XCeloSeq® cfDNA Library Preparation Kit with UDIs

For use with Unique Dual Indexing (UDI) Sets only

SEQ001

FOR RESEARCH USE ONLY

Store at -20 °C

or

Store at 2-8 °C - Component Dependant

Instructions for Use – English

IFU1852 Version 1.0 – January 2025

genefirst

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1. Copyright and Trademarks

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2. Notices

For **Research Use Only (RUO)**. 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, PC0034**) from the box and store at 2-8 °C.

All other components must be stored at -20 °C.

Please check for signs of damage. If damaged, please contact GeneFirst customer services or your local distributor. Do not use damaged kit components as they may not perform as expected.

4. Intended Use

The XCeloSeq cfDNA Library Prep Kit is intended for use along with any compatible XCeloSeq UDI Set (**See Section 7**) for the generation of 'whole genome', high-complexity, next-generation sequencing libraries suitable for use with Illumina next-generation sequencing (NGS) instruments.

This is a Research Use Only product.

5. Technological Principle

This XCeloSeq product is built on the strongest technical foundation,

Adaptor **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 'whole genome' capture 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 stranded, or highly damaged templates in a highly efficient process. This process is ideal for cell-free DNA samples.

ATOM-Seq's chemistry provides an advanced and superior method for capturing and enriching genetic sequences, not just from highly fragmented but also from single strand nucleic acid templates and from ultra-low quantities of starting material. ATOM-Seq is entirely ligation independent and as such can avoid inefficiencies associated with, as well as having advantages over, ligation-capture based methods and ligation-amplicon based methods.

The strength of ATOM-Seq is in the unique process of capturing the 3' ends of starting material, including single or double strand cell-free DNA, fragmented high quality genomic DNA (gDNA) or FFPE, or cDNA in a highly optimised chemistry. During this process both a Unique Molecular Identifier (UMI) and universal priming site are added directly to the 3' ends of the original DNA molecules.

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6. cfDNA Library Preparation Kit Contents

6.1. Materials supplied with the kit

Reagent	Tube Colour	Tube Cap Colour	Storage Conditions	Part Code
ATO - 1	Transparent	Blue	-20 °C	PC0028
ATO Reaction Mix	Transparent	Green	-20 °C	PC0199
ATO Treatment	Transparent	White	-20 °C	PC0031
Amplification Primers	Transparent	Yellow	-20 °C	PC0032
Universal Enzyme Mix	Transparent	Lilac	-20 °C	PC0033
ATO Purification Beads	Transparent	Red	⚠ 2-8 °C	PC0034
ATO - 2	Transparent	Orange	-20 °C	PC0035

Reagents are not interchangeable between different XCeloSeq kits.

Only use “XCeloSeq cfDNA Library Prep Kit” reagents with the following protocol.

Reagents are not interchangeable between different lots of the same XCeloSeq kit; ensure that reagents are used in the combination in which they were supplied.

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7. Additional Equipment and Reagents Required (Not Provided in the Kit)

At least one XCeloSeq UDI Set must be purchased to be used with this protocol.

7.1. Unique Dual Index Primers from UDI Sets

The XCeloSeq cfDNA Library Kit is designed to work with unique dual index combinations. These are purchased separately. To support multiplex sequencing, there are a range of UDI Sets available. Only XCeloSeq UDI Sets are recommended for use with XCeloSeq library preparation kits, as these have undergone design optimisations and validations to ensure quality and reliability of performance.

Up to 96 samples can be multiplexed together when purchasing all 12 kits. Each UDI combination contains enough reagent for 8 reactions for a total of 64 samples for every UDI set.

