Cell3™ Target V2

DNA Target Enrichment for Next Generation Sequencing (Illumina Sequencers)

Version 1.5

nonacus

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Revision history

Revision	Date	Revision description
1.0	May 2023	First version
1.1	August 2023	Updates to table 1
1.2	October 2023	Updates to intended use section
1.3	January 2024	Updates to Cell3™ Target Panel table in kit contents section. Updates to Appendix I, fragmentation protocol
1.4	February 2024	Updates to fragmentation table on page 51
1.5	July 2024	• Updates to workflow overview diagram • Updates to 'Required laboratory reagents and consumables not supplied' table • Updates to section 2.B Probe capture on streptavidin beads and washes to clarify procedure • Updates to Appendix II. Alternative procedure for magnetic bead clean-up steps • Updates to Appendix III. Alternative procedure for concentrating pooled sample libraries prior to hybridization using Target Pure™ NGS clean-up beads • Updates to Appendix V. Alternative post-hybridization capture procedure to follow when using all Cell3™ exome panels

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Intended use

This protocol explains how to use the Library Preparation Kit V2 (a) or (b) – 16, 48 or 96 reactions, (a) without enzymatic fragmentation, (b) with fragmentation, in conjunction with the Hybridization and Capture Enrichment Kit V2 – 4 or 12 reactions kits and Cell3™ Target Panels - 2, 4, or 12 reactions, to perform DNA target enrichment for next generation sequencing on Illumina platforms using cell free DNA (cfDNA) or genomic DNA (gDNA) as input material. This protocol is to be used for all Cell3™ Target products:

Product code	Description
NGS_C3T_###*	Cell3™ Target product group
NGS_C3C_###*	Cell3™ Target Custom product group

*The ### references a product specific 3 letter code which is followed by eitherNF and the reaction size (Library Preparation Kit V2 (a) without enzymatic fragmentation– 16, 48 or 96 reactions), or FR and the reaction size (Library Preparation Kit V2 (b) with enzymatic fragmentation – 16, 48 or 96 reaction). E.g., NGS_C3C_XXL_NF_96 for Cell3™ Target: Custom Panel XXL (Non-Frag – 96 samples) and NGS_GAL_BCP_FR_96 Cell3™ Target: Bladder Cancer Panel (Frag - 96 samples).

Key features

- Suitable for use with 1 – 1000 ng of cfDNA or gDNA from any tissue of origin as input material.
- Single tube solution for library preparation reduces the number of bead clean-up steps, maximises yield and facilitates automation.
- Protocol supports library preparation for both long and short fragment DNA with two formats:
- a) Without enzymatic fragmentation reagents for library preparation of cfDNA.
- b) With enzymatic fragmentation reagents for library preparation of gDNA, which avoids the need to physically shear gDNA by sonication.
- Included in the Library Preparation Kit V2 (a) or (b) – 16, 48 or 96 reactions are;
  - **Illumina adapters** containing **Unique Dual Indexes (UDI)** to identify and avoid sample index skipping.
  - **Unique Molecular Identifiers (UMI)** 9 bp long for PCR/sequencing error removal and single molecule counting in bioinformatic analysis.

When to use UMIs in your bio-informatics analysis

With UMIs

Recommended for low DNA input quantities of 1-100 ng

Without UMIs

Recommended when using high DNA input quantities of 100-1000 ng or when sequencing PCR-free libraries

- Pooling of libraries PRIOR to hybridization and capture limits the number of capture reactions and amount of panel required.
- Concentration of pre-capture pooled individual sample libraries by using Target Pure™ NGS clean-up beads avoids the requirement for a vacuum concentrator or freeze drier.

Workflow overview

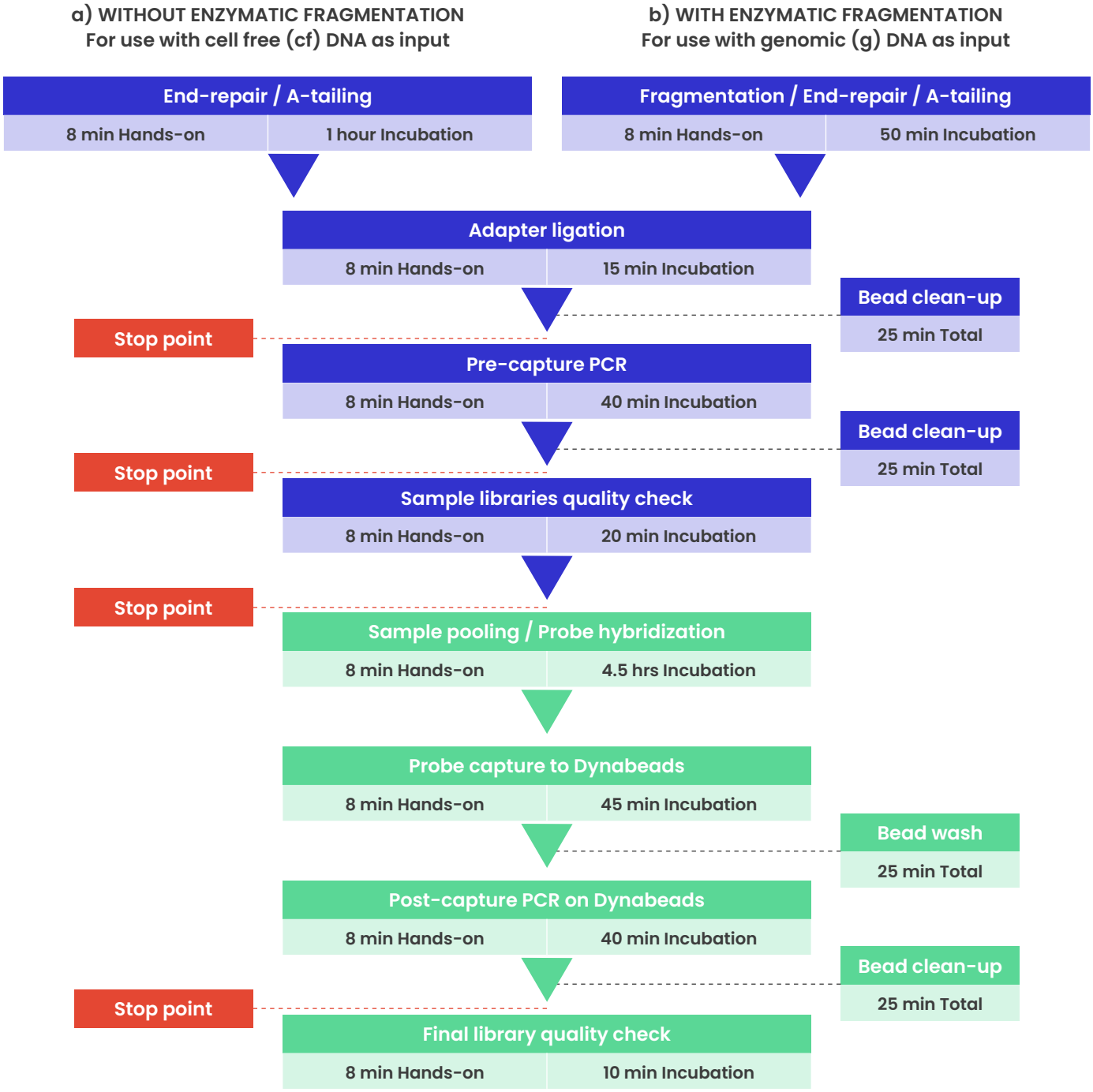

Figure 1. Flow chart outlining the main steps of the Cell3™ Target v2 workflow. Blue boxes refer to library preparation steps (3.5h); while green boxes refer to probe hybridization / capture and target enrichment steps (8h).

Kit contents

Library preparation kit V2 (a)

Reagent	Reagent volume and product codes						Storage	Reagent tube colour code
	16 samples NGS_ACC_LV2_NF_16		48 samples NGS_ACC_LV2_NF_48		96 samples NGS_ACC_LV2_NF_96			
ER-AT Enzyme	40 µl	C3TV2-ERE16	120 µl	C3TV2-ERE48	2x 120 µl	C3TV2-ERE48	-20°C	Red
ER-AT Buffer	93 µl	C3TV2-ERB16	280 µl	C3TV2-ERB48	2x 280 µl	C3TV2-ERB48	-20°C	Red
Ligation Mix	320 µl	C3TV2-LIG16	960 µl	C3TV2-LIG48	2x 960 µl	C3TV2-LIG48	-20°C	Blue
PreCap Amplification Mix	400 µl	C3TV2-PAM16	1.2 ml	C3TV2-PAM48	2x 1.2 ml	C3TV2-PAM48	-20°C	Green
PreCap Primer Mix	80 µl	C3TV2-PPM16	240 µl	C3TV2-PPM48	2x 240 µl	C3TV2-PPM48	-20°C	Black

Library preparation kit V2 (b)

Reagent	Reagent volume and product codes						Storage	Reagent tube colour code
	16 samples NGS_ACC_LV2_FR_16		48 samples NGS_ACC_LV2_FR_48		96 samples NGS_ACC_LV2_FR_96			
Fragmentation Enzyme	96 µl	C3TV2-FGE16	288 µl	C3TV2-FGE48	2x 288 µl	C3TV2-FGE48	-20°C	Red
Fragmentation Buffer	64 µl	C3TV2-FGB16	192 µl	C3TV2-FGB48	2x 192 µl	C3TV2-FGB48	-20°C	Red
Ligation Mix	320 µl	C3TV2-LIG16	960 µl	C3TV2-LIG48	2x 960 µl	C3TV2-LIG48	-20°C	Blue
PreCap Amplification Mix	400 µl	C3TV2-PAM16	1.2 ml	C3TV2-PAM48	2x 1.2 ml	C3TV2-PAM48	-20°C	Green
PreCap Primer Mix	80 µl	C3TV2-PPM16	240 µl	C3TV2-PPM48	2x 240 µl	C3TV2-PPM48	-20°C	Black

Adapter plates

Reagent	Product code	Storage
Adapter Plate (1-16 indexes)	NGS_ACC_ADP_1-16	-20°C
Adapter Plate (1-48 indexes)	NGS_ACC_ADP_1-48	-20°C
Adapter Plate (1-96 indexes)	NGS_ACC_ADP_1-96	-20°C
Adapter Plate (97-192 indexes)	NGS_ACC_ADP_97-192	-20°C
Adapter Plate (193-288 indexes)	NGS_ACC_ADP_193-288	-20°C
Adapter Plate (289-384 indexes)	NGS_ACC_ADP_289-384	-20°C

Cell3™ Target panel

Panel	Volume			Storage	No. of samples recommended for pre-capture pooling (see section 2.A Library pooling and probe hybridization, for more information)
	2 rxns	4 rxns	12 rxns		
Exome Panels (incl Whole exome, Tumor exome, ExomeCG and Nexome)	9 µl	-	54 µl	-20°C	8 samples
All other catalogue Cell3™ Target Panels	9 µl	-	54 µl	-20°C	8-16 samples
Custom Panel	-	18 µl	54 µl	-20°C	4-16 samples

Hybridization and capture enrichment kit V2

Reagent	Reagent volume and product codes				Storage	Reagent tube colour code
	4 rxns NGS_ACC_HV2_4		12 rxns NGS_ACC_HV2_12			
Hybridization Buffer (2x)	76 µl	C3TV2-THB04	228 µl	C3TV2-THB12	-20°C	**Blue**
Hybridization Enhancer	24 µl	C3TV2-THE04	72 µl	C3TV2-THE12	-20°C	**Brown**
Stringent Wash Buffer (10x)	160 µl	C3TV2-TSB04	480 µl	C3TV2-TSB12	-20°C	**White (S)**
Wash Buffer 1 (10x)	120 µl	C3TV2-TW104	360 µl	C3TV2-TW112	-20°C	**White (1)**
Wash Buffer 2 (10x)	80 µl	C3TV2-TW204	240 µl	C3TV2-TW212	-20°C	**White (2)**
Wash Buffer 3 (10x)	80 µl	C3TV2-TW304	240 µl	C3TV2-TW312	-20°C	**White (3)**
Bead Wash Buffer (2x)	1 ml	C3TV2-TWB04	2x 1.5 ml	C3TV2-TWB12	-20°C	**White (B)**
Universal Blockers	8 µl	C3TV2-TUB04	24 µl	C3TV2-TUB12	-20°C	**Orange**
COT-1 Human DNA	30 µl	C3TV2-TCO04	90 µl	C3TV2-TCO12	-20°C	**Red**
PostCap Amplification Mix*	100 µl	C3TV2-PCM04	300 µl	C3TV2-PCM12	-20°C	**Green**
PostCap Primer Mix*	10 µl	C3TV2-TPO04	30 µl	C3TV2-TPO12	-20°C	**Black**

***NOTE:** For large panels (all exome panels) the 12-capture reaction probe set kit will come with two vials of PostCap Amplification Mix and Primer Mix – PostCap in the Hybridization and Capture Enrichment Kit V2. This is to account for the two post-capture PCR reactions required for each capture reaction when using large probe panels.

Required laboratory reagents and consumables not supplied

Item	Recommended source
Buffer EB	Qiagen, Cat # 19086 (or equivalent: 10 mM Tris-HCl, pH 8.0)
Digital electrophoresis system consumables	**Agilent Technologies:** D1000 Reagents, Cat # 5067-5583; D1000 ScreenTape, Cat # 5067-5582 High Sensitivity D1000 Reagents, Cat # 5067-5585 High Sensitivity D1000 ScreenTape, Cat # 5067-5584 Genomic DNA ScreenTape, Cat # 5067-5365 Genomic DNA Reagents, Cat # 5067-5366 (Recommended: if not available, see appendix IV)
DNA low binding tubes, 1.5 ml PCR-clean	DNA LoBind 1.5 ml, Eppendorf, Cat # 022431021
Dynabeads® M-270 Streptavidin **IMPORTANT: we have validated our protocol with Dynabeads. Other beads are NOT recommended for use with the Cell3™ Target protocol**	Life Technologies, Cat# 65305
Ethanol (absolute, 100%)	Various sources available
Fluorometer consumables	**Invitrogen:** Qubit Assay Tubes, Cat # Q32856 Qubit dsDNA BR Assay kit, Cat # Q32853 Qubit dsDNA HS Assay kit, Cat # Q32854
Quantitative/Real-Time PCR library quantification kit	KAPA Library Quantification Kit – Illumina/Universal kit, KAPA Biosystems, Cat # KK4824 (optional)
Nuclease-free, molecular biology grade water	Various sources available
PCR-clean 0.2 ml PCR tubes / 8-well tube strips with caps / 96 well plates with caps/seals	Various sources available
PCR-clean 1.5-2 ml microcentrifuge tubes	Various sources available
Target Pure™ NGS Clean-up Beads	Nonacus, Cat # NGS_ACC_CUB_10 or equivalent (such as Agencourt®)
Ice or cold blocks	Various sources available
Low retention pipette tips	Various sources available

Required equipment

Item	Source
Digital electrophoresis system	Agilent 4200 TapeStation, Agilent Technologies, Cat # G2965AA (recommended: if not available, see appendix IV)
Fluorometer for DNA fluorometric quantitation	Qubit® 3.0 Fluorometer, Invitrogen, Cat # Q33216 Qubit™ 4 Fluorometer, Invitrogen, Cat # Q33238
Magnetic separation rack capable of accommodating 0.2 ml tubes / 8-well tube strips / 96 well plates	DynaMag™-96 Side Magnet, Invitrogen, Cat # 12331D (Recommended: if not available, see appendix II)
Magnetic separation rack capable of accommodating 1.5-2 ml tubes	DynaMag™ 2 Magnet, Invitrogen, Cat # 12321D (Optional, if a 96 well magnetic separation rack is not available)
Micro-centrifuge capable of accommodating 1.5-2 ml tubes	Various sources available
Mini-centrifuge capable of accommodating 0.2 ml PCR tubes / 8-well tube strips	Various sources available
Multichannel pipettes (10, 100, 200 µl capacity)	Various sources available
Plate centrifuge capable of accommodating 0.2 ml 96 well plates	Various sources available
Single channel pipettes (10, 100, 200, 1000 µl capacity)	Various sources available
Thermocycler with heated lid capable of accommodating 96 well plates	Various sources available
Vacuum concentrator	Concentrator Plus, Eppendorf, Cat # 5305000304 or vacuum lyophiliser / freeze-dryer (such as the ScanVac CoolSafe, Labogene) (Recommended: if not available, see appendix III)
Vortex mixer	Various sources available

Storage and handling

All kit components should be stored as indicated in the component list for each kit, as stated above. The enzyme mixes and Illumina UMI adapters in the Library Preparation Kit V2 (a) or (b); the PostCap Amplification Mix and PostCap Primer Mix in the Hybridization and Capture Enrichment Kit V2; and the probe set in the Cell3™ Target Panel should be thawed on ice and kept on ice during the relevant procedures. Briefly vortex mix all components after thawing and prior to use with the exception of the ER-AT Enzyme, the Fragmentation Enzyme, the Ligation Mix, PreCap Amplification Mix and PostCap Amplification Mix components in the Cell3™ Target kits, which should be mixed by light tapping. All components should be briefly spun down in a microcentrifuge after mixing.

