Experienced User

Protocol Guide v1.2.2

Cell3™ Target

DNA Target Enrichment for Next Generation Sequencing (Illumina Sequencers)

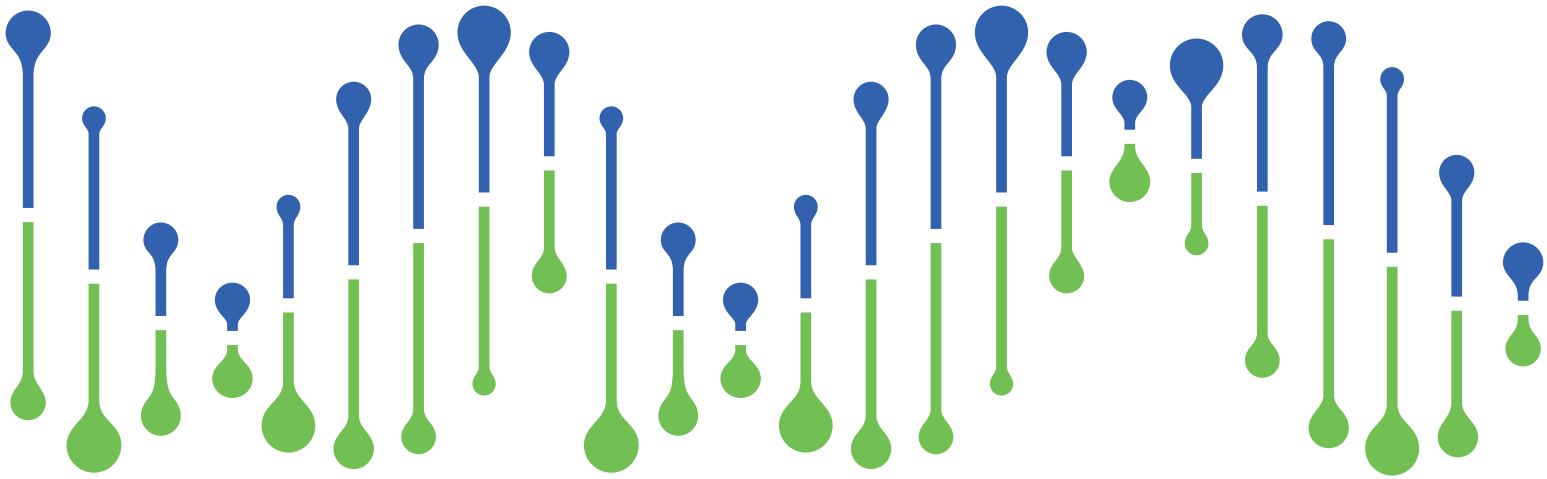

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Table of Contents

Workflow Overview

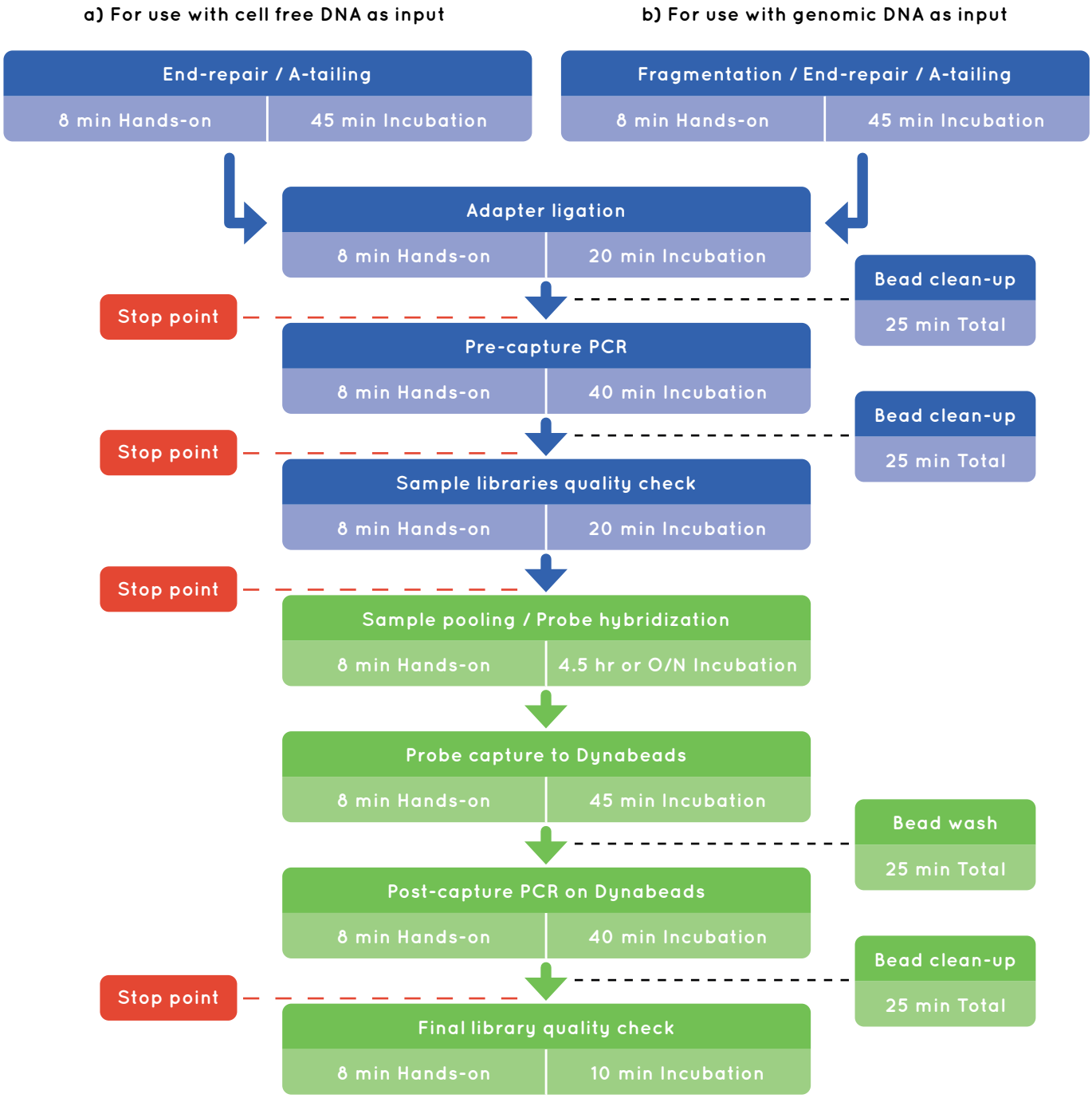


Figure 1. Flow chart outlining the main steps of the Cell3™ Target workflow. Blue boxes refer to library preparation steps (3h); while green boxes refer to probe hybridization / capture and target enrichment steps (8h).

Chapter 1: Library Preparation

Input DNA requirements

- Determine DNA concentration using a fluorometric method (e.g. Qubit assay, Invitrogen).
- Resuspend in molecular biology grade water or a low EDTA buffer.
- **1-100 ng** DNA is recommended when using UMIs
- **100-1000 ng** DNA is recommended if UMIs are not required or for PCR-free libraries

Input DNA requirements for FFPE samples

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

- A minimum input of **10 ng FFPE DNA** is recommended irrespective of the DIN score.

1.A Kit version (a): End-repair / A-tailing (non-fragmentation)

Before you start

- Thaw the End-repair / A-tailing Buffer (10x) (**red** cap) and Ligation Buffer (5x) (**blue** cap) at **RT** and mix by briefly **vortexing,** brief spin, **keep on ice.**
- Mix the End-repair / A-tailing Enzyme Mix (5x) (**red** cap) and DNA Ligase Enzyme (**blue** cap) by **tapping the tube,** brief spin, **keep on ice.**

Procedure

1. Set up the **ER/AT** program.

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

Set the heated lid at **70°C,** volume to **50 µl** and **start.**

Prepare the reaction, **keep on ice**. Mix by **vortexing or pipetting, spin.**

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

2. Add **10 µl of End-repair / A-tailing Enzyme Mix (5x)** for a total volume of **50 µl**. Mix by **vortexing or pipetting 10-15 times, spin.**
3. Transfer to the pre-chilled thermocycler (4°C) and "skip" to the next step.
4. Keep **on ice. Immediately proceed to the ligation step (1.C).**

1.B Kit version (b): Fragmentation and end-repair / A-tailing

- DNA input amounts lower than **50 ng**, add the **Fragmentation Enhancer.**
- For longer insert sizes see **Appendix I** of full protocol.