Product Name	Product Code
XCeloSeq UDI Set 2-01 for Illumina	IDX2-01
XCeloSeq UDI Set 2-02 for Illumina	IDX2-02
XCeloSeq UDI Set 2-03 for Illumina	IDX2-03
XCeloSeq UDI Set 2-04 for Illumina	IDX2-04
XCeloSeq UDI Set 2-05 for Illumina	IDX2-05
XCeloSeq UDI Set 2-06 for Illumina	IDX2-06
XCeloSeq UDI Set 2-07 for Illumina	IDX2-07
XCeloSeq UDI Set 2-08 for Illumina	IDX2-08
XCeloSeq UDI Set 2-09 for Illumina	IDX2-09
XCeloSeq UDI Set 2-10 for Illumina	IDX2-10
XCeloSeq UDI Set 2-11 for Illumina	IDX2-11
XCeloSeq UDI Set 2-12 for Illumina	IDX2-12

7.2. UDI Set Considerations for Sample Multiplexing

When determining how many UDI Sets are required to allow for different levels of sample multiplexing, please refer to the table below.

Number Of Samples to be Multiplexed in a Single Sequencing Run	Unique UDI Sets Required	Suggested Combination of UDI Sets
1 to 8	1	2-01 only
9 to 16	2	2-01 and 2-02
17 to 24	3	2-01 to 2-03
25 to 32	4	2-01 to 2-04
33 to 40	5	2-01 to 2-05
41 to 48	6	2-01 to 2-06
49 to 56	7	2-01 to 2-07
57 to 64	8	2-01 to 2-08
65 to 72	9	2-01 to 2-09
73 to 80	10	2-01 to 2-10
81 to 88	11	2-01 to 2-11
89 to 96	12	2-01 to 2-12

7.3. UDI Set Considerations for Total Number of Processed Samples

When determining UDI Set requirements for processing a fixed number of samples, please refer to the table below as an example. (Note: Please do still consider multiplexing requirements using the previous table).

Total Number of Samples to be Processed	Total Number of UDI Sets Required
1 to 64	1
65 to 128	2
129 to 192	3
193 to 256	4
257 to 320	5
321 to 384	6
385 to 448	7
449 to 512	8
513 to 576	9
577 to 640	10
641 to 704	11
705 to 768	12

7.4. Additional Required Equipment and Reagents Provided by the User

- Reagents and equipment for specimen collection, filtration, and nucleic acid extraction
- Distilled water (molecular biology grade)
- 10 mM tris-HCl pH 8.0 (molecular biology grade)
- 100% ethanol (molecular biology grade)
- DNase and RNase-free pipette tips with aerosol barriers
- AMPure® XP magnetic beads (Beckman Coulter, A63880 or equivalent)
- 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. Heated lid should always be on and set to ≥100 ^{o}C
- PCR tubes, plates, and accessories compatible with the PCR system used
- Ice

For library visualisation and quantification.

- Agilent® 2100 Bioanalyzer® (Agilent, cat. no. G2939BA) and Agilent High Sensitivity DNA Kit (Agilent cat. no. 5067-4626)

  or

  Agilent TapeStation® (Agilent, cat. no. G2991AA) and High Sensitivity D1000 ScreenTape Assay

- NEBNext® Library Quant Kit for Illumina® (NEB, catalogue # E7630L)

