



## **XCeloSeq<sup>®</sup> Pan Cancer Kit**

**SEQ002**

**FOR RESEARCH USE ONLY**

**Store at -20°C**

**Instructions for Use – English**

**Version 1.0 – March 2020**

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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 Intended Use

The XCeloSeq Pan Cancer Kit is intended for use for the targeted 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 XCelSeq Technological Principle

All XCelSeq products are built on the strongest technical foundation, **A**daptor **T**emplate **O**ligo **M**ediated **S**equencing – **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 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 Reagents

### 5.1 Reagents Supplied with the XCeloSeq Pan Cancer Kit

Reagent	Tube colour	Tube cap colour	Storage Conditions
ATO	Transparent	Blue	-20°C
ATO Reagents	Transparent	Amber	-20°C
ATO Enzyme	Transparent	Green	-20°C
Amplification One Mix	Transparent	Transparent	-20°C
Primers	Transparent	Yellow	-20°C
Master Mix	Transparent	Lilac	-20°C
Pool 1 – OUTER	Transparent	Orange	-20°C
Pool 1 – INNER	Transparent	Black	-20°C
Pool 2 – OUTER	Transparent	White	-20°C
Pool 2 – INNER	Transparent	Red cap	-20°C



Reagents between different XCeloSeq kits are not interchangeable.  
Only use “XCeloSeq Pan Cancer Kit” reagents with the following protocol.

### 5.2 Indexes Supplied with the XCeloSeq Pan Cancer Kit

Reagent	Tube Colour	Tube Cap Colour	Storage Conditions	Index	Machine Compatibility
i5-S-001 (i5 Primer)	Amber	Blue	-20°C	TCCGCTT	MiSeq, NovaSeq, HiSeq2500, HiSeq2000
				AAGCGGAA	MiniSeq, NextSeq, HiSeq4000, HiSeq3000
i7-001 (i7 Primer)	Amber	Green	-20°C	ATCACG	All

All SEQ005-i5-XN primers supplied in XCeloSeq Indexing Kits can directly replace the supplied i5-S-001 primer supplied with the kit.

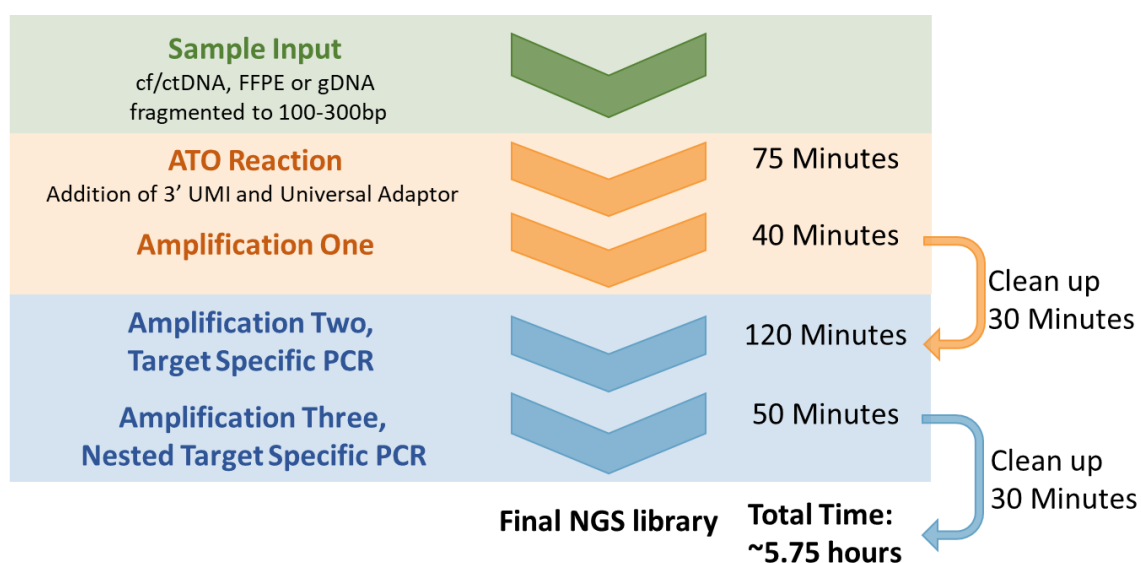


All SEQ005-i7-XN primers supplied in XCeloSeq Indexing Kits **MUST BE DILUTED 1:4** with the supplied i7 Dilution Buffer prior to replacing the i7-001 primer supplied with this kit.  
See section 7.2

