Roche

Sequencing Solutions Technical Note

September 2018

How To…

Prepare libraries for sequencing of circulating cell-free DNA inputs

Applications
Whole genome sequencing of ccfDNA
Targeted sequencing of ccfDNA

Products*
KAPA HyperPrep Kit
KAPA Pure Beads
KAPA Dual-Indexed Adapters
KAPA Library Quantification Kit
KAPA hgDNA Quantification and QC Kit
SeqCap EZ HyperCap Workflow v2.0

*For Research Use Only. Not for use in diagnostic procedures.

1. Overview

Circulating tumor DNA (ctDNA) is tumor-derived, fragmented, double-stranded DNA (dsDNA) found in blood along with circulating cell-free DNA (ccfDNA) from other sources. ctDNA serves as a biomarker for cancer mutation profiling. Detection of tumor-specific molecular mutations by analysis of bodily fluids, including peripheral blood, is termed “liquid biopsy”.

The KAPA HyperPrep Kit provides a streamlined protocol for the rapid construction of libraries for Illumina® sequencing across a wide range of inputs, down to 1 ng[1]. With inputs of ccfDNA ranging from 1 to 50 ng[2], the KAPA HyperPrep chemistry allows for maximal conversion of input molecules into unique library fragments leading to greater molecular complexity. This kit includes the KAPA HiFi DNA Polymerase for library amplification, resulting in lower duplication rates and improved coverage.

In this Technical Note, we provide a protocol illustrating the utility of the KAPA HyperPrep Kit[2] for generating sequencing-ready libraries from ccfDNA. Libraries produced with this protocol should be compatible with any target capture chemistry, however we recommend using the streamlined SeqCap EZ HyperCap v2.0[3] workflow. We also highlight reaction parameters that can be optimized to improve sequencing outcomes.

2. TABLE OF CONTENTS

3. REQUIRED REAGENTS

The following reagents are supplied by Roche:

- KAPA HyperPrep Kit
  - Roche PN: 07962312001 (8 libraries)
  - Roche PN: 07962347001 (24 libraries)
  - Roche PN: 07962363001 (96 libraries)
- KAPA Dual-Indexed Adapters (Roche PN: 08278555702)
- KAPA Pure Beads (Roche PN: 07983298001, 07983280001, 07983271001)
- KAPA Library Quantification Kit
  - Roche PN: 07960140001 / Universal qPCR Master Mix
  - Roche PN: 07960204001 / ABI Prism™ qPCR Master Mix
  - Roche PN: 07960255001 / Bio-Rad iCycler™ qPCR Master Mix
  - Roche PN: 07960336001 / ROX Low qPCR Master Mix
  - Roche PN: 07960298001 / qPCR Master Mix optimized for LightCycler® 480
- KAPA hgDNA Quantification and QC Kit
  - Roche PN: 07960590001 / Universal qPCR Master Mix
  - Roche PN: 07960603001 / ABI Prism™ qPCR Master Mix
  - Roche PN: 07960611001 / Bio-Rad iCycler™ qPCR Master Mix
  - Roche PN: 07960689001 / ROX Low qPCR Master Mix
  - Roche PN: 07960620001 / qPCR Master Mix optimized for LightCycler® 480

- HyperCap Target Enrichment Kit (including Roche Universal Blocking Oligos)
  - Roche PN: 08286370001 (24 reactions)
  - Roche PN: 08286345001 (96 reactions)
- HyperCap Bead Kit
  - Roche PN: 08286418001 (24 reactions)
  - Roche PN: 08286400001 (96 reactions)
- SeqCap EZ Prime Choice Probes
  - Off the shelf or custom options (See website for more information: *https://sequencing.roche.com/en/products-solutions/by-category/target-enrichment/hybridization/seqcap-ez-prime-choice-probes.html*)

The following reagents are not supplied by Roche, and must be sourced from general laboratory stocks or third-party suppliers:

- Freshly prepared 80% ethanol
- Elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5)
- If not using Roche-supplied adapters, any other full-length adapter designs compatible with "TA-ligation" sourced from a third-party vendor may be used.

  NOTE: Matched blocking oligonucleotides will also be required for targeted sequencing workflows.

