XCeloSeq® Targeted cfDNA Enrichment Protocol

FOR RESEARCH USE ONLY

Store at -20°C

Instructions for Use – English

IFU0627 Version 5.0 – November 2020

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1 Copyright and Trademarks

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 Intended Use

This XCeloSeq Targeted cfDNA Enrichment Protocol is intended for use with XCeloSeq Targeted cfDNA Core Reagents (Part Code: GF020) and any compatible XCeloSeq Targeted cfDNA Enrichment Primers (See Section 5.2). 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.

4 XCeloSeq Technological Principle

All XCeloSeq products are built on the strongest technical foundation:

Adaptor **T**emplate **O**ligo **M**ediated **Seq**uencing – **ATOM-Seq®**

ATOM-Seq is a patented technology which uses a unique, advanced capture chemistry designed for compatibility with 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 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 fragmented high quality genomic DNA (gDNA), FFPE, cfDNA 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 over comes 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.

5 GeneFirst XCeloSeq Targeted cfDNA Enrichment Kit Reagents

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 though 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 5.2 for a full list of compatible primer sets.

5.1 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**

Reagent	Tube Colour	Tube Cap Colour	Storage Conditions	Expected Index Sequence*	Machine Compatibility	Part Code
i5-001 (i5 Index Primer)	**Amber**	**Blue**	**-20°C**	**ATCCGTAC**	**MiSeq, NovaSeq, HiSeq2500, HiSeq2000**	**PC0006**
				GTACGGAT	**MiniSeq, NextSeq, HiSeq4000, HiSeq3000**	
i7-001 (i7 Index Primer)	**Amber**	**Green**	**-20°C**	**CATAGCCG**	**All**	**PC0007**

* Expected index sequence is dependent upon choice of Illumina Sequencing Platform.

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.

When using additional index primers supplied as part of XCeloSeq Indexing Kits be aware of the following. For more details see **Section 7**.

All additional i5 primers supplied as part of XCeloSeq Indexing Kits (which are purchased separately) can directly replace the i5-001 primer supplied with the XCeloSeq Targeted cfDNA Core Reagents.

All additional i7 primers supplied as part of XCeloSeq Indexing Kits (which are purchased separately) **MUST BE DILUTED 1:4** with the supplied i7 Dilution Buffer prior to replacing the i7-001 primer supplied with the XCeloSeq Targeted cfDNA Core Reagents. See **Section 7.2**.

5.2 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 Brest Cancer cfDNA Kit	12	245

