XCeloSeq® Targeted RNA 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

IFU1826 Version 1.0 – July 2022

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

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 RNA Enrichment Protocol is intended for use with XCeloSeq Targeted RNA Core Reagents (Part Code: **GF031**), any compatible XCeloSeq Targeted RNA Enrichment Primers (**See Section 6.2**), and, any compatible XCeloSeq UDI Set (**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 for the detection of both known and unknown fusions.

This is a Research Use Only product.

5. XCeloSeq Technological Principle

This XCeloSeq product is 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. This process is ideal for cell-free DNA.

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 cell-free DNA, 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. GeneFirst XCeloSeq Targeted RNA Enrichment Kit Reagents

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

- The first box contains the XCeloSeq Targeted RNA Core Reagents which include proprietary reagents for the patented approach of generating unique molecular identifiers and priming sites on all cDNA 3' ends through an **ATO Reaction**. The first box also contains the master mixes necessary for double-strand cDNA generation and all three amplification steps.
- The second box contains XCeloSeq Targeted RNA Enrichment Primers, which are used for the enrichment of cDNA regions during the construction of sequencing-ready libraries. See Section 5.2 for a full list of compatible primer sets.

6.1. XCeloSeq Targeted RNA Core Reagents (GF031)

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

Reagent	Tube colour	Tube cap colour	Storage Conditions	Part Code
FS Mix	Transparent	**Amber**	**-20°C**	**PC0038**
SS Enzyme	Transparent	**Yellow**	**-20°C**	**PC0039**
ATO	Transparent	**Blue**	**-20°C**	**PC0040**
ATO Reaction Mix	Transparent	**Green**	**-20°C**	**PC0041**
Amplification One Mix	Transparent	**Transparent**	**-20°C**	**PC0042**
Primers	Transparent	**Red**	**-20°C**	**PC0043**
Master Mix	Transparent	**Lilac**	**-20°C**	**PC0044**

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

6.2. XCeloSeq Targeted RNA Enrichment Primers

The XCeloSeq targeted RNA enrichment primers will be named according to the product purchased, see the table below.

This protocol can only be used with the following products:

Catalogue Number	Product Name	Target Genes	Target Primers
SEQ007	**XCeloSeq Fusion Research Kit**	**74**	**458**
SEQ008	**XCeloSeq Lung Cancer Fusion Kit**	**15**	**159**
SEQ012	**XCeloSeq Solid Cancer Fusion Kit**	**53**	**351**
SEQ014	**XCeloSeq Sarcoma Fusion Kit**	**26**	**146**
SEQ015	**XCeloSeq Actionable Fusion Kit**	**12**	**144**
SEQ017	**XCeloSeq Myeloid Fusion Kit**	**39**	**310**
SEQ018	**XCeloSeq Lymphoma Fusion Kit**	**33**	**241**
SEQ019	**XCeloSeq Acute Lymphoblastic Leukaemia (ALL) Fusion Kit**	**44**	**317**

Each of the above kits comes with the following two 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
OUTER Pool	Transparent	**Orange**	**-20°C**	**10.7**	**Refer to product insert**
INNER Pool	Transparent	**Black**	**-20°C**	**10.9**	

Reagents are not interchangeable amongst different XCeloSeq kits. Only use reagents supplied as part of XCeloSeq Targeted RNA Core Reagents and XCeloSeq Targeted RNA 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 RNA 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 16 reactions for a total of 128 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 Number of Processed Samples

