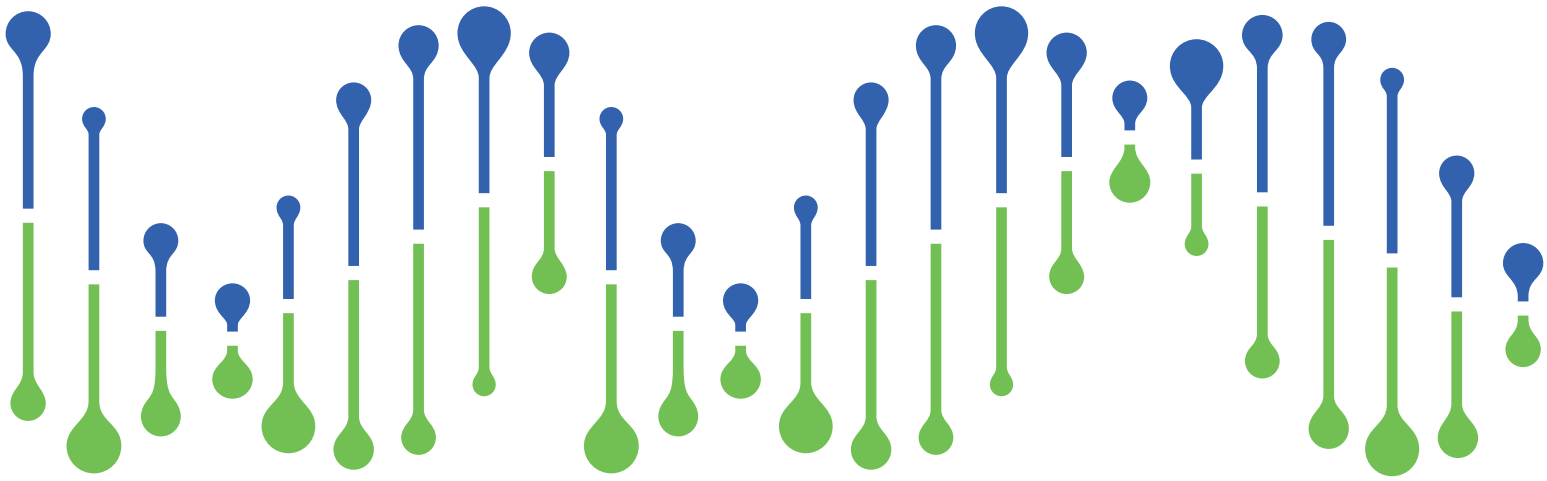
Quick Reference Guide

Protocol Guide v1.0

Cell3™ Target

DNA Target Enrichment for Next Generation Sequencing (Illumina Sequencers)



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Cell3™ Target: DNA Target Enrichment for Next Generation Sequencing