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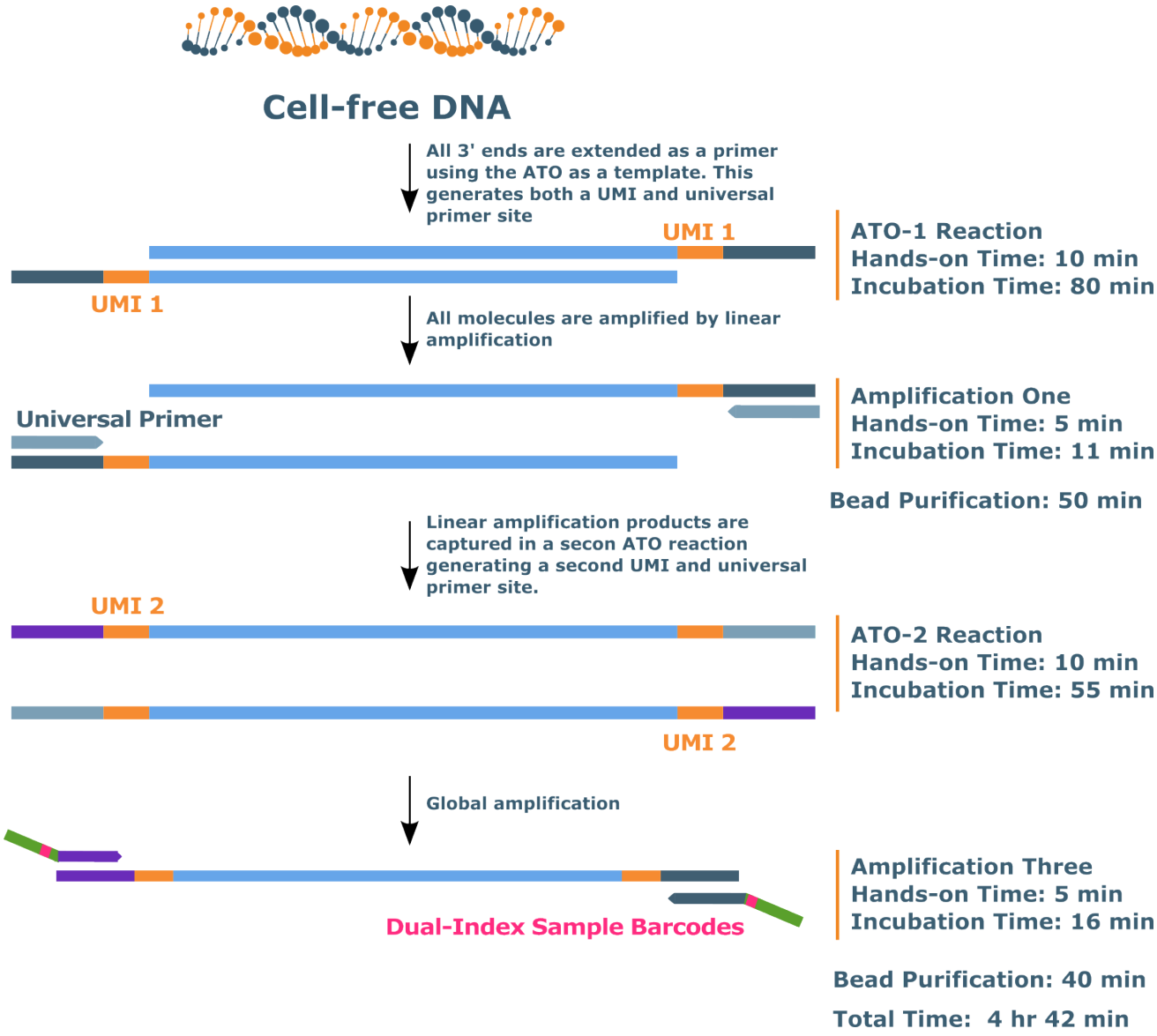
8. Protocol Overview



The above representative process is illustrative of the steps undertaken when following the Operating Procedure in Section 10.