### 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)
- TE buffer (10 mM Tris-HCl, 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
- Agencourt® AMPure® XP magnetic beads
- 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
- PCR tubes, plates, and accessories compatible with the PCR system used
- Ice

## 6 Protocol Overview



Times shown represent protocol duration for 10 ng of starting material.

## 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 strands 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/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.

*The recommended range of starting material is 5-50 ng of cfDNA. Larger quantities of DNA should be used where possible to improve maximum sensitivity. Starting quantities of DNA should not exceed 50 ng.*

### 7.1 Reagent Preparation

- Freshly prepared 80% ethanol. Ensure the vessel is tightly closed when not in use to prevent unwanted evaporation

### 7.2 Preparing Index Primers

The XCeloSeq Pan Cancer Kit is 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 indexing 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 Index Kits, they can directly replace the i5-S-001 indexing primer supplied with this Pan Cancer Kit.

If using additional XCeloSeq i7 Index Kits, ensure that prior to use the primers have been diluted 1:4 in the i7 Dilution Buffer provided with the Index Kit. Only once diluted can they be used with the XCeloSeq Pan Cancer Kit. Ensure diluted primers are only used with compatible XCeloSeq products. See the below table for an example.

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	10	30	40	-20°C	6 months from date of dilution
	SEQ005-i7-x2	10	30	40	-20°C	
	SEQ005-i7-x3	10	30	40	-20°C	
	SEQ005-i7-x4	10	30	40	-20°C	
	SEQ005-i7-x5	10	30	40	-20°C	
	SEQ005-i7-x6	10	30	40	-20°C	
	SEQ005-i7-x7	10	30	40	-20°C	
	SEQ005-i7-x8	10	30	40	-20°C	

## 8 Operating Procedure

### 8.1 ATO Reaction – Step 1: ATO Reaction Mix and Incubation

- In a PCR vessel, combine **2 µl ATO (transparent tube, blue cap)** and your DNA 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

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

### 8.2 ATO Reaction – Step 2: Enzyme and Reagent Addition

- Prepare the following mixture:

Order	Name	Tube colour	Tube Cap colour	Volume per single reaction (µl)
1	ATO Reagents	Transparent	Amber	3.3
2	ATO Enzyme	Transparent	Green	2.2
	<b>Total Volume</b>			<b>5.5</b>

- Enough reagents for a 10% excess of this mix are supplied
- Mix by vortexing, and centrifuge briefly
- Remove the sample from the thermocycler, carefully open the PCR vessel, and add **5.0 µl of the ATO Enzyme and ATO Reagents** mixture. 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



Stage	Cycles	Temperature	Duration
1	-	4 °C	Hold/Pause
2	1x	10 °C	1 minute
3	1x	26 °C	6 minutes
4		30 °C	<b>10 minutes</b>
5		65 °C	1 minute
6		10 °C	1 minute
7		26 °C	6 minutes
8		30 °C	<b>10 minutes</b>
9	2x	65 °C	1 minute
10		10 °C	1 minute
11		26 °C	6 minutes
12		30 °C	<b>5 minutes</b>
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 cap)** and **1.0 µl Primers (Yellow cap)** 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

