 and i7 oligos can directly substitute the oligos used in this protocol, as detailed in the table below.

- Prepare the Amplification 2 Mix by adding reagents to the sample from step 9.8 according to the order in the table below

Order	Name	Tube colour	Tube Cap colour	Volume per single reaction (µl)
1	**Product of Step 9.8**	N/A	N/A	**22.0**
2	**Universal Enzyme Mix**	**Transparent**	**Lilac**	**25.0**
3	**i7-001 (i7 Primer)**	**Amber**	**Green**	**1.5**
4	**i5-L-001 (i5 Primer)**	**Amber**	**Blue**	**1.5**
	Total Volume			**50.0**

- Seal the PCR vessel, mix by vortexing, and centrifuge briefly
- Incubate the mixture in a thermocycler as detailed in the table below

Stage	Cycles	Temperature	Duration
1	1x	98 °C	30 seconds
2	Various, see table below	98 °C	10 seconds
3		60 °C	30 seconds
4		65 °C	75 seconds
5	1x	65 °C	2 minutes
6	-	4 °C	Hold

The recommended PCR cycle number depends on the amount of starting material (see table below). It also depends upon the quality of the starting material and the desired library yield. The below table contains recommended starting values, but these may have to be adjusted by the individual user.

Input cfDNA (ng)	Recommended Cycle number
20-50	5 – 7
10	7 – 8
5	8 – 9

9.10 Bead Purification

1) Allow the AMPure Beads to come to room temperature, then completely resuspend the beads by vortexing. **Note:** All bead purification steps must be performed at room temperature and reagents should not be kept on ice.
2) Add **0.9X** volumes (**45 µl**) of AMPure beads to each reaction.
3) Vortex, or mix by pipetting each sample 15 times, to ensure a homogenous mixture of beads and sample.
4) Leave samples at room temperature for 5 minutes. If required spin down briefly to collect sample in the bottom of the vial.
5) Place the samples on the magnet for 3 minutes, or until all the beads have been collected.
6) Without disturbing the collected beads, use a pipette to collect and dispose of the supernatant. If the beads are disturbed, return the solution back into the vial and repeat the incubation to allow them to collect on the side of the tube again.
7) Add **50 µl** of distilled water and resuspend beads by pipetting each sample 15 times to ensure a homogenous mixture of bead and sample.
8) Add **0.7X** volumes (**35 µl**) of AMPure beads to each reaction.
9) Vortex, or mix by pipetting each sample 15 times, to ensure a homogenous mixture of beads and sample.
10) Leave samples at room temperature for 5 minutes. If required spin down briefly to collect sample in the bottom of the vial.
11) Place the samples on the magnet for 3 minutes, or until all the beads have been collected.
12) Without disturbing the collected beads, use a pipette to collect and dispose of the supernatant. If the beads are disturbed, return the solution back into the vial and repeat the incubation to allow them to collect on the side of the tube again
13) While leaving the vial on the magnet add **180 µl** of freshly prepared **80% ethanol**.
14) Incubate for 30 seconds. Ensure all beads remain gathered to the side of the vial.
15) Carefully discard the supernatant.
16) Repeat steps 13 - 15 one additional time.
17) After the second wash carefully remove as much of the residual ethanol as possible.
18) Allow the beads to air dry for 3 minutes. Take care not to over dry the beads, as this will have a significant effect on the overall yield of the purification.
19) Elute the DNA by resuspending the beads in **20 µl** of 10 mM Tris-HCL pH 8.0. Incubate the resuspended beads for 5 minutes at room temperature. **Note:** Higher elution volumes can be used depending on user requirements
20) Place the samples on the magnet for 3 minutes, or until all the beads have been collected.
21) Carefully transfer all the eluted amplification product into a clean tube.

The bead purified library is now ready for downstream processing, including bioanalyzer size analysis, qPCR quantification, and Illumina sequencing.

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10 Troubleshooting

10.1 Library yields are low or absent

When the kit reagents are stored as recommend, suitable starting material is used and the protocol is completed as stated in this IFU, the results are expected to be highly consistent and robust. Please ensure that the kit components are stored at the correct temperatures, that you are only using reagents supplied with the XCeloSeq cfDNA Library Preparation Kit (excluding the additional indexes), that the input quantity of the starting material is suitable, and that you carefully read and fully follow all steps in the IFU.

10.2 Low library yields when using sonicated DNA

Recommended starting material quantities are based off using cell free DNA or enzymatically fragmented gDNA. The XCeloSeq cfDNA Library Preparation Kit is compatible with DNA fragmented by sonication, however this can drastically reduce the proportion of DNA which is capturable. You should either use enzymatically fragmented DNA (if possible) or increase the starting quantity of sonicated DNA.

10.3 Low Library yields when using FFPE gDNA

Formalin Fixed Paraffin Embedded (FFPE) samples can be of highly variable quality. As such using larger quantities of enzymatically fragmented FFPE DNA may still result in low library yields, indicating that the proportion of capturable DNA in the sample is low. Using larger quantities of starting material can ensure that high quality libraries are generated.

11 Customer Contact Information

For all sales order processing, training and technical support enquiries, please contact the following:

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