



XCeloSeq[®] cfDNA Library Preparation Kit

SEQ001

FOR RESEARCH USE ONLY

Store at -20 °C

or

Store at 2-8 °C - Component Dependant

Instructions for Use – English

IFU1852 Version 2.0 – June 2026

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2. Revision History

Version	Date	Description
2.0	June 2026	<p>This kit is now sold in a two-box format, where Box 1 is stored at -20 °C and Box 2 is stored at 2-10 °C (See Sections 5 and 8).</p> <p>A larger volume of beads is provided, such that they can be used during both Bead Purification steps (Procol Steps 12.5 and 12.10). To reflect this change in usage, the name of this reagent has been changed from ATO Purification Beads to Purification Beads.</p> <p>Language and formatting revised for UDIs (Section 9.1) and Sample Requirements (Section 11.1).</p> <p>Updated document visual style and formatting throughout.</p>

3. Copyright and Trademarks

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4. 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.



5. Upon Delivery

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.

5.1. Items requiring storage at -20 °C

XCelSeq cfDNA Library Prep Kit (Box 1 of 2) (REF: SEQ001) must be stored at -20 °C upon arrival.

5.2. Items requiring storage at 2-10 °C

XCelSeq cfDNA Library Prep Kit (Box 2 of 2) (REF: SEQ001-BDX) must be stored at 2-10 °C upon arrival. This box must not be frozen.

6. Intended Use

The XCelSeq cfDNA Library Preparation Kit is intended for use along with any compatible XCelSeq UDI Set (**See Section 9.1**) for the generation of high-complexity whole-genome next-generation sequencing libraries suitable for use with Illumina next-generation sequencing (NGS) instruments.

This is a Research Use Only product.

7. Technological Principle

This XCelSeq product is built on the strongest technical foundation,

A adaptor Template Oligo Mediated Sequencing – **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-free 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.


8. XCeloSeq cfDNA Library Preparation Kit Contents

This product is provided in two boxes:

8.1. XCeloSeq cfDNA Library Prep Kit (Box 1 of 2) (REF: SEQ001)

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 - 2	Transparent	Orange	-20 °C	PC0035

8.2. XCeloSeq cfDNA Library Prep Kit (Box 2 of 2) (REF: SEQ001-BDX)

Reagent	Tube colour	Tube cap colour	Storage conditions	Part code
Purification Beads	Transparent	Red	 2-8 °C	PC0774

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

9.1. Unique Dual Index (UDI) Primers from UDI Sets



The i5 and i7 primers required for Amplification Two in this protocol (**Step 12.9**) are sold separately as XCeloSeq UDI Sets (Ref: IDX2-01 to IDX2-12).

The number of UDI Sets required depends on both the total number of reactions to be performed and the number of samples to be multiplexed on the same sequencing run. Each XCeloSeq UDI Set supports 64 reactions, divided between 8 unique combinations of i5 and i7 oligo. The table below explains how to determine which kits are required:

Total number of reactions	Maximum number of samples multiplexed	Quantity of UDI kits to purchase	Suggested product codes to order
Up to 64	Up to 8	1	IDX2-01 to 01
Up to 128	Up to 16	2	IDX2-01 to 02
Up to 192	Up to 24	3	IDX2-01 to 03
Up to 256	Up to 32	4	IDX2-01 to 04
Up to 320	Up to 40	5	IDX2-01 to 05
Up to 384	Up to 48	6	IDX2-01 to 06
Up to 448	Up to 56	7	IDX2-01 to 07
Up to 512	Up to 64	8	IDX2-01 to 08
Up to 576	Up to 72	9	IDX2-01 to 09
Up to 640	Up to 80	10	IDX2-01 to 10
Up to 704	Up to 88	11	IDX2-01 to 11
Up to 768	Up to 96	12	IDX2-01 to 12



Note that only XCeloSeq UDI Sets IDX2-01 to IDX2-12 are compatible with this workflow. Other XCeloSeq UDI Sets cannot be used with this workflow.

