XCeloSeq® Targeted cfDNA Enrichment V2 - Protocol

For Use with Second Generation ATOM-Seq Chemistry Only

FOR RESEARCH USE ONLY

Storage conditions are component dependent

Store at 2-10°C or -20°C. Please see box labels for details.

Instructions for Use – English

IFU2115 Version 2.0 – December 2024

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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

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.

Items requiring storage at -20 °C

XCeloSeq targeted cfDNA enrichment primers (See **Section 6.1**) must be stored at -20 °C upon arrival

XCeloSeq Targeted cfDNA Core Reagents V2 Box 1 of 2 (REF: GF020-V2) must be stored at -20 °C upon arrival.

Items requiring storage at 2-10 °C

XCeloSeq Targeted cfDNA Core Reagents V2 Box 2 of 2 (REF: GF020-BDX) must be stored at 2-10 °C upon arrival.

4. Intended Use

This XCeloSeq Targeted cfDNA Enrichment V2 Protocol is intended for use with XCeloSeq Targeted cfDNA Core Reagents V2, any compatible XCeloSeq Targeted cfDNA Enrichment Primers (**See Section 6.1**) and any compatible XCeloSeq UDI Set(s) (**See Section 7**). Together, these allow for the 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. Technological Principle

This XCeloSeq product is built on the strongest technological 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 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 stranded, or highly damaged templates in a highly efficient process. These include both cell-free DNA (cfDNA) and FFPE samples.

ATOM-Seq's chemistry provides an advanced and superior method for targeting and enriching specific 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 an ATO Reaction which involves capturing the 3' ends of starting material, including single or double strand cfDNA, fragmented high quality genomic or FFPE DNA, 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 molecule.

As opposed to amplicon-based approaches, where nucleic acid fragments must contain binding sites for two opposing primers for successful PCR amplification and capture to occur, ATOM-Seq has the advantage of needing only a single target specific primer. This, in combination with the universal primer site incorporated by ATOM-Seq, allows for amplification of both known and unknown sequences downstream of the target primer, even from highly fragmented samples. ATOM-Seq can therefore also be leveraged for the detection of unknown DNA combinations, such as those generated during genomic rearrangement events, including novel fusions, insertions, and deletions.

ATOM-Seq's unique method allows for independent targeting of sense and antisense strands of starting material for independent, dual-direction target coverage. ATOM-Seq overcomes the bias and errors introduced by DNA polymerases into NGS libraries by combining 1) the UMIs added onto the original starting material, which allows for correction of polymerase-introduced errors, and 2) the ability to independently target sense and antisense strands of DNA, to further enhance the confidence of identified variants.

The second-generation ATOM-Seq chemistry provides improved capture efficiency and useability though optimisations and refinement of the capture chemistry and enhancements to the downstream protocol.

6. XCeloSeq Targeted cfDNA Enrichment Kit V2 Reagents

XCeloSeq Targeted cfDNA Enrichment Kits V2 have a modular design comprising:

- The **XCeloSeq Targeted cfDNA Enrichment Primers** contains PCR enrichment primers specific to the DNA regions targeted in each individual kit.

- The **XCeloSeq Targeted cfDNA Core Reagents V2** has two (2) boxes:
  - **Box 1 of 2** contains all second-generation proprietary reagents for the ATO Reaction and downstream amplification steps.
  - **Box 2 of 2** contains sufficient purification beads for all recommended purification steps.

Reagents are **not interchangeable** between different XCeloSeq kits or lots. Only use reagents supplied as part of XCeloSeq Targeted cfDNA Core Reagents V2 and XCeloSeq Targeted cfDNA Enrichment Primers with this protocol.

6.1. XCeloSeq Targeted cfDNA Kits

The XCeloSeq targeted cfDNA enrichment primers are named according to the product purchased, see table below.

This protocol is for use with the following products:

Product number	XCeloSeq product name	Target genes	Target primers
Custom assays		Various	Various
SEQ030	Pan Cancer cfDNA Kit V2	100	1147
SEQ031	Colon Cancer cfDNA Kit V2	23	311
SEQ032	Lung Cancer cfDNA Kit V2	17	296
SEQ033	Breast Cancer cfDNA Kit V2	12	241

Please contact Customer Services if you would like to discuss our custom assay development services.