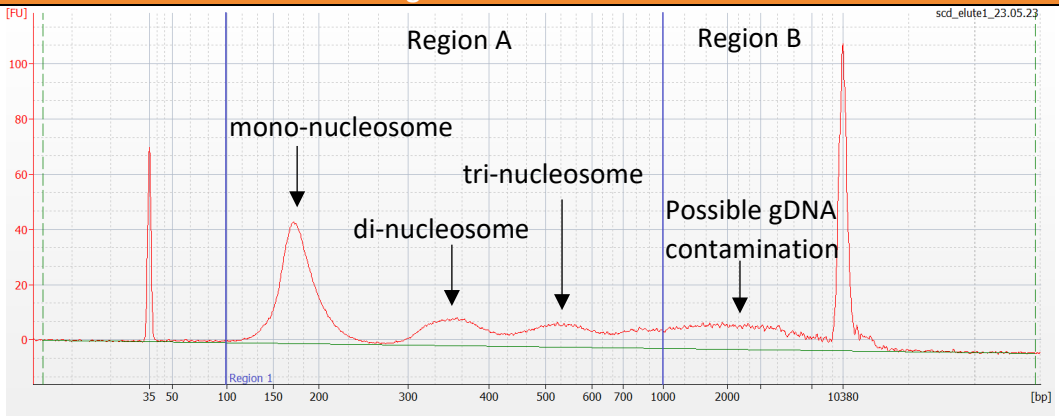
9. Before Starting

- Read this protocol in its entirety before beginning the library preparation to ensure everything is prepared and the process is clear prior to beginning
- Ensure good laboratory practice is used at all times to prevent contamination of the samples or kit by any double or single strand DNA (such as PCR products)
- Workstations and equipment should be cleaned, 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

9.1. Input Material

It is important to quantify the concentration of DNA in a sample to determine its suitability for use with the XCeloSeq cfDNA Library Prep Kit with UDIs. Unfortunately, approaches such as NanoDrop are not ideally suitable as a means of performing this quantification. They can overestimate nucleic acid concentrations, are only applicable with higher concentration samples, and can be adversely influenced by contaminants in the sample. We recommend therefore that nucleic acids are at minimum quantified by a fluorometric-based method such as Qubit.

This protocol has been optimised for use with cell-free DNA (cfDNA) or total cell-free nucleic acids and as such they are each recommended starting material for this protocol. This protocol is **not compatible** with uracil-containing DNA such as sodium bisulfite treated DNA. Note that cfDNA extractions may be contaminated with high molecular weight DNA, which artificially inflates the reported DNA concentration but will not contribute significantly to library generation. If the proportion of the sample which is cfDNA is low and this is not accounted for, this will have a significant negative impact on the sensitivity of the assay. It is important therefore to assess the size profile of the starting DNA sample by a suitable electrophoresis method such the Agilent Bioanalyzer or TapeStation. This should be used to determine the portion of DNA in a nucleic acid extraction which is cfDNA (between 140-510 bp) relative to the proportion of high molecular weight contaminating genomic-DNA (>1,000bp). This is then used to determine the effective cfDNA concentration of the sample.

Starting Material	
Visualisation of cfDNA with a High Sensitivity DNA Kit	

Input DNA Mass as Measured by Qubit.	Proportion of DNA Assessed to be cfDNA by High Sensitivity DNA Bioanalyzer Chip	Effective Useable cfDNA Input Mass
50 ng	100.0%	50.0 ng
50 ng	75.0%	37.5 ng
50 ng	50.0%	25.0 ng
50 ng	25.0%	12.5 ng
50 ng	12.5%	6.25 ng

Information in the above table is representative only.

The recommended input range of cfDNA is **up to 50 ng**; however, input should always be maximised within this range to obtain maximum sensitivity. If samples are too dilute users should consider either concentrating the starting material or adjusting purification methods to generate more concentrated nucleic acids. cfDNA must be in no more than 1mM EDTA solution, ideally 10 mM Tris 1mM EDTA pH 8.0, or ultra-pure water.

This workflow is compatible with FFPE derived DNA and high quality gDNA. These materials must be enzymatically fragmented prior to beginning this protocol. The use of high quality gDNA or FFPE DNA which has been fragmented by either using sonication or other similar physical sheering methods **-is not compatible with this product-** and should not be used. Recommended range of enzymatically fragmented FFPE DNA is **5-50 ng**; input should be maximised where possible within this range to obtain maximum sensitivity. Enzymatically fragmented DNA must be in no more than 1mM EDTA solution, ideally 10 mM Tris 1mM EDTA pH 8.0, or ultra-pure water.

9.2. Reagent Preparation

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

- Freshly prepared 70% and 80% ethanol. Ensure vessels are tightly closed when not in use to prevent unwanted evaporation.
- Please ensure that both AMPure XP beads and ATO Purification Beads are given sufficient time to reach room temperature prior to use. A minimum recommended time is 20 minutes.