9.2. 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
- 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 °C
- PCR tubes, plates, and accessories compatible with the PCR system used
- Ice

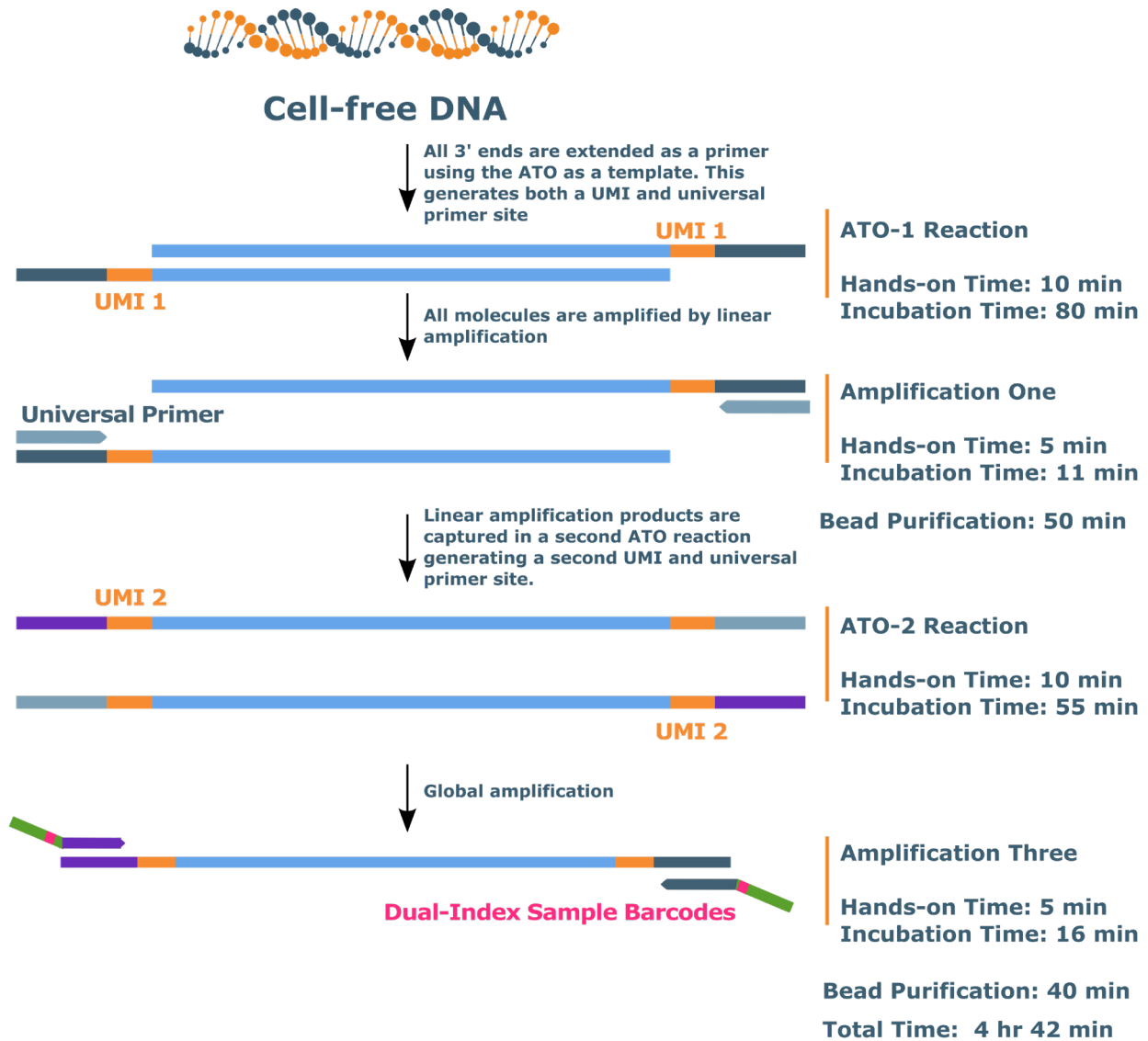
For visualisation of final libraries, either of:

- Agilent® 2100 Bioanalyzer® (Agilent, cat. no. G2939BA) and Agilent High Sensitivity DNA Kit (Agilent cat. no. 5067-4626)
- Agilent TapeStation® (Agilent, cat. no. G2991AA) and High Sensitivity D1000 ScreenTape Assay

For quantification of final libraries:

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

10. Protocol Overview



The above representative process is illustrative of the steps undertaken when following the Operating Procedure in **Section 12**.

11. 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
- Follow good laboratory practices to prevent contamination of the samples or kit by any double or single stranded 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

11.1. Input material

11.1.1. Compatible sample types

This workflow is intended and optimised for use with cell-free DNA, including DNA from cell-free total nucleic acid samples. It is also compatible high-quality DNA or DNA from FFPE-preserved tissue; note that all high-molecular-weight DNA samples require **enzymatic** fragmentation before use.

Samples should be stored in solutions of no more than 1mM EDTA, such as 10 mM Tris 1mM EDTA (pH 8.0) or ultra-pure water.

11.1.2. Incompatible sample types



This workflow is incompatible with:

- DNA containing uracil, such as sodium bisulphite-treated DNA,
- High-molecular-weight DNA that has not been fragmented,
- DNA fragmented by physical sheering methods such as sonication.

11.1.3. Recommended input mass

5 – 50ng of DNA can be used as input. The mutation sensitivity of the assay is proportional to the mass of DNA used, meaning it is always **highly recommended to maximise input up to 50ng**. Reducing the input mass reduces the mutation sensitivity of the final library.

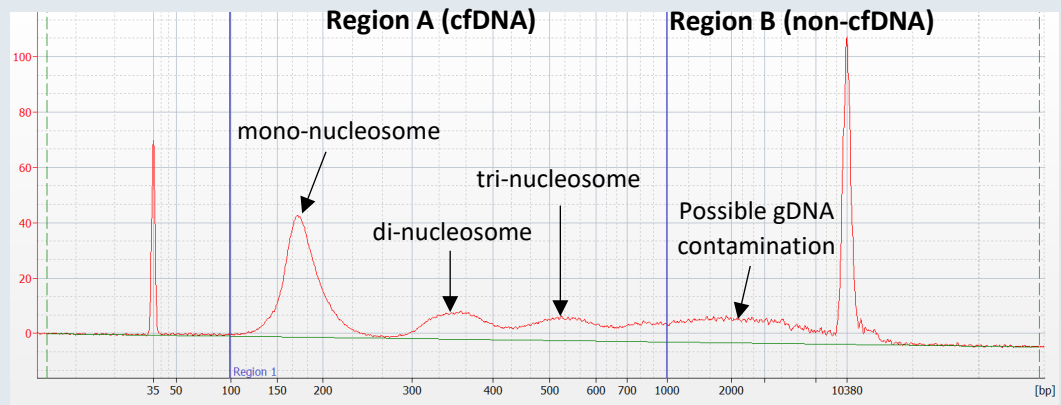
For dilute samples, consider concentrating existing samples or adjusting future purification methods to generate concentrated nucleic acids.

11.1.4. Recommendations for sample QC

We recommend quantifying sample DNA using fluorometric methods such as the Thermofisher Qubit, and visualising size distribution using the Agilent TapeStation/Bioanalyzer or equivalent.

When using cfDNA, be aware that gDNA contamination may be present and must be excluded when calculating the mass of the sample. As shown overleaf, cfDNA in **Region A** is recognisable as mono/di/tri-nucleosome peaks at approximately 140-510 bp. In contrast, most gDNA contamination is over 1000bp in **Region B**.