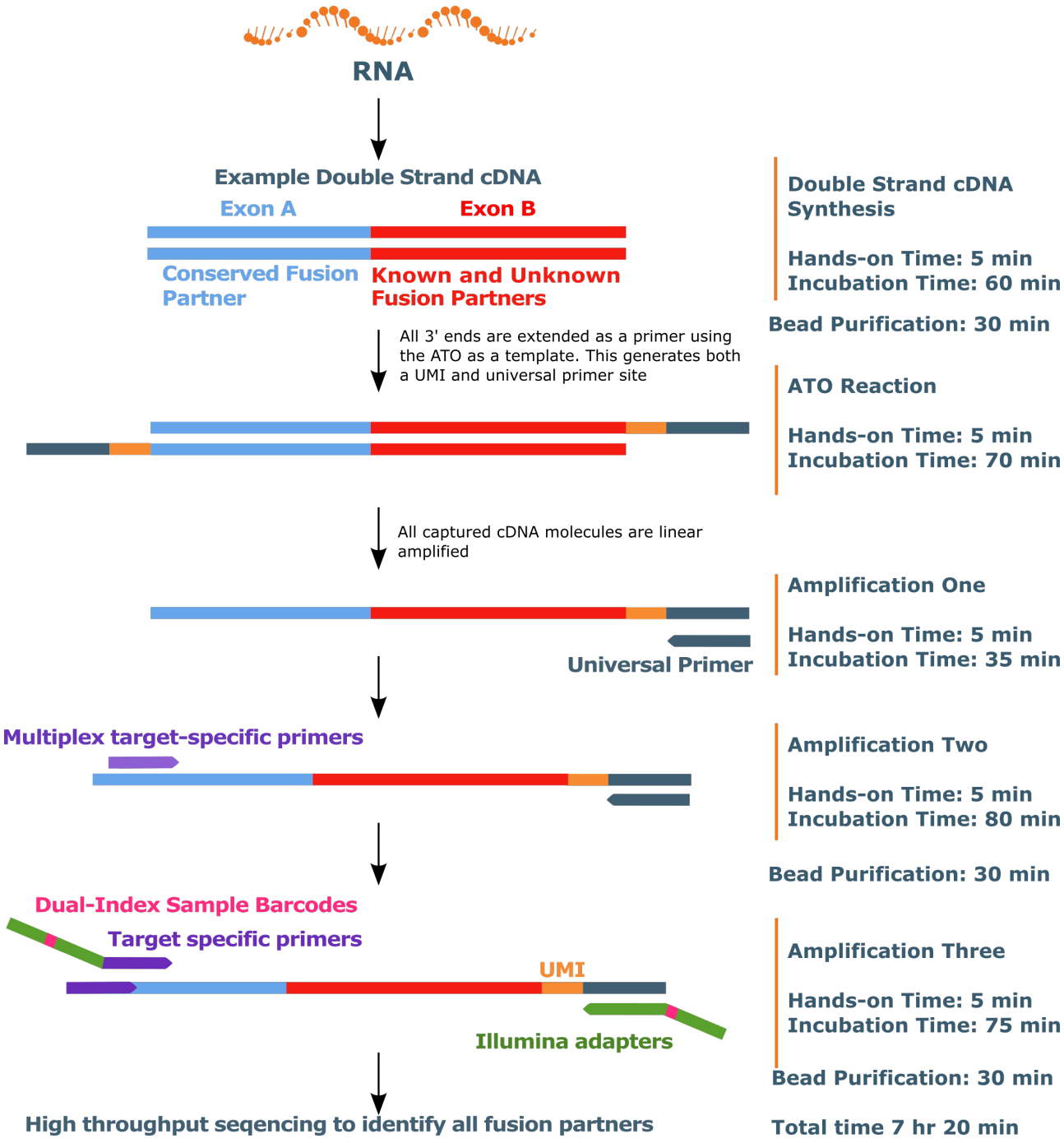
When determining UDI Set requirements for processing a fixed number of samples, please refer to the table below as an example. (Note: Please do still consider multiplexing requirements using the previous table).

Total Number of Samples to be processed	Total Number of UDI Sets Required
1 to 128	1
129 to 256	2
257 to 384	3
385 to 512	4
513 to 640	5
641 to 768	6
769 to 896	7
897 to 1024	8
1025 to 1152	9
1153 to 1280	10
1281 to 1408	11
1409 to 1536	12

7.4. Additional Required Equipment and Reagents Provided by the User

- Reagents and equipment for specimen collection, filtration, and nucleic acid extraction
- Distilled water (molecular biology grade)
- Quantification reagents, fluorometric-based method such as Qubit™ RNA High Sensitivity (HS).
- 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
- 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

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 25 ng of high quality total RNA, however all recommended starting mateiral will follow the same process. This includes RNA extracted from FFPE material.