Chapter 1: Library preparation

Library Preparation Kit V2 (a) or (b) – 16, 48 or 96 reactions kits are available in two versions:

a) WITHOUT ENZYMATIC FRAGMENTATION

This version can be used to prepare libraries from fragmented DNA, such as cfDNA originating from any tissue type (including foetal and tumour) or genomic DNA which has been previously sheared by sonication or similar mechanical methods.

b) WITH ENZYMATIC FRAGMENTATION

This version uses a fragmentation enzyme to allow enzymatic shearing of high molecular weight gDNA extracted from any tissue type including leukocytes, FFPE and other tissues.

Input DNA requirements

Only high-purity DNA samples which are free of residual salts, proteins, detergents, or other contaminants should be used as input material. Library preparation can be conducted using 1 – 1000 ng of DNA. Fluorometric methods (such as the Qubit assay, Invitrogen) are recommended to accurately determine DNA concentration, especially when using <100 ng of DNA as input.

IMPORTANT: We would advise against the use of a Nanodrop or similar spectrophotometry-based methods for DNA quantitation as these cannot accurately distinguish between DNA and RNA and have reduced sensitivity for <100 ng/ul concentrations.

DNA samples should be resuspended in molecular biology grade water, a low EDTA concentration Tris-HCl buffer (such as 0.1 mM EDTA TE buffer) or a 10 mM TrisHCl pH 8.0 saline buffer (such as QIAGEN Buffer EB or equivalent). If DNA samples are kept in a high EDTA concentration buffer (such as 1x TE), DNA needs to be purified using a commercially available kit or DNA Purification Beads (such as Target Pure™ NGS clean-up beads or equivalent; see 'Laboratory supplied reagents and consumables') and resuspended in one of the above-mentioned buffers.

Input DNA quantities and use of UMIs

Unique molecular identifiers (UMIs) enable PCR/sequencing error removal and high accuracy single molecule counting analysis. These 9bp molecular tags are unique in sequence and positioned directly adjacent to the i7 index within the adapters, which are ligated to the end of DNA fragments during library preparation. Sequencing reads with the same UMI that map to the identical genomic location, are assumed to originate from the same DNA molecule and are considered to be PCR duplicates. They can be grouped together to form consensus reads (molecular families) allowing for PCR sequencing error correction and ultra-low frequency mutation calling.

UMIs are built into Cell3™ Target libraries to enable a single workflow for all sample types and tests. Regardless of the input DNA amount, it is anticipated that 90% of sequenced reads will be duplicates, therefore, to make the best use of UMIs and achieve a 0.1% VAF, it is important to sequence at 10 X the consensus depth of coverage that you wish to achieve (assuming that a minimum of 2 consensus reads per variant are required to call a true variant).

EXAMPLE

Input DNA: 25 ng
Consensus depth of coverage (original molecules) aimed for: 3000x
Raw depth of coverage (total sequencing read depth prior to UMI demultiplexing) needed: 30,000x

Depending on the need for UMIs, the following DNA input quantities are recommended for Cell3™ Target libraries:

- 1-100 ng of input DNA when wanting to take advantage of UMIs
- 100-1000ng of input DNA if UMIs are not required or when preparing PCR-free libraries

Input DNA requirements for FFPE samples

DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue is generally more degraded than genomic DNA extracted from fresh tissue or cells and can be chemically modified to different degrees. Depending on the level of DNA degradation, increased quantities of input DNA need to be used during library preparation in order to achieve similar yields compared to high-quality DNA. The DNA integrity score (or DIN score) can be determined by running FFPE DNA samples on an Agilent Genomic DNA ScreenTape (Agilent Technologies). The following table provides a guideline on FFPE DNA input quantities to use according to the DIN score observed:

Input DNA guidelines for DNA samples extracted from FFPE			
DNA input parameters	**DIN score >8**	**DIN score 3-8**	**DIN score <3**
Fold increase compared to high-purity DNA	No increase required	Increase input DNA quantity by 1.5-4-fold	Increase input DNA quantity by 5-10-fold

NOTE: When using FFPE DNA as input material, a minimum of 10 ng is recommended irrespective of the DIN score.

In addition to increasing the amount of input material, an increased number of cycles is required in the pre-capture PCR amplification step during library preparation (see section 1.D).

1.A Kit version (a): Without enzymatic fragmentation

End-repair / A-tailing for cell free DNA and fragmented DNA samples

In this step, end-repair and dA-tailing reactions are combined in a single tube to convert fragmented DNA into 5'-phosphorylated and 3'-dA-tailed DNA fragments, enabling direct ligation of Illumina sequencing adapters.

Before you start

Thaw the ER-AT Buffer (**red** cap) from the Library Preparation Kit V2 (a) – 16, 48 or 96 reactions at room temperature and briefly vortex mix. Mix the ER-AT Enzyme (**red** cap) and the Ligation Mix (blue cap) from the Library Preparation Kit V2 (a) – 16, 48 or 96 reactions by lightly tapping the tube. Briefly centrifuge all 3 components in a microcentrifuge to collect the liquid to the bottom of the tube and keep on ice.

IMPORTANT: all library preparation reaction setup procedures should be conducted while keeping tubes / 8-well tube strips / 96 well plates on ice, unless stated otherwise.

Procedure

1. Set up the following thermocycler program

Step	Temperature	Time
1	4°C	Hold
2	20°C	30 min
3	65°C	30 min
4	4°C	Hold

NOTE: Set the thermocycler heated lid to 85°C (if possible), the sample volume is 50 µl.

2. In a 0.2 ml PCR tube / 8-well tube strip / 96 well plate, prepare the DNA sample in a total volume of 41.7 µl (according to the input amount). Use nuclease-free water to dilute the DNA, if required. **Make sure to keep the reaction on ice during the whole procedure.**

3. Prepare the following reaction mix for each DNA sample as indicated in the following table. Vortex briefly for 5 seconds to ensure appropriate mixing is achieved without formation of bubbles.

Components	Volume for 1 reaction
ER-AT Buffer	5.8 µl
ER-AT Enzyme	2.5 µl
DNA Sample	X µl
Nuclease-free water	(41.7-X) µl
Total	50 µl

NOTE: The ER-AT Buffer and ER-AT Enzyme can be combined in a master mix prior to adding the DNA Samples when processing multiple samples at the same time. Ensure that the master mix includes 10% overage to enable consistent pipetting of 8.3 µl to the 41.7 µl of DNA sample. The master mix should be vortexed at moderate speed for 5 seconds to ensure appropriate mixing is achieved without formation of bubbles.

4. Add 8.3 µl of prepared End-repair / A-tailing reaction mix to each reaction for a total final volume of 50 µl. Mix well by briefly vortex mixing or pipette mixing 10-15 times and briefly centrifuge the 0.2 ml PCR tube / 8-well tube strip / 96 well plate to collect the liquid at the bottom of the tube.

5. Immediately transfer the tube / 8-well tube strip / 96 well plate to the pre-chilled thermocycler (4ºC) and "skip" to the next step in the program.

6. When the program finishes, and the thermocycler has returned to 4ºC, remove the samples from the cycling block and place on ice. **Immediately proceed to the ligation step (1.C).**

1.B Kit version (b): With enzymatic fragmentation

Fragmentation and end-repair / A-tailing for intact genomic DNA samples

In this step, gDNA is sheared to a size of 180-200 bp by enzymatic fragmentation and the resulting fragments undergo end-repair and dA-tailing in a single reaction. This converts high molecular weight DNA into short 5'-phosphorylated and 3'-dA-tailed DNA fragments, enabling direct ligation of Illumina sequencing adapters.

NOTE: if longer insert sizes are required, refer to Appendix I for more details on how to achieve sizes of 250 – 550 bp by altering the incubation temperature and time in the fragmentation step.

Before you start

Thaw the Fragmentation Buffer (**red** cap) from the Library Preparation Kit V2 (b) – 16, 48 or 96 reactions at room temperature and briefly vortex mix. Mix the Fragmentation Enzyme (**red** cap) and the Ligation Mix (blue cap) from the Library Preparation Kit V2 (b) – 16, 48 or 96 reactions by lightly tapping the tube. Briefly centrifuge all 3 reagents in a microcentrifuge to collect the liquid to the bottom of the tubes and keep on ice.

IMPORTANT: all library preparation reaction setup procedures should be conducted while keeping tubes / 8-well tube strips / 96 well plates on ice, unless stated otherwise.

Procedure

1. Set up the following thermocycler program

Step	Temperature	Time
1	4°C	Hold
2	37°C	20 min
3	65°C	30 min
4	4°C	Hold

NOTE: Set the thermocycler heated lid to 105°C (if possible), the sample volume is 50 µl

2. Prepare the following reaction mix for each DNA sample (according to the input amount) in a 0.2 ml PCR tube / 8-well tube strip / 96 well plate as indicated in the table below, **keeping the reaction on ice during the whole procedure.** Mix well by briefly vortex mixing or pipette mixing 10-15 times and briefly centrifuge the 0.2 ml PCR tube / 8-well tube strip / 96 well plate to collect the liquid at the bottom of the tube.

Components	Volume for 1 reaction
Fragmentation Buffer	4 µl
Fragmentation Enzyme	6 µl
DNA sample	X µl
Nuclease-free water	(40 – X) µl
Total	50 µl

NOTE: The Fragmentation Buffer and Fragmentation Enzyme can be combined in a master mix prior to adding the DNA Samples when processing multiple samples at the same time. Ensure that the master mix includes 10% overage to enable consistent pipetting of 10 µl to the 40 µl of DNA sample. The master mix should be vortexed at moderate speed for 5 seconds to ensure appropriate mixing is achieved without formation of bubbles.

3. Immediately transfer the tube / 8-well tube strip / 96 well plate to the pre-chilled thermocycler (4ºC) and "skip" to the next step in the program.

4. When the program finishes, and the thermocycler has returned to 4ºC, remove the samples from the cycling block and place on ice. **Immediately proceed to the ligation step (1.C).**

1.C Ligation of Illumina UMI adapters

During the ligation step, Illumina UMI Adapters are ligated on both ends of the 5'-phosphorylated / 3'-dA-tailed DNA fragments. A clean-up step is performed immediately after adapter ligation using Target Pure™ NGS clean-up beads to purify the DNA library and remove residual non-ligated adapters, enzymes and buffers.

Before you start

Equilibrate the Target Pure™ NGS clean-up beads to room temperature for 20-30 minutes ready for use in step 9. Remove the Illumina UMI adapter-containing 96 well plate from the freezer and thaw on ice. Centrifuge the plate in a plate centrifuge to collect the liquid at the bottom of the tubes.

Refer to Appendix VI, Table-2 for the location of each adapter within the supplied Illumina UMI Adapter 96 well plate, (one adapter per sample library) containing wells with 16, 48 or 96 adapters.

Procedure

1. Set up the following thermocycler program.

Step	Temperature	Time
1	4°C	Hold
2	20°C	15 min

NOTE: Set the lid to **"not heated"** (or leave the lid open), the sample volume is 75 µl

2. Illumina UMI Adapters are provided at a concentration of 15 µM. Depending on the input and workflow the required adapter concentration needs to be adjusted. Refer to the guidelines on the appropriate adapter concentration below.

End-repair / A-tailing workflow (section 1.A)

- When using a DNA input quantity of <5 ng, prepare a 1:5 dilution of the UMI Adapters using molecular grade water, ready for use in the ligation step (i.e., a final concentration of 3 µM)
- When using a DNA input quantity of ≥5 ng of input DNA, use the adapters directly from the tube undiluted, at 15 uM.

Fragmentation workflow (section 1.B)

- When using a DNA input quantity of <10 ng, prepare a 1:5 dilution for the UMI Adapters using molecular grade water, ready for use in the ligation step (i.e., a final concentration of 3 µM)
- When using a DNA input quantity of ≥10 ng of input DNA, use the adapters directly from the tube undiluted, at 15 uM.

3. While keeping the tubes / 8-well tube strip / 96 well plate containing the end-repaired / A-tailed DNA samples on ice, add 5 µl of the selected Illumina UMI adapter (either the 15 µM or the 3 µM concentration depending on the initial input, as explained in the guidelines above) to each sample and mix gently by pipette mixing or briefly vortex mixing.

IMPORTANT: use only one Illumina UMI adapter-containing well from the 96 well plate at a time by piercing the aluminium seal to access the adapter. Adapters are single use only; diluted adapters cannot be stored for further use as they will degrade.

4. Add 20 µl of Ligation Mix (blue cap) into each reaction for a total final volume of 75 µl. **Keep on ice.**

5. Mix well by pipetting up and down 10 – 15 times **(do not vortex)**. Briefly centrifuge the tubes in a microcentrifuge or the 96 well plate in a plate centrifuge to collect all the liquid at the bottom of the tubes.

6. Immediately transfer the tube / 8-well tube strip / 96 well plate to the pre-chilled thermocycler (4ºC) and "skip" to the next step in the program.

7. After the program finishes, proceed immediately to the clean-up step using Target Pure™ NGS clean-up beads.

Clean-up of adapter ligated library

8. Add 67.5 µl of thoroughly vortex mixed room-temperature equilibrated Target Pure™ NGS clean-up beads to a new 0.2 ml PCR tube / 8-well tube strip / 96 well plate for each sample.