The following equipment and plastic ware is required for this workflow:

- Pipettes
- 96-well PCR thermocycler instrument
- 0.2 mL PCR tubes/96-well PCR plates
- Low DNA binding microtubes 1.5 mL
- Magnetic block e.g. DynaMag™-96 Side Magnet (ThermoFisher Catalog number: 12331D)
- Microcentrifuge (16,000 x g capability)
- Vortex mixer
- NanoDrop Spectrophotometer
- Agilent Bioanalyzer 2100
- Roche LightCycler® 480 Instrument II (optional)

4. WORKFLOW

The KAPA HyperPrep workflow, illustrated in **Figure 1**, outlines the steps required for library construction from total ccfDNA input into sequenceable library molecules. Following library construction, one can either perform targeted capture of regions of interest or alternatively, proceed directly to sequencing.

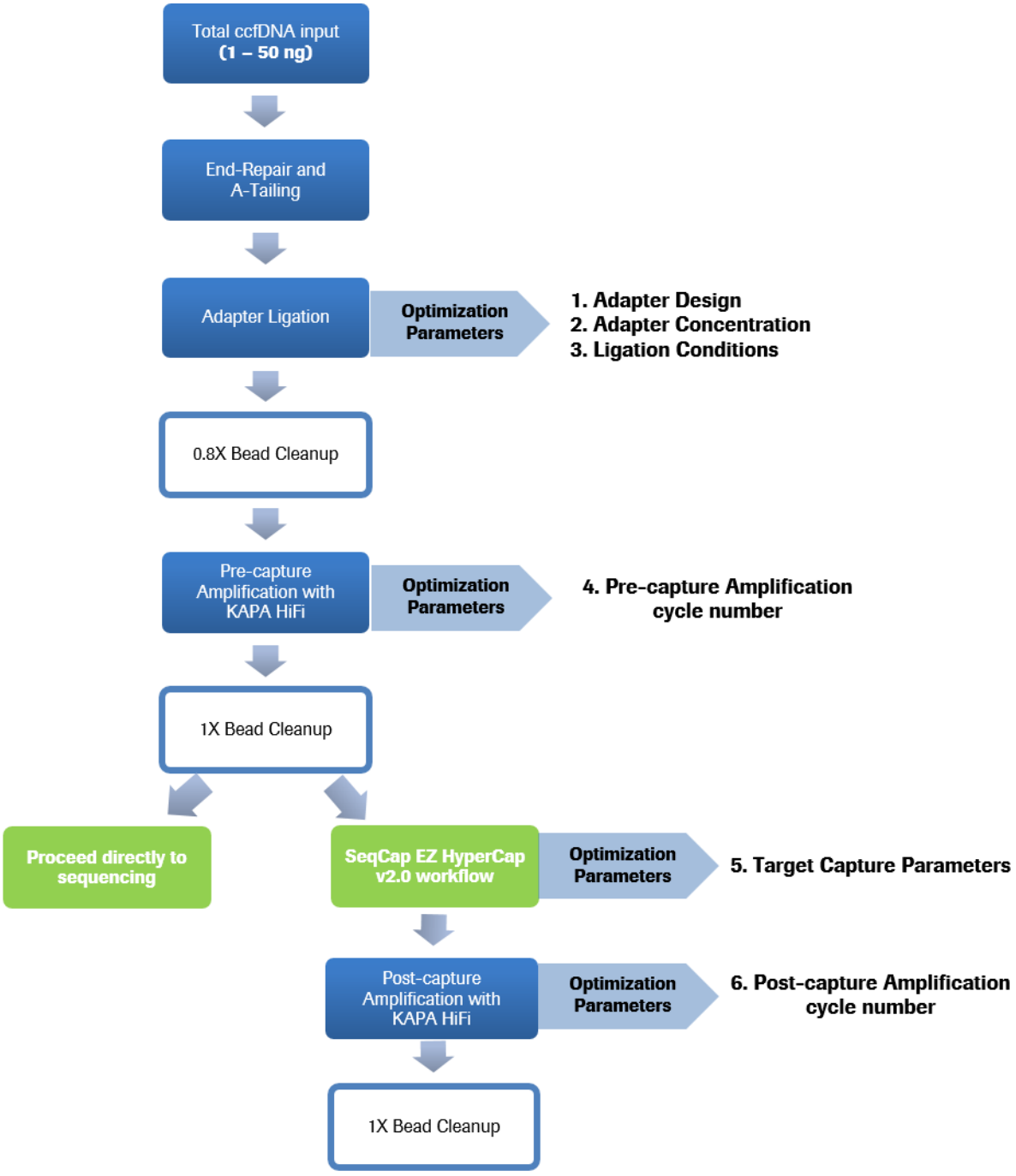

Figure 1: Overview of the KAPA HyperPrep library construction workflow for direct or targeted sequencing of ccfDNA. Total ccfDNA (1 – 50 ng) is required as input into this workflow. Optimization parameters are highlighted at each step in the workflow (See **Section 6: Optimization Parameters** for detailed information).