9. Before Starting

- Read this protocol in its entirety before beginning the library preparation to ensure everything is prepared and the process is clear prior to 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 RNA in a sample to determine its suitability for use with the XCeloSeq Targeted RNA 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 RNA samples believed or expected to be fragmented (such as FFPE-extracted RNA) we also recommend a quality assessment though the use capillary electrophoresis, for example a Bioanalyzer or TapeStation Systems. These instruments generate an RNA Integrity Number which indicates the level of degradation of the RNA and is out of a maximum of 10. XCeloSeq kits which utilise ATOM-Seq technology are able to work with shorter input material so are more resistant to levels of degradation, however we always recommend using the highest input mass possible, especially with low/poor quality samples.

A high-quality total RNA sample may have RIN scores >8. For these, a suggested starting point of **100 ng** of high-quality Total RNA as input should give high quality results.

An FFPE total RNA sample may have RIN score below 8.0. With lower quality material, such as from FFPE, a suggested starting point is **200 ng** of FFPE RNA.

Alternatively, cell-free RNA or total cell-free nucleic acids may be used, however fusion detection sensitivity will be lower due to cell-free RNA concentrations typically being very low in such samples. A cfRNA sample should also be expected to contain a significant mass of cfDNA which results in a relatively low proportion of the final library having originated from RNA molecules, the majority will be derived from DNA molecules.

For all materials, especially cell-free RNA, input should be maximised where possible within the above ranges to ensure the highest possible quality of data.

9.2. 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.

10. Operation Procedure

10.1. First Strand cDNA Synthesis

- In a PCR vessel, combine **4.0 µl FS Mix (Transparent Tube, Amber Cap, PC0038)** and your RNA sample. The total volume of this mixture must not exceed **20 µl**. If required add molecular biology grade water to a final volume of **20 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 ^{o}C:

First Strand cDNA Synthesis Incubation Conditions

Stage	Temperature	Duration
1	65 °C	5 minutes
2	4 °C	2 minutes
3	25 °C	2 minutes
4	55 °C	10 minutes
5	4 °C	Hold

10.2. Second Strand cDNA Synthesis

- Remove the sample from the thermocycler, carefully open the PCR vessel, and add **1.5 µl of the SS Enzyme (Transparent Tube, Yellow Cap, PC0039).** The total volume of each sample will now be **21.5 µl**
- Seal the PCR vessel, mix by vortexing, and centrifuge briefly
- Place your PCR vessel into the machine and continue to thermocycle as detailed below, with a heated lid ≥100^{o}C:

Second Strand cDNA Synthesis Incubation Conditions

Stage	Temperature	Duration
1	22 °C	30 minutes
2	4 °C	Hold

10.3. 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 **28.5 µl** of nuclease free water to your sample making the final volume **50.0 µl.**
2. Add **2.0X** volumes (**100 µl**) of AMPure XP beads to each reaction.
3. Mix samples by vortexing or 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 **15 µl** of either molecular biology grade H_2O 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 **13 µl** of the eluted amplification product into a clean PCR tube.

10.4. ATO Reaction – Step 1: ATO and cDNA Mixture

- Add **2.0 µl ATO (Transparent Tube, Blue Cap, PC0040)** with your bead purified cDNA sample. The total volume of this mixture is now **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

10.5. 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 ATO Reaction Mix (Transparent Tube, Green Cap, PC0041).** 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 below, with a heated lid ≥100 °C:

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

10.6. Amplification One

- Remove the samples from the thermocycler and briefly spin down. Add **27.0 µl Amplification One Mix (Transparent Tube, Transparent Cap, PC0042)** and **1.0 µl Primers (Transparent Tube, Red Cap, PC0043)** to each sample. The total volume per sample is now **48.0 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

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.