Each of the above kits comes with the following four reagents which should be used directly at the necessary point in this protocol:

Component	Tube cap colour	Storage conditions (°C)	Component REF
Pool 1 – Outer	Orange	-20	Refer to product insert
Pool 1 – Inner	Black	-20	
Pool 2 – Outer	White	-20	
Pool 2 – Inner	Yellow	-20	

6.2. XCeloSeq Targeted cfDNA Core Reagents V2

6.2.1. Box 1 of 2 (REF: GF020-V2)

The XCeloSeq Targeted cfDNA Core Reagents V2 Box 1 of 2 box contains the following reagents:

Component	Tube cap colour	Storage conditions (°C)	Tube REF
ATO	Blue	-20	PC0678
ATO Reaction Buffer	Amber	-20	PC0679
ATO Reaction Enzyme	Green	-20	PC0680
Amplification One Mix	Transparent	-20	PC0681
Primers	Red	-20	PC0682
Master Mix	Lilac	-20	PC0683

6.2.2. Box 2 of 2 (REF: GF020-BDX)

The XCeloSeq Targeted cfDNA Core Reagents V2 Box 2 of 2 box contains the following reagent:

Component	Tube cap colour	Storage conditions (°C)	Tube REF
Purification Beads	Amber	2-10	PC0728

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 targeted cfDNA enrichment V2 kits are designed to work with Unique Dual Index (UDI) combinations. These are purchased separately. To support multiplex sequencing, there are a range of UDI Sets. 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. Please see product insert of the associated product for storage conditions.

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

XCeloSeq product name	Product code
UDI Set 1-01 for Illumina	IDX1-01
UDI Set 1-02 for Illumina	IDX1-02
UDI Set 1-03 for Illumina	IDX1-03
UDI Set 1-04 for Illumina	IDX1-04
UDI Set 1-05 for Illumina	IDX1-05
UDI Set 1-06 for Illumina	IDX1-06
UDI Set 1-07 for Illumina	IDX1-07
UDI Set 1-08 for Illumina	IDX1-08
UDI Set 1-09 for Illumina	IDX1-09
UDI Set 1-10 for Illumina	IDX1-10
UDI Set 1-11 for Illumina	IDX1-11
UDI Set 1-12 for Illumina	IDX1-12

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	1-01 only
9 to 16	2	1-01 and 1-02
17 to 24	3	1-01 to 1-03
25 to 32	4	1-01 to 1-04
33 to 40	5	1-01 to 1-05
41 to 48	6	1-01 to 1-06
49 to 56	7	1-01 to 1-07
57 to 64	8	1-01 to 1-08
65 to 72	9	1-01 to 1-09
73 to 80	10	1-01 to 1-10
81 to 88	11	1-01 to 1-11
89 to 96	12	1-01 to 1-12

7.3. UDI Set Considerations for Total Numbers 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 Equipment and Reagents Provided by the User

For library preparation workflow:

- Reagents and equipment for specimen collection, filtration, and nucleic acid extraction
- Molecular biology grade water
- 10 mM Tris-HCl pH 8.0 (molecular biology grade)
- TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) (molecular biology grade)
- 100% Ethanol (molecular biology grade)
- DNase and RNase-free pipette tips with aerosol barriers
- DNase and RNase-free PCR tubes for preparing Reaction Mix
- 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 or other suitable temperature-controlled equipment for eppendorfs and PCR vessels

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) or equivalent

8. Protocol Overview

Cell-free DNA

All 3' ends are extended as a primer using the ATO as a template. This generates both a UMI and universal primer site

UMI

UMI

ATO Reaction
Hands-on Time: 5 min
Incubation Time: 101 min

All molecules are amplified by linear amplification

Amplification One
Hands-on Time: 5 min
Incubation Time: 29 min

Products are divded into two PCR reactions

Pool-1 enriches the sense DNA strand

Pool-2 enriches the antisense DNA strand

Multiplex target-specific primers

Amplification Two
Hands-on Time: 5 min
Incubation Time: 81 min

Bead Purification: 30 min

Nested PCR to further enrich the sense and antisense DNA strands

Dual-Index Sample Barcodes

Amplification Three
Hands-on Time: 5 min
Incubation Time: 70 min

Bead Purification: 30 min
Total Time: 6 hr

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 starting library preparation.
- It is vital to ensure all equipment and work areas are thoroughly cleaned to prevent contamination of the samples, kits or reactions by any double- or single-strand DNA. Potential sources of such contamination include PCR products from previous amplifications.
- To reduce the chance of contaminating a reaction ensure good laboratory practice is used at all times. The use of suitable cleaning products such as DNA AWAY™ (Thermofisher) can be used to clean laboratory surfaces and equipment including benchtops and pipettes.
- Where possible all equipment should be cleaned, calibrated and in good working order.
- 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.