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 **RT** and mix by **briefly vortexing,** spin, keep on ice
- Mix the Fragmentation Enzyme Mix (5x) (**red** cap) and the DNA Ligase Enzyme (**blue** cap) by **tapping** the tube, spin, keep on ice.

Procedure

1. Set up the program in a thermocycler.

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

Set the heated lid at **70°C,** volume to **50 µl** and **start.**

2. Prepare the reaction, **keep reaction on ice.** Mix well by **vortexing or pipetting, spin.**

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

3. Add **10 µl** Fragmentation Enzyme Mix (5x) for a total volume of **50 µl**. Mix by **vortexing or pipetting 10-15** times, **brief spin.**
4. Transfer to the thermocycler (4°C) "skip" to the next step.
5. Keep **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 required number of Illumina UMI Adapters, **thaw on ice** and spin.

Procedure

1. Set up the program in a thermocycler.

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

   Set the lid to **"not heated"** (or leave the lid open), volume **to 100 µl** and **start.**

2. Illumina UMI Adapters are provided at a concentration of 15 µM.
   - Use at **1.5 µM for DNA inputs less than 50ng** (1:10 dilution)
   - Use directly from the tube for ≥50ng.
3. While keeping the end-repaired / A-tailed DNA samples **on ice,** add 5 µl of Illumina UMI adapter to each sample and **mix** by **pipetting or briefly vortexing.**
4. Prepare the ligation buffer master mix. Mix by vortexing, spin 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 ligation master mix for a total volume **100 µl.**
6. Mix by pipetting **10 times or brief vortexing** and spin.
7. Transfer to the pre-chilled thermocycler (4°C) and "skip" to the next step.
8. Remove samples and proceed to the clean-up step using **Target Pure™ NGS clean-up beads.**

Clean-up of adapter ligated library

9. Add **90 µl** of **Target Pure™ NGS clean-up beads** to a fresh tube for each sample.
10. Transfer **100 µl of adapter ligation reaction** to the 90 µl of Target Pure™ NGS clean-up beads and mix well by pipetting **15-20 times.**
11. Incubate for **5 minutes** at **RT.**
12. Prepare a **fresh** solution of 80% ethanol / 20% molecular biology grade water (800 µl per sample).
13. Place tubes on the magnet for **5 minutes.**
14. Remove and discard the **supernatant,** without disturbing the pelleted beads.
15. Add **200 µl** of 80% ethanol to the tube/well and incubate for **30 seconds.**
16. Repeat steps 14-15 for a total of **two 80% ethanol washes.**
17. Keeping the tubes on the magnet, slowly remove and discard the **supernatant.**
18. Remove any residual liquid from the tubes/wells with a **10 µl** pipette.
19. Keeping the tubes on the magnet, incubate at **RT** with **open lids** for **5 minutes or until the beads are dry. Do not over dry.**
20. Remove the tubes from the magnet and resuspend in **27 µl of Buffer EB or molecular biology grade water.**
21. Incubate the tubes for **2 minutes** at **RT.**
22. Place the tubes on the magnet for **2 minutes** at **RT** to pellet beads.
23. Recover **24 µl** of **supernatant** and transfer it to a fresh **1.5 ml low-bind tube.**

Stop point: adapter ligated libraries can be stored at **-20°C**

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. Mix by **vortexing** and spin. Keep **on ice**.
- Equilibrate the **Target Pure™ NGS clean-up** beads to **RT** for **20-30 minutes.**

Procedure

1. Set up the following program in a thermocycler.

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

** as a general guideline:* ***4 cycles for 200 ng, 5 cycles for 100 ng, 6 cycles for 50 ng, 9 cycles for 10 ng, 12 cycles for 1 ng.***
For FFPE DNA increase the cycle number by ***1*** *(e.g.* ***7 cycles for 50 ng)****.*

Set the heated lid at **105°C,** volume at **50 µl** and **start.**

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

2. Prepare the PCR reaction master mix in a separate PCR tube for each sample **on ice.**
3. Mix by pipetting **10 times** or briefly **vortexing** and spin.
4. Transfer **22.5 µl** of adapter-ligated library to the **27.5 µl** of PCR reaction mix. Mix by pipetting **10 times** or brief **vortexing** and spin.
5. Transfer to the pre-heated thermocycler **(98°C)** and **skip** to the next step.
6. 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