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10.7. Amplification Two, Target Specific PCR

- Remove the samples from the thermocycler and briefly spin down. Add **2.0 µl OUTER Pool (Transparent Tube, Orange Cap, See Product Insert for Part Code)** to each sample. The total volume per sample is now **50.0 µl**. Refer to product insert for Part Code.

- Vortex the Amplification Two Mixture and centrifuge briefly

- Incubate the mixture in a thermocycler as detailed in the table below, with a heated lid ≥100 °C:

Amplification Two: Incubation Conditions

Stage	Cycles	Temperature	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

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10.8. 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. Mix samples by vortexing or by pipetting each sample 15 times to ensure a homogenous mixture of beads and sample.
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. Repeat steps 7 – 9 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.

Safe Stopping Point

10.9. Amplification Three, Nested Target Specific PCR

- Prepare the INNER-Pool Amplification Three Mix by adding reagents to the bead purified sample from step 10.8, according to the order in the table below.

Note: When preparing the sample mixes below, any of the compatible XCeloSeq UDI Sets may be used. See section 7.1 for details.

Order	Name	Tube Colour	Tube Cap Colour	Volume Per Single Reaction (µl)	Part Code
1	**OUTER-Pool Product of Step 10.8**	N/A	N/A	21.0	**N/A**
2	**INNER-Pool**	**Transparent**	**Black**	2.0	**See Product Insert**
3	**Master Mix**	**Transparent**	**Lilac**	25.0	**PC0044**
4	**Pre-mixed UDI Primers**	**Transparent**	**White**	2.0	**See Product Insert**
			Total Volume:	**50.0**	

- Vortex the Amplification Three Mixture 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 below	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 Amplification Three yields are too high or too low, or when using higher or lower quantities of RNA, 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. For example, additional cycles may be necessary when using RNA extracted from FFPE material.

High Quality Total RNA	
RNA Quantity	**Recommended Amplification Three Cycle Number**
5 ng	**14-15x**
10 ng	**13-14x**
25 ng	**12-13x**
50 ng	**11-12x**
100-200 ng	**10-11x**

10.10. 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 **1.2X** volumes (**60 µl**) of AMPure XP beads to each reaction.
2. Mix samples by vortexing or by pipetting each sample 15 times to ensure a homogenous mixture of beads and sample.
3. Leave samples at room temperature for 5 minutes. If required spin down briefly if required to collect the 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. Repeat steps 7 – 9 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.
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.

10.11. Library QC, Quantification, and Sequencing Recommendations

The bead purified Amplification 3 product is now ready for downstream processing.

We recommend the use of an Agilent Bioanalyzer High Sensitivity DNA Chip (or equivalent) for determining the size distribution of the generated libraries. As shown in Section 11.1 (below), when using large quantities of input RNA a peak will appear around or above 600bp. This peak should not be used when determining the average size of your libraries as they are far larger than the expected PCR library products and are non-sequenceable, non-specific products. As such an upper limit of approximately 500bp should be used when determining average insert size.

Library concentration should be determined by a suitable qPCR quantification method such as NEBNext® Library Quant Kit for Illumina (#E7630), prior to sequencing. Quantification by any other method such as direct DNA quantification or the use of a Bioanalyzer (or equivalent) to estimate library concentration is likely to give highly inaccurate results and should be avoided.