Visualisation of cfDNA with a High Sensitivity DNA Kit on the Agilent Bioanalyzer



The table below shows how the Qubit sample mass should be adjusted to compensate for gDNA contamination.

Input DNA mass as measured by Qubit.	Proportion of total DNA shown to be cfDNA by Tapestation / Bioanalyzer	Effective 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.

11.2. 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 Purification Beads are given sufficient time to reach room temperature prior to use. A minimum recommended time is 20 minutes.

12. Operating Procedure

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

- In a PCR vessel, combine the components according to the order in the table below. The combined volumes of cfDNA and molecular biology grade water must equal 6.5 μ l.

Order	Component	Tube cap colour	Volume per reaction (μ l)
1	cfDNA Sample	--	Up to 6.5
2	Molecular biology-grade water	--	Variable
3	ATO - 1	Blue	1
Total			7.5

- Seal the PCR vessel, 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

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

- Remove the sample from the thermocycler, briefly spin down and carefully open the PCR vessel.
- Add components to product from **Step 12.1** as detailed below.

Order	Component	Tube cap colour	Volume per reaction (μ l)
1	Product from Step 12.1	--	7.5
2	ATO Reaction Mix	Green	2.5
Total			10

- 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
3	1	26	6
4		30	10
5		65	1
6		10	1
7		26	6
8		30	10
9	2	65	1
10		10	1
11		26	6
12		30	5
13	Hold	4	Hold

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

- Remove the sample from the thermocycler, briefly spin down and carefully open the PCR vessel.
- Add components to product from **Step 12.2** as detailed below.

Order	Component	Tube cap colour	Volume per reaction (µl)
1	Product from Step 12.2	--	10
2	ATO Treatment	White	1
Total			11

- Seal the PCR vessel, mix by vortexing, and centrifuge briefly.
- 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

12.4. Amplification One

- Remove the sample from the thermocycler, briefly spin down and carefully open the PCR vessel.
- Add components to product from **Step 12.3** as detailed below.

Order	Component	Tube cap colour	Volume per reaction (µl)
1	Product from Step 12.3	--	11
2	Universal Enzyme Mix	Lilac	12.5
3	Amplification Primers	Yellow	1.5
Total			25

- Seal the PCR vessel, mix by vortexing, and centrifuge briefly.
- Vortex, centrifuge and incubate the mixture in a thermocycler as detailed in the table below, with a heated lid ≥ 100 °C.

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

12.5. Bead Purification One



Before starting, prepare fresh **70% Ethanol** and allow the **Purification Beads** (Red Cap, PC0774) to reach room temperature for at least 20 minutes.

All bead purification steps must be performed at room temperature. Reagents should not be kept on ice.

Purification Beads should always be vortexed immediately before use to ensure they are completely resuspended – if they are allowed to settle, vortex them again.

Bind Amplification One products to Purification Beads

- 1) Add **25 µl** of **molecular biology grade water** into the product from **Step 12.4**. The volume of the sample will now be 50 µl
- 2) Add **90 µl** of room temperature **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**.
Avoid centrifuging the sample until the incubation is complete, as this can cause the beads and sample to separate.
- 4) Once the incubation is complete, spin down briefly to collect the sample at the bottom of the vial. Place the samples on a magnet until the solution is completely clear, typically for **3 minutes**.
- 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.

Wash beads

Wash the samples by repeating steps 6-8 three times: Wash 1 (✓) Wash 2 (✓) Wash 3 (✓)

- | | | | | |
|----|---|--------------------------|--------------------------|--------------------------|
| 6) | Whilst leaving the vial on the magnet, add 180 µl of freshly prepared 70% ethanol . | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 7) | Incubate for 30 seconds . Ensure all beads remain gathered to the side of the vial. | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 8) | Carefully discard the supernatant without disturbing the beads. | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

Dry beads and remove residual ethanol

- 9) After carefully discarding the third supernatant, leave the vials for **2 minutes** at room temperature to allow residual ethanol to collect at the bottom of the vessel.
- 10) Carefully inspect each well and completely remove all traces of the ethanol without disturbing the beads.
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.
- 11) 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**.

