XCeloSeq® Targeted cfDNA Enrichment Protocol with UDIs

For use with Unique Dual Indexing (UDI) Sets only

FOR RESEARCH USE ONLY

Store at -20°C

Instructions for Use – English

IFU1851 Version 1.0 – September 2022

genefirst

Contents

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

All 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

This XCeloSeq Targeted cfDNA Enrichment Protocol is intended for use with XCeloSeq Targeted cfDNA Core Reagents (**Part Code: GF020**), any compatible XCeloSeq Targeted cfDNA Enrichment Primers (**See Section 6.2**) 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. XCeloSeq Technological Principle

The XCeloSeq products which use this protocol are built on a strong 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 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 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 molecules.

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.

6. XCeloSeq Technological Principle

XCeloSeq Targeted cfDNA Enrichment Kits have a dual component modular design:

- The first box contains the XCeloSeq Targeted cfDNA Core Reagents which include proprietary reagents for the patented approach of generating unique molecular identifiers and priming sites on DNA 3' ends through an **ATO Reaction**. The first box also contains the master mixes necessary for all three amplification steps.
- The second box contains XCeloSeq Targeted cfDNA Enrichment Primers, which are used for the enrichment of DNA regions during the construction of sequencing-ready libraries. See Section 6.2 for a full list of compatible primer sets.

6.1. XCeloSeq Targeted cfDNA Core Reagents (GF020)

The **XCeloSeq Targeted cfDNA Core Reagents** box contains the following reagents:

Reagent	Tube Colour	Tube Cap Colour	Storage Conditions	Part Code
ATO	Transparent	**Blue**	**-20°C**	**PC0001**
ATO Reaction Mix	Transparent	**Green**	**-20°C**	**PC0002**
Amplification One Mix	Transparent	**Transparent**	**-20°C**	**PC0003**
Primers	Transparent	**Red**	**-20°C**	**PC0004**
Master Mix	Transparent	**Lilac**	**-20°C**	**PC0005**

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

6.2. XCeloSeq Targeted cfDNA Enrichment Primers

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

This protocol can only be used with the following products:

Catalogue Number	Product Name	Target Genes	Target Primers
SEQ002	**XCeloSeq Pan Cancer cfDNA Kit**	**100**	**1147**
SEQ009	**XCeloSeq Colon Cancer cfDNA Kit**	**23**	**315**
SEQ010	**XCeloSeq Lung Cancer cfDNA Kit**	**15**	**300**
SEQ011	**XCeloSeq Breast Cancer cfDNA Kit**	**12**	**245**
SEQ016	**XCeloSeq Actionable cfDNA Kit**	**34**	**252**

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

Reagent	Tube Colour	Tube Cap Colour	Storage Conditions	Step Used	Part Code
Pool 1 – OUTER	Transparent	**Orange**	**-20°C**	**10.4**	**Refer to product insert**
Pool 1 – INNER	Transparent	**Black**	**-20°C**	**10.6**	
Pool 2 – OUTER	Transparent	**White**	**-20°C**	**10.4**	
Pool 2 – INNER	Transparent	**Yellow**	**-20°C**	**10.6**	

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

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 Kits are design 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.

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

Product Name	Product Code
XCeloSeq UDI Set 1-01 for Illumina	IDX1-01
XCeloSeq UDI Set 1-02 for Illumina	IDX1-02
XCeloSeq UDI Set 1-03 for Illumina	IDX1-03
XCeloSeq UDI Set 1-04 for Illumina	IDX1-04
XCeloSeq UDI Set 1-05 for Illumina	IDX1-05
XCeloSeq UDI Set 1-06 for Illumina	IDX1-06
XCeloSeq UDI Set 1-07 for Illumina	IDX1-07
XCeloSeq UDI Set 1-08 for Illumina	IDX1-08
XCeloSeq UDI Set 1-09 for Illumina	IDX1-09
XCeloSeq UDI Set 1-10 for Illumina	IDX1-10
XCeloSeq UDI Set 1-11 for Illumina	IDX1-11
XCeloSeq 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