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Workflow overview

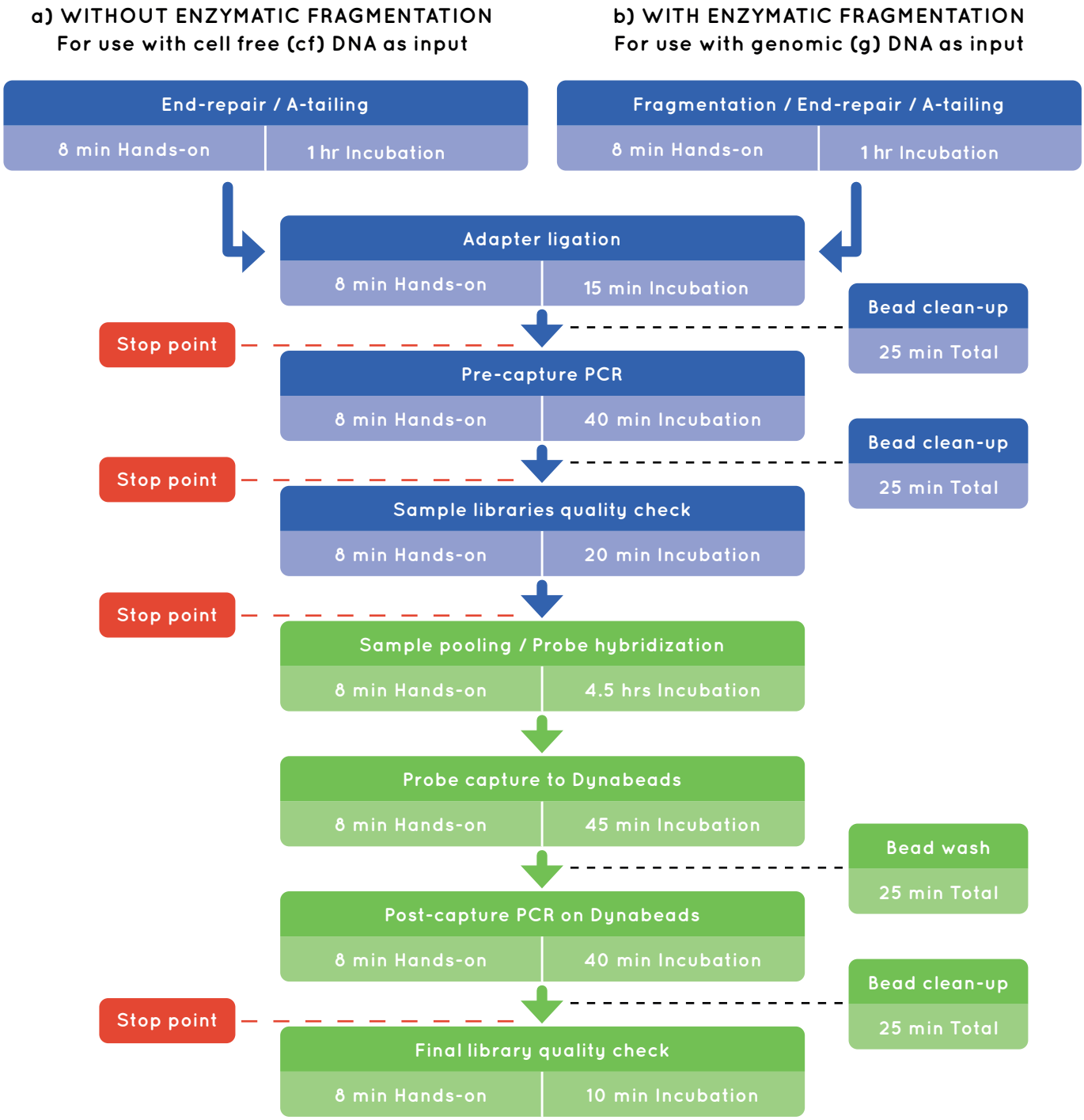

Figure 1. Flow chart outlining the main steps of the Cell3™ Target workflow. Blue boxes refer to library preparation steps (3h); while green boxes refer to probe hybridization / capture and target enrichment steps (8h).

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Chapter 1: Library preparation

This protocol explains how to use the Cell3™ Target: Library Preparation kit – 16, 48 or 96 reactions (a or b version; with or without enzymatic fragmentation) in conjunction with the Cell3™ Target: Capture Enrichment Reagents – 4 or 12 reaction kits and the Cell3™ Target: Probe Set – 2, 4, or 12 capture reaction kits, to perform DNA target enrichment for next generation sequencing on Illumina platforms using cell free DNA (cfDNA) or genomic DNA (gDNA) as input material.

Cell3™ Target: Library Preparation – 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.

Included in the Cell3™ Target: Library Preparation – 16, 48 or 96 reactions kits 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

Input DNA requirements

Library preparation can be conducted using 1 – 1000 ng of high purity DNA resuspended in molecular biology grade water, or low EDTA TE Buffer (0.1 mM EDTA)

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

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.

Input DNA requirements for FFPE samples

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).