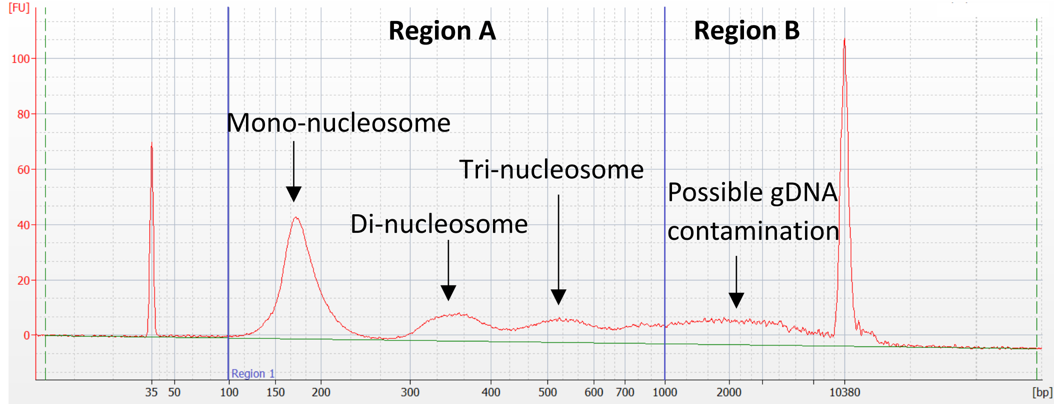
9.1. Input Material Considerations

It is important to quantify the concentration of cfDNA in a sample to determine its suitability for use with the XCeloSeq Targeted cfDNA Enrichment V2 protocol. Unfortunately, approaches such as NanoDrop are not ideal as a means of performing this quantification. They can overestimate nucleic acid concentrations, are only applicable to higher concentration samples, and can be adversely influenced by contaminants in the sample.

We recommend that nucleic acids are at minimum quantified by a fluorometric-based method such as Qubit. For cfDNA samples we also recommend a quality assessment though the use capillary electrophoresis, for example a Bioanalyzer or TapeStation System, as these instruments allow for the visualisation of the size distribution of the nucleic acids in your sample. As cfDNA can have high molecular weight DNA contamination, it is important to assess the size profile of the starting cfDNA sample to determine if there is sufficient material before starting.

Capillary electrophoresis allows the determination of the proportion of the sample which is capturable cfDNA. Pure cfDNA is expected to have a major peak at approximately 160-170 bp (a mono-nucleosome peak) and a smaller secondary peak at 300-310 bp (a di-nucleosome peak). A small proportion of cfDNA may be longer than 400 bp, however large quantities of longer DNA (especially greater than 1,000 bp) are likely to be high molecular weight contaminating genomic-DNA. If the proportion of the sample which is cfDNA is low and this is not accounted for when determining input mass, this will have a significant negative impact on the sensitivity of the assay.

Visualisation of cfDNA with a High Sensitivity DNA Kit



Input DNA mass as measured by Qubit	Proportion of DNA assessed to be cfDNA by capillary electrophoresis	Adjusted useable cfDNA input mass	Uncapturable gDNA contamination
50 ng	100%	~50.0 ng	~0.0 ng
50 ng	75.0%	~37.5 ng	~12.5 ng
50 ng	50.0%	~25.0 ng	~25.0 ng
50 ng	25.0%	~12.5 ng	~37.5 ng
50 ng	12.5%	~6.25 ng	~43.75 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 to obtain maximum sensitivity. If samples are too dilute, users should consider methods to concentrate the starting material or adjust purification methods to generate more concentrated nucleic acids, such as by reducing elution volumes (please refer to manufacture's protocol for available options). cfDNA must be in no more than 1 mM EDTA solution, ideally 10 mM Tris 1mM EDTA pH 8.0 or alternatively ultra-pure molecular biology grade water.

9.2. Reagent Preparation

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

- Freshly prepared 80% ethanol. Ensure the vessel is tightly closed when not in use to prevent unwanted evaporation
- Please ensure that all XCeloSeq 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 Reaction V2 – Step 1: cfDNA, ATO, and Buffer 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 17 µl:

Order	Component	Tube cap colour	Volume per reaction (µl)
1	cfDNA Sample	--	Up to 17.0
2	Molecular Biology Grade Water	--	Variable
3	ATO	Blue	1.5
4	ATO Reaction Buffer	Amber	1.5
	Total		20.0

- Seal the PCR vessel using PCR caps, mix by vortexing, and centrifuge briefly. Every well 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 Reaction – Step 1: Incubation Conditions

Stage	Temperature (°C)	Duration (min)
1	98	2
2	10	1
3	10	Hold

10.2. ATO Reaction V2 – Step 2: Addition of ATO Reaction Enzyme

- Remove the sample from the thermocycler, briefly spin down and carefully open the PCR vessel. Add components to product from **Step 10.1** as detailed below:

Order	Component	Tube cap colour	Volume per single reaction (µl)
1	Product from **Step 10.1**	--	20.0
2	ATO Reaction Enzyme	Green	2.0
	Total		22.0

- 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 table at the top of the following page, with a heated lid ≥100 °C.

ATO Reaction

ATO Reaction V2 – Step 2: Incubation Conditions

Step	Cycles	Temperature (°C)	Duration (min)
1	1	4	Hold/Pause
2		10	1
3		26	12
4		30	20
5	2	65	1
6		10	1
7		26	12
8		30	20
9	Hold	4	Hold

Amplification One

10.3. Amplification One

- Remove the samples from the thermocycler, briefly spin down and carefully open the PCR vessel. Add components to product from **Step 10.2** as detailed below:

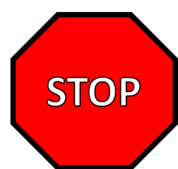
Order	Component	Tube cap colour	Volume per single reaction (µl)
1	Product from **Step 10.2**	--	22.0
2	Amplification One Mix	Transparent	26.0
3	Primers	Red	2.0
	Total		**50.0**

- Vortex the Amplification One Mixture and centrifuge briefly.
- 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	37	10 min
2	1	98	30 s
3	15	98	5 s
4		60	30 s
5		72	30 s
6	1	72	2 min
7	Hold	4	Hold

Safe Stopping Point



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

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

10.4. Amplification Two: Target-Specific PCR

WARNING!

