XCeloSeq® cfDNA Library Preparation Kit

SEQ001

FOR RESEARCH USE ONLY

Store at -20°C or 4°C - Component Dependant

Instructions for Use – English

IFU0613 Version 3.0 – November 2020

genefirst

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1 Copyright and Trademarks

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2 Notices

For **Research Use Only (RUO)**. Not for use in diagnostic procedures. Not intended to be used for treatment of human or animal diseases.

Safety data sheets pertaining to this product are available upon request.

The use of **caution** symbols identifies steps in the procedure where there is risk of assay failure if the protocol is not fully understood and followed.

The use of **stop** symbols indicates points in the protocol where it is safe to stop.

3 Upon Delivery

Immediately upon delivery remove the ATO Purification Beads (**Transparent Tube, Red Cap**) from the box and store at 2-8°C.

4 Intended Use

The XCeloSeq cfDNA Library Prep Kit is intended for use for the generation of 'whole genome', high-complexity, next-generation sequencing libraries suitable for use with Illumina next-generation sequencing (NGS) instruments.

This is a Research Use Only product.

5 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 'whole genome' capture of nucleic acid fragments to generate a sequencing-ready NGS library. The chemistry underpinning ATOM-Seq has been developed to be specifically capable of capturing nucleic acid molecules from ultra-low input, highly fragmented, single and double stranded, or highly damaged templates in a highly efficient process. 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.

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6 Kit Contents

6.1 Materials supplied with the kit

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

Reagents are not interchangeable between different XCeloSeq kits.
Only use "XCeloSeq cfDNA Library Prep Kit" reagents with the following protocol.

6.2 Indexes supplied with the kit

Reagent	Tube Colour	Tube Cap Colour	Storage Conditions	Expected Index Sequence*	Machine Compatibility	Part Code
i5-002 (i5 Index Primer)	Amber	Blue	-20°C	ATCCGTAC	MiSeq, NovaSeq, HiSeq2500, HiSeq2000	PC0200
				GTACGGAT	MiniSeq, NextSeq, HiSeq4000, HiSeq3000	
i7-002 (i7 Index Primer)	Amber	Green	-20°C	CATAGCCG	All	PC0201

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

Indexes are not interchangeable between different XCeloSeq kits.
Only use "XCeloSeq cfDNA Library Prep Kit" Indexes with the following protocol.

When using additional index primers supplied as part of XCeloSeq Indexing Kits be aware of the following:

All additional **i5** primers supplied as part of XCeloSeq Indexing Kits (which are purchased separately) can directly replace the i5-002 primer supplied with the XCeloSeq cfDNA Library Prep Kit.

All additional **i7** primers supplied as part of XCeloSeq Indexing Kits (which are purchased separately) can directly replace the i7-002 primer supplied with the XCeloSeq cfDNA Library Prep Kit. Some XCeloSeq kits require addition i7 oligos to be diluted, **DO NOT** use diluted oligos with this protocol they will affect protocol performance.

6.3 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 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

6.4 Use of additional index primers

The XCeloSeq cfDNA Library Preparation Kit is provided with a single i5 index and a single i7 index. These indexes are both 8bp in length. To support multiplex sequencing, we offer a range of XCeloSeq Indexing kits (catalogue number SEQ005, sold separately) which provide additional unique 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 and reliability of performance.