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1.A Kit version (a): Without Enzymatic fragmentation End-repair / A-tailing for cell free DNA and fragmented DNA samples

Before you start

- Thaw the End-repair / A-tailing Buffer (10x) (**red** cap) and the Ligation Buffer (5x) (blue cap) at room temperature and briefly vortex mix.
- Mix the End-repair / A-tailing Enzyme Mix (5x) (**red** cap) and the DNA Ligase Enzyme (blue cap) by lightly tapping the tube. Briefly centrifuge all 4 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 70°C (if possible), the sample volume is 50 µl

2. Prepare the reaction mix for each DNA sample in a tube / 8-well tube strip / 96 well plate. Mix well by briefly vortex mixing or pipette mixing 10-15 times and briefly centrifuge.

Components	Volume for 1 reaction
End-repair / A-tailing Buffer (10x)	5 µl
DNA sample	X µl
Nuclease-free water	(35 – X) µl
Total	40 µl

3. Add 10 µl of End-repair / A-tailing Enzyme Mix (5x) 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

4. Immediately transfer to the pre-chilled thermocycler (4°C) and "skip" to the next step in the program.

5. When the program finishes, keep the samples on ice. **Immediately proceed to the ligation step (1.C).**

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1.B Kit version (b): With enzymatic fragmentation
Fragmentation and end-repair / A-tailing for intact genomic DNA samples

NOTE: for DNA input amounts lower than 50 ng, use of the Fragmentation Enhancer is recommended.

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

Before you start

- Thaw the Fragmentation Buffer (10x) (**red** cap), the Ligation Buffer (5x) (**blue** cap) and the Fragmentation Enhancer (**orange** cap) (if required) at room temperature and briefly vortex mix.

- Mix the Fragmentation Enzyme Mix (5x) (**red** cap) and the DNA Ligase Enzyme (**blue** cap) by lightly tapping the tube.

- Briefly centrifuge all 5 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	32°C	30 min
3	65°C	30 min
4	4°C	Hold

NOTE: Set the thermocycler heated lid to 70°C (if possible), the sample volume is 50 µl

Components	Volume for 1 reaction (DNA input ≥ 50 ng)	Volume for 1 reaction (DNA input < 50 ng)
Fragmentation Buffer (10x)	5 µl	5 µl
DNA sample	X µl	X µl
Fragmentation Enhancer	-	2.5 µl
Nuclease-free water	(35 – X) µl	(32.5 – X) µl
Total	40 µl	40 µl

2. Prepare the 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, **keeping the reaction on ice during the whole procedure.** Mix well by briefly vortex mixing or pipette mixing 10-15 times and briefly centrifuge

3. Add 10 µl of Fragmentation Enzyme Mix (5x) to each reaction for a total final volume of 50 µl. Mix well by briefly vortex mixing or pipette mix 10-15 times and briefly centrifuge.

4. Immediately transfer to the pre-chilled thermocycler (4°C) and "skip" to the next step in the program.

5. When the program finishes, 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

Before you start

Equilibrate the Target Pure™ NGS clean-up beads to room temperature for 20-30 minutes. 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.

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 is100 µl

2. Illumina UMI Adapters are provided at a concentration of 15 µM

   - When using a DNA input quantity of <10 ng, prepare a 1:10 dilution for the UMI Adapters using molecular grade water, ready for use in the ligation step (i.e., a final concentration of 1.5 µM)

   - When using a DNA input quantity of ≥10 ng but <50 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 ≥50 ng of input DNA, use the adapters directly from the tube undiluted, at 15 uM

3. While keeping the end-repaired / A-tailed DNA samples on ice, add 5 µl of the selected Illumina UMI adapter 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. Prepare the following ligation buffer master mix immediately before use in a separate 1.5 ml tube. For multiple samples, prepare the ligation master mix in a 1.5 ml tube by multiplying the volume of each reagent by the number of samples, adding extra volume (overage) as necessary. Briefly vortex mix and centrifuge to collect the liquid at the bottom of the tube and **keep on ice**.

Components	Volume for 1 reaction
Ligation Buffer (5x)	20 µl
DNA Ligase Enzyme	10 µl
Nuclease-free Water	15 µl
Total	45 µl

5. Add 45 µl of freshly prepared ligation master mix to each reaction for a total final volume of 100 µl. Mix well by pipetting up and down 10 times or briefly vortex mixing. Briefly centrifuge to collect all the liquid at the bottom of the tubes.