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

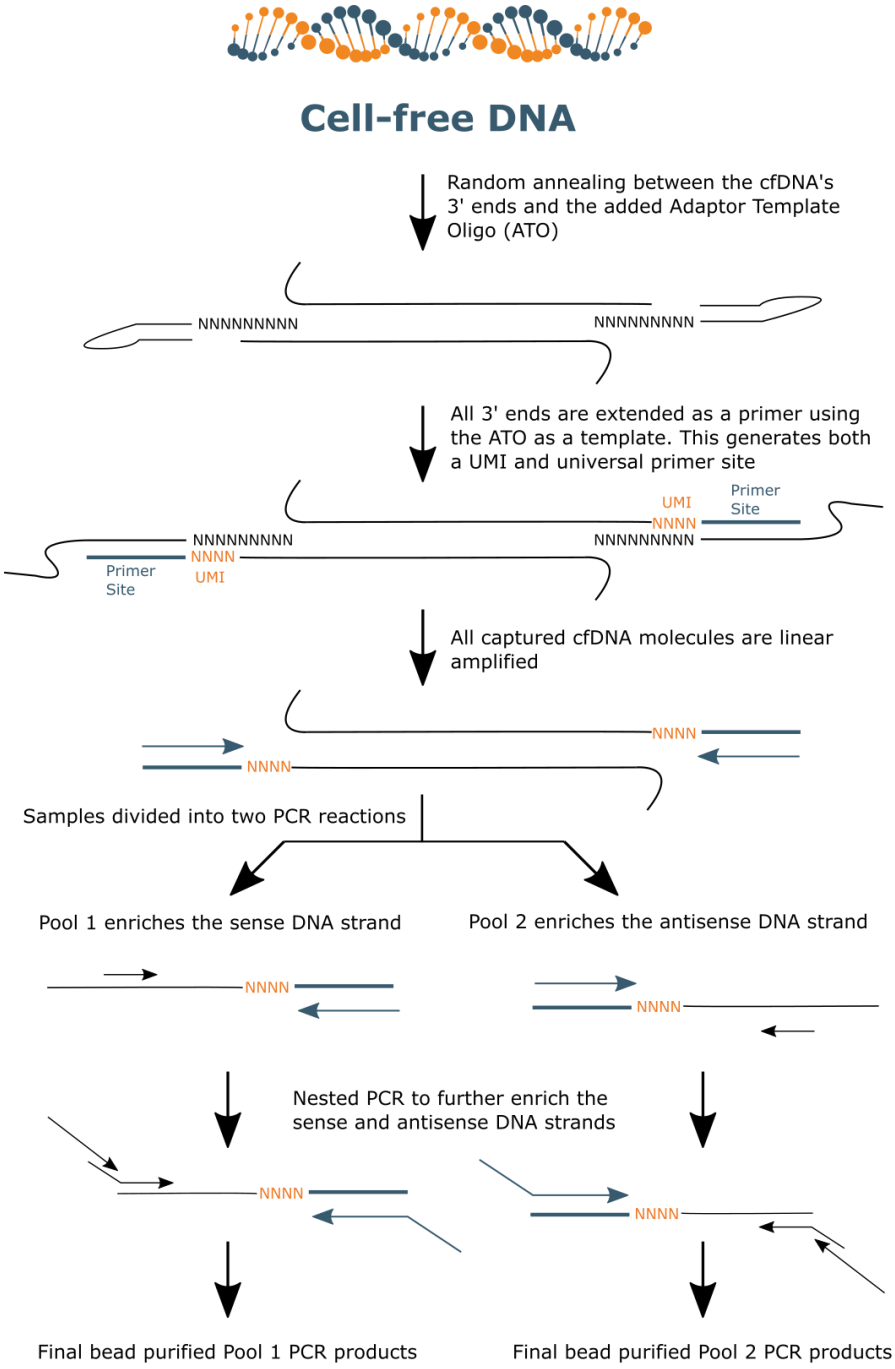
Reagent	Tube Colour	Tube Cap Colour	Storage Conditions	Step Used	Part Code
Pool 1 – OUTER	Transparent	Orange	-20°C	8.4	Refer to product insert
Pool 1 – INNER	Transparent	Black	-20°C	8.6	
Pool 2 – OUTER	Transparent	White	-20°C	8.4	
Pool 2 – INNER	Transparent	Yellow	-20°C	8.6	

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.

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

- Reagents and equipment for specimen collection, filtration, and nucleic acid extraction
- Distilled water (molecular biology grade)
- 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

6 Protocol Overview



ATO Reaction

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

Amplification One

Hands-on Time: 5 min
Incubation Time: 35 min

Amplification Two

Hands-on Time: 5 min
Incubation Time: 80 min

Bead Purification: 30 min

Amplification Three

Hands-on Time: 5 min
Incubation Time: 75 min

Bead Purification: 30 min

Total Time: 5 hr 40 min

The above process is illustrative of the steps undertaken when following the Operating Procedure in Section 8. 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.

7 Before Starting

- Read this protocol in its entirety before beginning the library preparation to ensure everything is prepared and the process is clear prior to beginning
- Ensure good laboratory practice is used at all time 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

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

*Recommended range of cfDNA is **5-50 ng**; input should be maximised where possible within this range to obtain maximum sensitivity. Minimum cfDNA starting quantity is 1 ng. Starting quantities of cfDNA 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, ultra-pure water.*

7.1 Reagent Preparation

- 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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7.2 Preparing Index Primers from Additional XCeloSeq Indexing Kits

The XCeloSeq Targeted cfDNA Enrichment Kits are provided with a single i5 index and a single i7 index. These indexes are both 8bp in length. For additional indexes there are a range of XCeloSeq Indexing kits which provide additional 8bp i5 and i7 index primers. Only XCeloSeq Indexing kits are recommended for use with XCeloSeq library preparation kits as these have undergone design optimisations and validations to ensure quality.