NOTE: DNA clean-up with Target Pure NGS clean-up beads can also be performed in 1.5 ml tubes, as explained in Appendix II.

9. Transfer the whole 75 µl of adapter ligation reaction to the 67.5 µl of Target Pure™ NGS clean-up beads and mix well by pipetting up and down 15-20 times, taking care to avoid the formation of bubbles.

10. Incubate the mixture for 5 minutes at room temperature.

11. Prepare a solution of 80% ethanol / 20% molecular biology grade water (400 µl per sample is required for each clean-up step). For two washes and including an overage, 1000 µl per sample should be prepared for the entire library preparation procedure.

12. Place the tubes / 8-well tube strip / 96 well plate on the magnetic stand for 5 minutes at room temperature to pellet the beads on the side of the tubes/wells.

13. Keeping the tubes / 8-well tube strip / 96 well plate on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

14. Add 200 µl of 80% ethanol to the tube/well and incubate at room temperature for 30 seconds.

15. Repeat steps 14-15 for a total of two 80% ethanol washes.

16. Keeping the tubes / 8-well tube strip / 96 well plate on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

17. Use a 10 µl multichannel or single channel pipette to remove any residual liquid from the tubes/wells.

18. Keeping the tubes / 8-well tube strip / 96 well plate on the magnetic stand, incubate at room temperature with open lids for 3- 5 minutes or until the beads are dry.

IMPORTANT: Avoid over-drying of beads, as this can result in a significant loss of DNA recovered. When dry, beads will appear matt in appearance, but should not be cracked

19. Remove the tubes / 8-well tube strip / 96 well plate from the magnetic stand and resuspend the dried beads in 22 µl of Buffer EB or equivalent buffer saline solution (10 mM Tris-HCl, ph8.0) by pipette mixing up and down 10-15 times, taking care to avoid the formation of bubbles.

NOTE: If proceeding immediately to pre-capture library amplification (chapter 1.D), molecular biology grade water can also be used to elute the library DNA from beads.

20. Incubate the tubes / 8-well tube strip / 96 well plate for 2 minutes at room temperature.

21. Place the tubes / 8-well tube strip / 96 well plate on the magnetic stand for 2 minutes at room temperature to pellet the beads on the side of the tubes/wells.

22. Carefully recover 20 µl of supernatant and transfer it to a new 1.5 ml low-bind tube.

STOPPING POINT: at this point, adapter ligated libraries can be stored at -20°C, if not proceeding immediately to the library amplification step.

1.D Library amplification

A high-fidelity amplification step is performed to ensure that sufficient library yield is available for the following targeted enrichment procedure. This is conducted using primers that bind to the adapter ligated DNA fragments at the start of the standard P5 and P7 sequences, which are present in all Illumina adapters.

Before you start

Thaw the PreCap Amplification Mix (**green** cap) and the PreCap Primer Mix (**black** cap) from the Library Preparation Kit V2 (a) or (b) – 16, 48 or 96 reactions kit on ice. Once thawed, lightly tap the tube containing the PreCap Amplification Mix to ensure adequate mixing of the reagent **(do not vortex)**. Briefly vortex mix the PreCap Primer Mix. Centrifuge all reagents to collect the liquid at the bottom of the tubes. Keep both tubes **on ice** for the whole procedure.

Equilibrate the Target Pure™ NGS clean-up beads to room temperature for 20-30 minutes for use in step 6 and prepare 80% ethanol (500 µl per sample to allow for overage), if not done so already in section 1.C, step 12.

Procedure

1. Set up the following thermocycler program.

Step	Temperature	Time	Cycles
1	98°C	Hold	1
2	98°C	45 sec	1
3	98°C	15 sec	3-12*
4	60°C	30 sec	
5	72°C	30 sec	
6	72°C	1 min	1
7	4°C	Hold	1

NOTE: Set the thermocycler heated lid to 105°C, the sample volume is 50 µl.

***IMPORTANT:** Recommended number of amplification cycles

Starting DNA input	Recommended number of amplification cycles	
	High quality DNA	FFPE DNA
1 ng	12-13	An input quantity of <10 ng is not recommended for FFPE DNA samples
5 ng	9-10	
10 ng	8-9	9-11
50 ng	5-6	6-8
100 ng	4-5	5-7
200 ng	3-4	4-6

NOTE: Some optimisation may be required for FFPE amplification cycles. For further guidance, please contact us at **support@nonacus.com.**

2. Prepare the following PCR master mix on ice in a separate 1.5 ml tube as indicated in the following table. Mix well by pipette mixing up and down 10 times or briefly vortex mix for 4 seconds. Centrifuge in a microcentrifuge to collect the liquid at the bottom of the tube. For multiple samples, prepare PCR master mix in a 1.5 ml tube by multiplying the volume of each reagent by the number of samples, add extra volumes (overage) to compensate for volume loss due to pipetting. Aliquot 30 µl of the prepared mix in a new 0.2 ml PCR tube / 8-well tube strip / 96 well plate for each sample.

NOTE: Do not store the PCR master mix for periods of time exceeding 2 hours

Components	Volume for 1 reaction
PreCap Amplification Mix	25 µl
PreCap Primer Mix	5 µl
Total	30 µl

3. Transfer 20 µl of adapter-ligated and purified sample library to the 30 µl of PCR reaction master mix from step 2 and mix well by pipette mixing up and down 10 times or briefly vortex mixing for 4 seconds. Centrifuge using microcentrifuge to collect liquid at the bottom of he tube.

4. Transfer the 0.2 ml PCR tubes / 8-well tube strip / 96 well plate to the pre-heated thermocycler (98ºC) and skip to the next step in the program.

5. When the program finishes, and the thermocycler has returned to 4ºC, remove the samples from the cycling block and **proceed immediately to library clean-up using Target Pure™ NGS clean-up beads.**

Clean-up of amplified library

6. Add 50 µl of thoroughly vortex mixed room-temperature equilibrated Target Pure™ NGS clean-up beads to a new 0.2 ml PCR tube / 8-well tube strip / 96 well plate for each sample.

NOTE: DNA clean-up with Target Pure™ NGS clean-up beads can also be performed in 1.5 ml tubes, as explained in Appendix II.

7. Transfer the entire 50 µl volume of PCR amplified library to the 50 µl of Target Pure™ NGS clean-up beads and mix well by pipette mixing up and down 15-20 times, taking care to avoid the formation of bubbles.

8. Incubate the mixture for 5 minutes at room temperature.

9. Place the tubes / 8-well tube strip / 96 well plate on the magnetic stand for 5 minutes at room temperature to pellet the beads on the side of the tubes/wells.

10. Keeping the tubes / 8-well tube strip / 96 well plate on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

11. Add 200 µl of 80% ethanol to the tube/well and incubate at room temperature for 30 seconds.

12. Repeat steps 10-11 for a total of two 80% ethanol washes.

13. Keeping the tubes / 8-well tube strip / 96 well plate on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

14. Use a 10 µl multichannel or single channel pipette to remove any residual liquid from the tubes/wells.

15. Keeping the tubes / 8-well tube strip / 96 well plate on the magnetic stand, incubate at room temperature with open lids for 3-5 minutes or until the beads are dry.

IMPORTANT: Avoid over-drying of beads, as this can result in a significant loss of DNA recovered. When dry, beads will appear matt in appearance, but should not be cracked

16. Remove the tubes / 8-well tube strip / 96 well plate from the magnetic stand and resuspend the dried beads in 32.5 µl of **nuclease-free water** by pipette mixing up and down 10-15 times, taking care to avoid the formation of bubbles.

17. Incubate the tubes / 8-well tube strip / 96 well plate for 2 minutes at room temperature.

18. Place the tubes / 8-well tube strip / 96 well plate on the magnetic stand for 2 minutes at room temperature to pellet the beads on the side of the tubes/wells.

19. Carefully recover 30 µl of supernatant and transfer it to a new 1.5 ml low-bind tube.

STOPPING POINT: at this point, amplified libraries can be stored at 4°C overnight or at -20°C for long term storage, if not proceeding immediately to the library quality check step.

1.E Library quality check

Libraries are assessed by determining:

- DNA **quantity** in terms of concentration (ng/µl) and total yield (ng)
- DNA **quality** in terms of expected fragment size distribution and absence of additional lower or higher molecular weight peaks (recommended)

Library DNA quantity

Libraries prepared from high-purity DNA usually generate >500 ng of total DNA yield (i.e., >16 ng/µl in a volume of 30 µl). Use of fluorometric assays for dsDNA (such as the Qubit dsDNA BR assay kit, Invitrogen) is recommended for accurate determination of DNA concentration.

Library DNA quality

By ligating dual indexed adapters containing UMIs to DNA fragments, the library preparation procedure adds 144 bp to the fragment length. This can be assessed by analysing libraries with digital electrophoresis systems (such as the Agilent 4200 TapeStation with D1000 reagents and screentape, Agilent Technologies) and determining the peak size within the fragment distribution. Library yield can also be assessed using a digital electrophoresis system, but the measurement is not as accurate as that obtained with fluorometric assays (such as the Qubit), as it tends to underestimate DNA quantity. However, if the discrepancy between the measurement taken with a fluorometric assay and a digital electrophoresis assay is higher than 50%, then this might indicate PCR over-amplification of the library (refer to the troubleshooting guide to learn about this issue and how to fix it). Presence of carried-over adapters, adapter-dimers and primer-dimers can also be observed in the 60-160 bp range (refer to the troubleshooting guide). Note that adapter-dimers are generally removed during probe hybridization and therefore do not affect the targeted enrichment procedure. While this quality control procedure is recommended, it is not mandatory and correct fragment size can be obtained by performing size selection, as explained in appendix IV. See examples below for reference on how to check library quality.

The Library Preparation Kit V2 (a) – 16, 48 or 96 reactions, can be used for library preparation of cell free DNA samples, which are highly fragmented in nature. Most of these fragments are 166 bp in length, while a smaller portion are present in sizes which are multiples of 166 (329, 506, 682) in ever decreasing quantities. Therefore, libraries prepared using cell free DNA as input material yield fragment size distributions containing multiple peaks (see Figure-2, below).

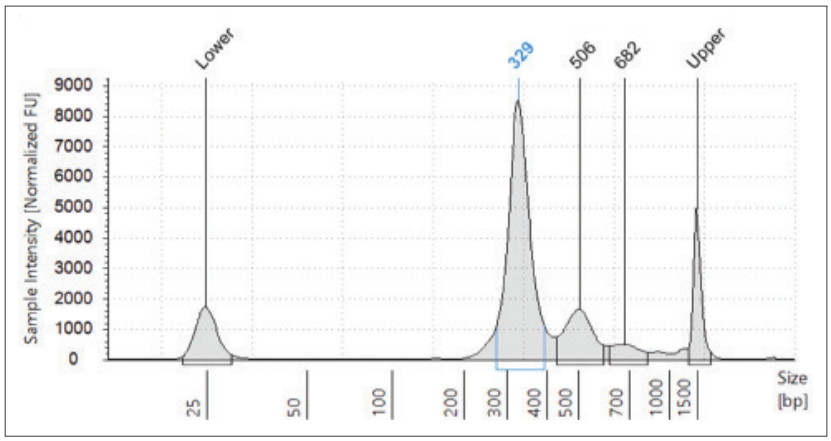

Figure 2. Fragment size distribution of library prepared with 5 ng of input cell free DNA.

The Library Preparation Kit V2 (b) – 16, 48 or 96 reactions, enables the preparation of libraries using high molecular weight genomic DNA. The enzymatic fragmentation procedure included in the kit shears the DNA to the required fragment length. Libraries successfully prepared using this kit show a single peak in the fragment size distribution graph (see Figure-4, below). Libraries which have not been completely sheared show a tail of variable size in the long fragment range (see Figure-5). In these cases, fragmentation incubation time should be adjusted according to input DNA quantity to achieve complete shearing. Note that the small peak at 160 bp in Figure-5 represents carry-over of adapter dimers (see the troubleshooting guide to learn more about adapter-dimers and how they may affect downstream applications.

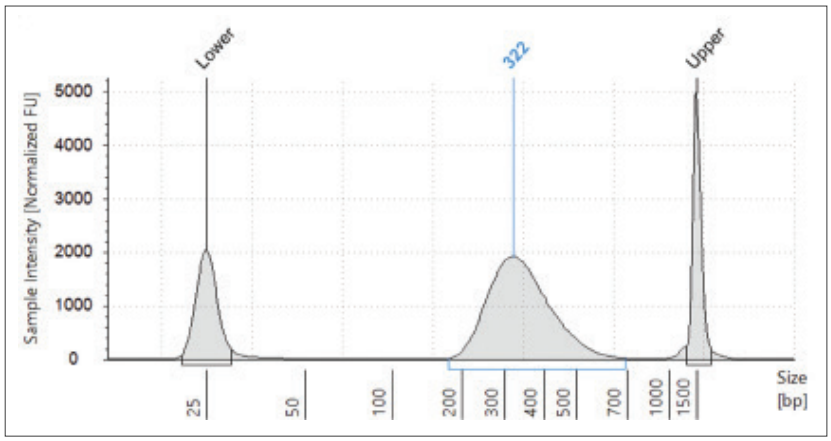

Figure 3. Fragment size distribution of library prepared with 25 ng of input high molecular weight genomic DNA

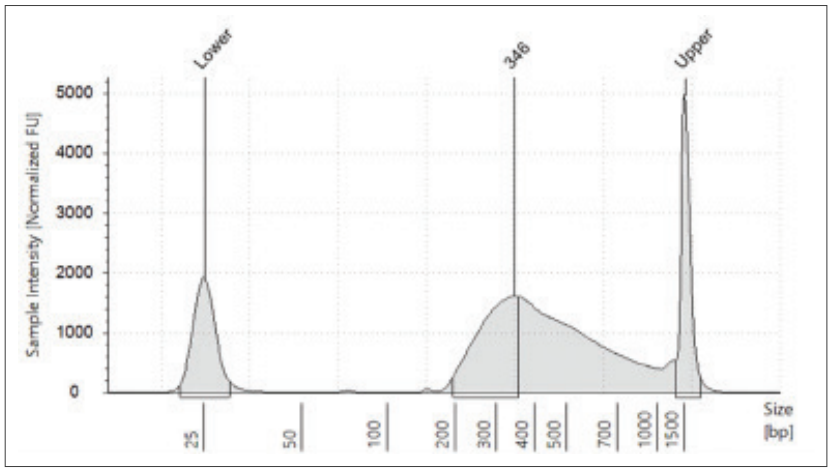

Figure 4. Fragment size distribution of unsuccessful library prepared with 100 ng of input high molecular weight genomic DNA

- The presence of a tail in the long fragment size range suggests that the sample was not entirely sheared during enzymatic fragmentation.

STOPPING POINT: at this point, amplified libraries can be stored at 4°C overnight or at -20°C for long term storage, if not proceeding immediately to hybridization and capture.

Chapter 2: Probe hybridization and capture enrichment

The Hybridization and Capture Enrichment Kit V2– 4 or 12 reactions, enables probe hybridization-based targeted enrichment of Illumina sequencing libraries (i.e., containing Illumina adapters) prepared from cfDNA or gDNA as input material in combination with the Cell3™ Target Panel – 2, 4, and 12 reactions.