6. Immediately transfer to the pre-chilled thermocycler (4°C) 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 90 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 of the main protocol.

9. Transfer the whole 100 µl of adapter ligation reaction to the 90 µl of Target Pure™ NGS clean-up beads and mix well by pipetting up and down 15-20 times.

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

11. Prepare a solution of 80% ethanol / 20% molecular biology grade water (800 µl per sample are needed for the entire library preparation procedure).

12. Place the tubes/strip/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 samples 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.

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16. Keeping the samples 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 samples 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

19. Remove the samples from the magnetic stand and resuspend the dried beads in 27 µl of Buffer EB/ equivalent buffer saline solution/molecular grade water, by pipette mixing up and down 10-15 times.

20. Incubate for 2 minutes at room temperature.

21. Place the samples on the magnetic stand for 2 minutes at room temperature to pellet the beads on the side of the tubes/wells.

22. Carefully recover 24 µl of supernatant and transfer it to a new 1.5 ml low-bind tube.

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

1.D Library amplification

Before you start

- Thaw the PCR Master Mix – PreCap (2x) (green cap) and the Primer Mix – PreCap (10 µM) (black cap on ice and briefly vortex mix. Keep both tubes on ice.

- Equilibrate the Target Pure™ NGS clean-up beads to room temperature for 20-30 minutes.

- Prepare 80% ethanol (400 µl per sample), 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	2 min	1
3	98°C	20 sec	4-12*
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

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

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

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2. Prepare the following PCR reaction master mix on ice in a in a separate tube / 8-well tube strip / 96 well plate per sample. Mix well by pipette mixing up and down 10 times or briefly vortex mix. Centrifuge in a microcentrifuge. 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 samples, add extra volume(overage) as necessary, then aliquot 27.5 µl to a new tube / 8-well tube strip / 96 well plate for each sample.

Components	Volume for 1 reaction
PCR Master Mix – PreCap (2x)	25 µl
Primer Mix – PreCap (10 µM)	2.5 µl
Total	27.5 µl

3. Transfer 22.5 µl of adapter-ligated and purified sample library to the 27.5 µl of PCR reaction master mix. Pipette mix up and down 10 times or briefly vortex mix then centrifuge.

4. Transfer to the pre-heated thermocycler (98°C) and skip to the next step in the program.

5. When the program finishes, 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 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 of the main protocol.

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.

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

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

10. Keeping the samples 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 samples 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 samples 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 samples 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.

17. Incubate the samples for 2 minutes at room temperature.

18. Place the samples 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

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1.E Library quality check

Libraries are assessed by determining:

- DNA **quantity** in terms of concentration (ng/µl) and total yield (ng). Use of fluorometric assays for dsDNA (such as the Qubit dsDNA BR assay kit, Invitrogen) is recommended for accurate determination of DNA concentration. If libraries yield is <500 ng in total, refer to the troubleshooting guide in the main protocol.

- DNA **quality** in terms of expected fragment size distribution and absence of additional lower or higher molecular weight peaks (recommended), by analysing libraries with digital electrophoresis systems (such as the Agilent 2200 TapeStation with D1000 reagents and screentape, Agilent Technologies) and determining the peak size within the fragment distribution

Chapter 2: Probe Hybridization and Capture Enrichment

2.A Library pooling and probe hybridization

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 less 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 less 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 of the main protocol
- 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) at room temperature.
- Thaw the Cell3™ Target: Probe Set on ice
- Vortex mix, then microcentrifuge all components

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).

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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 to 100°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.

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

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 of the main protocol

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: Probe Set	4 µl
Nuclease-free water	1.8 µl
Total	17 µl

7. Gently pipette mix up and down 10 times, briefly centrifuge 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

9. Place 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, or for up to 16 hours overnight

2.B Probe capture on Streptavidin beads and washes

Before you start

- Equilibrate the Dynabeads® M-270 Streptavidin to room temperature for 30 minutes

- 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) at room temperature. Thoroughly vortex mix and centrifuge

IMPORTANT: If using a panel (custom or catalogue) with a total covered region size >30Mb refer to **Appendix V** in the main protocol to proceed with the alternative post-hybridization capture protocol. Information for panel total covered region size can be located in the **Kit Contents** section of the main protocol.