10. Operating Procedure

10.1. ATO 1 Reaction – Step 1: ATO and cfDNA Mixture

- In a PCR vessel, add **1 µl 'ATO – 1'** (Blue Cap, PC0028) 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, with a heated lid ≥100 °C:

ATO 1 Reaction – Step 1: Incubation Conditions

Stage	Temperature (°C)	Duration (min)
1	65	2.5
2	10	1
3	4	Hold

10.2. ATO 1 Reaction – Step 2: Addition of ATO Reaction Mix

- Remove the sample from the thermocycler, carefully open the PCR vessel, and add **2.5 µl of the ATO Reaction Mix** (Green Cap, PC0199). 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 in the following table, with a heated lid ≥100 °C

ATO 1 Reaction – Step 2: Incubation Conditions

Stage	Cycles	Temperature (°C)	Duration (min)
1	Hold	4	Hold
2	1	10	1
2	1	26	6
3		30	**10**
4		65	1
5		10	1
6		26	6
7		30	**10**
8	2	65	1
9		10	1
10		26	6
11		30	**5**
12	Hold	4	Hold

10.3. ATO 1 Reaction – Step 3: ATO 1 Treatment

- Remove the samples from the thermocycler and briefly spin down. Add **1 µl ATO Treatment** (White Cap, PC0031) 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, with a heated lid ≥100 °C:

ATO 1 Reaction – Step 3: Incubation Conditions

Stage	Temperature (°C)	Duration
1	37	15 min
2	4	Hold

After the incubation step has completed, samples can be stored at -20 °C overnight. Samples must proceed to Amplification One within 24 hours.

If stored at –20 °C, ensure samples are at room temperature and are briefly centrifuged before proceeding

10.4. Amplification One

- Remove the samples from the thermocycler and briefly spin down. Add **12.5 µl Universal Enzyme Mix** (Lilac Cap, PC0033) and **1.5 µl Amplification Primers** (Yellow Cap, PC0032) 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

Amplification One: Incubation Conditions

Stage	Cycles	Temperature (°C)	Duration
1	1	98	30 s
2	6	98	10 s
		65	75 s
3	1	65	2 min
4	Hold	4	Hold

10.5. Bead Purification

Before starting it is necessary to ensure that fresh **70%** Ethanol is prepared ready for use.

Also ensure the **ATO Purification Beads** (Red Cap, PC0034) have already come to room temperature and have been completely resuspended by vortexing prior to use. **Note:** All bead purification steps must be performed at room temperature and reagents should not be kept on ice.

1. First, add 25 µl of **molecular biology grade water** into the **Amplification 1** product from **Step 10.4**. The volume of the sample will now be 50 µl.
2. Add **90 µl** of **ATO Purification Beads** to each reaction. Vortex well or mix by pipetting each sample 15 times to generate a homogenous mixture of beads and sample that is consistent in colour.
3. Leave samples at room temperature for **20 minutes**. Once the incubation is complete, spin down briefly to collect sample in the bottom of the vial.
4. Place the samples on a magnet for **3 minutes**, or until all the beads have been collected.
5. Without disturbing the collected beads, use a pipette to collect and dispose of the supernatant. If the beads are disturbed, dispense the supernatant back into the vessel and repeat the incubation to allow them to collect on the side of the tube again.
6. Whilst leaving the vial on the magnet, add **180 µl** of freshly prepared **70% ethanol**.
7. Incubate for **30 seconds**. Ensure all beads remain gathered to the side of the vial.
8. Carefully discard the supernatant without disturbing the beads.
9. Wash the samples twice more, by repeating steps 7 – 9 two additional times.
10. After carefully discarding the second supernatant leave vials for **2 minutes** at room temperature to allow residual ethanol to collect at the bottom of the vessel.
11. Carefully inspect each well and without disturbing the beads completely remove all traces of the ethanol.