Two separate reactions are required **per sample** to independently target the sense and antisense DNA strands, referred to as Pool 1 and Pool 2 reactions.

Before continuing, use the 50 µl Amplification One Product produced in **Step 10.3** to **prepare two 22 µl aliquots in fresh PCR vessels**, to be used in the Pool 1 and Pool 2 reactions. If the sample is not split before continuing to this step (**Step 10.4**), it will not be possible to continue with the library preparation.

- Prepare the Pool 1 - Outer Amplification Two Reaction Mixture by adding reagents to **one aliquot of Step 10.3 product**, according to the order in the table below:

Order	Component	Tube cap colour	Volume per single reaction (µl)
1	**Aliquot 1** of **Step 10.2** Product	--	22.0
2	Pool 1 – Outer	Orange	3.0
3	Master Mix	Lilac	25.0
	Total		50.0

- Prepare the Pool 2 - Outer Amplification Two Reaction Mixture by adding reagents to the **second aliquot of Step 10.3 product**, according to the order in the table below:

Order	Component	Tube cap colour	Volume per single reaction (µl)
1	**Aliquot 2** of **Step 10.2** Product	--	22.0
2	Pool 2 – Outer	White	3.0
3	Master Mix	Lilac	25.0
	Total		50.0

- Vortex the Amplification Two Mixtures 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		98	5 s
3	14	65	5 min
4		72	30 s
5	1	72	2 min
6	Hold	4	Hold

Amplification Two – Target Specific PCR

10.5. Bead Purification

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

Also ensure XCeloSeq Purification 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.

Bind Amplification Two products to Purification Beads

1)	Add **90 µl** of **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**. 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.

Wash beads twice with 80% ethanol

	Wash the samples by repeating steps 5 – 7 two times:	Wash 1	Wash 2
5)	While leaving the vial on the magnet, add **150 µl** of freshly prepared **80% ethanol.**	A	A
6)	Incubate for **30 seconds.** Ensure all beads remain gathered to the side of the vial.	B	B
7)	Carefully discard the supernatant without disturbing the beads.	C	C

Dry beads and remove residual ethanol

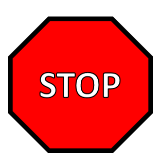
8)	After carefully discarding the second supernatant, leave the vials for **2 minutes** at room temperature to allow residual ethanol to collect at the bottom of the vessel.
9)	Carefully inspect each well and without disturbing the beads completely remove all traces of the ethanol. **Note:** Residual 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.
10)	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 then incubate for a further **2 minutes**.

Elute samples from beads

11)	To elute samples, remove from the magnet and add **23 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.
12)	Place the samples on the magnet for **3 minutes**, or until all the beads have been collected.
13)	Carefully transfer **21 µl** of the eluted amplification product into a clean PCR tube

Bead Purification

Safe Stopping Point



After the bead purification samples can be stored at –20 °C overnight.
Samples must proceed to Amplification Three within 24 hours.
If stored at –20 °C, ensure samples are at room temperature, vortexed and centrifuged before proceeding.

10.6. Amplification Three: Nested Target-Specific PCR

Two different reactions are required to target the sense and antisense DNA strands.

The reaction which used '**Pool 1** – Outer' in **Step 10.4** **must now use** '**Pool 1** – Inner'.

The reaction which used '**Pool 2** – Outer' in **Step 10.4** **must now use** '**Pool 2** – Inner'**.**

Using the wrong pool will result in a failure of the library preparation.

Note: When preparing the sample mixes below, the same UDI can be used in both Pool 1 and Pool 2 from the sample initial sample. Different samples must use different UDIs to allow for sample multiplexing. See **Section 7** for details.

- Prepare the Pool 1 - Inner Amplification Three Reaction Mixture by adding reagents to the bead purified sample from **Step 10.5**, according to the order in the table below:

Order	Component	Tube cap colour	Volume per single reaction (µl)
1	**Pool 1 – Outer** product of **Step 10.4**	--	21.0
2	Pool 1 – Inner	Black	2.0
3	Master Mix	Lilac	25.0
4	Pre-mixed UDI Primers	White	2.0
	Total		50.0

- Prepare the Pool 2 - Inner Amplification Three Reaction Mixture by adding reagents to the bead purified sample from **Step 10.5**, according to the order in the table below:

Order	Component	Tube cap colour	Volume per single reaction (µl)
1	**Pool 2 – Outer** product of **Step 10.4**	--	21.0
2	Pool 2 – Inner	Yellow	2.0
3	Master Mix	Lilac	25.0
4	Pre-mixed UDI Primers	White	2.0
	Total		50.0

- Vortex the Amplification Three Reaction Mixtures and centrifuge briefly.
- Incubate the mixture in a thermocycler as detailed in the table below, with a heated lid ≥100 °C:

Amplification Three: Incubation Conditions

Stage	Cycles	Temperature (°C)	Duration
1	1	98	30 s
2	Variable, See Next Table	98	5 s
3		65	5 min
4		72	30 s
5	1	72	2 min
6	Hold	4	Hold

Yields are expected to be high with this workflow. The number of cycles can be adjusted depending on users' needs. If the final yields are too low or too high, then the cycle number can be adjusted accordingly.