7. Add **50 µl** of **Target Pure™ NGS clean-up beads** to a fresh tube for each sample.
8. Transfer the **50 µl** of PCR amplified library to the 50 µl of Target Pure™ NGS clean-up beads and mix well by pipetting **15-20 times.**
9. Incubate for **5 minutes** at RT.
10. Place the tubes on the magnet for **5 minutes** at **RT.**
11. Keeping the tubes on the magnet, remove and **discard** the **supernatant.**
12. Add **200 µl of 80% ethanol** to the tube/well and incubate for **30 seconds.**
13. Repeat steps 10-11 for a total of **two 80% ethanol** washes.
14. Keeping the tubes on the magnet, remove and discard the **supernatant.**
15. Remove any residual liquid from the tubes/wells with a 10 µl pipette.
16. Keeping the tubes on the magnet, incubate at **room temperature, lids open** for **5 minutes or until the beads are dry. Do not over dry.**
17. Remove the tubes from the magnet and resuspend in **32.5 µl** of **nuclease-free water.**
18. Incubate the tubes for **2 minutes** at **RT.**
19. Place the tubes on the **magnet** for **2 minutes** at **RT.**
20. Carefully recover **30 µl** of **supernatant** and transfer it to a fresh **1.5 ml low-bind tube.**

Stop point: **4°C overnight or at -20°C for long term storage.**

1.E Library QC

- DNA concentration (ng/µl) and total yield (ng) using fluorometric method (e.g. dsDNA BR assay kit, Invitrogen **Qubit**)
- DNA quality in terms of expected fragment size distribution and absence of additional lower or higher molecular weight peaks (recommended) **TapeStation.**

Chapter 2: Probe Hybridization and Capture Enrichment

Before you start

1. Switch on a vacuum concentrator and set the temperature to **70°C or lower.**
2. 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 **RT.** Thaw the Cell3™ Target: Probe Set **on ice.**
3. Mix each component by **vortexing** and **spin.**

NOTE: Heat the Hybridization Buffer (2x) (**blue** cap) at **65°C in a heat block** and vortex every few minutes if crystallisation is seen

Procedure

1. Set up the following program on a thermocycler.

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

   Set the lid to heated at **100°C,** the sample volume to **17 µl** and **start.**

2. Pool equal amounts individual libraries into **1.5 ml low-bind** tube to a total of **1000 ng.**
3. Add **5 µl of COT-1 Human DNA** and **2 µl of Universal Blockers** to the library pool. Mix by vortexing and spin.
4. Place the tube (lid open) in the vacuum concentrator until pool is **completely dry.**

Stop point: the dried down library pool can be stored **overnight at 4°C.**

5. Prepare the hybridization reaction mix by adding to the dried down pool.

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

6. **Mix** by pipetting **10 times,** briefly centrifuge and incubate at **RT** for **10 minutes.**
7. Transfer hybridization reaction mix to a **0.2 ml PCR tube,** brief spin.
8. Place the hybridization reaction mix in the thermocycler (95°C), skip to the next step.
9. Incubate the hybridization reaction mix at **65°C** on the thermocycler for **4-16 hours.**

2.B Probe capture on Streptavidin beads and washes

Before you start

- Equilibrate the **Dynabeads® M-270** Streptavidin to **RT for 30 minutes.**
- Thaw all Wash Buffers (S, 1, 2, 3, B) at **RT, vortex** and spin.
- Heat Wash Buffer 1 at **65°C** to resuspend precipitated particles.

IMPORTANT: for **Cell3™ Target Exome Panel** or **custom probe sets of Tier-4** and above, refer **to Appendix V** to proceed with the alternative post-hybridization capture protocol.