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. Consider 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

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 65°C for 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 in a thermocycler at 65°C for 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 required

Preparation of Dynabeads® M-270 Streptavidin

6. 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.

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 incubate 20-30 seconds or until all beads have pelleted

9. Carefully remove and discard the supernatant

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 pelleted on the side of the tube

13. Carefully remove and discard the supernatant

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

15. Transfer 100 µl of resuspended beads into a new tube for each capture reaction. Proceed to the next step **only when the hybridization (section 2.A, step 10) incubation ends.**

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 of the main protocol. In this case, turn on a heat block and set to 65°C.

16. Place the tube on a magnetic stand and incubate for 1-2 minutes or until all beads have pelleted on the side of the tube/well.

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

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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

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

19. Transfer the entire hybridization reaction mix (from section 2.A, step 10) to the pelleted Dynabeads® M-270 Streptavidin

20. Remove from the magnetic stand and mix the hybridization reaction mix with the Dynabeads® M-270 Streptavidin by pipette mixing up and down 10 times.

21. Transfer back to the thermocycler set to 65°C (with the heated lid set to 70°C) and incubate 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 of the main protocol

22. Every 12 minutes during the 45-minute incubation at 65°C, remove from the thermocycler, quickly vortex mix for 3 seconds and place back on the thermocycler

23. Remove 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 on a magnetic stand for 10-15 seconds to allow the beads to separate from the supernatant and pellet on the side of the tube/well

25. Carefully remove the supernatant

26. Remove from the magnetic stand and add 200 µl of pre-heated **1x Stringent Wash Buffer** (from step 4)

27. Pipette mixing up and down 10 times

28. Transfer to a thermocycler set to 65°C (with the heated lid set to 70°C) and incubate for 5 minutes

29. After incubation, remove from the thermocycler and place on a magnetic stand for 10-15 seconds to allow the beads to separate from the supernatant and pellet on the side of the tube/well

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

31. Carefully remove the supernatant

32. Remove from the magnetic stand and add 200 µl of **room temperature 1x Wash Buffer 1**

33. Vortex mix for 2 minutes and briefly centrifuge

34. Place 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

35. Carefully remove the supernatant

36. Remove from the magnetic stand and add 200 µl of **room temperature 1x Wash Buffer 2**

37. Vortex mix for 1 minute and briefly centrifuge

38. Place 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

39. Carefully remove the supernatant

40. Remove from the magnetic stand and add 200 µl of **room temperature 1x Wash Buffer 3**

41. Vortex mix for 30 seconds and briefly centrifuge

42. Place 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

43. Carefully remove the supernatant

44. Remove 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

2.C Captured library amplification and clean-up

Before you start

- Thaw the PCR Master Mix – PostCap (2x) (**green** cap) and the Primer Mix – PostCap (10 µM) (**black** cap) on ice. Briefly vortex mix and centrifuge. 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)

- Prepare a solution of 80% Ethanol / 20% molecular biology grade water (400 µl required per capture reaction, 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	2 min	1
3	98°C	20 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.

Components	Volume for 1 reaction
PCR Master Mix – PostCap (2x)	25 µl
Primer Mix – PostCap (10 µM)	2.5 µl
Total	27.5 µl

3. Transfer 22.5 µl of resuspended Dynabeads® M-270 Streptavidin with captured library DNA to the 27.5 µl of PCR reaction master mix from step 2 and pipette mix up and down 10-15 times

4. Transfer to the pre-heated thermocycler (98°C) and skip to the next step in the program

5. When the program finishes, proceed immediately to library clean-up using Target Pure™ NGS clean-up beads

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Clean-up of amplified captured library

6. Add 75 µl of thoroughly vortex mixed room-temperature equilibrated Target Pure™ NGS clean-up beads to a new tube for each captured library.