Suggested initial cycle numbers are shown in the table below.

Amplification Three: Recommended Amplification Cycles

Input cfDNA quantity	Recommended amplification three cycle number for cfDNA
5 ng	16
10 ng	15
20 ng	14
50 ng	13

10.7. Bead Purification

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

Also ensure XCeloSeq Purification 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.

Bind Amplification Three products to Purification Beads

1)	Add **60 µl** of **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**. 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.

Wash beads twice with 80% ethanol

	Wash the samples repeating steps 5 – 7 two times:	Wash 1	Wash 2
5)	While leaving the vial on the magnet add **150 µl** of freshly prepared **80% ethanol.**	A	A
6)	Incubate for **30 seconds.** Ensure all beads remain gathered to the side of the vial.	B	B
7)	Carefully discard the supernatant without disturbing the beads.	C	C

Dry beads and remove residual ethanol

8)	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.
9)	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.
10)	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 then incubate for a further **2 minutes**.

Elute samples from beads

11)	To elute samples, remove from the magnet and add **32 µl** of **TE** (10 mM Tris-HCL pH 8.0 containing 0.1 to 1mM 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. **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.
12)	Place the samples on the magnet for **3 minutes**, or until all the beads have been collected.
13)	Carefully transfer **30 µl** of the eluted amplification product into a clean PCR tube

11. Library QC, Visualisation, and Sequencing

11.1. Final Library Information

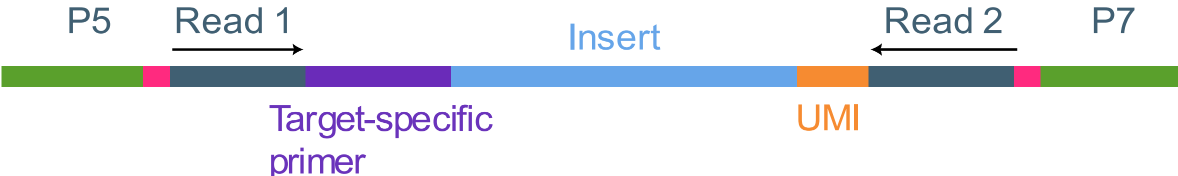
The average library size for cfDNA is expected to be 250-300 bp, and is composed of the following elements:

i) Necessary Illumina sequencing adaptors for a total of 134 bp.
   a. The P5 and P7 flow cell grafting sequences (green)
   b. Dual-unique 8 bp indexes (pink)
   c. Read 1 and Read 2 primer binding sites (dark blue)

ii) Variable length UMI (orange)
   Designed to be at least 8 bp, can be up to 20 bp in length