Elute samples from beads

- 12) Remove the samples from the magnet and add **14 µl** of either **molecular biology-grade H₂O** or **10 mM Tris-HCL pH 8.0**. Thoroughly resuspend the beads in the eluent by vortexing or pipetting to form a homogenous suspension.

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.

- 13) Incubate the fully resuspended beads for **5 minutes** at room temperature.
- 14) Place the samples on a magnet until the solution is completely clear, typically for **3 minutes**.
- 15) Carefully transfer **13 µl** eluted amplification product into a clean tube

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

- Briefly spin down the sample if necessary and then carefully open the PCR vessel.
- Add components to product from **Step 12.5** as detailed below.

Order	Component	Tube cap colour	Volume per reaction (µl)
1	Product from Step 12.5	--	13
2	ATO – 2	Orange	2
Total			15

- 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.

ATO 2 Reaction – Step 1: Incubation Conditions

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

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

- Remove the sample from the thermocycler, briefly spin down and carefully open the PCR vessel.
- Add components to product from **Step 12.6** as detailed overleaf.

Order	Component	Tube cap colour	Volume per reaction (µl)
1	Product from Step 12.6	--	15
2	ATO Reaction Mix	Green	5
Total			20

- 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

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

- Remove the sample from the thermocycler, briefly spin down and carefully open the PCR vessel.
- Add components to product from **Step 12.7** as detailed below.

Order	Component	Tube cap colour	Volume per reaction (µl)
1	Product from Step 12.7	--	20
2	ATO Treatment	White	2
Total			22

- Seal the PCR vessel, mix by vortexing, and centrifuge briefly.
- Incubate the mixture in a thermocycler as detailed below, with a heated lid ≥ 100 °C.

ATO 2 Reaction – Step 3: Incubation Conditions

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

12.9. Amplification Two

- Remove the sample from the thermocycler, briefly spin down and carefully open the PCR vessel.
- Add reagents to the sample from **Step 12.8** according to the order in the table below.

Order	Component	Tube cap colour	Volume per reaction (µl)
1	Product from Step 12.8	--	22.0
2	Universal Enzyme Mix	Lilac	25.0
3	UDI Primers* (See Section 9.1)	White	3.0
Total			50.0

** If multiplexing, use a different UDI for each sample.*

- 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

12.10. Bead Purification Two



Before starting, prepare fresh **80% Ethanol** and allow the **Purification Beads** (Red Cap, PC0774) to reach room temperature for at least 20 minutes.

All bead purification steps must be performed at room temperature. Reagents should not be kept on ice.

Purification Beads should always be vortexed immediately before use to ensure they are completely resuspended – if they are allowed to settle, vortex them again.

Bind Amplification Two products to Purification Beads

1) Add **45 µl** of room temperature **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.

2) Leave samples at room temperature for **5 minutes**.

Avoid centrifuging the sample until the incubation is complete, as this can cause the beads and sample to separate.

3) Once the incubation is complete, spin down briefly to collect the sample at the bottom of the vial. Place the samples on a magnet until the solution is completely clear, typically for **3 minutes**.

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 room temperature **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.

7) Leave samples at room temperature for **5 minutes**.

Avoid centrifuging the sample until the incubation is complete, as this can cause the beads and sample to separate.

8) Once the incubation is complete, spin down briefly to collect the sample at the bottom of the vial. Place the samples on a magnet until the solution is completely clear, typically for **3 minutes**.

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.

Wash beads

Wash the samples by repeating steps 10-12 two times:

Wash 1 (✓)

Wash 2 (✓)

10) Whilst 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

Dry beads and remove residual ethanol

- 13) After carefully discarding the second supernatant, leave the vials for **2 minutes** at room temperature to allow residual ethanol to collect at the bottom of the vessel.
- 14) Carefully inspect each well and completely remove all traces of the ethanol without disturbing the beads.
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.
- 15) 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**.