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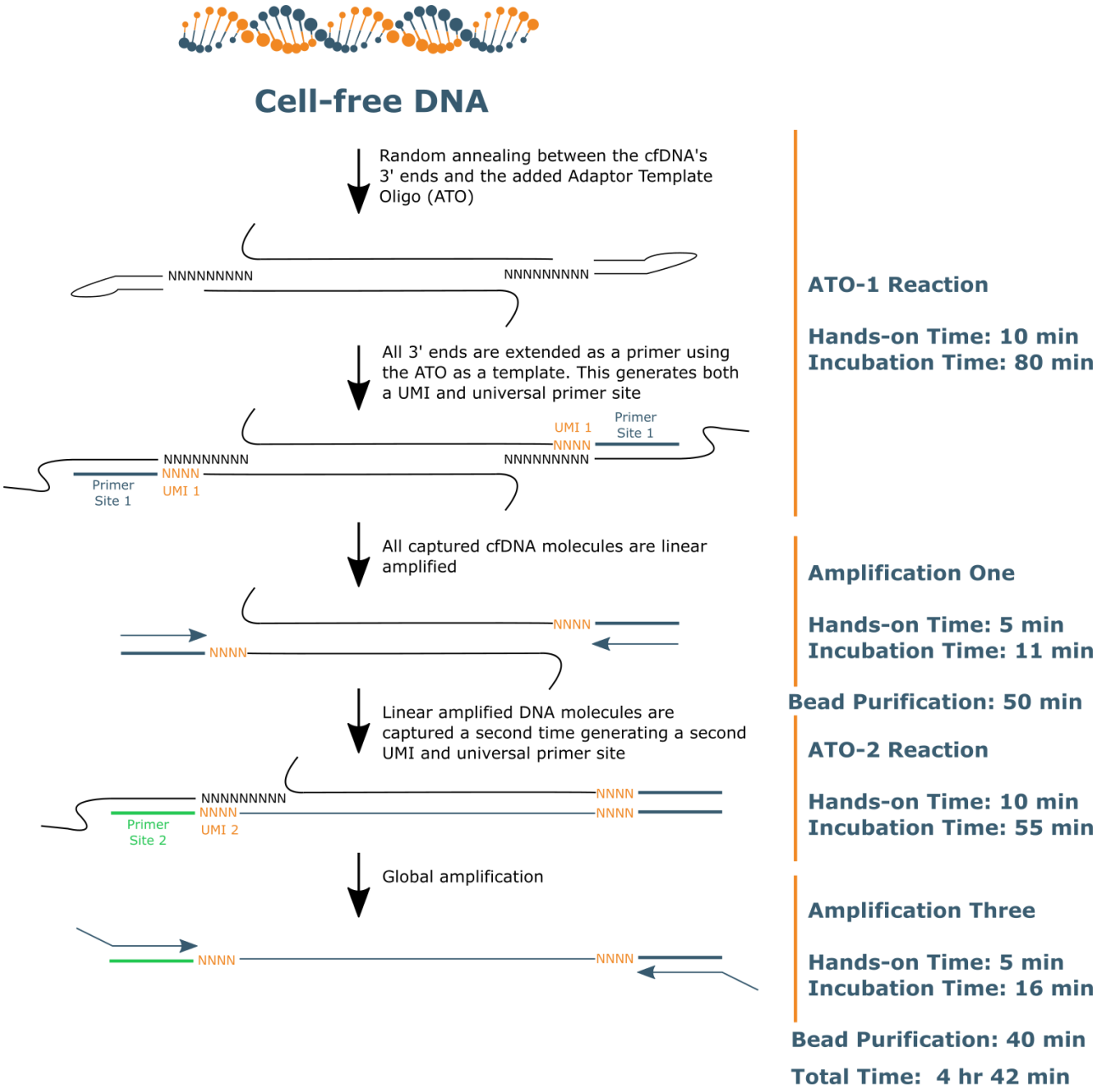
7 Protocol Overview



The above process is illustrative of the steps undertaken when following the Operating Procedure in Section 9. The times in the above protocol are repersenataive for 5 ng of cfDNA. Double strand DNA is shown as an example, however all recommended starting material will follow the same process. This includes enzymatically fragmented FFPE and gDNA.

8 Before Starting

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

*Minimum cfDNA starting quantity is 1 ng. The recommended range of starting material is **5-50 ng** of cfDNA. Starting quantities of DNA should not exceed 50 ng. When using greater than or less than 5 ng of starting material Amplification Two cycle number can be adjusted depending on the desired library yields. cfDNA must be in no more than 1mM EDTA solution, ideally 10 mM Tris 1mM EDTA pH 8.0, or, ultra-pure water.*

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

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

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9 Operating Procedure

9.1 ATO 1 Reaction – Step 1: ATO and cfDNA Mixture

- In a PCR vessel, add **1 µl 'ATO – 1' (Transparent Tube, Blue Cap, PC0028)** to your DNA sample. The total volume of this mixture must not exceed 7.5 µl. If required add molecular biology grade water to a final volume of 7.5 µl
- Seal the PCR vessel using PCR caps, mix by vortexing, and centrifuge briefly. Samples should be sealed tightly to avoid evaporation and sample loss
- Incubate the mixture in a thermocycler as detailed in the table below, with a heated lid ≥100 °C:

ATO 1 Reaction – Step 1: Incubation Conditions

Stage	Temperature	Duration
1	65 °C	2.5 minutes
2	10 °C	1 minute
3	4 °C	Hold

9.2 ATO 1 Reaction – Step 2: Addition of ATO Reaction Mix

- Remove the sample from the thermocycler, carefully open the PCR vessel, and add **2.5 µl of the ATO Reaction Mix (Transparent Tube, Green Cap, PC0199).** The total volume of each sample will now be 10 µl
- Seal the PCR vessel, mix by vortexing, and centrifuge briefly
- Ensure that the thermocycler is precooled to 4 °C. Place your PCR vessel into the machine and continue to thermocycle as detailed in the following table, with a heated lid ≥100 °C