If using additional XCeloSeq i5 Indexing Kits, these index oligos can directly replace the i5-001 indexing primer supplied with XCeloSeq Targeted cfDNA Enrichment Kits.

If using additional XCeloSeq i7 Indexing Kits, ensure that prior to use the **primers have been diluted 1:4 in the i7 Dilution Buffer** provided with the Indexing Kit. Only once diluted can they be used with this XCeloSeq Targeted cfDNA Enrichment Kit Protocol. Ensure diluted primers are only used with compatible XCeloSeq products. See the below table for an example of diluting a new SEQ005-i7 Indexing Kit. A check box is included on all i7 index oligo vials to allow recording of their dilution.

Kit Name	Oligo Name	Volume to mix (µl)		Final Volume (µl)	Storage Conditions	Shelf Life
		Index Oligo	i7 Dilution Buffer			
SEQ005-i7-x	**SEQ005-i7-x1**	15	45	60	-20°C	6 months from date of dilution
	SEQ005-i7-x2	15	45	60	-20°C	
	SEQ005-i7-x3	15	45	60	-20°C	
	SEQ005-i7-x4	15	45	60	-20°C	
	SEQ005-i7-x5	15	45	60	-20°C	
	SEQ005-i7-x6	15	45	60	-20°C	
	SEQ005-i7-x7	15	45	60	-20°C	
	SEQ005-i7-x8	15	45	60	-20°C	

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8 Operating Procedure

8.1 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

8.2 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

8.3 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

Amplification One

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 before proceeding

WARNING!

Amplification Two – Target Specific PCR

8.4 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 8.3** 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 **Step 8.4** it will not be possible to continue with the library preparation.

- Prepare the Pool-1 OUTER Amplification Two Mix by adding reagents to one aliquot of Step 8.3 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 8.3 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 Mix by adding reagents to the remaining aliquot of Step 8.3 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 8.3 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 Mixtures and centrifuge briefly
- Incubate the mixtures in a thermocycler as detailed in the table below, with a heated lid ≥100 °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

8.5 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.

Bead Purification

Safe Stopping Point

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.

8.6 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 8.4 **must now use** '**Pool 1** – INNER'.

The reaction which used '**Pool 2** – OUTER' in step 8.4 **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, different combinations of i7 and i5 primers can be used to allow for sample multiplexing. See Section 7.2 for details.

- Prepare the Pool - 1 INNER Amplification Three Mix by adding reagents to the bead purified sample from step 8.5, 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 8.5**	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	**i5-001**	**Amber**	**Blue**	1.0	**PC0006**
5	**i7-001**	**Amber**	**Green**	1.0	**PC0007**
	Total Volume			50.0	

- Prepare the Pool - 2 INNER Amplification Three Mix by adding reagents to the bead purified sample from step 8.5, 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 8.5**	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	**i7-001**	**Amber**	**Green**	1.0	**PC0006**
5	**i5-001**	**Amber**	**Blue**	1.0	**PC0007**
	Total Volume			50.0	

- Vortex the Amplification Three 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

8.7 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.

1) Add **1.2X** volumes (**60 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 **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
13) Place the samples on the magnet for 3 minutes, or until all the beads have been collected.
14) Carefully transfer **30 µl** of the eluted amplification product into a clean tube.

8.8 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 both individually processed.