Elute samples from beads

- 16) Remove the samples from the magnet and add **20 µl of TE Buffer** (10 mM Tris-HCL pH 8.0 with 0.1-1 mM EDTA). Thoroughly resuspend the beads in the eluent by vortexing or pipetting to form a homogenous suspension.
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.
- 17) Incubate the fully resuspended beads for **5 minutes** at room temperature.
- 18) Place the samples on a magnet until the solution is completely clear, typically for **3 minutes**.
- 19) Carefully transfer all the eluted amplification product into a clean tube.

The bead-purified Amplification Two product is the final sequencing library. It is now ready for downstream processing such as library QC, visualisation, sequencing and analysis.

13. Library QC, Visualisation, and Sequencing

13.1. Final Library Structure

The average library size for cfDNA is expected to be 250-300 bp. Each library molecule is composed of the following elements:

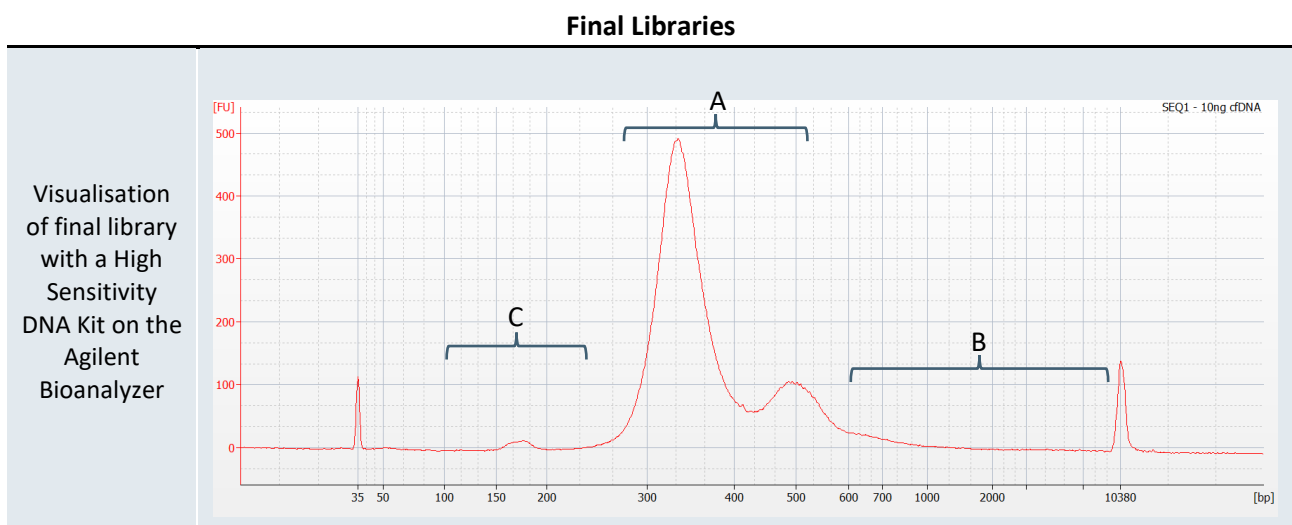
- i) Necessary Illumina sequencing adaptors for a total of 134 bp
 - Green: The P5 and P7 flow cell grafting sequences
 - Pink: Unique dual i5 and i7 indexes, each 8 bp in length
 - Blue: Read 1 and Read 2 primer binding sites
- ii) Variable length UMIs (orange)
 - Designed to be at least 8 bp, can be up to 20 bp in length
- iii) The insert (cyan)
 - Variable length, on average 100 bp



13.2. Final library visualisation

Capillary electrophoresis is recommended for the visualisation of the final libraries. This can be performed using an Agilent Bioanalyzer High Sensitivity DNA Chip, Agilent High Sensitivity D1000 ScreenTape Assay, or an equivalent assay.