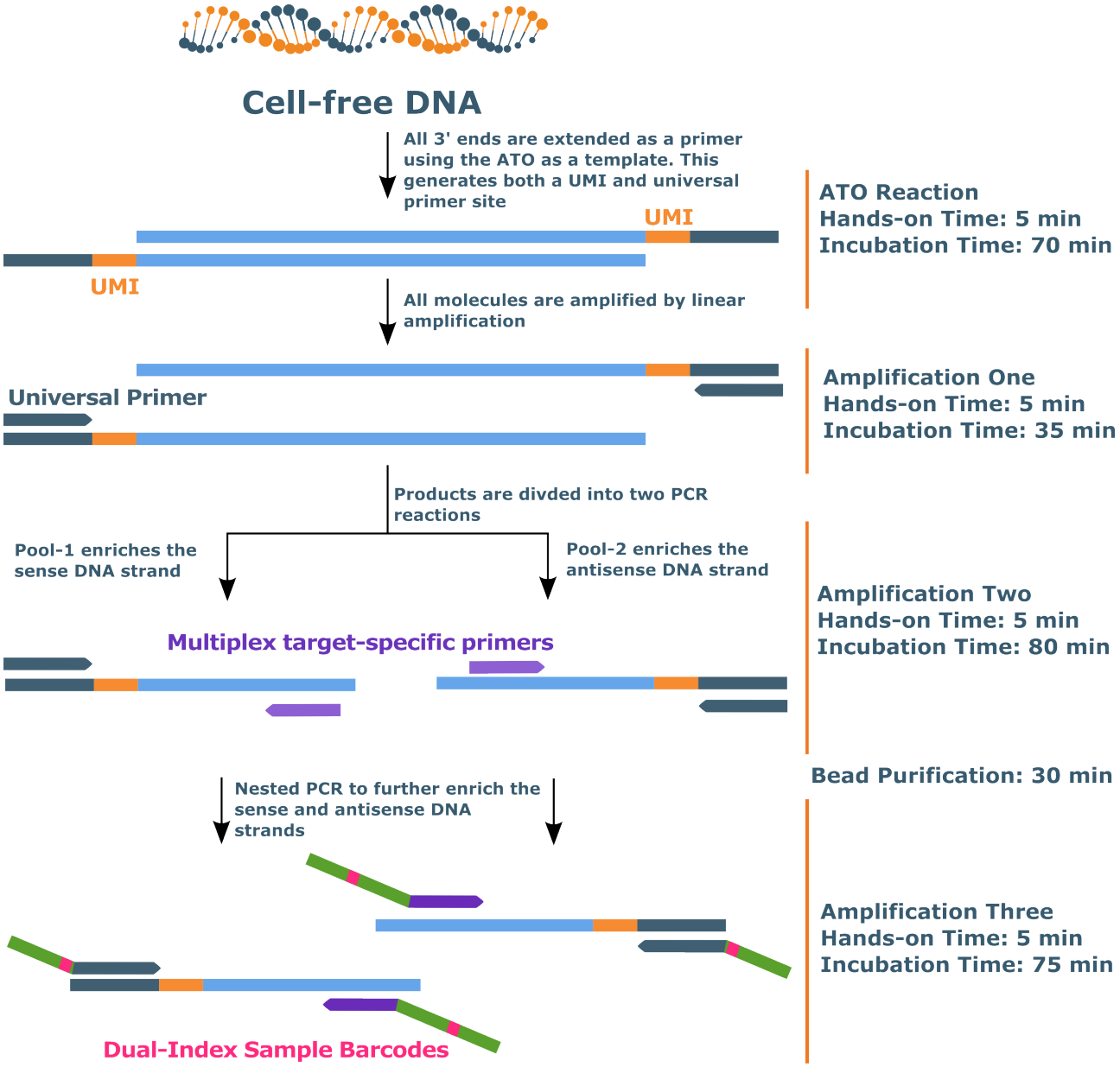
When determining UDI Set requirements for processing a fixed number of samples, please refer to the table below as an example. (Note: Please do still consider multiplexing requirements using the previous table).