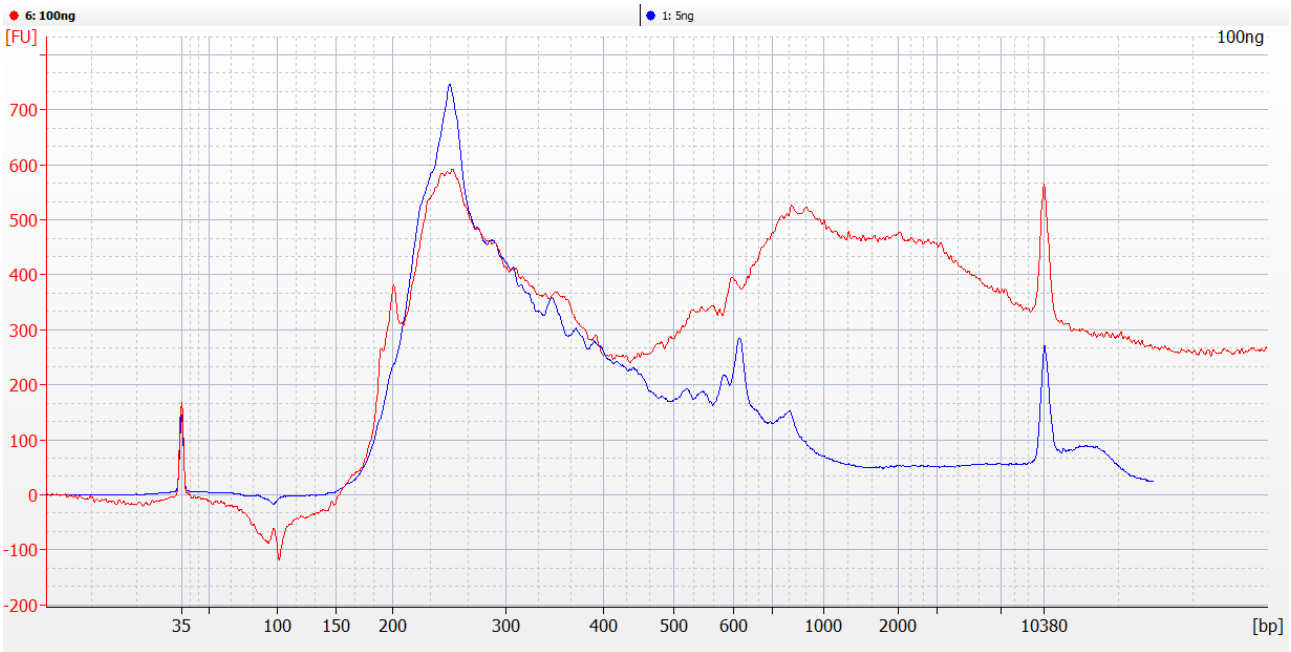
The total number of recommended reads per sample should be used as detailed in the individual product inserts. For sequencing use the read length for each sequencing stage as set out in the table below.

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

11.1. High quality RNA

Representative final libraries generated using 5 ng or 100 ng of total high-quality RNA:



12. Troubleshooting

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 reagents supplied with the XCeloSeq Fusion Research 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.

12.2. Low Library yields when using FFPE-extracted RNA

Formalin Fixed Paraffin Embedded (FFPE) samples can be of highly variable quality. As such using larger quantities of total FFPE RNA may still result in low library yields, indicating that the proportion of capturable RNA in the sample is low. Using larger quantities of starting material can help ensure that high quality libraries are generated.

12.3. 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 between 600-1000bp in Section 11.1. This is not expected to influence the quality of the library and you should continue to quantify and sequence the library as normal.

12.4. 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 through 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 **Error! Reference source not found.**) will reduce their abundance.

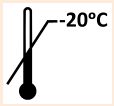
12.5. Library yields are high and there is a 'wavy' secondary bump following the main library peak

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.

12.6. Do XCeloSeq kits use standard Illumina sequencing primers?

The indexing PCR primers supplied in XCeloSeq Targeted RNA Core Reagents and in XCeloSeq Indexing Kits contain standard Illumina sequences and therefore no custom sequencing primers are required for libraries generated with these reagents. We recommended that only XCeloSeq indexing primers are used because they have been specially formulated and optimised to ensure high quality libraries are obtained when using this protocol.

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:

GeneFirst Limited
Unit 2 The Quadrant,
Abingdon Science Park,
Abingdon,
Oxfordshire,
OX14 3YS
United Kingdom

Customer Service & Sales Enquiries:
Telephone: +44 (0)1865 407 400
Email: sales@genefirst.com