2.A Library pooling and probe hybridization

In this step, individual libraries prepared with the Library Preparation Kit V2 (a) or (b) – 16, 48 or 96 reactions, are pooled together in equal amounts and hybridized with DNA biotin-labelled probes, to enrich for the targeted region of interest.

IMPORTANT: We recommend pooling 8 samples per hybridization and capture reaction. Further considerations are noted below;

- For catalogue panels, enough reagents to perform a minimum of 8 libraries per capture are provided. If pooling more than 8 libraries per capture, not all the reactions in the kit will be utilised
- For custom panels, enough reagents to perform a minimum of 4 libraries per capture are provided. If pooling more than 4 libraries per capture, not all the reactions in the kit will be utilised
- If pooling less than 4 libraries per capture and sequencing the final captured library on a single run (less than 4 libraries in the sequencing run overall), there will be issues with lack of complexity in the indices on Illumina sequencers, resulting in low quality data
- When using larger panels (≥300,000 probes) with more than 8 libraries per capture, depth of coverage may be impacted
- For deep sequencing (≥20,000x) and to ensure efficiency when using UMI's, sequencing more than 8 samples per capture may impact the amount of duplicates per library (a 90% duplication rate or higher is required when sequencing deep with UMI's)

For further support, please email us at **support@nonacus.com.**

Before you start

Switch on a vacuum concentrator and set the temperature to 70°C or lower. Alternatively, switch on a vacuum lyophiliser / freeze dryer. If this equipment is not available, pooled libraries can be concentrated using Target Pure™ NGS clean-up beads as described in Appendix III.

Thaw the Hybridization Buffer (2x) (**blue** cap), the Hybridization Enhancer (**brown** cap), the Universal Blockers (**orange** cap) and the COT-1 Human DNA (**red** cap) from the Hybridization and Capture Enrichment Kit V2 – 4 or 12 reactions, at room temperature. Thaw the the Cell3™ Target Panel – 2, 4, and 12 reactions on ice. Mix each component vigorously by vortex mixing, then microcentrifuge to collect the liquid at the bottom of the tube.

NOTE: Inspect the Hybridization Buffer (2x) (**blue** cap) for crystallization of salts. If crystals are present, heat the tube at 65°C in a heat block and vortex every few minutes until the buffer is completely homogenised (this may require heating for 30-60 minutes).

Procedure

1. Set up the following thermocycler program.

Step	Temperature	Time	Cycles
1	95°C	Hold	1
2	95°C	30 sec	1
3	65°C	4 hours	1
4	65°C	Hold	1

NOTE: Set the thermocycler heated lid to100°C, the sample volume is 17 µl.

2. If individual sample libraries were frozen, ensure that they are completely thawed and briefly vortex mixed.

3. Pool equal concentrations (in ng) of individual sample libraries into a new 1.5 ml low-bind tube to reach a total combined quantity of 1000 ng.

4. Add 5 µl (equivalent to 5 µg) of COT-1 Human DNA and 2 µl of Universal Blockers to the library pool. Briefly vortex mix and centrifuge in a microcentrifuge to collect the liquid at the bottom of the tube.

5. Place the tube with the lid open in the vacuum concentrator or vacuum lyophiliser / freeze drier and press start.

NOTE: Depending on the amount of liquid present in the tube, the drying procedure may take from 10 to 60 minutes in a vacuum concentrator; and from 30 to 90 minutes in a vacuum lyophiliser / freeze drier. Ensure that all liquid has evaporated from the tube before proceeding to the next step.

NOTE: If a vacuum concentrator or vacuum lyophiliser / freeze drier are not available, pooled libraries can be concentrated using Target Pure™ NGS clean-up beads as described in Appendix III.

STOPPING POINT: at this point, the dried down library pool / COT-1 Human DNA / Universal Blockers can be stored overnight at 4°C, if not proceeding immediately to probe hybridization and capture enrichment.

6. Prepare the hybridization reaction mix by adding the components in the table below to the 1.5 ml low-bind tube containing the dried-up library pool / COT-1 Human DNA / Universal Blockers.

Components	Volume for 1 reaction
Hybridization Buffer (2x)	8.5 µl
Hybridization Enhancer	2.7 µl
Cell3™ Target Panel	4 µl
Nuclease-free water	1.8 µl
Total	17 µl

7. Gently pipette mix up and down 10 times, then briefly centrifuge to ensure the liquid is collected at the bottom of the tube and incubate at room temperature for 10 minutes.

8. Transfer the whole volume of hybridization reaction mix to a 0.2 ml PCR tube and briefly centrifuge to ensure that the liquid is collected at the bottom of the tube.

9. Place the 0.2 ml PCR tube containing the hybridization reaction mix in the pre-heated thermocycler (95°C) and skip to the next step in the program.

10. Leave the hybridization reaction mix at 65°C on the thermocycler to incubate for 4 hours.

NOTE: Alternatively, and if it aids the efficiency of the workflow, the hybridization reaction can be incubated for 16 hours or overnight. This may improve performance for GC-rich or small panels (< 100 Kb in size).

2.B Probe capture on streptavidin beads and washes

Biotin-labelled probes hybridized to their DNA targets are captured on streptavidin-coated beads. The beads are then washed multiple times to remove non-targeted DNA.

Before you start

Equilibrate the Dynabeads® M-270 Streptavidin to room temperature for 30 minutes for use in step 6.
Thaw the Stringent Wash Buffer (10x) (white cap, S), the Wash Buffer 1 (10x) (white cap, 1), the Wash Buffer 2 (10x) (white cap, 2), the Wash Buffer 3 (10x) (white cap, 3) and the Bead Wash Buffer (2x) (white cap, B) from the Hybridization and Capture Enrichment Kit V2 – 4 or 12 reactions, at room temperature. Thoroughly vortex mix and centrifuge in a microcentrifuge to collect the liquid at the bottom of the tube.

Set a thermocycler to 65°C on hold with the heated lid set at 70°C in advance to prepare for steps 18 onwards.

In the event you have access to only one thermocycler, use a heated block to store the hybridization reaction mix at 65°C until the heated lid of your thermocycler has reached temperature; the temperature of the thermocycler block may be impacted whilst the heated lid achieves the correct temperature.

IMPORTANT: If using a panel (custom or catalogue) with a total covered region size >30Mb refer to Appendix V to proceed with the alternative post-hybridization capture protocol.

NOTE: If necessary, heat the Wash Buffer 1 (10x) at 65°C in a heat block to completely resuspend precipitated particles.

NOTE: Dynabeads® M-270 Streptavidin washes can also be performed in a 1.5 ml tube using a magnetic stand capable of accommodating 1.5-2 ml tubes, as outlined in Appendix II. In this case, turn on a heat block and set to 65°C.

Preparation of wash buffers

1. Dilute the following components for each capture reaction to prepare a 1x working solution in 1.5 ml tubes, as indicated in the table below. For multiple samples, prepare the buffers by multiplying the volume of each reagent by the number of samples, add extra volume (overage) to compensate for pipetting loss.

Components	Stock solution	Nuclease-free water	Total
Stringent Wash Buffer (10x)	40 µl	360 µl	400 µl
Wash Buffer 1 (10x)	30 µl	270 µl	300 µl
Wash Buffer 2 (10x)	20 µl	180 µl	200 µl
Wash Buffer 3 (10x)	20 µl	180 µl	200 µl
Bead Wash Buffer (2x)	250 µl	250 µl	500 µl

2. Mix each diluted component thoroughly by vortex mixing and centrifuge in a microcentrifuge to collect liquid at the bottom of the tube.

3. Transfer 100 µl of 1x Wash Buffer 1 into a fresh 0.2 ml PCR tube and pre-heat it in a thermocycler at 65ºC for at least 15 minutes before use.

4. Split the 1x Stringent Wash Buffer into two 0.2 ml PCR tubes, transferring 200 µl in each tube, and pre-heat both aliquots in a thermocycler at 65ºC for at least 15 minutes.

NOTE: Both the 100 µl aliquot of 1x Wash Buffer 1 and the two 200 µl aliquots of 1x Stringent Wash Buffer can be pre-heated on the same thermocycler where the hybridization reaction is taking place.

5. Store the 200 µl of 1x Wash Buffer 1 and the remaining 1x wash buffers at room temperature until needed.

Preparation of Dynabeads® M-270 Streptavidin

6. After equilibration at room temperature, mix the Dynabeads® M-270 Streptavidin thoroughly by vortex mixing for 15 seconds.

7. Aliquot 50 µl of Dynabeads® M-270 Streptavidin per capture reaction into a fresh 1.5 ml tube. If using Cell3™ Target exome panels, refer to Appendix V for alternative post-hybridization capture procedure for this step.

NOTE: If preparing more than one capture reaction, up to 600 µl of Dynabeads® M-270 Streptavidin can be aliquoted into a single 1.5 ml tube for bead preparation.

8. Place the 1.5 ml tube in a magnetic stand and incubate20-30 seconds until all beads have separated from the supernatant and have pelleted on the side of the tube.

9. Carefully remove and discard the supernatant, taking care not to disturb the bead pellet.

10. Add 200 µl of 1x Bead Wash Buffer per capture reaction, remove the tube from the magnetic stand and vortex for 10 seconds.

11. Repeat steps 8-10 once more for a total of two washes.

12. Place the 1.5 ml tube in a magnetic stand and incubate 20-30 seconds or until all beads have separated from the supernatant and have pelleted on the side of the tube.

13. Carefully remove and discard the supernatant, taking care not to disturb the bead pellet.

14. Add 100 µl of 1x Bead Wash Buffer per capture reaction, remove the tube from the magnetic stand an vortex briefly.

15. Transfer 100 µl of resuspended beads into a new 0.2 ml PCR tube / 8-well tube strip for each capture reaction.

NOTE: At this stage, Dynabeads® M-270 Streptavidin resuspended in 100 µl of Bead Wash Buffer can be transferred to a 1.5 ml tube to conduct bead capture and washes on a magnetic stand capable of accommodating 1.5-2 ml tubes, as explained in Appendix II.

NOTE: Washed Dynabeads® M-270 Streptavidin can be kept in solution at room temperature. Proceed to the next step **only when the hybridization (section 2.A, step 10) incubation ends.**

NOTE: Small amounts of residual 1x Bead Wash Buffer will not interfere with downstream binding of the biotin-labelled probes to the Dynabeads® M-270 Streptavidin.

Procedure

16. Set a thermocycler at 65°C on hold with the heated lid set at 70°C.

17. Carefully remove and discard the supernatant, taking care not to disturb the bead pellet, and **proceed immediately to the next step.**

18. Place the tube on a magnetic stand capable of accommodating 0.2 ml PCR tubes / 8-well tube strips and incubate for 1-2 minutes or until all beads have separated from the supernatant and have pelleted on the side of the tube/well.

IMPORTANT: It is important that the heated lid is set to 70°C during the washes of Dynabeads® M-270 Streptavidin post-capture. Ensure that the hybridization reaction is kept at 65°C throughout the hybridization, capture and washes with 1x Stringent Wash Buffer steps to avoid unspecific binding of non-target DNA to the probes.

19. Transfer the 0.2 ml PCR tube / 8- well tube strip containing the pelleted Dynabeads® M-270 Streptavidin adjacent to the thermocycler set to 65°C (with the heated lid set to 70°C).

20. Immediately transfer the whole amount of hybridization reaction mix on the thermocycler (from section 2.A, step 10) to the 0.2 ml PCR tube / 8-well tube strip containing the pelleted Dynabeads® M-270 Streptavidin. Pipette mix the Dynabeads and hybridization reaction mix up and down 10 times on the thermocycler.

21. Incubate the hybridization reaction mix with the Dynabeads® M-270 Streptavidin on the thermocycler for 45 minutes.

NOTE: At this stage, if bead capture and washes are conducted in 1.5-2 ml tubes, incubate the Dynabeads® M-270 Streptavidin mixed with the hybridization reaction mix in a heat block set at 65°C, as explained in Appendix II.

22. Every 12 minutes during the 45-minute incubation at 65°C, carefully pipette mix whilst on the thermocycler. If its not possible to pipette mix on the thermocycler, briefly vortex mix on a low speed to avoid splashing, for 3 seconds, to ensure the beads remain suspended in solution and then place back on the thermocycler.

23. Remove the 0.2 ml PCR tube / 8-well tube strip from the thermocycler and add 100 µl of pre-heated 1x Wash Buffer 1 (from step 3).

24. Pipette mix up and down 10 times and place the 0.2 ml PCR tube / 8-well tube strip on a magnetic stand for to allow the beads to separate from the supernatant and pellet on the side of the tube/well, this should happen within 2-5 seconds.

25. Once the liquid is clear, Immediately remove the supernatant, taking care not to disturb the bead pellet. Remove the 0.2 ml PCR tube / 8-well tube strip from the magnetic stand and add 200 µl of pre-heated 1x Stringent Wash Buffer (from step 4).

26. Mix well by pipette mixing up and down 10 times, taking care to avoid the formation of bubbles.

27. Transfer the 0.2 ml PCR tube / 8-well tube strip to a thermocycler set to 65°C (with the heated lid set to 70°C) and incubate for 5 minutes.

28. After incubation, remove the 0.2 ml PCR tube / 8-well tube strip from the thermocycler and place on a magnetic stand to allow beads to separate from supernatant. and pellet on the side of the tube/well. As soon as the liquid is clear, remove the supernatant, this should happen within 2-5 seconds from placing samples on the magnet.

29. Repeat steps 25-29 for a total of two washes with pre-heated 1x Stringent Wash Buffer.

30. As soon as steps 25-29 are complete immediately remove the supernatant, taking care not to disturb the bead pellet.

31. Remove the 0.2 ml PCR tube / 8-well tube strip from the magnetic stand and add 200 µl of room temperature 1x Wash Buffer 1.

32. Vortex mix thoroughly for 2 minutes and briefly centrifuge to collect the liquid at the bottom of the tube.

33. Place the 0.2 ml PCR tube / 8-well tube strip on a magnetic stand for 20-30 seconds to allow the beads to separate from the supernatant and pellet on the side of the tube/well.

34. Carefully remove the supernatant, taking care not to disturb the bead pellet.

35. Remove the 0.2 ml PCR tube / 8-well tube strip from the magnetic stand and add 200 µl of room temperature 1x Wash Buffer 2.

36. Vortex mix thoroughly for 1 minute and briefly centrifuge to collect the liquid at the bottom of the tube.

37. Place the 0.2 ml PCR tube / 8-well tube strip on a magnetic stand for 20-30 seconds to allow the beads to separate from the supernatant and pellet on the side of the tube/well.

38. Carefully remove the supernatant, taking care not to disturb the bead pellet.

39. Remove the 0.2 ml PCR tube / 8-well tube strip from the magnetic stand and add 200 µl of room temperature 1x Wash Buffer 3.

40. Vortex mix thoroughly for 30 seconds and briefly centrifuge to collect the liquid at the bottom of the tube.

41. Place the 0.2 ml PCR tube / 8-well tube strip on a magnetic stand for 1-2 minutes to allow the beads to separate from the supernatant and pellet on the side of the tube/well.