5. QUALITY CONTROL OF INPUT MATERIAL

ccfDNA has an average length of ~170 bp[4] and typically occurs in low abundance in peripheral blood resulting in limited amounts available for input into library construction. Following the isolation of ccfDNA, the quality and quantity of the extracted material should be assessed.

A fluorometric assay, such as the Qubit dsDNA HS Assay Kit (ThermoFisher Scientific), is recommended to quantify ccfDNA as it provides an accurate concentration of double-stranded DNA (dsDNA) molecules in the sample. In addition, an electrophoretic method should be employed where the profile of the ccfDNA should display a sharp peak around ~170 bp. This can also be used to assess the presence of high molecular weight species, which may indicate genomic DNA (gDNA) carryover.

A more sensitive approach to assessing gDNA contamination is to make use of the KAPA hgDNA Quantification and QC Kit[5]. This qPCR-based kit determines the amplification efficiency derived from three amplicons of different lengths (41 bp, 129 bp and 305 bp). For this application, amplification of the 305 bp amplicon is indicative of residual gDNA contamination since ccfDNA are shorter than 305 bp in length.

6. OPTIMIZATION PARAMETERS

Additional optimization may be necessary for ccfDNA inputs to ensure high conversion of input molecules into adapter-ligated library. Higher conversion rates typically translate to higher library complexity. While best laboratory practice and precision is key with challenging samples, such as ccfDNA, the following parameters may also be considered for optimization of conversion rates:

Optimization Parameter 1: Adapter Design

(See Section 7.3 Adapter Ligation p. 10)

KAPA Dual-Indexed Adapters are recommended for use with the KAPA HyperPrep Kit. However, this workflow is also compatible with dual-indexed adapters from alternate sources, either with or without unique molecular identifiers (UMIs), designed for use in Illumina® sequencing workflows.

UMIs are unique molecular tags that can be used to distinguish between low frequency mutations and PCR or sequencing errors based on the consensus of the reads sharing the same tagged sequence. This allows for a higher confidence in variant calls specifically where variant allele frequencies exist near the limit of detection. In addition to low frequency variant detection, UMIs provide more accurate deduplication to increase genomic complexity of sequencing data from samples with non-random fragmentation.

NOTE: If using adapters from a third-party supplier and performing targeted sequencing, the adapter sequences must be matched with appropriate blocking oligonucleotides (Section 7.7). For assistance with adapter compatibility, please contact your local Roche Technical Support representative.

Adapter Design	Blocking Oligos	Catalog Number
KAPA Dual-Indexed Adapters	Roche Universal Blocking Oligos (UBOs)	Provided with the HyperCap Target Enrichment Kit (08286370001; 08286345001). (UBOs also available separately)
Other adapter design	User-supplied blocking oligonucleotides	Third-party vendors

Optimization Parameter 2: Adapter Concentration

(See Section 7.3 Adapter Ligation p. 10)

Adapter concentration affects ligation efficiency, as well as adapter-dimer carry-over during the post-ligation cleanup. Adapter quality has an impact on the effective concentration of adapter available for ligation. Always source the highest quality adapters from a reliable supplier, dilute and store adapters in a buffered solution with the requisite ionic strength, and avoid excessive freezing and thawing of adapter stock solutions.

High adapter: insert molar ratios (>200:1) are beneficial for low-input applications, such as ccfDNA. When optimizing workflows for DNA inputs ≤25 ng, two or three adapter concentrations should be evaluated: try the recommended adapter concentration, as well as one or two additional concentrations in a range that is 2 – 10 times higher than the recommended concentration. The optimal adapter: insert molar ratio will be a tradeoff between post-ligation yield and adapter-dimer carry-over.

For whole genome applications, a second post-ligation or post-amplification cleanup (using a 1X bead-to-DNA ratio) may be performed if analysis reveals unacceptable levels of adapter and/or adapter-dimer carry-over after the first cleanup. The sample volume should be adjusted (with elution buffer) to at least 50 µL for a second cleanup.

For targeted capture workflows, adapter dimer may not be as much of a concern as the hybrid capture and subsequent wash steps may remove excess adapter dimer. Again, a second post-capture amplification cleanup may be performed. It is important to note that cleanup steps inherently result in loss of yield, therefore a second post-ligation cleanup should be assessed empirically.