iii) The target specific primer (purple)
   On average is 25 bp in length

iv) The insert (cyan)
   Variable length between 80-100 bp

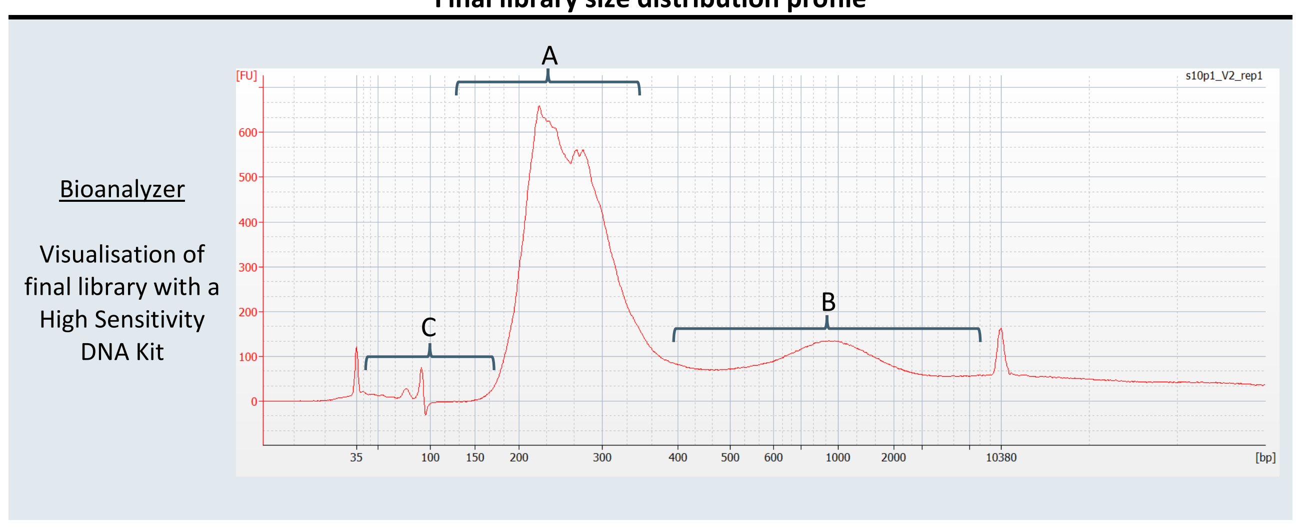


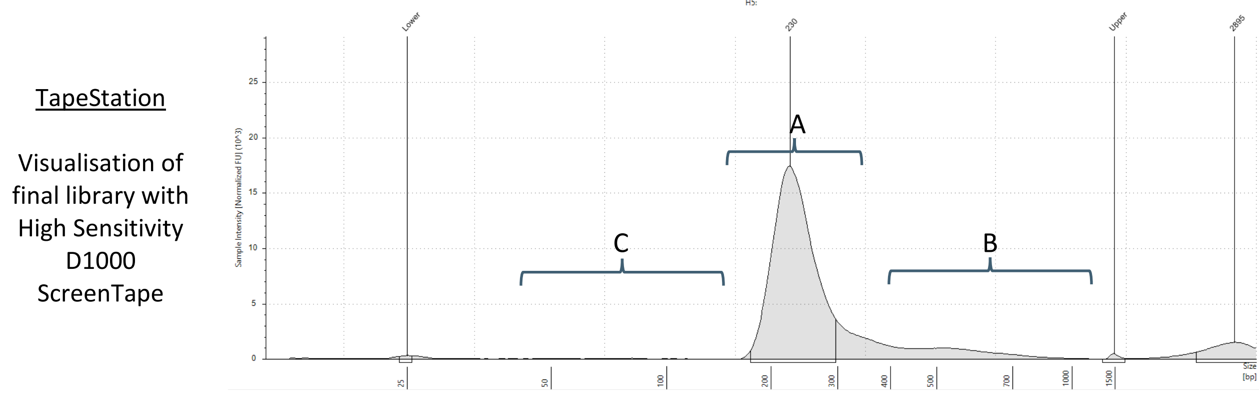
11.2. Final Library Visualisation

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 equivalent.

Below are representative profiles of a final library.

Final library size distribution profile





A) Major final library peak. For cfDNA this peak should be between the 200 bp and 300 bp markers on both visualization platforms.

B) Varying height secondary “bump” or “wave”. For samples with high input masses, 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 and give a false indication of larger double-strand DNA being present in your sample. These should not be considered when determining library size or concentration.

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

11.3. Determination of average insert size – Bioanalzyer

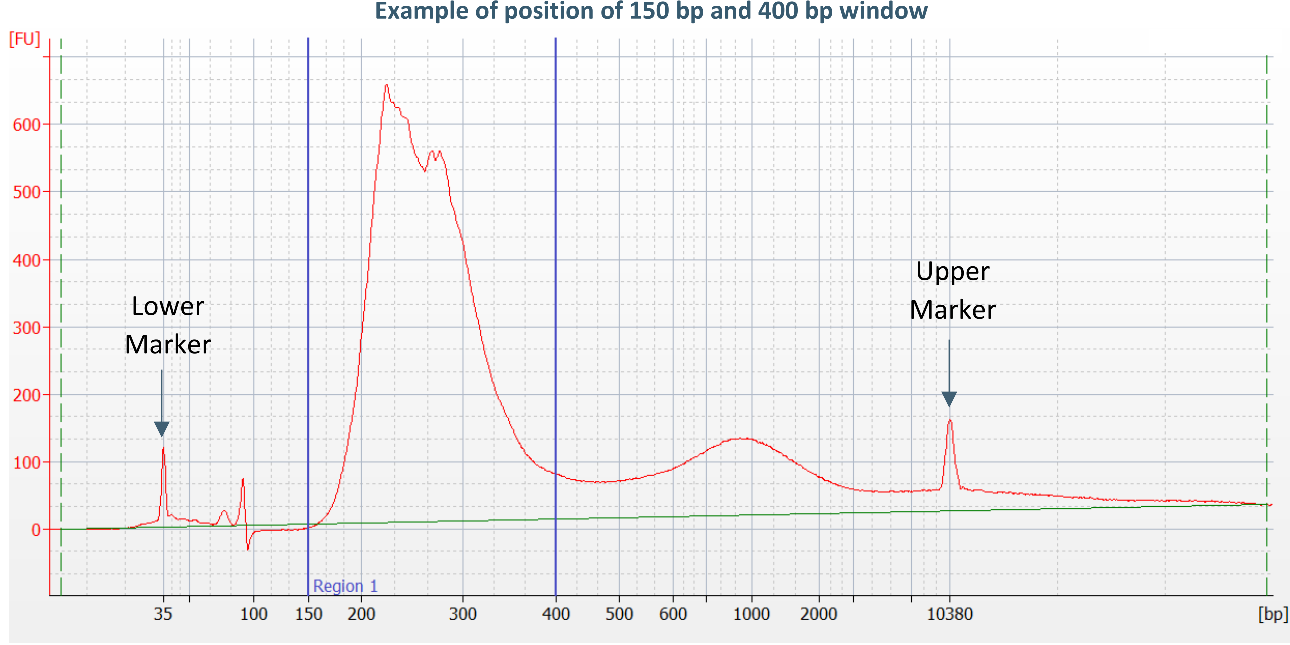
When determining average insert sizes, a size window of 150 bp to 400 bp should be used. Average insert size is highly consistent between cfDNA samples, in the range of 240-280 bp.