Preparation of wash buffers

1. Dilute for each capture reaction. Prepare a 1x working solution in **1.5 ml** tubes:

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. **Vortex** each component and spin.
3. Transfer 100 µl of **1x Wash Buffer 1** into a fresh 0.2 ml PCR tube and pre-heat at **65°C**
4. Split the **1x Stringent Wash Buffer** into **two 200** µl **aliquots, in 0.2 ml PCR tubes,** pre-heat at **65°C.**
5. Store the 200 µl of **1x Wash Buffer 1** and the **remaining 1x wash buffers at RT.**

Preparation of Dynabeads® M-270 Streptavidin

6. Mix **RT Dynabeads® M-270 Streptavidin** by vortexing for **15 seconds.**
7. Aliquot **50 µl** of Dynabeads® M-270 Streptavidin per capture reaction into a 1.5 ml tube.
8. Place the 1.5 ml tube on a magnet and incubate **20-30 seconds.**
9. Remove and discard the **supernatant**.
10. Add 200 µl of **1x Bead Wash Buffer** per capture reaction 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.**
13. Remove and discard the **supernatant.**
14. Add 100 µl of **1x Bead Wash Buffer** and vortex briefly.
15. Transfer 100 µl of resuspended beads into a new tube. **Proceed to the next step only when the hybridization incubation ends.**
16. Place the tube on a magnet and incubate **1-2 minutes** or until all beads have separated.
17. Remove and discard the **supernatant** and **proceed immediately to the next step.**

Procedure

18. Set a thermocycler at **65°C to hold** with heated lid **at 70°C.**
19. Transfer the hybridization reaction mix to the tube containing the pelleted **Dynabeads® M-270 Streptavidin.**
20. Remove the 0.2 ml PCR tube from the magnet and mix the hybridization reaction mix with the Dynabeads® M-270 Streptavidin by pipetting **10 times.**
21. Transfer to the **65°C** thermocycler (heated lid at **70°C**), incubate for **45 minutes.**
22. **Every 12 minutes** during the 45-minute incubation at 65°C, vortex for **3 seconds.**
23. Remove the tube from the thermocycler, add **100 µl** of **pre-heated 1x Wash Buffer 1.**
24. Mix by pipetting **10 times** and place on a magnet for **10-15 seconds.**
25. Remove the **supernatant.**
26. Remove the tube from the magnet, add **200 µl** of pre-heated **1x Stringent Wash Buffer**
27. Mix by pipetting **10 times.**
28. Transfer to **65°C** thermocycler (heated lid at to **70°C**) and incubate **for 5 minutes.**
29. Remove from the thermocycler and place on a magnet stand for **10-15 seconds.**
30. Repeat steps 25-29 for a total of **two washes** with **heated 1x Stringent Wash Buffer.**
31. Remove the **supernatant.**
32. Remove from the magnet, add **200 µl of RT 1x Wash Buffer 1.**
33. **Vortex** for **2 minutes** and briefly spin.
34. Place the tube on a magnet for **20-30 seconds.**
35. Remove the **supernatant.**
36. Remove from the magnet, add **200 µl of RT 1x Wash Buffer 2.**
37. **Vortex** for **1 minute** and briefly spin.
38. Place the tube on a magnet for **20-30 seconds.**
39. Remove the **supernatant.**
40. Remove from the magnet, add **200 µl of RT 1x Wash Buffer 3.**
41. **Vortex** for **30 seconds** and briefly spin.
42. Place the tube on a magnet **for 1-2 minutes.**
43. Remove the **supernatant.**
44. Remove the tube from the magnet, resuspend in **24 µl of nuclease-free water** by pipetting **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,** vortex, spin down. Keep **on ice.**
- Equilibrate the **Target Pure™ NGS clean-up beads** to **RT** for **20-30 minutes**
- Prepare a solution of 80% Ethanol / 20% molecular biology grade water (400 µl per capture reaction)

Procedure

1. Set up the program.

Step	Temperature	Time	Cycles
1	98°C	Hold	1
2	98°C	2 min	1
3	98°C	20 sec	7-16*
4	60°C	30 sec	
5	72°C	30 sec	
6	72°C	1 min	1
7	4°C	Hold	1

** as a general guideline: 7 cycles for exome, 9 cycles for probe sets >5 Mb in size, 10 cycles for 3-5 Mb, 12 cycles for 0.2-3 Mb, 14 cycles for 0.2-0.04 Mb, 16 cycles for <0.04 Mb.*

Set the lid to heated at **105°C,** volume to **50 µl** and start.