Optimization Parameter 3: Ligation Conditions

(See Section 7.3 Adapter Ligation p. 10)

The recommended ligation reaction parameters (20°C for 15 min) are a good starting point for high-quality DNA, however may require optimization of both time and temperature for lower inputs, such as ccfDNA. It is important to optimize ligation parameters to ensure the highest recovery of ccfDNA molecules. When evaluating ccfDNA as input into the KAPA HyperPrep Kit for the first time, it is recommended that the optimal ligation parameters are determined empirically. Set up optimization reactions with the desired input of a non-precious, bulk sample that is representative of the actual samples to be processed.

Increased ligation time, typically overnight, may lead to increased conversion of input molecules into adapter-ligated library, with the caveat that adapter dimer carryover may also be increased. The ligation temperature can be decreased to 16°C or 4°C to mitigate the formation of adapter dimers when increased ligation times are assessed.

Optimization Parameter 4: Pre-capture Amplification cycle number

(See Section 7.5 Pre-Capture Library Amplification p. 12)

Excessive library amplification can result in other unwanted artefacts such as amplification bias, PCR duplicates, chimeric library inserts and nucleotide substitutions. The extent of library amplification should therefore be limited as much as possible, while ensuring that sufficient material is generated for QC and downstream target capture.

Optimization Parameter 5: Target Enrichment Parameters

(See Section 7.7 Target Enrichment Workflow p. 13)

For target enrichment workflows, we recommend the protocol outlined in the SeqCap EZ HyperCap Workflow User's Guide (v2.3 or later)[3]. If using target enrichment chemistry from an alternate source, then follow the manufacturer's guidelines.

For challenging samples, including ccfDNA, certain key parameters may be considered for optimization. The amount of library required for hybridization may be decreased to limit the amount of pre-capture amplification cycles performed. Pre-capture multiplexing also reduces the amount of individual library required for capture where a subset of libraries are pooled and processed in a single target capture. Although pre-capture multiplexing is possible, this approach is not recommended for ccfDNA applications as this may lead to reduced genome recovery rates.

The universal blocking oligos (UBOs) must be appropriately matched to the adapter design used. The optimal concentration of UBOs relative to the amount of library molecules in the hybridization reaction should be determined empirically to ensure minimal off-target depletion.

Optimization Parameter 6: Post-capture Amplification cycle number

(See Section 7.7 Target Enrichment Workflow p. 13)

The extent of post-capture library amplification should also be kept to a minimum. The number of post-capture amplification cycles required is dependent on the sequencing multiplexing strategy, target capture panel size and the sequencing instrument and chemistry used. This should be optimized to ensure that enough material is available for QC and sequencing, whilst using the lowest number of amplification cycles.

7. LIBRARY CONSTRUCTION PROTOCOL

7.1 Input Material

7.1.1 This protocol is suitable for library construction from 1 – 50 ng of total ccfDNA resuspended in 50 µL of 10 mM Tris-HCl, pH 8.0 – 8.5. However, the maximum available input of ccfDNA should be used to ensure the requisite coverage and complexity of the final library. For inputs > 50 ng, please refer to the KAPA HyperPrep Kit Technical Data Sheet (v6.17 or later)[1].

7.2 End Repair and A-tailing

7.2.1 Assemble each end repair and A-tailing reaction in a tube or well of a PCR plate as follows:

Component	Volume
Double-stranded ccfDNA input	50 µL
End Repair & A-tailing Buffer*	7 µL
End Repair & A-tailing Enzyme Mix*	3 µL
Total Volume	**60 µL**

* The buffer and enzyme mix should preferably be pre-mixed and added in a single pipetting step. Premixes are stable for ≤24 hrs at room temperature, for ≤3 days at 4°C, and for ≤4 weeks at -20°C.

7.2.2 Vortex gently and spin down briefly. Return the plate/tube(s) to ice.

7.2.3 Proceed immediately to the next step.

7.2.4 Incubate in a thermocycler programmed as outlined below:

Step	Temp	Time
End Repair and A-tailing	20°C	30 min
	65°C	30 min
HOLD	4°C	∞

* A heated lid is required for this incubation. If possible, set the temperature of the lid at 85°C, instead of the usual ~105°C. If proceeding to the adapter ligation reaction setup without any delay, the reaction may be cooled to 20°C instead of 4°C.