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. Additionally 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
- DNase and RNase-free PCR tubes for preparing Reaction Mix
- 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

8. Protocol Overview



The above process is illustrative of the steps undertaken when following the Operating Procedure in Section 10. The times in the above protocol are repersenataive for 10 ng of cfDNA. Double strand DNA is shown as an example, however all recommended starting mateiral will follow the same process. This includes enzymatically fragmented FFPE and gDNA.

9. Before Starting

- Read this protocol in its entirety before beginning the library preparation to ensure everything is prepared and that 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 cfDNA in a sample to determine its suitability for use with the XCeloSeq Targeted cfDNA Enrichment Protocol 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 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.

Input DNA Mass as Measured by Qubit	Proportion of DNA Assessed to be cfDNA by Capillary Electrophoresis	Effective 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 **5-50 ng**; however, input should always be maximised within this range 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). Starting quantities should not exceed 50 ng. cfDNA must be in no more than 1mM EDTA solution, ideally 10 mM Tris 1mM EDTA pH 8.0 or alternatively ultra-pure water.

This workflow is compatible with FFPE derived DNA and high quality gDNA, but 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 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 alternatively ultra-pure 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 AMPure XP beads are given sufficient time to reach room temperature prior to use. A minimum recommended time is 20 minutes.

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10. Operating Procedure

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

In a PCR vessel, combine **2 µl ATO (Transparent Tube, Blue Cap, PC0001)** and your cfDNA sample. The total volume of this mixture must not exceed 15 µl. If required add molecular biology grade water to a final volume of **15 µl**

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

10.2. ATO 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 (Transparent Tube, Green Cap, PC0002).** 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 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

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ATO Reaction – Step 2: Incubation Conditions

Stage	Cycles	Temperature	Duration
1	-	4 °C	Hold/Pause
2	1x	10 °C	1 minute
3	1x	26 °C	6 minutes
4		30 °C	**10 minutes**
5		65 °C	1 minute
6		10 °C	1 minute
7		26 °C	6 minutes
8		30 °C	**10 minutes**
9	2x	65 °C	1 minute
10		10 °C	1 minute
11		26 °C	6 minutes
12		30 °C	**5 minutes**
13	-	4 °C	Hold

10.3. Amplification One

- Remove the samples from the thermocycler and briefly spin down. Add **25 µl ATO Amplification One Mix (Transparent Tube, Transparent Cap, PC0003)** and **1.0 µl Primers (Transparent Tube, Red Cap, PC0004)** to each sample. The total volume per sample is now **46 µl**
- 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 One: Incubation Conditions

Stage	Cycles	Temperature	Duration
1	1x	37 °C	10 minutes
2	1x	98 °C	30 seconds
3	10x	98 °C	5 seconds
4		60 °C	1 minute
5		72 °C	1 minute
6	1x	72 °C	2 minutes
7	-	4 °C	Hold

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 before proceeding

WARNING!

Amplification Two – Target Specific PCR

10.4. Amplification Two, Target Specific PCR

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 46 µl 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	Name	Tube colour	Tube Cap colour	Volume per single reaction (µl)	Part Code
1	**Aliquot 1 of Step 10.3 Product**	N/A	N/A	22.0	-
2	**Pool 1 – OUTER**	**Transparent**	**Orange**	3.0	**See product insert**
3	**Master Mix**	**Transparent**	**Lilac**	25.0	**PC0005**
		Total Volume:		**50.0**	

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

Order	Name	Tube colour	Tube Cap colour	Volume per single reaction (µl)	Part Code
1	**Aliquot 2 of Step 10.3 Product**	N/A	N/A	22.0	-
2	**Pool 2 – OUTER**	**Transparent**	**White**	3.0	**See product insert**
3	**Master Mix**	**Transparent**	**Lilac**	25.0	**PC0005**
		Total Volume:		**50.0**	