    Note: Residual ethanol is a PCR inhibitor and must not be carried over into subsequent reactions. A well-dried bead pellet appears dry but retains a rich, solid brown colour.
12. Allow the beads to air dry for **3 minutes**. If surfaces of the well do not appear dry, remove any droplets of ethanol with a fresh pipette tip, and incubate for a further 2 minutes.
13. To elute samples, remove from the magnet and add **14 µl** of either molecular biology grade H_2O or 10 mM Tris-HCL pH 8.0. Thoroughly resuspend the beads in the eluent by vortexing or pipetting to form a homogenous suspension. Incubate the fully resuspended beads for 5 minutes at room temperature.

    Note: Dry beads should resuspend quickly and easily. Over-dry beads appear cracked, dry, and flaky and may require a longer time to resuspend by extending the time spent vortexing or being pipetted.
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.

10.6. ATO 2 Reaction – Step 1: ATO 2 and Amplification One Mixture

- Add **2 µl 'ATO – 2'** (Orange Cap, PC0035) 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, with a heated lid ≥100 °C:

ATO 2 Reaction – Step 1: Incubation Conditions

Stage	Temperature (°C)	Duration (min)
1	65	2.5
2	10	1
3	10	Hold

10.7. ATO 2 Reaction – Step 2: Addition of ATO Reaction Mix

- Remove the sample from the thermocycler, carefully open the PCR vessel, and add **5.0 µl of the ATO Reaction Mix** (Green Cap, PC0199). 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, with a heated lid ≥100 °C:

ATO 2 Reaction – Step 2: Incubation Conditions

Stage	Temperature (°C)	Duration (min)
1	4	Hold/Pause
2	10	1
3	26	6
4	30	10
5	65	1
6	10	1
7	26	6
8	30	10
9	4	Hold

10.8. ATO 2 Reaction – Step 3: ATO 2 Treatment

- Remove the samples from the thermocycler and briefly spin down. Add **2 µl ATO Treatment** (White Cap, PCPC0031) 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, with a heated lid ≥100 °C

ATO 2 Reaction – Step 3: Incubation Conditions

Stage	Temperature (°C)	Duration (min)
1	37	15
2	4	Hold

10.9. Amplification Two

When preparing the sample mixes, different UDI Sets can be used to allow for sample multiplexing. When using compatible XCeloSeq UDI Sets (see Section 7.1), the oligos can directly substitute the oligos used in this step, as detailed in the table below.

- Prepare the Amplification Two Reaction Mixture by adding reagents to the sample from step 10.8 according to the order in the table below

Order	Name	Tube Cap Colour	Volume per Single Reaction (µl)	Part Code
1	Product of Step 10.8	N/A	22.0	NA
2	Universal Enzyme Mix	Lilac	25.0	PC0033
3	Pre-mixed UDI Primers	White	3.0	See Product Insert
		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, with a heated lid ≥100 °C

Amplification Two: Incubation Conditions

Stage	Cycles	Temperature (°C)	Duration
1	1	98	30 s
2	Various, see table below	98	10 s
3		60	30 s
4		65	75 s
5	1	65	2 min
6	Hold	4	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

10.10. Bead Purification

Before starting it is necessary to ensure that fresh **80%** Ethanol is prepared ready for use.

Also ensure AMPure XP beads have already come to room temperature and have been completely resuspended by vortexing prior to use. **Note:** All bead purification steps must be performed at room temperature and reagents should not be kept on ice.