7.2.5 Proceed immediately to ***Adapter Ligation***.

7.3 Adapter Ligation

Optimization Parameters that may be considered in this step:

- Parameter 1: Adapter Design
- Parameter 2: Adapter Concentration
- Parameter 3: Ligation Conditions (time/temperature)

7.3.1. Dilute adapter stocks to appropriate concentration to obtain an adapter: insert molar ratio of 200:1, based on table below:

ccfDNA input	Adapter Stock Concentration*	Adapter: insert molar ratio
50 ng	15 µM	200:1
25 ng	7.5 µM	
10 ng	3 µM	
5 ng	1.5 µM	
2.5 ng	750 nM	
1 ng	300 nM	

* The adapter stock concentration may be increased or decreased to obtain optimal adapter: insert molar ratio.

7.3.2. In the same plate/tube(s) in which end repair and A-tailing was performed, assemble each adapter ligation reaction as follows:

Component	Volume
End repair and A-tailing reaction product	60 µL
Adapter stock	5 µL
PCR-Grade water*	5 µL
Ligation Buffer*	30 µL
DNA Ligase*	10 µL
Total Volume	**110 µL**

* The water, buffer and ligase enzyme should preferably be premixed and added in a single pipetting step. Premixes are stable for ≤24 hrs at room temperature, for ≤3 days at 4°C, and for ≤4 weeks at -20°C.

7.3.3. Mix thoroughly and centrifuge briefly.

7.3.4. Incubate the ligation reaction, based on the table below:

Temperature	Time
20°C	15 min – 4 hrs
4 – 16°C	overnight

7.3.5. Proceed immediately to ***Post-Ligation Cleanup***.

7.4 Post-Ligation Cleanup

7.4.1. In the same plate/tube(s), perform a 0.8X bead-based cleanup by combining the following:

Component	Volume
Adapter ligation reaction product	110 µL
KAPA Pure Beads	88 µL
Total Volume	**198 µL**

7.4.2. Mix thoroughly by vortexing and/or pipetting up and down multiple times.

7.4.3. Incubate the plate/tube(s) at room temperature for 5 – 15 min to bind DNA to the beads.

7.4.4. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

7.4.5. Carefully remove and discard the supernatant.

7.4.6. Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

7.4.7. Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

7.4.8. Carefully remove and discard the ethanol.

7.4.9. Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

7.4.10. Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

7.4.11. Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

7.4.12. Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated.

Caution: over-drying the beads may result in reduced yield.

7.4.13. Remove the plate/tube(s) from the magnet.

7.4.14. Thoroughly resuspend the beads in 22 µL of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5).

7.4.15. Incubate the plate/tube(s) at room temperature for 2 min to elute DNA off the beads.

7.4.16. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

7.4.17. Transfer 20 µL of the clear supernatant to a new plate/tube(s) and proceed to ***Pre-Capture Library Amplification*.**

Safe stopping point: The supernatant can be stored at 2°C to 8°C for ≤ 24 hrs, or at -15°C to -25°C.

7.5 Pre-Capture Library Amplification

Optimization Parameters that may be considered in this step:

- Parameter 4: Pre-capture amplification cycle number

7.5.1. Assemble the library amplification reaction as follows and add 30 µL of the library amplification master mix* to 20 µL of purified, adapter-ligated DNA:

Component	Volume
Purified, adapter-ligated DNA	20 µL
KAPA HiFi HotStart ReadyMix (2X)*	25 µL
Library Amplification Primer Mix (10X)*	5 µL
Total volume	**50 µL**

*The ReadyMix and Primer Mix should be premixed and added in a single pipetting step.

7.5.2. Mix well by pipetting up and down several times.

7.5.3. Amplify the library using the following thermocycling profile:

Step	Temperature	Duration	Cycles
Initial denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	Refer to table below
Annealing	60°C	30 sec	
Extension	72°C	30 sec	
Final extension	72°C	1 min	1
HOLD	4°C	∞	1

ccfDNA Input (into ER and AT)	Number of cycles required to generate*	
	100 ng library	1 µg library
25 ng	5 – 7	8 – 10
10 ng	7 – 9	11 – 13
5 ng	9 – 11	13 – 14
2.5 ng	11 – 13	14 – 16
1 ng	13 – 15	17 – 19

* The number of pre-capture amplification cycles may require optimization.