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 of the full protocol

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

8. Incubate the mixture for 5 minutes at room temperature

9. Place on the magnetic stand for 5 minutes at room temperature to pellet the beads

10. Keeping the tube/tubes on the magnetic stand, slowly remove and discard the supernatant

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

12. Repeat steps 8-9 for a total of two 80% ethanol washes

13. Keeping the tube/tubes on the magnetic stand, slowly remove and discard the supernatant

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

15. 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

16. Remove from the magnetic stand and resuspend the dried beads in 32.5 µl of Buffer EB or equivalent buffer saline solution by pipette mixing up and down 10-15 times, taking care to avoid the formation of bubbles.

17. Incubate for 2 minutes at room temperature

18. Place 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). 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 guides in the main protocol

- DNA **quality** in terms of DNA average fragment size and absence of additional lower or higher molecular weight peaks. 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. Alternatively, 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 (KAPA Biosystems). 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.

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 using the following ways:

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

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

2. 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. Chapter 3, Table 1 in the main protocol 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.

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 of the main Cell3 Target Protocol.

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 SampleSheet** 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 SampleSheet A or B** for use with bcl2fastq or on device demultiplexing of Illumina machines that do not include Dragen based demultiplexing.

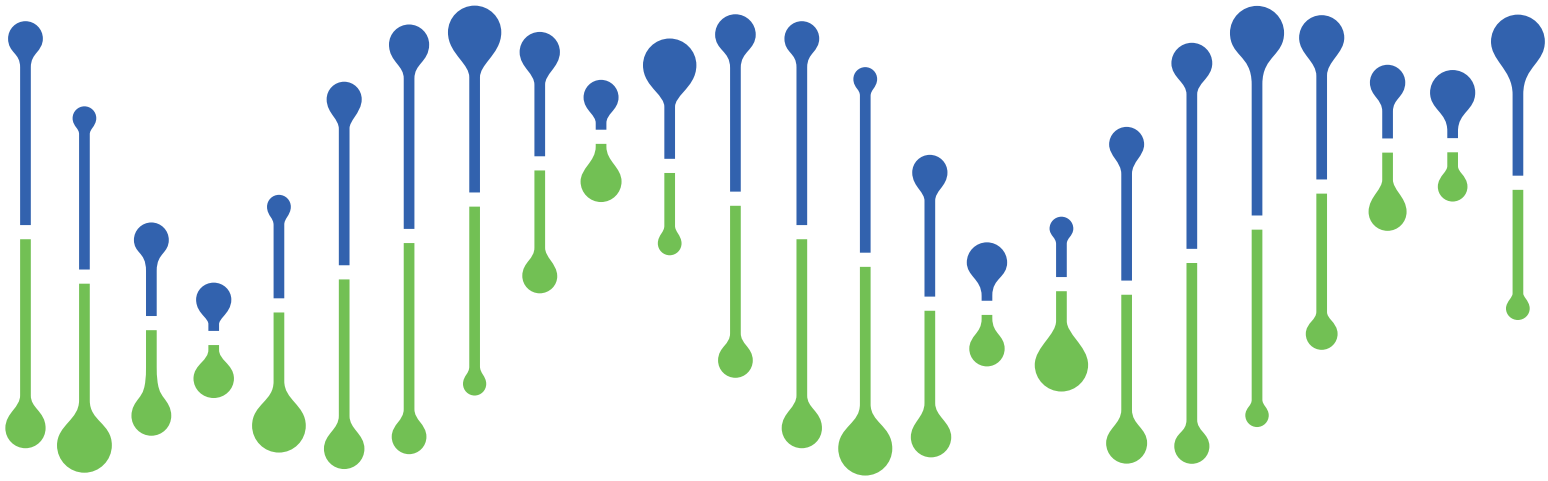
Note: The sample sheet templates can be downloaded from **https://nonacus.com/cell3tm-target/**. Further guidance for preparation of sample sheets and demultiplexing can be found in Chapter 3, section 3.C of the main Cell3 Target Protocol

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 needed.

For further information please refer to the main Cell3 Target Protocol. For further support, please email us at **support@nonacus.com**.

Quick Reference Guide



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