42. Carefully remove the supernatant, taking care not to disturb the bead pellet.

43. Remove the 0.2 ml PCR tube / 8-well tube strip from the magnetic stand and resuspend the bead pellet in 24 µl of nuclease-free water by pipette mixing up and down 10-15 times. If using Cell3(TM) Target exome panels, r efer to Appendix V for alternative post-hybridization capture procedure for this step.

2.C Captured library amplification and clean-up

Targeted library DNA sequences hybridized to the biotin-labelled probes and captured on Dynabeads® M-270 Streptavidin are amplified by PCR using primers that specifically bind to the P5-P7 sequences on Illumina adapters. Target Pure™ NGS clean-up beads are then used to clean-up the amplified captured library.

Before you start

Thaw the PostCap Amplification Mix (**green** cap) and the PostCap Primer Mix (**black** cap) from the Hybridization and Capture Enrichment Kit V2 – 4 or 12 reactions, on ice. Briefly vortex mix and centrifuge to collect the liquid at the bottom of the tubes. Keep both tubes on ice for the whole procedure.

Equilibrate the Target Pure™ NGS clean-up beads to room temperature for 20-30 minutes (for use in step 6) and prepare a solution of 80% Ethanol / 20% molecular biology grade water (500 µl required per capture reaction including overage, for use in step 11).

Procedure

1. Set up the following thermocycler program

Step	Temperature	Time	Cycles
1	98°C	Hold	1
2	98°C	45 sec	1
3	98°C	15 sec	*Determined from below tables
4	60°C	30 sec	
5	72°C	30 sec	
6	72°C	1 min	1
7	4°C	Hold	1

NOTE: Set the thermocycler heated lid to 105°C, the sample volume is 50 µl

***IMPORTANT:** Refer to the tables below to determine the recommended post-capture PCR cycles.

For further optimisation guidance please contact us at **support@nonacus.com.**

Design ID	Post-cap PCR cycles recommended
Cell3™ Target Cancer 50 Panel v1.1	12
Cell3™ Target Hereditary Cancer Panel	12
Cell3™ Target Bladder Cancer Panel	17
Cell3™ Target Nexome Panel	7
Cell3™ Target Exome Panel	7
Cell3™ Target Pan Cancer 524 TMB Panel v1.0	10
Cell3™ Target SNP Identity Tracking Panel v1.0	21
Cell3™ Target Tumour Exome Panel v1.0	7
Cell3™ Target Actionable Mutation Panel EGFR	21

Custom design capture size (Mb)	Post-cap PCR cycles recommended
0.002-0.004	21-20
0.004-0.008	20-19
0.008-0.016	19-18
0.016-0.032	18-17
0.032-0.064	17-16
0.064-0.128	16-15
0.128-0.256	15-14
0.256-0.512	14-13
0.512-1	13-12
1-2	12-11
2-4	11-10
4-8	10-9
8-16	9-8
16-32	8-7
32-64	7-6

2. Prepare the PCR reaction mix in a new 1.5 ml tube on ice. For each capture reaction, prepare one PCR reaction mix in a 0.2 ml PCR tubes / 8-well tube strip as indicated in the table below. For multiple samples, prepare the PCR master mix in a 1.5 ml tube by multiplying the volume of each reagent by the number of capture reactions, add extra volume (overage) to compensate for pipetting loss. Mix well by pipette mixing up and down 10 times or briefly vortex mixing. Centrifuge in a microcentrifuge to collect the liquid at the bottom of the tube. If using Cell3™ Target exome panels, refer to Appendix V for alternative post-hybridization capture procedure for this step.

Components	Volume for 1 reaction
PostCap Amplification Mix	25 µl
PostCap Primer Mix	2.5 µl
Total	27.5 µl

3. Transfer 22.5 µl of resuspended Dynabeads® M-270 Streptavidin with captured library DNA (from section 2. B, step 42) to the 27.5 µl of PCR reaction master mix from step 2 and mix well by pipette mixing up and down 10-15 times.

4. Transfer the 0.2 ml PCR tube / 8-well tube strip to the pre-heated thermocycler (98°C) and skip to the next step in the program.

5. When the program finishes, and the thermocycler has returned to 4°C, remove the samples from the cycling block and proceed **immediately to library clean-up using Target Pure™ NGS clean-up beads.**

Clean-up of amplified captured library

6. Add 75 µl of thoroughly vortexed room-temperature equilibrated Target Pure™ NGS clean-up beads to a new 0.2 ml PCR tube / 8-well tube strip for each captured library. If using Cell3™ Target exome panels, refer to Appendix V for alternative post-hybridization capture procedure for this step.

NOTE: DNA clean-up with Target Pure NGS clean-up beads can also be performed in 1.5 ml tubes, as explained in Appendix II.

7. Transfer the entire 50 µl of PCR product for each captured library to the 75 µl of Target Pure™ NGS clean-up beads and mix well by pipette mixing up and down 15-20 times, taking care to avoid the formation of bubbles. If using Cell3(TM) Target exome panels, refer to Appendix V for alternative post-hybridization capture procedure for this step.

8. Incubate the mixture for 5 minutes at room temperature.

9. Place the 0.2 ml PCR tube / 8-well tube strip on the magnetic stand for 5 minutes at room temperature to pellet the beads on the side of the tubes/wells.

10. Keeping the 0.2 ml PCR tube / 8-well tube strip on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

11. Add 200 µl of 80% ethanol to the tube/well and incubate at room temperature for 30 seconds.

12. Repeat steps 10-11 for a total of two 80% ethanol washes.

13. Keeping the 0.2 ml PCR tube / 8-well tube strip on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

14. Use a 10 µl pipette to remove any residual liquid from the tube/well.

15. Keeping the 0.2 ml PCR tube / 8-well tube strip on the magnetic stand, incubate at room temperature with open lids for 5 minutes or until the beads are dry.

IMPORTANT: Avoid over-drying of beads, as this can result in a significant loss of DNA recovered. When dry, beads will appear matt in appearance, but should not be cracked

16. Remove the 0.2 ml PCR tube / 8-well tube strip from the magnetic stand and resuspend the dried beads in 32.5 µl of Buffer EB or equivalent buffer saline solution (10 mM Tris-HCl, ph 8.0) by pipette mixing up and down 10-15 times, taking care to avoid the formation of bubbles.

17. Incubate the 0.2 ml PCR tube / 8-well tube strip for 2 minutes at room temperature.

18. Place the 0.2 ml PCR tube / 8-well tube strip on the magnetic stand for 2 minutes at room temperature to pellet the beads on the side of the tube/well.

19. Carefully recover 30 µl of supernatant and transfer it to a fresh 1.5 ml low-bind tube.

STOPPING POINT: at this point, the captured DNA library can be stored at -20°C, if not proceeding immediately to the library quality check step.

2.D Captured library quality check

Libraries are assessed by determining:

- DNA **quantity** in terms of concentration (ng/µl) and total yield (ng)
- DNA **quality** in terms of expected fragment size distribution and absence of additional lower or higher molecular weight peaks

Captured library DNA quantity

Captured libraries should yield 60-300 ng of total DNA. Use of high sensitivity fluorometric assays for dsDNA (such as the Qubit dsDNA HS assay kit, Invitrogen) is recommended for accurate determination of DNA concentration. If libraries yield <60 ng or >300 ng in total, refer to the troubleshooting guide.

Captured library DNA quantification by quantitative PCR (Optional)

Quantitative PCR (qPCR) is widely regarded as the most accurate way of measuring library concentration.

This assumption is based on the principle that only DNA fragments correctly ligated with the Illumina P5 and P7 adapters will amplify in the qPCR reaction and will therefore be quantified. Therefore, the calculated DNA concentration is relevant only to the fraction of properly adapted DNA fragments which can be sequenced.

Library quantification kits by qPCR are commercially available, such as the KAPA Library Quantification – Illumina/ Universal kit (Roche). To ensure an accurate measurement of library DNA concentration when using these kits, follow the manufacturer's guidelines and use a 1:10,000 – 1:40,000 dilution of the captured library as input material.

Captured library DNA quality

A high sensitivity digital electrophoresis system (such as the Agilent 4200 TapeStation with High Sensitivity D1000 reagents and screentape, Agilent Technologies) should be used to determine the peak size within the fragment distribution and the average fragment size. The latter is required to calculate the molar concentration of the captured library, which is essential for final library dilution and preparation for sequencing. See examples of captured libraries below for reference.

- Library obtained after targeted enrichment with a 60 kb capture probe set of a pool of 5 individual libraries prepared from cell free DNA as input material.

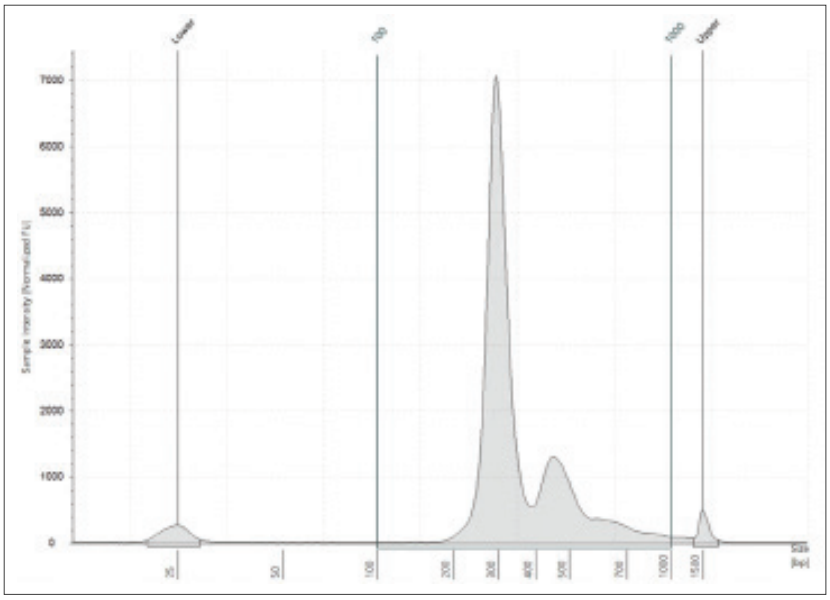

Figure 5. Fragment size distribution showing the range of 100-1000 bp within which the average fragment size is calculated. Average fragment size: 365 bp.

- Library obtained after targeted enrichment with a 60Kb capture probe set of a pool of 16 individual libraries prepared from genomic DNA as input material.

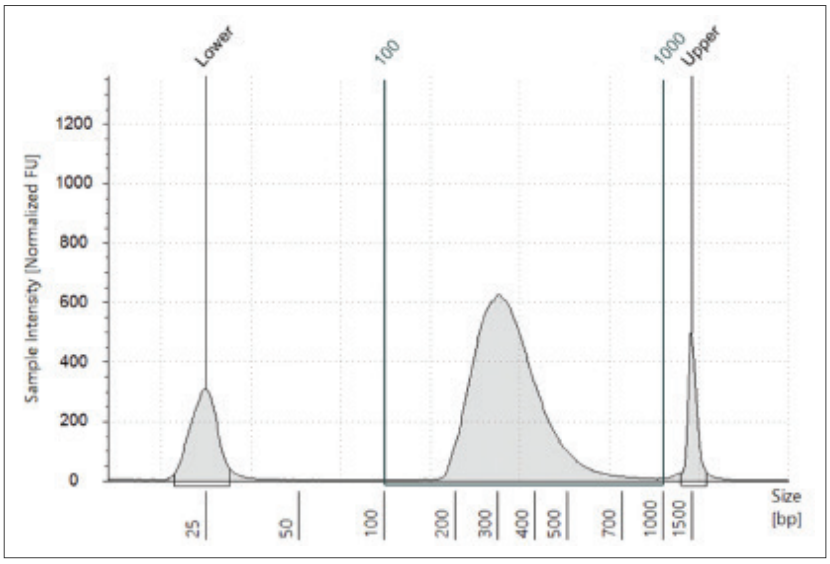

Figure 6. Fragment size distribution showing the range of 100-1000 bp within which the average fragment size is calculated. Average fragment size: 338 bp.

Chapter 3: Sequencing of captured libraries

Libraries enriched by targeted capture using Cell3™ Target technology are ready for sequencing on Illumina platforms (such as MiniSeq, MiSeq, NextSeq, HiSeq and NovaSeq instruments).

3.A Calculate captured library molar concentration

An accurate molar concentration can be calculated in the following ways:

- In combination with fluorometric assay reading: use the following formula to calculate molarity.

$$\text{concentration in nM} = \frac{\text{concentration in ng/ul}}{\left(660 \frac{g}{mol} \times \text{average library size in bp}\right)} \times 10^6$$

- In combination with the KAPA Library Quantification – Illumina/Universal kit or equivalent: insert the average fragment size in bp into the required field of the KAPA Library Quantification Data Analysis worksheet (or equivalent from other supplier) to determine library molar concentration.

3.B Choice of Illumina sequencing platform and kit size

Cell3™ Target technology is suitable for sequencing on the Illumina MiniSeq, MiSeq, NextSeq, HiSeq and NovaSeq, NextSeq2000 and NovaSeqX platforms. The recommended cycling parameters for cfDNA libraries are 2x 75 paired end sequencing, given that the average cfDNA fragment length is 166 bp. However, longer sequencing reads can be chosen for gDNA and FFPE DNA libraries prepared at larger fragment sizes (see section 1.B). Irrespective of the cycling parameters chosen, the Cell3™ Target technology requires paired end sequencing with dual indexing to be performed. The latter is necessary for sample de-multiplexing and use of UMIs and requires 25 sequencing cycles (17 for I7 index/barcode sequencing + 8 for I5 index sequencing). In every Illumina sequencing kit, a certain quantity of reagent excess is provided to allow for sequencing of indexes. However, the amount of excess reagent varies between kit sizes, so it is important to be aware of the maximum number of sequencing cycles which can be performed for the selected sequencing kit. Table-1 (below) outlines available kit sizes for each compatible Illumina platform; the excess number of cycles included and the maximum sequencing read length which can be selected when using Cell3™ Target technology in combination with the Illumina UMI Adapters – 16, 48 or 96 reactions format.

Table 1: Breakdown of kit sizes, excess cycles provided and maximum number of cycles usable for Illumina MiniSeq, MiSeq, NextSeq, HiSeq, NovaSeq sequencing platforms when using Cell3™ Target technology in combination with the Illumina UMI Adapters.