Pool 1 and Pool 2 are expected to have similar yields and average insert sizes.

Library	Size window	Expected average insert size
Pool 1	150-400 bp	240-280 bp
Pool 2	150-400 bp	240-280 bp

XCeloSeq Targeted cfDNA Enrichment libraries do not require any special handling when visualised using a High Sensitivity DNA Assay (Agilent Part Number 5067-4626; Agilent High Sensitivity DNA Kit). The Agilent protocol should be followed (Agilent High Sensitivity DNA Kit Guide).

After completion of a run ensure that all upper and lower markers have been correctly detected by the software, their position is indicated below. Please refer to the "Agilent 2100 Bioanalyzer System" manual for instructions. Failure to identify markers will result in inaccurate estimation of average insert size.



11.4. Determination of average insert size – TapeStation

When determining average insert sizes, a size window of 150 bp to 400 bp should be used. Average insert size is highly consistent between cfDNA samples, in the range of 240-280 bp.

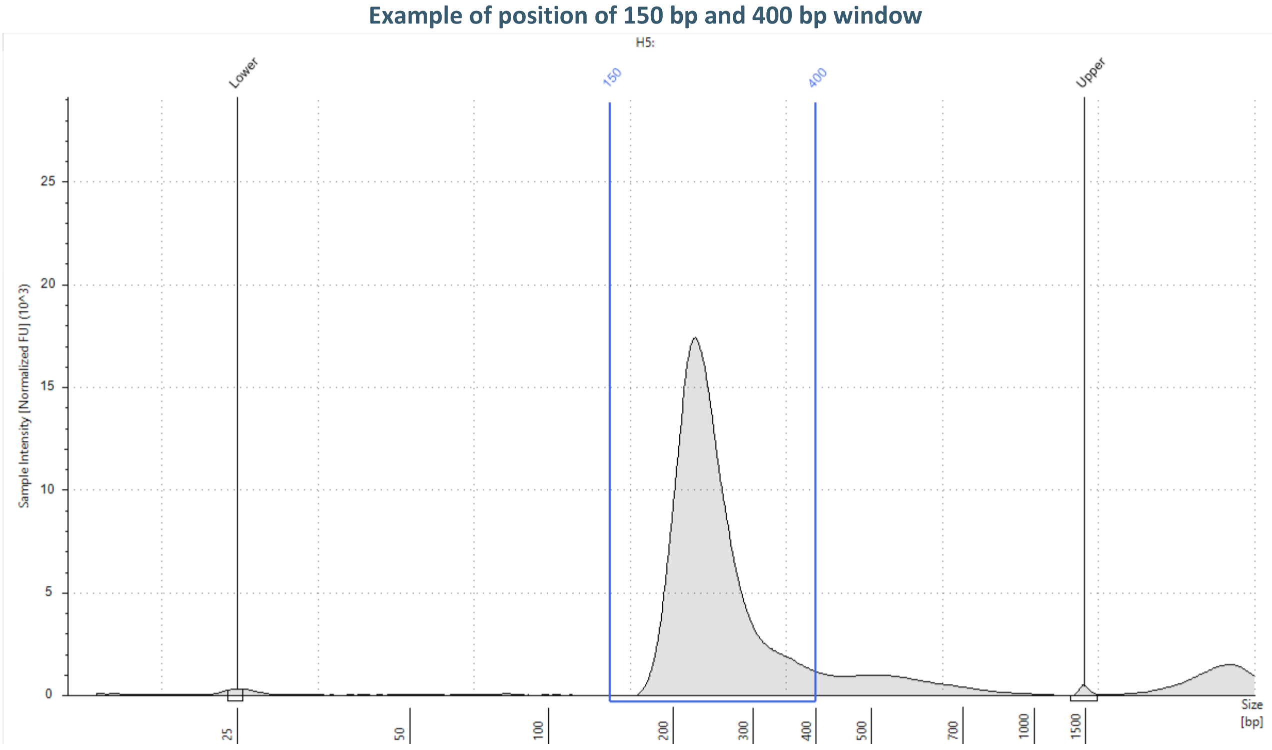
Pool 1 and Pool 2 are expected to have similar yields and average insert sizes.