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.*

## 8.4 Amplification Two, Target Specific PCR



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

Before continuing, use the 46  $\mu$ l produced in **Step 8.3** to **prepare two 22  $\mu$ l 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 ( $\mu$ l)
1	<u>Aliquot 1</u> of Step 8.3 Product	N/A	N/A	22.0
2	<u>Pool 1 – OUTER</u>	Transparent	Orange	3.0
3	Master Mix	Transparent	Lilac	25.0
	<b>Total Volume</b>			<b>50.0</b>

- 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 ( $\mu$ l)
1	<u>Aliquot 2</u> of Step 8.3 Product	N/A	N/A	22.0
2	<u>Pool 2 – OUTER</u>	Transparent	White	3.0
3	Master Mix	Transparent	Lilac	25.0
	<b>Total Volume</b>			<b>50.0</b>

- Vortex the Amplification Two Mixtures and centrifuge briefly
- Incubate the mixtures in a thermocycler as detailed in the table below:

Stage	Cycles	Temperature	Duration
1	1x	98 °C	30 seconds
2	20x	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.

- 1) Allow AMPure Beads to come to room temperature, then completely resuspend the beads by vortexing. **Note:** All bead purification steps must be performed at room temperature and reagents should not be kept on ice.
- 2) Add **1.8X** volumes (**90 µl**) of AMPure beads to each reaction.
- 3) Vortex or mix by pipetting each sample 15 times to ensure a homogenous mixture of beads and sample.
- 4) Leave samples at room temperature for 5 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 the 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) While leaving the vial on the magnet add **150 µl** of freshly prepared **80%** ethanol.
- 8) Incubate for 30 seconds. Ensure all beads remain gathered to the side of the vial.
- 9) Carefully discard the supernatant.
- 10) Repeat steps 7 – 9 one additional time.
- 11) After the second 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 23 µl of either molecular biology grade H<sub>2</sub>O or 10 mM Tris-HCL pH 8.0. Incubate the resuspended beads for 5 minutes.
- 14) Place the samples on the magnet for 3 minutes, or until all the beads have been collected.
- 15) Carefully transfer 21.0 µl of the eluted amplification product into a clean PCR tube.



*After the bead purification samples can be store 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 strands of the DNA. 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)
1	Pool 1 – OUTER Product of Step 8.5	N/A	N/A	21.0
2	<u>Pool 1 – INNER</u>	Transparent	Black	2.0
3	Master Mix	Transparent	Lilac	25.0
4	i7-001	Amber	Green	1.0
5	i5-S-001	Amber	Blue	1.0
	<b>Total Volume</b>			<b>50.0</b>

- 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)
1	Pool 2 – OUTER Product of Step 8.5	N/A	N/A	21.0
2	<u>Pool 2 – INNER</u>	Transparent	Red cap	2.0
3	Master Mix	Transparent	Lilac	25.0
4	i7-001	Amber	Green	1.0
5	i5-S-001	Amber	Blue	1.0
	<b>Total Volume</b>			<b>50.0</b>

- Vortex the Amplification Three Mixtures and centrifuge briefly.
- Incubate the mixture in a thermocycler as detailed in the table below:

Stage	Cycles	Temperature	Duration
1	1x	98 °C	30 seconds
2	Variable, see table on 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.

Input DNA Quantity	Recommended Amplification Three cycle number by input DNA type		
	Enzymatically Fragmented High Quality gDNA	cfDNA	Enzymatically Fragmented FFPE DNA
5 ng	9-10x	10-11x	11-12x
10 ng	8-9x	9-10x	10-11x
20 ng	7-8x	8-9x	9-10x
50 ng	6-7x	7-8x	8-9x