Reagent type	Kit size	Excess cycles provided	Max number of cycles	Cell3™ Target dual indexing cycles	Max usable sequencing cycles
MiniSeq	75	16	91	25	2x 33
	150	16	166	25	2x 70
	300	16	316	25	2x 145
MiSeq – v2	50	25	75	25	2x 25
	300	25	325	25	2x 150
	500	25	525	25	2x 250
MiSeq – v3	150	25	175	25	2x 75
	600	25	625	25	2x 300
NextSeq 500 / 550 – v2	75	16	91	25	2x 33
	150	16	166	25	2x 70
	300	16	316	25	2x 145
HiSeq TruSeq SBS v3	50	8	58	25	2x 16
	200	9	209	25	2x 92
HiSeq TruSeq SBS v4	50	25	75	25	2x 25
	250	25	275	25	2x 125
HiSeq Rapid SBS v2	50	24	74	25	2x 24
	200	25	225	25	2x 100
	500	25	525	25	2x 250
HiSeq 3000 / 4000 SBS	50	24	74	25	2x 24
	150	24	174	25	2x 74
	300	25	325	25	2x 150
NovaSeq 6000 S2	100	25	125	25	2x 50
	200	25	225	25	2x 100
	300	25	325	25	2x 150
NovaSeq 6000 S4	300	25	325	25	2x 150
NextSeq 1000 / 2000	P1 100	38	138	25	2x50
	P1 300	38	338	25	2x150
	P1 600	38	638	25	2x150
	P2 100	38	138	25	2x50
	P2 200	38	238	25	2x100
	P2 300	38	338	25	2x150
	P3 50	38	88	25	2x25
	P3 100	38	138	25	2x50
	P3 200	38	238	25	2x100
	P3 300	27	327	25	2x150

3.C Preparing sample sheets for Illumina sequencing and demultiplexing Nonacus library kit data

Captured libraries are compatible with the Illumina TruSeqHT protocol and sample sheets for dual indexed libraries. Depending on the ID of the adapters used, DNA library fragments contain the indexes listed in Appendix VI, Table-2.

Options for demultiplexing

Settings are provided by the user which enable the BCL conversion software to understand which cycles correspond with index, template and UMI data, which indexes correspond with which samples for proper demultiplexing and how to export the output of this conversion as new FASTQ files. These options can be provided in a sample sheet or through the software interface.

Currently there are two main pathways for demultiplexing Illumina sequencing data.

- A **v2 Sample Sheet** for use with the on-device Dragen BCL converter or standalone bcl-convert with BCL files produced from any Illumina machine. **This is the recommended method.**
- A **v1 Sample Sheet A or B** for use with bcl2fastq or on device demultiplexing of Illumina machines that do not include Dragen based demultiplexing.

NOTE: The sample sheet templates can be downloaded from **https://nonacus.com/cell3tm-target/.**

Using a v2 Sample Sheet

The following instructions are for using bcl-convert or the Dragen bcl-conversion software. The instructions for performing this on-instrument using the same software will be similar but care should be taken to follow the specific instrument instructions. Users should review the documentation for bcl-convert and the Dragen BCL conversion software before use.

Setting up Sequencing Samples and v2 Sample Sheet

The Illumina instrument will generate a Sample Sheet populated with user defined data during sequencing set up. You can start from this file, or the existing template provided with this documentation. You must supply or correct three pieces of data in this file to enable correct demultiplexing and handling of UMI sequences.

1. **Add sample indexing:** Open the v2 Sample Sheet template and add the sample library IDs in column A (under "Sample_ID") in the BCLConvert_Data section. Add the correct indexes as listed in Appendix VI, Table-2. For a v2 Sample Sheet, only include indexes in forward orientation.

2. **Add the correct cycle information:** Input the correct number of sequencing cycles required for Read1Cycles, Read2Cycles, Index1Cycles and Index2Cycles (under "[Reads]"). Typically, the number of Index cycles will be 17 and 8. Note, although the Index 1 is 8bp long, there are 17 cycles. The extra cycles correspond with sequencing the UMI data.

3. **Add the correct BCL conversion settings:** In the [Settings] section, correct the Override cycles parameter to Y<read length>;I8U9;I8;Y<read length>. In addition, set TrimUMI to 0 if you wish to save UMI data. Finally, add any additional settings for BCL conversion (see software documentation).

The text in the Sample Sheet should look like this (in this example, read length is 101bp):

```
[Header]
FileFormatVersion,2,

[Reads]
Read1Cycles,101
Read2Cycles,101
Index1Cycles,17
Index2Cycles,8

[BCLConvert_Settings]
FastqCompressionFormat,gzip
OverrideCycles,Y101;I8U9;I8;Y101
TrimUMI,0

[BCLConvert_Data]
Sample_ID,Index,Index2
sample1,ACGTTCAG,GCACAACT
```

Save the sample sheet with a new name as a .csv file to use on the Illumina sequencer. For sequencing platforms that can only be set up using BaseSpace or in standalone mode (such as the NextSeq), select standalone mode and make sure to select the correct number of cycles for the indexes: 8 cycles for the I5 index and 17 cycles for the I7 index.

NOTE: The prefix "BCLConvert_" before "Settings" and "Data" in the section headers can be omitted in certain situations but is left here for maximum compatibility.

Running bcl-convert software

The bcl-convert software (or Dragen equivalent) should be run via command line using the sample sheet provided above. To run, use the command:

Variables:

DATAFOLDER: Path to output of sequencing machine containing BCL files. Should be the base directory including RunInfo.xml.

OUTPUTFOLDER: Path to folder to write FASTQ files and logs to.

SAMPLESHEET: Path to Sample Sheet created in the above step. This should not exist, but if it does use the additional option --force to overwrite the existing folder.

```
# Using bcl-convert software
bcl-convert \
    --bcl-input-directory $DATAFOLDER \
    --output-directory $OUTPUTFOLDER \
    --sample-sheet $SAMPLESHEET


# Using Dragen software
dragen \
    --bcl-conversion-only true \
    --bcl-input-directory $DATAFOLDER \
    --output-directory $OUTPUTFOLDER \
    --sample-sheet $SAMPLESHEET
```

The bcl-convert software should proceed to create the relevant FASTQs using the correct sample ID values provided by the Sample Sheet.

Verifying correct demultiplexing of data

The software will create a pair of FASTQ files per sample, with additional FASTQs for Undetermined reads. If demultiplexing has run correctly, sample FASTQ files should be the largest files with Undetermined remaining relatively small. It is typical to see the file size of undetermined reads to be roughly equivalent to an additional sample.

To check correct handling of UMIs, it is necessary to inspect the contents of the FASTQ files. If the UMI sequence has been handled correctly, it will have a 9bp sequence in the header of each read. This can be seen in the header of any read in the FASTQ file, which should look like the below example (UMI sequence bold and underlined):

Read 1:
@VH01265:2:AACCN2MM5:1:1101:36940:1000:**GCAGAATAA** 1:N:0:GATGTGTG+GAGCCATG

Read 2:
@VH01265:2:AACCN2MM5:1:1101:36940:1000:**GCAGAATAA** 2:N:0:GATGTGTG+GAGCCATG

NOTE: The FASTQ header matches for both read 1 and read 2, the index sequences are both 8bp long and the UMI sequence is 9bp. This should be compatible with a normal sequencing workflow without any modifications or the UMIs can be used in a UMI aware workflow.

Using a v1 Sample Sheet

The following instructions are for use with BCL conversion and demultiplexing on older Illumina instruments or with the bcl2fastq software. This is no longer recommended but instructions are provided to enable compatibility. Users should familiarise themselves with the relevant software prior to use.

Setting up Sequencing Samples and v1 Sample Sheet

From the provided template or sample sheet generated by the Illumina software add sample specific information.

Some Illumina platforms sequence the I5 index on the opposite strand. Therefore, reverse complement sequences for the I5 index are provided in the Illumina adapter table (appendix VI). The following sample sheet templates can be downloaded from **https://nonacus.com/cell3tm-target/** and used according to the Illumina platform of interest:

- When sequencing libraries on the NovaSeq 6000 with v1.0 reagent kits, MiniSeq with Rapid Reagent Kits, MiSeq, HiSeq 2500 or HiSeq 2500 the I5 index is in the forward orientation and **sample sheet v1 template A** should be used.

- When sequencing libraries on the iSeq 100, MiniSeq with Standard Reagent kits, NextSeq systems, NovaSeq 6000 with v1.5 reagent kits, HiSeq X, HiSeq 4000 and HiSeq 3000 the I5 sequence is in the reverse complement form and **sample sheet v1 template B** should be used.

All Ns should be removed from the sample sheet prior to use as the software expects the index sequence to be exactly 8bp long. Unlike when using bcl-convert, all settings should be provided via the command line.

Running bcl2fastq software

The bcl2fastq software should be run from the command line using the sample sheet created above. To run, use the following bcl2fastq command:

Variables:

DATAFOLDER: Path to output of sequencing machine containing BCL files. Should be the base directory including RunInfo.xml.

OUTPUTFOLDER: Path to folder to write FASTQ files and logs to.

SAMPLESHEET: Path to Sample Sheet created in the above step. This should not exist, but if it does use the additional option --force to overwrite the existing folder.

```
bcl2fastq \
--input-dir $DATAFOLDER/Data/Intensities/BaseCalls \
--runfolder-dir $DATAFOLDER \
--output-dir $OUTPUTFOLDER \
--sample-sheet $SAMPLESHEET \
--barcode-mismatches 1 \
--use-bases-mask Y*,I8Y9,I8,Y* \
--no-lane-splitting \
--mask-short-adapter-reads 0 \
--ignore-missing-bcls
```

Confirm the software runs and completes correctly.

Verifying correct demultiplexing of data

The software will create three FASTQ files per sample, with additional FASTQ files for Undetermined reads. If demultiplexing has run correctly, sample FASTQ files should be the largest files with Undetermined remaining relatively small. It is typical to see the file size of undetermined reads to be roughly equivalent to an additional sample.

If the UMI sequences have been handled correctly the three UMI files will be;

- R1: forward sequence FASTQ file
- R2: UMI sequence FASTQ file
- R3: reverse UMI sequence FASTQ file

To check correct handling of UMIs it is necessary to inspect the contents of the FASTQ files. If the UMI sequence has been handled correctly, the R2 UMI file will be a smaller file size than the R1 and R3 files and when inspecting the content of the three files they will contain data similar to the below example:

Contents of R1 (forward) file:
@NB501034:457:H23HVAFX5:1:11101:16054:1183 1:N:0:CTGATCGT+GCGCATAT
CTGCCCCATGGTAGGCACGGGGACTCTCTTCCTCACAAGTAACATTCACGCATCTGTCTGCCCGAGCGTCATATGCCTCAAT
GGCATTGGTGGGGCTCCCACCACTCCAGCCGCCAATTGCAAAGAGGATGGCATAGGGCAAGCG

+

AAAAA/AEEEEEEEEE/EE6EE/EE/EEEEE/AEE/EEEE/EEEEEEE//EEAEEEE6EE/A<EEEEEEEEE6EA/E/E//EEEEEEEE/AEEEEE6E/E/
EEA<AE/<EEE/<AE/<A//EEAEEEEAEAEE/E/EEE<A/EE/

Contents of R2 (UMI) file:
@NB501034:457:H23HVAFX5:1:11101:16054:1183 2:N:0:CTGATCGT+GCGCATAT

ACTCCGCCA

+

/E/EEEAE/

Contents of R3 (reverse) file:
@NB501034:457:H23HVAFX5:1:11101:16054:1183 3:N:0:CTGATCGT+GCGCATAT

TTCAAAGGCAGATCGAAAAATGGGAGTTAAGGATGTGGGATTTCCTGGCACTCTCACGTCCCCTGAATGACTTTCTGCGT
TTGCTTCTTAGGTTCGCCTGGCCCTAATGCATGCTGAGTACTTCATGAACAATGTTAAGATGAAT

+

AAAAAEEEEEEEEAEEAEEEEEEEEEEEAEEEEEAE/AEEEEEEEEEEEE/EEEEEAEEE//EE</AEAE6EEEE/E<AEEEA/EE<<EEE<EEA<E<EAE/E/A/
EEAAAEE</<A//<AEEAAEEEAEEAEEEE<EEE<<EE<

NOTE: The UMI read is 9bp long but has the same header as the matching forward and reverse read. The forward and reverse read can be used in a standard workflow while the UMI read can be incorporated into a UMI aware workflow.

3.D Prepare captured library for Illumina sequencing

Following Illumina guidelines for the chosen sequencing platform, denature and dilute the captured library to the recommended concentration and load onto the cartridge. Primers for sequencing are included in Illumina sequencing reagents and no additional custom sequencing primers are required.

Troubleshooting guide

The following guide is meant to address the most common issues which might arise during library preparation and targeted capture enrichment. For further guidance, please contact us at **support@nonacus.com.**

A) Individual sample library yield <500 ng

- Library preparation reaction setup should be conducted on ice, in order to ensure that enzymatic activity does not start before all components have been added to the reaction mix.
- After thawing, all components should be thoroughly vortex mixed or tubes tapped for enzyme mixes, (as indi cated in the protocol) to ensure that salts and/or enzymes are homogenously mixed and in solution.
- Prior to incubation, all reaction mixes should be thoroughly vortex mixed or pipette mixed (as indicated in the protocol) to ensure maximum enzymatic activity.
- The number of cycles in the pre-capture PCR amplification step may need to be optimized. Repeat the library preparation procedure increasing the number of cycles by 1 or 2.
- Ensure that Target Pure™ NGS clean-up beads have been equilibrated at room temperature for 20-30 min utes prior to use, as the beads DNA binding capacity is reduced at low temperatures.
- During bead clean-up steps, 80% ethanol solution should be prepared fresh on the same day, as evaporation of ethanol over time can increase the water fraction and cause elution of DNA from the Target Pure™ NGS clean-up beads during washes.
- Over-drying of bead pellet during bead clean-up can significantly reduce DNA recovery in eluate. After drying beads at room temperature for 5 minutes, inspect the bead pellet frequently to ensure it does not over-dry. Bead pellets that show signs of cracking have been dried for too long, beads should matt in appearance.

B) Larger than expected fragment size in individual sample library from genomic DNA input

- Optimize fragmentation time. Find the optimal fragmentation parameters by increasing the fragmentation time by intervals of 5 minutes.
- If the DNA sample is kept in buffer containing >0.1 mM EDTA, use a bead or column clean-up procedure and elute the DNA sample in nuclease-free water or a 10 mM Tris-HCl, pH 8.0 solution (such as Buffer EB, Qiagen), then repeat the library preparation procedure.

C) Discrepancy between qubit and tapestation measured sample library yield

The sample library may have been over-amplified. When a considerable discrepancy between Qubit and digital electrophoresis measurement of sample library yield is observed, this may be due to an excess in PCR cycles post-ligation. This causes the formation of a secondary population of fragments of around 800-900 bp in length which is often difficult to notice on a Tapestation D1000 screentape electropherogram (see figure 9) or a Bioanalyser 7500 assay but becomes apparent on a genomic DNA screentape electropherogram (see figure 10) or a Bioanalyser High Sensitivity assay. Sample library over-amplification does not have a negative impact on the yield of viable DNA fragments used in the hybridization and capture stage, as the secondary peak is caused by fragments containing different insert sequences which hybridize at the adapter sequences, thus creating a "bubble" in the fragment which causes it to migrate slower during electrophoresis. However, this causes under-estimation of the sample library yield by Qubit and digital electrophoresis methods, which will impact on the amount of sample library pooled in the hybridization reaction. Reducing the number of PCR cycles post-ligation will solve the issue.