- Vortex the Amplification Two Reaction Mixtures and centrifuge briefly
- Incubate the mixtures in a thermocycler as detailed in the table below, with a heated lid ≥100 ^{o}C:

Amplification Two: Incubation Conditions

Stage	Cycles	Temperature	Duration
1	1x	98 °C	30 seconds
2	14x	98 °C	5 seconds
3		65 °C	5 minutes
4		72 °C	30 seconds
5	1x	72 °C	2 minutes
6	-	4 °C	Hold

10.5. 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 **1.8X** volumes (**90 µl**) of AMPure XP beads to each reaction.
2) Vortex well or mix by pipetting each sample 15 times. Ensure a homogenous mixture of beads and sample before continuing.
3) Leave samples at room temperature for 5 minutes. If required spin down briefly to collect sample in the bottom of the vial.
4) Place the samples on the 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, return the solution back into the vial and repeat the incubation to allow them to collect on the side of the tube again.
6) While leaving the vial on the magnet add **150 µl** of freshly prepared **80% ethanol**.
7) Incubate for 30 seconds. Ensure all beads remain gathered to the side of the vial.
8) Carefully discard the supernatant.
9) Wash the samples again by repeating the preceding 3 steps (6 – 8) one additional time.
10) After the second wash carefully remove as much of the residual ethanol as possible.
11) Allow the beads to air dry for 3 minutes. Take care not to over dry the beads as this will have a significant effect on the overall yield of the purification.
12) Elute the DNA by resuspending the beads in **23 µl** of either molecular biology grade H_2O or 10 mM Tris-HCL pH 8.0. Incubate the resuspended beads for 5 minutes.
13) Place the samples on the magnet for 3 minutes, or until all the beads have been collected.
14) Carefully transfer **21 µl** of the eluted amplification product into a clean PCR tube.

After the bead purification samples can be stored at 4 °C or –20 °C. If stored at 4 °C samples should ideally proceed to Amplification Three within 24 hours.

If stored at –20 °C, ensure samples are at room temperature 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, unique pre-mixed combinations of i7 and i5 primers can be used to allow for sample multiplexing. See Section 7.1 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	Name	Tube colour	Tube Cap colour	Volume per single reaction (µl)	Part Code
1	**Pool 1 – OUTER Product of Step 10.5**	N/A	N/A	21.0	-
2	**Pool 1 – INNER**	**Transparent**	**Black**	2.0	**See product insert**
3	**Master Mix**	**Transparent**	**Lilac**	25.0	**PC0005**
4	**Pre-mixed UDI Primers**	**Transparent**	**White**	2.0	**See Product Insert**
			Total Volume:	**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	Name	Tube colour	Tube Cap colour	Volume per single reaction (µl)	Part Code
1	**Pool 2 – OUTER Product of Step 10.5**	N/A	N/A	21.0	-
2	**Pool 2 – INNER**	**Transparent**	**Yellow**	2.0	**See product insert**
3	**Master Mix**	**Transparent**	**Lilac**	25.0	**PC0005**
4	**Pre-mixed UDI Primers**	**Transparent**	**White**	2.0	**See Product Insert**
			Total Volume:	**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	Duration
1	1x	98 °C	30 seconds
2	Variable, see table at top of next page	98 °C	5 seconds
3		65 °C	5 minutes
4		72 °C	30 seconds
5	1x	72 °C	2 minutes
6	-	4 °C	Hold

If the Amplification Three yields are too high or low, or when using higher or lower quantities of DNA, the cycle number can be adjusted accordingly. Suggested initial cycle numbers are shown in the table below. These may need adjusting on a sample-by-sample basis.