### 8.7 Bead Purification

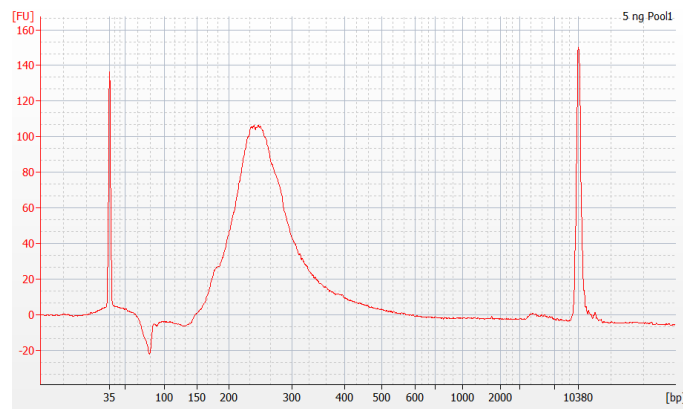
- 1) Allow the AMPure Beads to come to room temperature, then completely resuspend the beads by vortexing. **Note:** All bead purification steps must be performed at room temperature, and reagents should not be kept on ice.
- 2) Add **1.2X** volumes (**60 µl**) of AMPure beads to each reaction.
- 3) Vortex or mix by pipetting each sample 15 times to ensure a homogenous mixture of beads and sample.
- 4) Leave samples at room temperature for 5 minutes. If required spin down briefly if required to collect sample in the bottom of the vial.
- 5) Place the samples on the magnet for 3 minutes, or until all the 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) While leaving the vial on the magnet add 150 µl of freshly prepared 80% ethanol.
- 8) Incubate for 30 seconds. Ensure all beads remain gathered to the side of the vial.
- 9) Carefully discard the supernatant.
- 10) Repeat steps 7 – 9 one additional time.
- 11) After the second 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 32 µl of 10 mM Tris-HCL pH 8.0. Incubate the resuspended beads for 5 minutes.
- 14) Place the samples on the magnet for 3 minutes, or until all the beads have been collected.
- 15) Carefully transfer 30 µl of the eluted amplification product into a clean tube.

The bead purified Amplification 3 product is now ready for downstream processing including, bioanalyzer size analysis, qPCR quantification, and Illumina sequencing.

## 9 Representative Data

Representative final libraries generated using enzymatically fragmented gDNA

### 9.1 5 ng high quality enzymatically fragmented gDNA

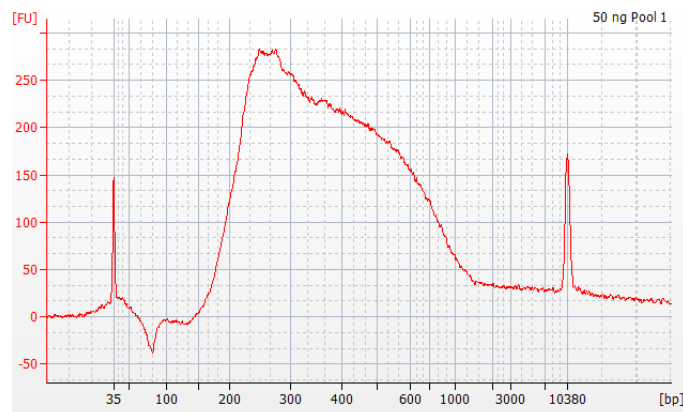


### 9.2 50 ng high quality enzymatically fragmented gDNA

When using starting quantities at the upper supported limits, 20-50 ng, a large “shoulder” or “hump” may be visible on visualising final library on a bioanalyzer.

This is unlikely to be an over amplified library and as such we do not recommend reducing the cycle number too far, as this will disproportionately reduce the final library products. The nature of the protocol results in generation of these products. You should continue normally and quantify your library, for example by qPCR (NEBNext® Library Quant Kit for Illumina®).

For the libraries below the average peak size used was 260bp, the shoulder was ignored when determining library insert size.



## 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 XCelSeq Pan Cancer 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 XCelSeq Pan Cancer 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.

### 10.4 Low library yields or libraries with a 'shoulder' when using XCelSeq indexing kits

Failing to correctly dilute i7 index oligos supplied in XCelSeq 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 Bionalyzer 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 Bionalyzer profile indicate that the primers from Amplification Three have carried over though the final bead purification. When performing 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.



## 11 Customer Contact Information

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

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