Below is a representative profile of a final library generated using cfDNA.



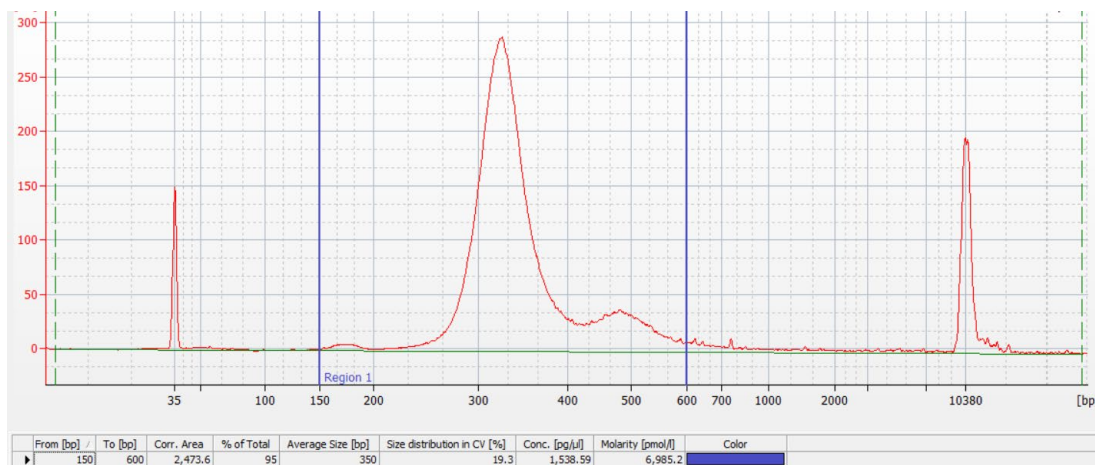
The regions of the profile highlighted in the image are:

- A) The major final library peak(s). For cfDNA, an initial larger peak should be between the 300 bp and 400 bp, representing mono-nucleosomal cfDNA.
- B) A secondary “bump” or “wave” of varying height, most common in samples prepared with large input masses. This occurs if UDI primers become exhausted during Amplification Two, creating single stranded DNA products whose size is reported incorrectly by dsDNA electrophoresis assays.
- C) ATOM-Seq workflows are expected to have very little “primer dimer.” If it is present, it will be visible in this region.

13.3. Determination of average library molecule length

After visualising the final library by capillary electrophoresis in **Section 13.2**, the average library molecule length should be calculated using a 150 – 600bp size window in the Bioanalyzer or TapeStation software.

Example of position of 150 bp to 600 bp window (cell-free DNA)



13.4. Library quantification

We recommend qPCR quantification using the Illumina P5 and P7 primers, using products such as the NEBNext® Library Quant Kit for Illumina (#E7630). This allows the greatest accuracy when loading samples to the Illumina flowcell.

Alternatively, the library concentration (determined by Qubit) and average molecule length (determined by Agilent TapeStation/Bioanalyzer) can be used to calculate library concentration. This is less accurate than qPCR, especially if the secondary bump (“Region B” in **Section 13.2**) is present.

Although the Agilent TapeStation and Bioanalyzer report library concentrations directly, these values are significantly less accurate. It is not recommended to use these values to quantify the libraries for the purposes of loading the Illumina flowcell.

13.5. Sequencing

The recommended sequencing depth is dependent upon your experimental requirements.

The read length for each sequencing stage is given in the table below.

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

14. Troubleshooting

14.1. 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 XCelSeq cfDNA Library Preparation Kit (**Section 8**) and XCelSeq UDI Sets (**Section 9.1**); that the input quantity of the starting material is suitable; and that you carefully read and fully follow all steps in this IFU.

14.2. 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.











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

The XCelSeq cfDNA Library Preparation Kit is not compatible with DNA fragmented by sonication. You should ensure the starting material has been enzymatically fragmented.

14.4. 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.

15. 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

16. Customer Support Information

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

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