Amplification Three: Recommended Amplification Conditions

Input DNA Quantity	Recommended Amplification Three cycle number by input DNA type		
	Enzymatically Fragmented High Quality gDNA	cfDNA	Enzymatically Fragmented FFPE DNA
5 ng	14-15x	15-16x	16-17x
10 ng	13-14x	14-15x	15-16x
20 ng	12-13x	13-14x	14-15x
50 ng	11-12x	12-13x	13-14x

10.7. 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.

15) Add **1.2X** volumes (**60 µl**) of AMPure XP beads to each reaction.
16) Vortex well or mix by pipetting each sample 15 times. Ensure a homogenous mixture of beads and sample before continuing.
17) Leave samples at room temperature for 5 minutes. If required spin down briefly to collect sample in the bottom of the vial.
18) Place the samples on the magnet for 3 minutes, or until all the beads have been collected.
19) Without disturbing the collected beads, use a pipette to collect and dispose of the supernatant. If the beads are disturbed, return the solution back into the vial and repeat the incubation to allow them to collect on the side of the tube again.
20) While leaving the vial on the magnet add **150 µl** of freshly prepared **80% ethanol**.
21) Incubate for 30 seconds. Ensure all beads remain gathered to the side of the vial.
22) Carefully discard the supernatant.
23) Wash the samples again by repeating the preceding 3 steps (6 – 8) one additional time.
24) After the second wash carefully remove as much of the residual ethanol as possible.
25) Allow the beads to air dry for 3 minutes. Take care not to over dry the beads as this will have a significant effect on the overall yield of the purification.
26) Elute the DNA by resuspending the beads in **32 µl** of 10 mM Tris-HCL pH 8.0. Incubate the resuspended beads for 5 minutes. **Note:** Higher elution volumes can be used depending on user requirements
27) Place the samples on the magnet for 3 minutes, or until all the beads have been collected.
28) Carefully transfer **30 µl** of the eluted amplification product into a clean tube.

10.8. Library QC, Quantification, and Sequencing Recommendations

The two separate bead purified Amplification 3 products are now ready for downstream processing. The Pool 1 and Pool 2 products should be kept separate and be processed individually.

We recommend the use of an Agilent Bioanalyzer High Sensitivity DNA Chip (or equivalent) for determining both the success and 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).

When multiplexing samples for sequencing we recommend that Pool 1 and Pool 2 products should be mixed such that they are both at an equal final concentration. The total number of recommended reads per sample, as detailed in the individual product inserts, must be equally shared between the Pool 1 and Pool 2 products.

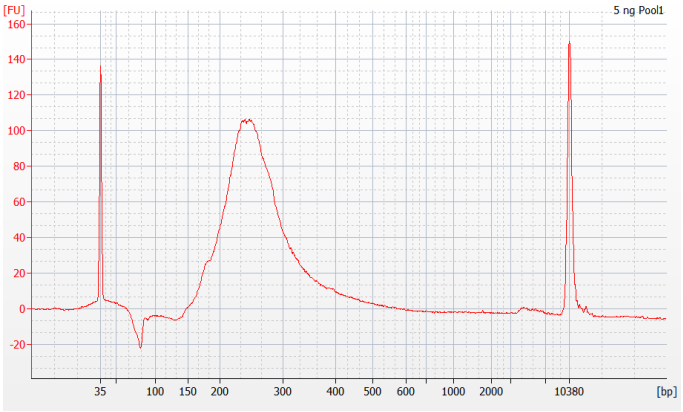
For sequencing use the read length for each sequencing stage as set out in the table below.