1. Add **45 µl** of AMPure XP beads to each reaction. Vortex well or mix by pipetting each sample 15 times to generate a homogenous mixture of beads and sample that is consistent in colour.
2. Leave samples at room temperature for **5 minutes**. Once the incubation is complete, spin down briefly to collect sample in the bottom of the vial.
3. Place the samples on a magnet for **3 minutes**, or until all the beads have been collected.
4. Without disturbing the collected beads, use a pipette to collect and dispose of the supernatant. If the beads are disturbed, dispense the supernatant back into the vessel and repeat the incubation to allow them to collect on the side of the tube again.
5. Add **50 µl** of distilled water and resuspend beads by vortexing or by pipetting each sample 15 times to ensure a homogenous mixture of bead and sample. Beads should resuspend easily, however take care to thoroughly break up the bead pellet by vortexing or pipetting to form a homogenous suspension. Spin down very briefly to collect sample in the bottom of the vial – the beads may begin to form a pellet after centrifugation, but this is not cause for concern.
6. Add **35 µl** of AMPure XP beads to each reaction. Vortex well or mix by pipetting each sample 15 times to generate a homogenous mixture of beads and sample that is consistent in colour. If any bead pellet formed due to previous centrifugation steps, ensure now that it is resuspended thoroughly.
7. Leave samples at room temperature for **5 minutes**. Once the incubation is complete, spin down briefly to collect sample in the bottom of the vial.
8. Place the samples on a magnet for **3 minutes**, or until all the beads have been collected.
9. Without disturbing the collected beads, use a pipette to collect and dispose of the supernatant. If the beads are disturbed, dispense the supernatant back into the vessel and repeat the incubation to allow them to collect on the side of the tube again.
10. While leaving the vial on the magnet add **180 µl** of freshly prepared **80% ethanol**.
11. Incubate for **30 seconds**. Ensure all beads remain gathered to the side of the vial.
12. Carefully discard the supernatant without disturbing the beads.
13. Wash the samples twice more, by repeating steps 10-12 two additional times.
14. After carefully discarding the second supernatant leave vials for **2 minutes** at room temperature to allow residual ethanol to collect at the bottom of the vessel.

15. Carefully inspect each well and without disturbing the beads completely remove all traces of the ethanol.

    Note: Residual ethanol is a PCR inhibitor and must not be carried over into subsequent reactions. A well-dried bead pellet appears dry but retains a rich, solid brown colour.

16. Allow the beads to air dry for **3 minutes**. If surfaces of the well do not appear dry, remove any droplets of ethanol with a fresh pipette tip, and incubate for a further 2 minutes.
17. To elute samples, remove from the magnet and add **20 µl** of TE Buffer (10 mM Tris-HCL pH 8.0 and with 1 mM or 0.1 mM EDTA). Thoroughly resuspend the beads in the eluent by vortexing or pipetting to form a homogenous suspension. Incubate the fully resuspended beads for 5 minutes at room temperature
18. Place the samples on the magnet for 3 minutes, or until all the beads have been collected.
19. Carefully transfer all the eluted amplification product into a clean tube.

11. Library QC, Visualisation, and Sequencing

11.1. Final Library Information

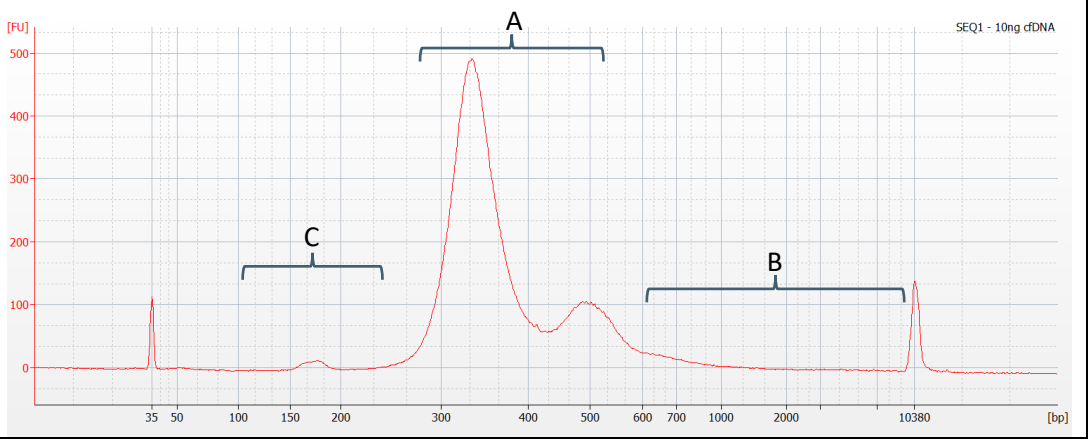
The average library size for cfDNA is expected to be 250-300 bp, and is composed of the following elements;