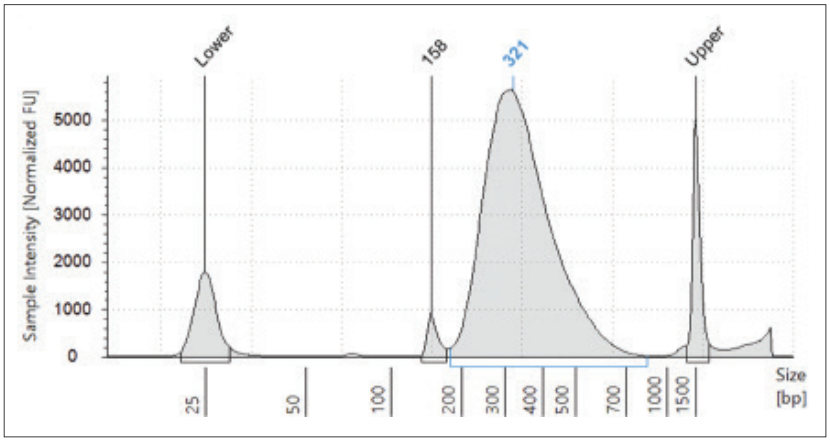

Figure 8. D1000 screentape electropherogram of a sample library prepared from 10 ng input gDNA and amplified for 8 cycles in the post-ligation PCR.

D) Larger than expected fragment size in individual sample library from cell free DNA input

Incorrect pre-analytical processing of blood can lead to increased nucleated blood cell degradation and shedding of genomic DNA into the plasma. Make sure that blood used for cell free DNA applications is collected in the correct tube (i.e., EDTA tube if plasma isolation is conducted within 24 hours from blood draw; or blood cell stabilizing tube, such as the Cell3™Preserver (Product code - PRE_C3P_WBS_50) – Whole Blood Stabilization Tube from Nonacus, if plasma isolation is conducted after >24 hours). Follow recommended guidelines for plasma isolation (i.e., centrifuge blood at 2,000 g for 10 minutes, remove the plasma taking care not to disturb the blood cell portion and centrifuge again at ≥10,000 g for 10 minutes). Extract cell free DNA using a specific kit for extraction of cell free DNA from plasma and/or other bodily fluids, such as the Cell3™Xtract (Product code - PRE_EXT_C3X_16 or PRE_EXT_C3X_48) kit from Nonacus. To read more about recommended pre-analytical blood processing guidelines for cfDNA applications, visit our News & Blog page at **www.nonacus.com/news-blog.**

E) Low molecular weight peaks present in individual sample library

- A low molecular weight peak of 150-160 bp in size indicates the presence of adapter-dimers carried over from the adapter ligation reaction. Adapter-dimers are generally lost during the targeted capture enrichment procedure and therefore will not affect downstream processes. If individual sample libraries are used for whole genome sequencing, large quantities of adapter-dimers may affect sequencing yield by sequestering space on the flow cell. Perform a size selection clean-up using SPRI-purification to remove adapter-dimers. Small quantities of adapter-dimers, similar to the peak seen in figure-5 (see section 1.E), do not significantly affect downstream sequencing.

- Make sure that the right quantity of adapters is used according to DNA input quantity. Excess amount of adapters increases the formation of adapter-dimers. Refer to the adapter concentration adjustment based on DNA input guidelines in section 1.C.

- Make sure that the correct amount of Target Pure™ NGS clean-up beads is used in the clean-up of amplified library step (see section 1.D). Use of a higher bead to sample volume ratio leads to the additional purification of smaller DNA fragments, such as adapters and adapter-dimers from the ligation reaction step (see section 1.C); and primer-dimers from the library amplification step (see section 1.D).

F) Captured library yield is lower than expected

- PCR cycle number in post-capture amplification may require optimization. Increase the cycle number by 1 or 2 cycles.

- Ensure that individual sample libraries are eluted in nuclease-free water and not in saline solutions, such as Buffer EB or TE, during the clean-up of amplified library step (section 1.D). Use of saline buffers to elute library DNA at this stage may interfere with probe hybridization (see section 2.A).

- Follow protocol recommendations when capturing hybridized probes to Dynabeads® M-270 Streptavidin and target DNA to Target Pure™ NGS clean-up beads. Make sure that Dynabeads® M-270 Streptavidin are equilibrated to room temperature for 20-30 minutes prior to use, as the biotin binding capacity is reduced at low temperatures. Ensure that target DNA clean-up using Target Pure™ NGS clean-up beads is conducted as recommended in the protocol (see relevant tips for DNA clean-up outlined in section A of the troubleshooting guide).

G) Low molecular weight peaks present in the captured library

- A low molecular weight peak of 150-160 bp in size indicates the presence of adapter-dimers, which are formed during the ligation reaction step in the library preparation procedure (see section 1.C). Adapter dimers should not hybridize to the probes and therefore are usually removed during the probe capture step (see section 2.B). Make sure 1x Wash Buffer 1 and 1x Stringent Wash Buffer are pre-heated at 65°C prior to use in steps 21-28 in section 2.B; and that these steps are performed as quickly as possible to ensure that the capture reaction does not considerably cool down below 65°C. This is to ensure the removal of non-hybridized DNA fragments, including adapter-dimers.

- Make sure that the correct amount of Target Pure™ NGS clean-up beads is used in the clean-up of amplified captured library step (see section 2.C), as explained in section D of the troubleshooting guide.

Appendix

The following information is intended to help users with the technical procedures described in this guide. For further support, please email us at support@nonacus.com.

I. Fragmentation protocol to achieve alternative size of the inserts

Enzymatic DNA fragmentation is influenced by reaction time, temperature and quantity of input DNA.

In order to obtain alternative length of the insert the fragmentation temperature and time can be altered. Fragmentation may be performed at 30 °C or 37 °C. Additionally, the time of the fragmentation can be adjusted based on the desired insert length. We highly recommend optimizing the reaction time by using the same DNA sample or very similar samples and to start with multiple timepoints. Choose the initial fragmentation time and temperature appropriate for desired length using the below tables as a guideline.

Fragment size				
250 bp	**300 bp**	**350 bp**	**400 bp**	**550 bp**
15	10	5	4	3
Fragmentation time at 30°C (minutes)				

Fragment size			
300 bp	**250 bp**	**200 bp**	**150 bp**
5	10	20	30
Fragmentation time at 37°C (minutes)			

NOTE: the table above should only be used as a guideline for optimization of fragmentation time in order to achieve the desired insert size.

1. Set up the following thermocycler program.

Step	Temperature	Time
1	4°C	Hold
2	30°C or 37 °C*	3-30min*
3	65°C	30 min
4	4°C	Hold

* Fragmentation time and temperature as determine above.

NOTE: Set the thermocycler heated lid to 105°C (if possible), the sample volume is 50 µl.

2. Prepare the following reaction mix for each DNA sample (according to the input amount) in a 0.2 ml PCR tube / 8-well tube strip / 96 well plate as indicated in the table below, **keeping the reaction on ice during the whole procedure.** Mix well by briefly vortex mixing or pipette mixing 10-15 times and briefly centrifuge the 0.2 ml PCR tube / 8-well tube strip / 96 well plate to collect the liquid at the bottom of the tube.

Components	Volume for 1 reaction
Fragmentation Buffer	4 µl
Fragmentation Enzyme Mix	6 µl
DNA sample	X µl
Nuclease-free water	(40 – X) µl
Total	50 µl

NOTE: The Fragmentation Buffer and Fragmentation Enzyme can be combined in a master mix prior to adding the DNA Samples when processing multiple samples at the same time. Ensure that the master mix includes 10% overage to enable consistent pipetting of 10 µl to the 40 µl of DNA sample. The master mix should be vortexed at moderate speed for 5 seconds to ensure appropriate mixing is achieved without formation of bubbles.

3. Immediately transfer the tube / 8-well tube strip / 96 well plate to the pre-chilled thermocycler (4°C) and "skip" to the next step in the program.

4. When the program finishes, and the thermocycler has returned to 4°C, remove the samples from the cycling block and place on ice. **Immediately proceed to the ligation step (1.C).**

II. Alternative procedure for magnetic bead clean-up steps

All handling of magnetic beads described in this protocol (ie Dynabeads® M-270 Streptavidin and Target Pure™ GS clean-up beads) requires the use of a magnetic rack capable of accommodating 0.2 ml PCR tubes, 8-well tube strips or 96 well plates.

Alternatively, all clean-up steps performed with Target Pure™ NGS clean-up beads (see sections 1.C, 1.D and 2.C) and Dynabeads® M-270 Streptavidin capture and bead washes (see section 2.B) can be performed in 1.5 ml tubes on a magnetic rack capable of accommodating 1.5-2 ml tubes. Follow the standard protocol but also ensure that when using 1.5-2ml tubes with magnetic beads , enough ethanol is used to completely submerge the bead pellet. This may be greater than the 200ul stated volume in the main protocol.

For Dynabeads® M-270 Streptavidin capture and washes at 65°C, set a heat block at 65°C and incubate for 45 minutes for capture (see section 2.B, step 19); and for 5 minutes for washes (see section 2.B, step 26). Centrifuge the 1.5-2 ml tube containing the Dynabeads® M-270 Streptavidin before vortex mixing during capture (see section 2.B, 20) to ensure that any condensation present on the cap is recovered at the bottom of the tube. After each 5-minute incubation of the Dynabeads® M-270 Streptavidin during washes with Stringent Wash Buffer (see section 2.B, 26), centrifuge the 1.5-2 ml tube to ensure that any condensation present on the cap is recovered at the bottom of the tube.

III. Alternative procedure for concentrating pooled sample libraries prior to hybridization using Target Pure™ NGS clean-up beads

If a vacuum concentrator or vacuum lyophiliser / freeze drier are not available for use, pooled sample libraries can be concentrated using Target Pure™ NGS clean-up beads. However, please note that this procedure does introduce a minor GC bias.

Before you start, equilibrate the Target Pure™ NGS clean-up beads to room temperature for 20-30 minutes (for use in step 4) and prepare a solution of 80% Ethanol / 20% molecular biology grade water (400 µl required per capture reaction, for use in step 8).

Proceeding from section 2.A, step 2:

1. Prepare the hybridization reaction mix (for use in step 13 below) in a new 1.5 ml tube as indicated in the table below.

Components	Volume for 1 reaction
Hybridization Buffer (2x)	9.5 µl
Hybridization Enhancer	3 µl
Universal Blockers	2 µl
Cell3™ Target Panel	4.5 µl
Total	19 µl

2. Pool equal concentrations (in ng) of individual sample libraries into a fresh 1.5 ml low-bind tube to reach a total combined quantity of 1000 ng.

3. Add 7.5 µl (equivalent to 7.5 µg) of COT-1 Human DNA to the library pool. Briefly vortex mix and centrifuge in a microcentrifuge to collect the liquid at the bottom of the tube.

4. Add 1.8x volume of Target Pure™ NGS clean-up beads and mix thoroughly by pipette mixing 15-20 times, taking care to avoid the formation of bubbles.

5. Incubate the mixture for 10 minutes at room temperature.

6. Place the tube on the magnetic stand for 5 minutes at room temperature to pellet the beads on the side of the tube.

7. Keeping the tube on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

8. Add enough volume of ethanol to ensure the bead pellet is fully submerged, then incubate at room temperature for 30 seconds

9. Keeping the tube on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

10. Use a 10 µl multichannel or single channel pipette to remove any residual liquid from the tube.

11. Keeping the tube on the magnetic stand, incubate at room temperature with open lids for 5 minutes or until the beads are dry.

IMPORTANT: Avoid over-drying of beads, as this can result in a significant loss of DNA recovered. When dry, beads will appear matt in appearance, but should not be cracked

12. Remove the tube from the magnetic stand and resuspend the dried beads in 19 µl of hybridization reaction mix.

13. Incubate the tube for 5 minutes at room temperature.

14. Place the tube on the magnetic stand for 5 minutes at room temperature to pellet the beads on the side of the tubes/wells.

15. Carefully recover 17 µl of supernatant and transfer it to a fresh 0.2 ml PCR tube / 8-well tube strip / 96 well plate.

NOTE: make sure to avoid bead carryover during the transfer process.

16. Place the 0.2 ml PCR tube / 8-well tube strip / 96 well plate containing the hybridization mix into the pre-heated (95°C) thermocycler and skip to the next step on the program.

17. Incubate for 4 hours until the thermocycler program reaches the hold step.

NOTE: for GC-rich or small panels (< 100 Kb in size), longer hybridization times (up to 16 hours) may improve performance.

18. Proceed to section 2.B in the protocol.

IV. Size selection protocol

If the fragment length in sample libraries is not assessed by digital electrophoresis (see chapter 1.E) after library amplification, then a size selection step should be performed instead of a standard clean-up (in chapter 1.D) to ensure that libraries only contain fragments within the required length range. Depending on the selected fragment length chosen during library preparation of genomic or FFPE DNA (independently on whether this is achieved through mechanical shearing or the use of the Library Preparation Kit V2 (b) – 16, 48 or 96 reactions), the fragment length range may vary. The following protocol explains how to perform size selection and lists the amount of beads needed to obtain determined ranges of fragment lengths.

1. Depending on the selected library size, use the following bead-to-sample ratios:
   - 250 – 450 bp library fragment length: use a 0.6x – 0.9x bead ratio
   - 300 – 750 bp library fragment length: use a 0.5x – 0.8x bead ratio
2. After library amplification (chapter 1.D, step 5), perform the first size cut by adding the following quantity of Target Pure™ NGS clean-up beads to a fresh 0.2 ml PCR tube / 8-well tube strip / 96 well plate for each sample:
   - When using the 0.6x – 0.9x bead ratio, add 30 µl of beads
   - When using the 0.5x – 0.8x bead ratio, add 25 µl of beads
3. Transfer the whole 50 µl of PCR amplified library to the 25 – 30 µl of Target Pure™ NGS clean-up beads and mix well by pipetting up and down 15-20 times, taking care to avoid the formation of bubbles.
4. Incubate the mixture for 5 minutes at room temperature.
5. Place the tubes / 8-well tube strip / 96 well plate on the magnetic stand for 5 minutes at room temperature to pellet the beads on the side of the tubes/wells.
6. Keeping the tubes / 8-well tube strip / 96 well plate on the magnetic stand, slowly recover the supernatant (taking care not to disturb the pelleted beads) and transfer it to a fresh 0.2 ml PCR tube / 8-well tube strip / 96 well plate.
7. Discard the 0.2 ml PCR tube / 8-well tube strip / 96 well plate containing the beads to which the larger library fragments are bound.
8. Perform the second size cut by adding 15 µl of Target Pure™ NGS clean-up beads to the recovered supernatant of each sample and mix well by pipette mixing up and down 15-20 times, taking care to avoid the formation of bubbles.
9. Incubate the mixture for 5 minutes at room temperature.

10. Place the tubes / 8-well tube strip / 96 well plate on the magnetic stand for 5 minutes at room temperature to pellet the beads on the side of the tubes/wells.

11. Keeping the tubes / 8-well tube strip / 96 well plate on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

12. Add 200 µl of 80% ethanol to the tube/well and incubate at room temperature for 30 seconds.

13. Repeat steps 11-12 for a total of two 80% ethanol washes.

14. Keeping the tubes / 8-well tube strip / 96 well plate on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

15. Use a 10 µl multichannel or single channel pipette to remove any residual liquid from the tubes/wells.

16. Keeping the tubes / 8-well tube strip / 96 well plate on the magnetic stand, incubate at room temperature with open lids for 3-5 minutes or until the beads are dry.