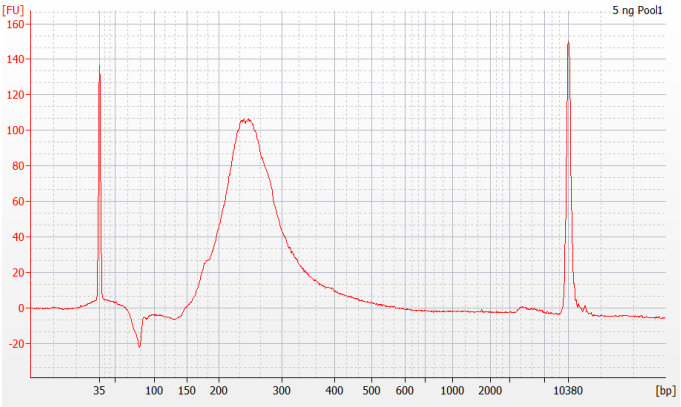
We recommend the use of an Agilent Bioanalyzer High Sensitivity Chip (or equivalent) for determining both the success and the size distribution of the generated libraries. Library concentration should be determined by a suitable method, we recommend a qPCR quantification method such as NEBNext® Library 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 assigned to a sample must be equally shared between the Pool 1 and Pool 2 products. The total read depth to be shared is detailed in the product inserts.

9 Representative Data

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

9.1 5 ng high quality enzymatically fragmented gDNA



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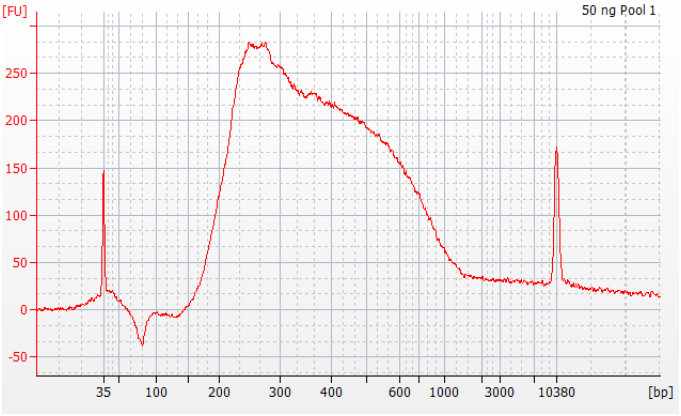
9.2 50 ng high quality enzymatically fragmented gDNA

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 8.6), 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 can be ignored entirely which results in an average fragment size of approximately 260bp, which should be used during library quantification. If you should require help in this situation, please contact us (see Section 11 for contact information).

10 Troubleshooting and FAQ

10.1 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.

10.2 Low library yields when using sonicated DNA

Recommended starting material quantities are based off extensive research and development work using cell free DNA or enzymatically fragmented gDNA. The XCeloSeq Targeted cfDNA Enrichment Kits are compatible with DNA fragmented by sonication, however this should be expected to drastically reduce the proportion of DNA which is capturable. You should either use enzymatically fragmented DNA (if possible) or increase the starting quantity of sonicated DNA if no other options are available.

10.3 Low Library yields when using FFPE gDNA

Formalin Fixed Paraffin Embedded (FFPE) samples can be of highly variable quality. As such using larger quantities of enzymatically fragmented FFPE DNA may still result in low library yields, indicating that the

proportion of capturable DNA in the sample is low. Using larger quantities of starting material can ensure that high quality libraries are generated. 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.

10.4 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 9.2. This is not expected to influence the quality of the library and you should continue to quantify and sequence the library as normal.

10.5 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.

10.6 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 8.7) will reduce their abundance.

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

This is indicative of primer exhaustion during Amplification Three. This is not expected to have a negative effect on sequencing quality, and therefore libraries can be quantified by qPCR and sequenced as normal.

10.8 Can you combine the Pool 1 and Pool 2 primers into a single PCR reaction?

The XCeloSeq Targeted cfDNA Enrichment Kit protocol has been optimised to maximise the quality and complexity of sequencing library, to allow for the highest levels of sensitivity and specificity from precious liquid biopsy samples. The recommended protocol for cfDNA is detailed above. It is possible to perform a single PCR with combined Pool 1 and Pool 2 primers, however this should be expected to lead to a reduction of library quality and quantity. For details please contact us visit our website to see the "XCeloSeq Single Tube Targeted cfDNA Enrichment Workflow".

11 Customer Contact Information

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

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