i) The Read 1 and Read 2 primer binding sites (dark blue), two 8 bp indexes (pink), and the P5 and P7 flow cell grafting sequences (green), for a total of 134 bp
ii) The UMIs, which are designed to be at least 8 bp in length each
iii) The insert which is variable length and on average 100 bp

11.2. Final Library Visualisation

Capillary electrophoresis is recommended for the visualisation of the final libraries. Either an Agilent Bioanalyzer High Sensitivity DNA Chip, Agilent High Sensitivity D1000 ScreenTape Assay, or equivalent.

Below are representative profiles of cell-free DNA and of a final library generated using cfDNA.

Final Libraries	
Visualisation of final library with a High Sensitivity DNA Kit	

A) Major final library peak. For cfDNA an initial larger peak should be between the 300 bp and 400 bp, with a secondary smaller markers on both visualization platforms.

B) Varying height secondary “bump” or “wave”. For sample with the highest input mass it is possible that primers may start to become exhausted. This leads to the generation of single-strand products which migrate differently to double-strand products.

C) ATOM-Seq workflows are expected to have very little “primer dimer”. If it is present it will be visible in this region.

The bead-purified Amplification 2 product is now ready for downstream processing.

We recommend the use of an Agilent Bioanalyzer High Sensitivity Chip (or equivalent) for determining the size distribution of the generated libraries. Library concentration should be determined by a suitable method, we recommend a qPCR quantification method such as NEBNext® Library Quant Kit for Illumina (#E7630) prior to sequencing. Any other method such as assessing library concentration from DNA concentration or directly from Bioanalyzer data may lead to significantly inaccurate concentrations and lower cluster densities and poor sequencing output.

For sequencing use the read length for each sequencing stage as set out in the table below. Sequencing depth should be determined by the user depending on experiment requirements.

Sequencing Stage	Read Length
Read 1 (R1)	151
Index Read 1 (I1)	8
Index Read 2 (I2)	8
Read 2 (R2)	151

12. Troubleshooting

12.1. 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 and XCeloSeq UDI Sets (see Section 7.1 for further explanation), that the input quantity of the starting material is suitable, and that you carefully read and fully follow all steps in this IFU.

12.2. Low library yields when using FFPE gDNA

Formalin Fixed Paraffin Embedded (FFPE) samples can be of highly variable quality. As such 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 help ensure that high quality libraries are generated.

12.3. Low library yields or failed library prep when using sonicated DNA

Recommended starting material quantities are based off using cell-free DNA or enzymatically fragmented high quality genomic DNA or FFPE DNA. The XCeloSeq cfDNA Library Preparation Kit is not compatible with DNA fragmented by sonication. You should ensure the starting material has been enzymatically fragmented.

12.4. Size distribution of starting material

This library preparation kit has been designed for using cfDNA as input, which has a peak size of approximately 171 bp. When using enzymatically fragmented DNA, ideal size distributions should be determined on an individual case basis by the user. The average insert size can be up to 400-600 bp.

13. Symbols

Symbol	Description	Symbol	Description
	Consult instructions for use		Upper limit of storage temperature
	Catalogue number		Storage temperature range
	Date of manufacture		Batch code
	Manufacturer		Number of supplied reactions
	Use-by-date		Do not use if package damaged

14. Customer Contact Information

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

GeneFirst Limited
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Abingdon Science Park,
Abingdon,
Oxfordshire,
OX14 3YS
United Kingdom

Customer Service & Sales Enquiries:
Telephone: +44 (0)1865 407 400
Email: sales@genefirst.com