7.5.4. Proceed immediately to ***Library Amplification Cleanup***.

7.6 Library Amplification Cleanup

7.6.1. In the same plate/tubes(s), perform a 1X bead-based cleanup by combining the following:

Component	Volume
Amplified library DNA	50 µL
KAPA Pure Beads	50 µL
Total volume	**100 µL**

7.6.2. Thoroughly resuspend the beads pipetting up and down multiple times.

7.6.3. Incubate the plate/tube(s) at room temperature for 5 – 15 min to bind D to the beads.

7.6.4. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

7.6.5. Carefully remove and discard 95 µL of supernatant.

7.6.6. Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

7.6.7. Incubate the plate/tube(s) on the magnet at room temperature for ≥ 30 sec.

7.6.8. Carefully remove and discard the ethanol.

7.6.9. Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

7.6.10. Incubate the plate/tube(s) on the magnet at room temperature for ≥ 30 sec.

7.6.11. Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

7.6.12. Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated.

Caution: over-drying the beads may result in reduced yield.

7.6.13. Remove the plate/tube(s) from the magnet.

7.6.14. Thoroughly resuspend the beads in 22 µL of 10 mM Tris-HCl (pH 8.0 – 8.5).

7.6.15. Incubate the plate/tube(s) at room temperature for 2 min to elute DNA off the beads.

7.6.16. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

7.6.17. Transfer 20 µL of the clear supernatant to a new plate/tube(s).

Safe stopping point: The supernatant can be stored at 2°C to 8°C for ≤ 24 hrs, or at -15°C to -25°C. Proceed to Target Enrichment workflow (Section 7.7) or alternatively, proceed directly to sequencing for WGS applications.

7.7 Target Enrichment Workflow (optional)

Optimization Parameters that may be considered in this step:

- Parameter 5: Target Capture Parameters
- Parameter 6: Post-capture amplification cycle number

Libraries produced with this protocol should be compatible with any target capture chemistry, however we recommend using the streamlined SeqCap EZ HyperCap v2.02 workflow. Refer to SeqCap EZ HyperCap Workflow User's Guide (v2.3 or later)[3] and proceed from **Chapter 5: Hybridize the Sample and SeqCap EZ Probe Pool** to continue with enrichment of targeted regions using SeqCap EZ Prime Choice Probes.

8. EVALUATING THE SUCCESS OF LIBRARY CONSTRUCTION

Library Size Distribution

The size distribution of final, adapter-ligated libraries should be confirmed with an electrophoretic method. A LabChip GX, GXII or GX Touch (PerkinElmer) instrument, Bioanalyzer or TapeStation instrument (Agilent Technologies), Fragment Analyzer (Advanced Analytical) instrument or similar instrument is recommended over conventional gels.

Library Quantification

KAPA Library Quantification Kits are recommended for the absolute, qPCR-based quantification of final libraries, flanked by the P5 and P7 Illumina flow cell oligo sequences, prior to sequencing. Standard methods used for NGS library quantification such as fluorometry (as employed in Qubit/PicoGreen assays), spectrophotometry (on which the NanoDrop instrument is based) and electrophoretic methods (e.g. those performed using an Agilent Bioanalyzer or TapeStation instrument) measure total nucleic acid concentrations. In contrast, qPCR is inherently well-suited for NGS library quantification, as it measures only those library fragments that can serve as templates during cluster generation. Moreover, because qPCR is extremely sensitive, it allows for the quantification of dilute libraries and consumes only small amounts of library. For a detailed protocol, please refer to the KAPA Library Quantification Kit for Illumina platforms Technical Data Sheet (KR0405 v9.17 or later)[6].

9. ALTERNATIVES

- AMPure XP reagent (Beckman Coulter) may be used instead of KAPA Pure Beads for all bead cleanups

10. REFERENCES

1. KAPA HyperPrep Kit Technical Data Sheet, v6.17 or later. Roche document number: KR0961.
2. Devonshire, A, Whale, AS, Gutteridge, A et al. Towards standardisation of cell-free DNA measurement in plasma: controls for extraction efficiency, fragment size bias and quantification. Anal Bioanal Chem. 2014; doi: 10.1007/s00216-014-7835-3.
3. SeqCap EZ HyperCap Workflow User's Guide (v2.3 or later). Roche document number: 07939493001
4. Newman, A, Bratman, S, To, J et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med. 2014; doi: 10.1038/nm.3519.
5. KAPA Human Genomic DNA Quantification and QC Kit Technical Data Sheet, v3.17 or later. Roche document number: KR0454.
6. KAPA Library Quantification Kit for Illumina platforms Technical Data Sheet, v9.17 or later. Roche document number: KR0405.

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