ATO 1 Reaction – Step 2: Incubation Conditions

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

9.3 ATO 1 Reaction – Step 3: ATO 1 Treatment

- Remove the samples from the thermocycler and briefly spin down. Add **1 µl ATO Treatment (Transparent Tube, White Cap, PC0031)** to each sample. The total volume of each sample is now 11 µl
- Vortex, centrifuge and incubate the mixture in a thermocycler as detailed in the table below, with a heated lid ≥100 °C:

ATO 1 Reaction – Step 3: Incubation Conditions

Stage	Temperature	Duration
1	37 °C	15 minutes
2	4 °C	Hold

Safe Stopping Point

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

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

9.4 Amplification One

- Remove the samples from the thermocycler and briefly spin down. Add **12.5 µl Universal Enzyme Mix (Transparent Tube, Lilac Cap, PC0033)** and **1.5 µl Amplification Primers (Transparent Tube, Yellow Cap, PC0032)** to each sample. The total volume per sample is now 25 µl
- Seal the PCR vessel, mix by vortexing, and centrifuge briefly
- Incubate the mixture in a thermocycler as detailed in the table at the top of the following page

Amplification One: Incubation Conditions

Stage	Cycles	Temperature	Duration
1	1x	98 °C	30 seconds
2	6x	98 °C	10 seconds
		65 °C	75 seconds
3	1x	65 °C	2 minutes
4	-	4 °C	Hold

9.5 Bead Purification

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

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

1) First, 25 µl of **molecular biology grade water** must be added into the **Amplification 1** product from **step 9.4**. The volume of the sample will now be 50 µl.

2) Add 1.8X volumes (**90 µl**) of **ATO Purification Beads** to each reaction.

3) Vortex well or mix by pipetting each sample 15 times. Ensure a homogenous mixture of beads and sample before continuing.

4) Leave samples at room temperature for 20 minutes. If required, spin down briefly to collect sample in the bottom of the vial.

5) Place the samples on the magnet for 3 minutes, or until all beads have been collected.

6) 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.

7) Whilst leaving the vial on the magnet, add **180 µl** of freshly prepared **70% ethanol**.

8) Incubate for 30 seconds at room temperature.

9) Carefully discard the supernatant, without disturbing the beads.

10) Repeat steps 7 – 9 two additional times.

11) After the third wash carefully remove as much of the residual ethanol as possible.

12) 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.

13) Elute the DNA by resuspending the beads in **14 µl** of distilled water. Incubate the resuspended beads for 5 minutes at room temperature.

14) Place the samples on the magnet for 3 minutes, or until all the beads have been collected.

15) Carefully transfer **13 µl** eluted amplification product into a clean tube.

9.6 ATO 2 Reaction – Step 1: ATO 2 and Amplification One Mixture

- Add **2 µl 'ATO – 2' (Transparent Tube, Orange Cap, PC0035)** to the purified Amplification 1 product. Mix by vortexing, and centrifuge briefly, ensuring lids remain tightly sealed. The total volume of each sample is now 15 µl
- Incubate the mixture in a thermocycler as detailed in the table below, with a heated lid ≥100 °C:

ATO 2 Reaction – Step 1: Incubation Conditions

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

9.7 ATO 2 Reaction – Step 2: Addition of ATO Reaction Mix

- Remove the sample from the thermocycler, carefully open the PCR vessel, and add **5.0 µl of the ATO Reaction Mix (Transparent Tube, Green Cap, PC0199).** The total volume of each sample will now be 20 µl
- Seal the PCR vessel, mix by vortexing, and centrifuge briefly
- Ensure that the thermocycler is precooled to 4 °C. Place the PCR vessel into the machine and continue to thermocycle as detailed below, with a heated lid ≥100 °C:

ATO 1 Reaction – Step 2: Incubation Conditions

Stage	Temperature	Duration
1	4 °C	Hold/Pause
2	10 °C	1 minute
3	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	4 °C	Hold