IMPORTANT: Avoid over-drying of beads, as this can result in a significant loss of DNA recovered. When dry, beads will appear matt in appearance, but should not be cracked

17. Remove the tubes / 8-well tube strip / 96 well plate from the magnetic stand and resuspend the dried beads in 32.5 µl of nuclease-free water by pipetting up and down 10-15 times, taking care to avoid the formation of bubbles.

18. Incubate the tubes / 8-well tube strip / 96 well plate for 2 minutes at room temperature.

19. Place the tubes / 8-well tube strip / 96 well plate on the magnetic stand for 2 minutes at room temperature to pellet the beads on the side of the tubes/wells.

20. Carefully recover 30 µl of supernatant and transfer it to a fresh 1.5 ml low-bind tube.

V. Alternative post-hybridization capture procedure to follow when using all Cell3™ exome panels

Due to the size of the Cell3™ Target Exome Panel and panels with >30 Mb captured size, additional Dynabeads® M-270 Streptavidin need to be used during the capture procedure. When using these large probe panels, the following changes to chapter 2.B and 2.C of the protocol should be applied:

- **Chapter 2.B, step 7:** aliquot **100 µl** of Dynabeads® M-270 Streptavidin per capture reaction into a fresh 1.5 ml tube.
- **Chapter 2.B, step 43:** remove the 0.2 ml PCR tube / 8-well tube strip from the magnetic stand and resuspend the bead pellet in **46 µl** of nuclease-free water by pipetting up and down 10-15 times.
- **Chapter 2.C, step 2:** prepare the PCR reaction mix in a fresh 1.5 ml tube **on ice.** For each capture reaction, prepare **enough PCR reaction mix for two separate PCR reactions in two 0.2 ml PCR tubes / 8-well tube strip** as indicated in the table associated with **Chapter 2.C, step 2.** For multiple samples, prepare the PCR master mix in a 1.5 ml tube by multiplying the volume of each reagent by **2x the number of capture reactions,** add extra volume (overage) to compensate for pipetting loss. Mix well by pipette mixing up and down 10 times or briefly vortex mixing, centrifuge in a microcentrifuge to collect the liquid at the bottom of the tube.
- **Chapter 2.C, step 6:** add **150 µl** of thoroughly vortex mixed room-temperature equilibrated Target Pure™ NGS clean-up beads to a fresh 0.2 ml PCR tube / 8-well tube strip for each captured library.
- **Chapter 2.C, step 7: transfer both 50 µl PCR reactions for each captured library to the 150 µl of Target Pure™ NGS clean-up beads** and mix well by pipette mixing up and down 15-20 times, taking care to avoid the formation of bubbles.

VI. Index sequences of Illumina UMI adapters

Table 2: List of adapters contained in the Library Preparation Kit V2 (a) or (b) – 16, 48 or 96 reactions. I7 index and I5 index sequences are listed for each adapter. The reverse and complement sequence of the I5 index is also shown for the relevant Illumina platforms. Sequences are unique in the I5 and I7 position to detect sample index skipping. The 9 bp "NNNNNNNNN" sequence stands for the Unique Molecular Identifier (UMI), which is sequenced on the same read as the I7 index and allows PCR/sequencing error removal and single molecule counting.

IMPORTANT:

- If demultiplexing with bcl2fastq2 or bcl-convert, do not include the 'NNNNNNNNN' sequence in the I7 index.
- If using bcl2fastq to demultiplex, use a v1 Sample Sheet. If using Dragen or bcl-convert to demultiplex, then use a v2 Sample Sheet.

Well position	Adapter ID	I7 index	I5 index forward IMPORTANT: Please check which orientation is required for your sequencer	I5 index reverse IMPORTANT: Please check which orientation is required for your sequencer
A1	UMIRC_AN01	CTGATCGTNNNNNNNNN	ATATGCGC	GCGCATAT
B1	UMIRC_AN02	ACTCTCGANNNNNNNNN	TGGTACAG	CTGTACCA
C1	UMIRC_AN03	TGAGCTAGNNNNNNNNN	AACCGTTC	GAACGGTT
D1	UMIRC_AN04	GAGACGATNNNNNNNNN	TAACCGGT	ACCGGTTA
E1	UMIRC_AN05	CTTGTCGANNNNNNNNN	GAACATCG	CGATGTTC
F1	UMIRC_AN06	TTCCAAGGNNNNNNNNN	CCTTGTAG	CTACAAGG
G1	UMIRC_AN07	CGCATGATNNNNNNNNN	TCAGGCTT	AAGCCTGA
H1	UMIRC_AN08	ACGGAACANNNNNNNNN	GTTCTCGT	ACGAGAAC
A2	UMIRC_AN09	CGGCTAATNNNNNNNNN	AGAACGAG	CTCGTTCT
B2	UMIRC_AN10	ATCGATCGNNNNNNNNN	TGCTTCCA	TGGAAGCA
C2	UMIRC_AN11	GCAAGATCNNNNNNNNN	CTTCGACT	AGTCGAAG
D2	UMIRC_AN12	GCTATCCTNNNNNNNNN	CACCTGTT	AACAGGTG
E2	UMIRC_AN13	TACGCTACNNNNNNNNN	ATCACACG	CGTGTGAT
F2	UMIRC_AN14	TGGACTCTNNNNNNNNN	CCGTAAGA	TCTTACGG
G2	UMIRC_AN15	AGAGTAGCNNNNNNNNN	TACGCCTT	AAGGCGTA
H2	UMIRC_AN16	ATCCAGAGNNNNNNNNN	CGACGTTA	TAACGTCG
A3	UMIRC_AN17	GACGATCTNNNNNNNNN	ATGCACGA	TCGTGCAT
B3	UMIRC_AN18	AACTGAGCNNNNNNNNN	CCTGATTG	CAATCAGG
C3	UMIRC_AN19	CTTAGGACNNNNNNNNN	GTAGGAGT	ACTCCTAC
D3	UMIRC_AN20	GTGCCATANNNNNNNNN	ACTAGGAG	CTCCTAGT
E3	UMIRC_AN21	GAATCCGANNNNNNNNN	CACTAGCT	AGCTAGTG
F3	UMIRC_AN22	TCGCTGTTNNNNNNNNN	ACGACTTG	CAAGTCGT
G3	UMIRC_AN23	TTCGTTGGNNNNNNNNN	CGTGTGTA	TACACACG
H3	UMIRC_AN24	AAGCACTGNNNNNNNNN	GTTGACCT	AGGTCAAC
A4	UMIRC_AN25	CCTTGATCNNNNNNNNN	ACTCCATC	GATGGAGT

Well position	Adapter ID	I7 index	I5 index forward IMPORTANT: Please check which orientation is required for your sequencer	I5 index reverse IMPORTANT: Please check which orientation is required for your sequencer
B4	UMIRC_AN26	GTCGAAGANNNNNNNNN	CAATGTGG	CCACATTG
C4	UMIRC_AN27	ACCACGATNNNNNNNNN	TTGCAGAC	GTCTGCAA
D4	UMIRC_AN28	GATTACCGNNNNNNNNN	CAGTCCAA	TTGGACTG
E4	UMIRC_AN29	GCACAACTNNNNNNNNN	ACGTTCAG	CTGAACGT
F4	UMIRC_AN30	GCGTCATTNNNNNNNNN	AACGTCTG	CAGACGTT
G4	UMIRC_AN31	ATCCGGTANNNNNNNNN	TATCGGTC	GACCGATA
H4	UMIRC_AN32	CGTTGCAANNNNNNNNN	CGCTCTAT	ATAGAGCG
A5	UMIRC_AN33	GTGAAGTGNNNNNNNNN	GATTGCTC	GAGCAATC
B5	UMIRC_AN34	CATGGCTANNNNNNNNN	GATGTGTG	CACACATC
C5	UMIRC_AN35	ATGCCTGTNNNNNNNNN	CGCAATCT	AGATTGCG
D5	UMIRC_AN36	CAACACCTNNNNNNNNN	TGGTAGCT	AGCTACCA
E5	UMIRC_AN37	TGTGACTGNNNNNNNNN	GATAGGCT	AGCCTATC
F5	UMIRC_AN38	GTCATCGANNNNNNNNN	AGTGGATC	GATCCACT
G5	UMIRC_AN39	AGCACTTCNNNNNNNNN	TTGGACGT	ACGTCCAA
H5	UMIRC_AN40	GAAGGAAGNNNNNNNNN	ATGACGTC	GACGTCAT
A6	UMIRC_AN41	GTTGTTCGNNNNNNNNN	GAAGTTGG	CCAACTTC
B6	UMIRC_AN42	CGGTTGTTNNNNNNNNN	CATACCAC	GTGGTATG
C6	UMIRC_AN43	ACTGAGGTNNNNNNNNN	CTGTTGAC	GTCAACAG
D6	UMIRC_AN44	TGAAGACGNNNNNNNNN	TGGCATGT	ACATGCCA
E6	UMIRC_AN45	GTTACGCANNNNNNNNN	ATCGCCAT	ATGGCGAT
F6	UMIRC_AN46	AGCGTGTTNNNNNNNNN	TTGCGAAG	CTTCGCAA
G6	UMIRC_AN47	GATCGAGTNNNNNNNNN	AGTTCGTC	GACGAACT
H6	UMIRC_AN48	ACAGCTCANNNNNNNNN	GAGCAGTA	TACTGCTC
A7	UMIRC_AN49	GAGCAGTANNNNNNNNN	ACAGCTCA	TGAGCTGT
B7	UMIRC_AN50	AGTTCGTCNNNNNNNNN	GATCGAGT	ACTCGATC
C7	UMIRC_AN51	TTGCGAAGNNNNNNNNN	AGCGTGTT	AACACGCT
D7	UMIRC_AN52	ATCGCCATNNNNNNNNN	GTTACGCA	TGCGTAAC
E7	UMIRC_AN53	TGGCATGTNNNNNNNNN	TGAAGACG	CGTCTTCA
F7	UMIRC_AN54	CTGTTGACNNNNNNNNN	ACTGAGGT	ACCTCAGT
G7	UMIRC_AN55	CATACCACNNNNNNNNN	CGGTTGTT	AACAACCG
H7	UMIRC_AN56	GAAGTTGGNNNNNNNNN	GTTGTTCG	CGAACAAC
A8	UMIRC_AN57	ATGACGTCNNNNNNNNN	GAAGGAAG	CTTCCTTC
B8	UMIRC_AN58	TTGGACGTNNNNNNNNN	AGCACTTC	GAAGTGCT
C8	UMIRC_AN59	AGTGGATCNNNNNNNNN	GTCATCGA	TCGATGAC
D8	UMIRC_AN60	GATAGGCTNNNNNNNNN	TGTGACTG	CAGTCACA
E8	UMIRC_AN61	TGGTAGCTNNNNNNNNN	CAACACCT	AGGTGTTG
F8	UMIRC_AN62	CGCAATCTNNNNNNNNN	ATGCCTGT	ACAGGCAT
G8	UMIRC_AN63	GATGTGTGNNNNNNNNN	CATGGCTA	TAGCCATG
H8	UMIRC_AN64	GATTGCTCNNNNNNNNN	GTGAAGTG	CACTTCAC
A9	UMIRC_AN65	CGCTCTATNNNNNNNNN	CGTTGCAA	TTGCAACG

Well position	Adapter ID	I7 index	I5 index forward IMPORTANT: Please check which orientation is required for your sequencer	I5 index reverse IMPORTANT: Please check which orientation is required for your sequencer
B9	UMIRC_AN66	TATCGGTCNNNNNNNNN	ATCCGGTA	TACCGGAT
C9	UMIRC_AN67	AACGTCTGNNNNNNNNN	GCGTCATT	AATGACGC
D9	UMIRC_AN68	ACGTTCAGNNNNNNNNN	GCACAACT	AGTTGTGC
E9	UMIRC_AN69	CAGTCCAANNNNNNNNN	GATTACCG	CGGTAATC
F9	UMIRC_AN70	TTGCAGACNNNNNNNNN	ACCACGAT	ATCGTGGT
G9	UMIRC_AN71	CAATGTGGNNNNNNNNN	GTCGAAGA	TCTTCGAC
H9	UMIRC_AN72	ACTCCATCNNNNNNNNN	CCTTGATC	GATCAAGG
A10	UMIRC_AN73	GTTGACCTNNNNNNNNN	AAGCACTG	CAGTGCTT
B10	UMIRC_AN74	CGTGTGTANNNNNNNNN	TTCGTTGG	CCAACGAA
C10	UMIRC_AN75	ACGACTTGNNNNNNNNN	TCGCTGTT	AACAGCGA
D10	UMIRC_AN76	CACTAGCTNNNNNNNNN	GAATCCGA	TCGGATTC
E10	UMIRC_AN77	ACTAGGAGNNNNNNNNN	GTGCCATA	TATGGCAC
F10	UMIRC_AN78	GTAGGAGTNNNNNNNNN	CTTAGGAC	GTCCTAAG
G10	UMIRC_AN79	CCTGATTGNNNNNNNNN	AACTGAGC	GCTCAGTT
H10	UMIRC_AN80	ATGCACGANNNNNNNNN	GACGATCT	AGATCGTC
A11	UMIRC_AN81	CGACGTTANNNNNNNNN	ATCCAGAG	CTCTGGAT
B11	UMIRC_AN82	TACGCCTTNNNNNNNNN	AGAGTAGC	GCTACTCT
C11	UMIRC_AN83	CCGTAAGANNNNNNNNN	TGGACTCT	AGAGTCCA
D11	UMIRC_AN84	ATCACACGNNNNNNNNN	TACGCTAC	GTAGCGTA
E11	UMIRC_AN85	CACCTGTTNNNNNNNNN	GCTATCCT	AGGATAGC
F11	UMIRC_AN86	CTTCGACTNNNNNNNNN	GCAAGATC	GATCTTGC
G11	UMIRC_AN87	TGCTTCCANNNNNNNNN	ATCGATCG	CGATCGAT
H11	UMIRC_AN88	AGAACGAGNNNNNNNNN	CGGCTAAT	ATTAGCCG
A12	UMIRC_AN89	GTTCTCGTNNNNNNNNN	ACGGAACA	TGTTCCGT
B12	UMIRC_AN90	TCAGGCTTNNNNNNNNN	CGCATGAT	ATCATGCG
C12	UMIRC_AN91	CCTTGTAGNNNNNNNNN	TTCCAAGG	CCTTGGAA
D12	UMIRC_AN92	GAACATCGNNNNNNNNN	CTTGTCGA	TCGACAAG
E12	UMIRC_AN93	TAACCGGTNNNNNNNNN	GAGACGAT	ATCGTCTC
F12	UMIRC_AN94	AACCGTTCNNNNNNNNN	TGAGCTAG	CTAGCTCA
G12	UMIRC_AN95	TGGTACAGNNNNNNNNN	ACTCTCGA	TCGAGAGT
H12	UMIRC_AN96	ATATGCGCNNNNNNNNN	CTGATCGT	ACGATCAG

NOTE: to view the list of all 384 adapters available with Library Preparation Kit V2 (a) or (b), please download it at **https://nonacus.com/cell3tm-target/.**

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