Library	Size window	Expected average insert size
Pool 1	150-400 bp	240-280 bp
Pool 2	150-400 bp	240-280 bp

XCeloSeq Targeted cfDNA Enrichment libraries do not require any special handling when visualised using a High Sensitivity DNA ScreenTape assays, the Agilent protocol should be followed (4200 TapeStation System Manual).

After completion of a run ensure that all upper and lower markers have been correctly detected by the software, please refer to the "4200 TapeStation System Manual" manual for instructions. Failure to identify markers will result in inaccurate estimation of average insert size.

Example of position of 150 bp and 400 bp window



11.5. Determination of Library Concentration

We recommend that both the Pool 1 and Pool 2 Amplification Three products should have their concentration determined individually by qPCR. Quantify the concentration of each library following the instructions provided with the NEBNext® Library Quant Kit for Illumina (NEB; #E7630).

The Library Quant Kit contains 6 reference standards at 100 pM, 10 pM, 1 pM, 0.1 pM, 0.01 pM, and 0.001 pM. The XCeloSeq Targeted cfDNA Enrichment libraries are expected to be at a high concentration, therefore only the highest 4 standards need to be used (100-0.1 pM).

11.6. Recommendations for Illumina Sequencing

The Pool 1 and Pool 2 Amplification Three products are now ready for downstream processing.

The Pool 1 and Pool 2 products should be kept separate and be processed individually. Both Pool 1 and Pool 2 must be sequenced to the same depth. Please see product inserts for information on required depths.

When sequencing, use the read length for each sequencing stage as set out in the table below.

Sequencing stage	Read length
(R1) Read 1	151
(I1) Index Read 1	8
(I2) Index Read 2	8
(R2) Read 2	151

12. Troubleshooting Guide

This guide may be helpful in understanding and solving problems which may arise during use of an XCeloSeq Targeted cfDNA Enrichment kit.

The scientists at GeneFirst are always happy to support you with your work and answer questions you may have about this protocol or its underlying ATOM-Seq technology.

Please see **Section 14** for contact information.

Comments and suggestions	
Observation: Low Library Yield	
Suboptimal reaction conditions due to low DNA quality	Make sure to use high-quality DNA to ensure optimal activity of library enzymes.
Suboptimal reaction conditions due to low DNA quality	Where possible maximise the input mass of DNA, as the yield and sensitivity of the assay are directly related to the mass of starting material.
Inefficient PCR	During each purification step, XCeloSeq Beads need to be completely dried before elution. Any carryover of ethanol to Amplification Two or Three will significantly affect PCR reaction efficiency. In severe instances this could cause complete failure of the PCR reaction.
Observation: High Library Yield	
Libraries over amplified (See Section 11.2, annotated area B)	Over amplification will not affect the quality of the final library. Any secondary “bump” or “wave” in annotated area B should not be considered when determining library size or concentration. If libraries are regularly over-amplified, reduce cycle number for Amplification Three by 2 cycles in the first instance. This can be reduced further if required.
The average library insert size within the 150-400 bp window is higher than expected.	Over amplification may affect the assessed average insert size. This is more apparent on Bioanalyzer systems as they are more sensitive to ssDNA.

Observation: Unexpected signal peaks

Short peaks approx. below 100 bp (See Section 11.2, annotated area C)	These may be short, unexpected dimer products or primer carry over. Their presence may indicate that that there was too little DNA input, that PCR was inefficient or that bead purifications were not performed correctly. If library yields are high, then an additional bead purification as detailed in Section 14.7 can be completed to reduce the amount of unexpected nucleic acids.
Short sharp peaks approx. between 100-150 bp (See Section 11.2, annotated area C)	These may be short, unexpected dimer products. Their presence may indicate that that there was too little DNA input, that PCR was inefficient or that bead purifications were not performed correctly. If library yields are high, then an additional bead purification as detailed in Section 13.1 can be completed to reduce the amount of unexpected nucleic acids.

Observation: Sequencing issues

Cluster density is lower or higher than expected	Accurate library quantification is the key for optimal cluster density on any sequencing instrument. It is recommended to use a PCR-based quantification method. Other methods may lead to the incorrect quantification of the library especially when there is over amplification.
Imbalanced sequencing between Pool 1 and Pool 2	Sufficient sequencing depth for Pool 1 and Pool 2 products is important to maximise the sensitivity of the assay. It is recommended to use a PCR-based quantification method. Other methods may lead to the incorrect quantification of the library especially when there is over amplification.
Imbalanced Sequencing between multiplexed samples	It is recommended to use a PCR-based quantification method. Other methods may lead to the incorrect quantification of the library, especially when there is over amplification.

13. Symbols

Symbol	Description	Symbol	Description
	Consult instructions for use	-20°C	Upper limit of storage temperature -20°C
REF	Catalogue number	LOT	Batch code
	Date of manufacture	Σ	Number of supplied reactions
	Manufacturer		Do not use if package damaged
	Use-by-date		

14. Customer Contact Information

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

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