2. Prepare the PCR reaction mix **on ice.** Mix by **pipetting 10 times** or **vortex** and **spin.**

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 and mix by pipetting **10-15 times.**
4. Transfer to the pre-heated thermocycler **(98°C)** and skip to the next step.
5. 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

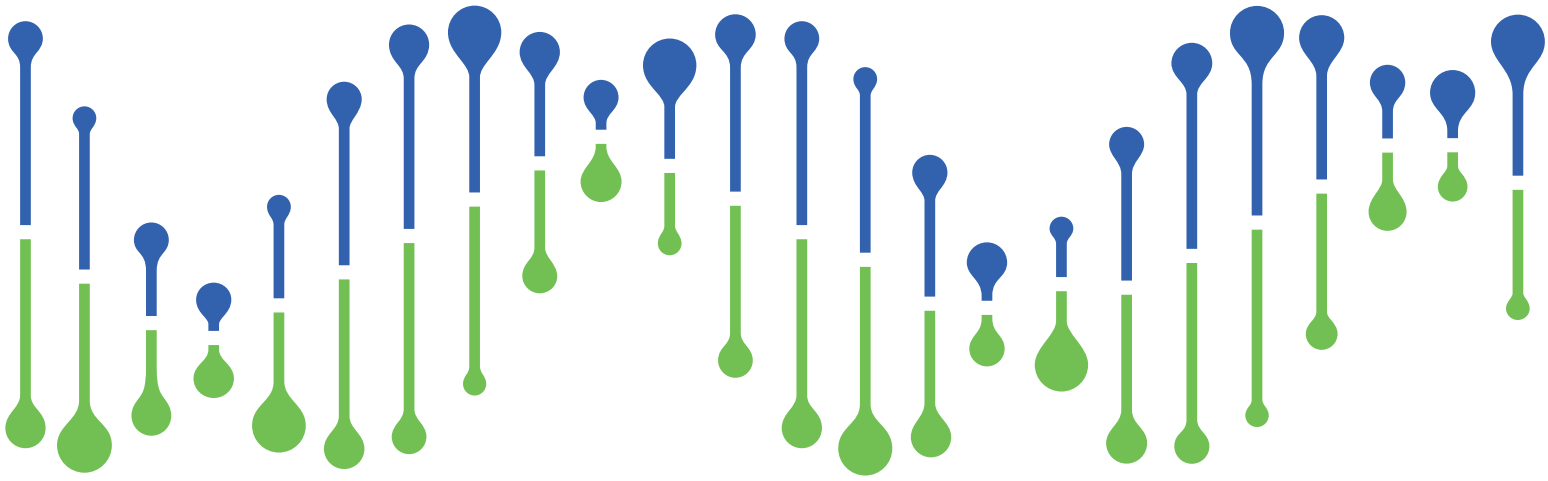
6. Add **75 µl** of vortexed **RT Target Pure™ NGS clean-up beads** to a fresh tube.
7. Transfer the whole **50 µl** of PCR product to the 75 µl of Target Pure™ NGS clean-up beads and mix by **pipetting 15-20 times,** taking care to avoid the formation of bubbles.
8. **Incubate** the mixture for **5 minutes** at **RT.**
9. Place the tube on the magnet for **5 minutes** at **RT.**
10. Keeping the tube on the magnet, remove and **discard** the **supernatant.**
11. Add 200 µl of 80% ethanol to the tube at **RT** for **30 seconds.**
12. Repeat steps 10-11 for a total of **two** 80% ethanol washes.
13. Keeping the tube on the magnet, remove and **discard** the **supernatant.**
14. Use a **10 µl pipette** to remove any residual liquid.
15. Keeping the tube on the magnet, incubate at **RT** with **open lids** for **5 minutes** or until the beads are dry. **Avoid over-drying of beads.**
16. Remove the tube from the magnet and resuspend the dried beads in **32.5 µl** of **Buffer EB** or equivalent pipetting **10-15 times.**
17. **Incubate** the tube for **2 minutes** at **RT.**
18. Place the tube on the magnet for **2 minutes** at **RT.**
19. **Recover 30 µl** of **supernatant** and transfer to a fresh 1.5 ml **low-bind tube.**

Stop 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 QC

- DNA **concentration** (ng/µl) and total yield (ng) (Qubit HS)
- DNA **average** fragment size and absence of additional lower or higher molecular weight peaks (TapeStation HS)

Experienced User



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