9.8 ATO 2 Reaction – Step 3: ATO 2 Treatment

- Remove the samples from the thermocycler and briefly spin down. Add **2 µl ATO Treatment (Transparent Tube, White Cap, PCPC0031)** to each sample. The total volume of each sample is now 22 µl
- Seal the PCR vessel, mix by vortexing, and centrifuge briefly
- Incubate the mixture in a thermocycler as detailed in the table at the top of the following page, with a heated lid ≥100 °C

ATO 1 Reaction – Step 3: Incubation Conditions

Stage	Temperature	Duration
1	37 °C	15 minutes
2	4 °C	Hold

9.9 Amplification Two

When preparing the sample mixes, different combinations of i7 and i5 primers can be used to allow for sample multiplexing. When using XCeloSeq i5 or i7 Indexing Kits the supplied i5 and i7 oligos can directly substitute the oligos used in this protocol, as detailed in the table below.

- Prepare the Amplification 2 Mix by adding reagents to the sample from step 9.8 according to the order in the table below

#	Name	Tube colour	Tube Cap colour	Volume per single reaction (µl)	Part Code
1	**Product of Step 9.8**	N/A	N/A	22.0	**NA**
2	**Universal Enzyme Mix**	**Transparent**	**Lilac**	25.0	**PC0033**
3	**i7-002 (i7 Primer)**	**Amber**	**Green**	1.5	**PC0201**
4	**i5-002 (i5 Primer)**	**Amber**	**Blue**	1.5	**PC0200**
	Total Volume	-	-	50.0	

- Seal the PCR vessel, mix by vortexing, and centrifuge briefly
- Incubate the mixture in a thermocycler as detailed in the table below, with a heated lid ≥100 ^{o}C

Amplification One: Incubation Conditions

Stage	Cycles	Temperature	Duration
1	1x	98 °C	30 seconds
2	Various, see table below	98 °C	10 seconds
3		60 °C	30 seconds
4		65 °C	75 seconds
5	1x	65 °C	2 minutes
6	-	4 °C	Hold

The recommended PCR cycle number depends on the amount of starting material (see table below). It also depends upon the quality of the starting material and the desired library yield. The below table contains recommended starting values, but these may have to be adjusted by the individual user.

Input cfDNA (ng)	Recommended Cycle number
20-50	**5 – 7**
10	**7 – 8**
5	**8 – 9**

9.10 Bead Purification

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

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

1) Add **0.9X** volumes (**45 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) Add **50 µl** of distilled water and resuspend beads by pipetting each sample 15 times to ensure a homogenous mixture of bead and sample.
7) Add **0.7X** volumes (**35 µl**) of AMPure XP beads to each reaction.
8) Vortex, or mix by pipetting each sample 15 times, to ensure a homogenous mixture of beads and sample.
9) Leave samples at room temperature for 5 minutes. If required spin down briefly to collect sample in the bottom of the vial.
10) Place the samples on the magnet for 3 minutes, or until all the beads have been collected.
11) 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
12) While leaving the vial on the magnet add **180 µl** of freshly prepared **80% ethanol**.
13) Incubate for 30 seconds. Ensure all beads remain gathered to the side of the vial.
14) Carefully discard the supernatant.
15) Wash the sample again by repeat the preceding 3 steps (13 – 15) one additional time.
16) After the second wash carefully remove as much of the residual ethanol as possible.
17) 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.
18) Elute the DNA by resuspending the beads in **20 µl** of 10 mM Tris-HCL pH 8.0. Incubate the resuspended beads for 5 minutes at room temperature. **Note:** Higher elution volumes can be used depending on user requirements
19) Place the samples on the magnet for 3 minutes, or until all the beads have been collected.
20) Carefully transfer all the eluted amplification product into a clean tube.

9.11 Library QC, Quantification, and Sequencing Recommendations

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

We recommend the use of an Agilent Bioanalyzer High Sensitivity Chip (or equivalent) for determining the size distribution of the generated libraries. Library concentration should be determined by a suitable method, we recommend a qPCR quantification method such as NEBNext® Library Quant Kit for Illumina (#E7630) prior to sequencing.

10 Troubleshooting

10.1 Library yields are low or absent

When the kit reagents are stored as recommend, suitable starting material is used and the protocol is completed as stated in this IFU, the results are expected to be highly consistent and robust. Please ensure that the kit components are stored at the correct temperatures, that you are only using reagents supplied with the XCeloSeq cfDNA Library Preparation Kit (excluding the additional indexes), 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 using cell free DNA or enzymatically fragmented gDNA. The XCeloSeq cfDNA Library Preparation Kit is compatible with DNA fragmented by sonication, however this can 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.

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.

11 Customer Contact Information

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

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