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

11. Representative Data

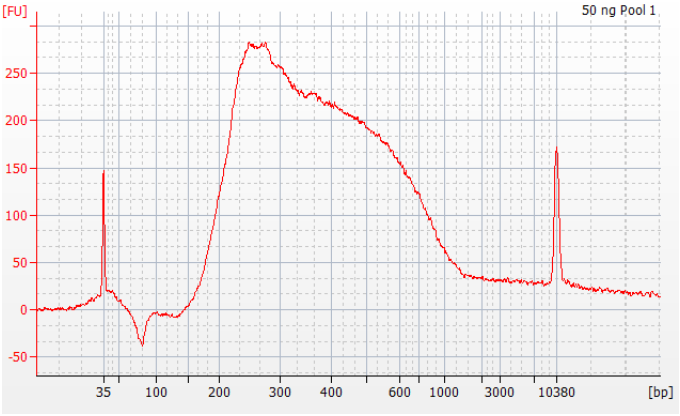
Representative final libraries which were generated using enzymatically fragmented gDNA are shown below.

11.1. 5 ng high quality enzymatically fragmented gDNA – SEQ002



11.2. 50 ng high quality enzymatically fragmented gDNA – SEQ002

When using starting quantities at the upper supported limits (20-50 ng), a large “shoulder” or “hump” may be visible on visualising final libraries on a bioanalyzer, such as seen in the example below in the range of 400bp to 1,000bp.



Although similar library profiles to the one shown above can be a result of over amplification during Amplification Three (see Section 9.6), the presence of the “shoulder” when using high input DNA amounts is not typically a reason for concern. Larger input amounts can result in the generation of by-products which, although they are visible on the Bioanalyzer, will not interfere with qPCR-based library quantification or with library sequencing. This is because these by-products are single strand DNA and will not contain the Illumina adaptor necessary for sequencing.

In this example, it is therefore not recommended to reduce PCR cycle number during Amplification Three as this will disproportionally reduce generation of final library products. You should continue and quantify your library normally, for example by qPCR (NEBNext® Library Quant Kit for Illumina®). When quantifying the example library shown above, the shoulder would be ignored entirely, and this would result in an average fragment size of approximately 260bp. This value would then be used during library quantification. If you should require help in this situation, please contact us (see Section 13 for contact information).

12. Troubleshooting and FAQ

12.1. Library yields are low or absent

When the kit reagents are stored as recommended, 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 compatible reagents as detailed in this IFU, excluding the additional indexes (which must be handled as detailed above); that the input quantity of the starting material is suitable; and that you carefully read and fully follow all steps in the 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. We recommend initially using the maximum of 50 ng of enzymatically fragmented FFPE DNA if quality is uncertain. This can later be optimised if samples are of consistent quality.

12.3. Low library yields 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 Targeted cfDNA Enrichment Kits are not compatible with DNA fragmented by sonication. You should ensure the starting material has been enzymatically fragmented.

12.4. Low library yields or libraries with a 'shoulder' when using XCeloSeq indexing kits

Failure to correctly dilute i7 index oligos supplied in XCeloSeq Indexing Kits can produce libraries with a large 'shoulder' that follows the main library peak, similar to the profile shown in Section 10.2. This is not expected to influence the quality of the library and you should continue to quantify and sequence the library as normal.

12.5. Final library profiles have many sharp peaks

If you have added too little 'capturable DNA' this can result in a low complexity library, which appears in an Agilent Bioanalyzer profile as a library with many sharp peaks.

12.6. Final library profiles have lots of peaks around and below 100bp

Peaks around and below 100bp in an Agilent Bionalayzer profile indicate that the primers from Amplification Three have carried over though the final bead purification. When preforming the bead purification please ensure you are following the directions correctly. If primer carryover persists, performing another 1.2x bead purification (as per Section 9.7) will reduce their abundance.

12.7. Library yields are high and there is a 'wavy' secondary bump following the main library peak

This is indicative of primer exhaustion and over amplification during Amplification Three. As long as a defined primary library peak can be visualised between 200-300 bp, this is not expected to have a negative effect on sequencing quality, and therefore libraries can be quantified by qPCR and sequenced as normal. Reducing Amplification Three cycle number should be considered in subsequent experiments.

13. Symbols

Symbol	Description	Symbol	Description
	Consult instructions for use		Upper limit of storage temperature -20°C
	Catalogue number		Batch code
	Date of manufacture		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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