Sequencing Library Preparation from Cell-Free DNA for Illumina® Sequencing Platforms

This protocol describes an optimized procedure for preparing sequencing libraries from minimal amounts of circulating cell-free DNA (cfDNA) using the GeneRead™ DNA Library I Core Kit (QIAGEN, cat. no. 180432), GeneRead Adapters (QIAGEN, cat. no. 180985 or 180986) and the GeneRead DNA I Amp Kit (QIAGEN, cat. no. 180455).

IMPORTANT: Please read the handbooks supplied with each required kit for general information on the handling and storage of kit components. Pay careful attention to the "Safety Information" and "Important Notes" sections before beginning this procedure.

Equipment and reagents

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- GeneRead DNA Library I Core Kit (QIAGEN, cat. no. 180432)
- GeneRead Adapters (QIAGEN, cat. no. 180985 or 180986)
- GeneRead DNA I Amp Kit (QIAGEN, cat. no. 180455)
- Buffer EB (QIAGEN, cat. no. 19086)
- Agencourt® AMPure® XP Beads (Beckman Coulter, cat. no. A63880)
- 80% ethanol
- PCR tubes or plates
- Pipet tips and pipets
- DNA LoBind tubes (from Axygene or Eppendorf)
- Vortexer
- Microcentrifuge
- Thermocycler
- Magnetic racks for separation of magnetic beads (e.g., Thermo Fisher Scientific/Life Technologies, DynaMag™-2 Magnet, cat.no. 12321D)

For NGS library QC:

- Agilent® DNA Chip 7500 (Agilent, cat. no. 5067-150)
- GeneRead Library Quant Kit (QIAGEN, cat. no. 180612)

Important points before starting

- This protocol is for constructing sequencing libraries for Illumina NGS platforms. The following QIAGEN products are required for this protocol: GeneRead DNA Library I Core Kit (QIAGEN, cat. no. 180432 or 180434), GeneRead Adapters (QIAGEN, cat. nos. 180985 or 180986) and GeneRead DNA I Amp Kit (QIAGEN, cat. no. 180455). Compared to the standard GeneRead Library Prep protocol, the following modifications are introduced in this library construction protocol to achieve optimal results with cfDNA: adapter concentration used in the ligation step is reduced and Agencourt AMPure XP is used for reaction cleanup and removal of adapter-dimers.
- Agencourt AMPure XP Beads (Beckman Coulter, cat. no. A63880) are required and should be ordered separately. Please follow the manufacturer's instructions for storage and handling of the AMPure XP Reagent.
- This protocol is verified with circulating DNA extracted using the QIAamp® Circulating Nucleic Acid Kit (QIAGEN, cat. no. 55114) and works with a broad range of input DNA amounts (1–100 ng). Typically, 10 µl circulating DNA extracted with the QIAamp Circulating Nucleic Acid Kit can be used directly with the following protocol without adjusting the input amount.
- The majority of circulating DNA from serum or plasma is present as short fragments smaller than 1000 bp and no further DNA fragmentation is needed prior to library construction.
- GeneRead Adapters are fully compatible with Illumina instruments, such as MiSeq®, NextSeq® or HiSeq® instruments. The enrichment step is not required to complete the adapter sequences.
- No heated lid required during the ligation step.

Procedure

cfDNA and reagent preparation

1. Prepare cfDNA for library construction.

   Note: We recommend the QIAamp Circulating Nucleic Acid Kit for circulating DNA extraction. The typical yield of circulating DNA from 1 ml plasma is 1–100 ng. This protocol works with a broad range of input DNA amounts (1–100 ng). Typically, 10 µl circulating DNA extracted with QIAamp Circulating Nucleic Acid Kit can be used directly with the following protocol without adjusting the input amount.
2. Thaw all reagents thoroughly on ice and vortex briefly before use to ensure thorough mixing.

End-repair

3. Prepare a reaction mix for end-repair according to Table 1, dispensing the reagents into a PCR tube or the well of a PCR plate.

   Note: The reaction mix should be prepared on ice.

Table 1. Reaction mix for end-repair

Component	Volume/reaction (µl)
cfDNA*	10
RNase-free water	10.5
End-Repair Buffer, 10x*	2.5
End-Repair Enzyme Mix	2
Total reaction volume	**25**

* If using cfDNA prepared with the QIAamp Circulating Nucleic Acid Kit (QIAGEN, cat. no. 55114). Otherwise, use 1–100 ng cfDNA as input.

4. Mix thoroughly.
5. Program a thermocycler to incubate for 30 min at 25°C, followed by 20 min at 75°C to inactivate the enzyme.

A-addition

6. Prepare a reaction mix for A-addition according to Table 2, adding the components to the PCR tube containing the end-repaired DNA from step 5.

Table 2. Reaction mix for A-addition

Component	Volume/reaction (µl)
End-repaired DNA (from step 5)	25
A-Addition Buffer, 10x	3
Klenow Fragment (3'→5' exo–)	3
Total reaction volume	**31**

7. Mix thoroughly.
8. Program a thermocycler to incubate for 30 min at 37°C, followed by 10 min at 75°C to inactivate the enzyme.

Adapter ligation

9. Make a 1:10 dilution of the GeneRead Adapter with RNase-free water. Use 2.5 µl of the diluted adapter for library prep.
10. Prepare a reaction mix for adapter ligation according to Table 3, adding the components to the PCR tube containing DNA that has undergone end-repair and A-addition (step 8).

    Note: When using barcode adapters, open one adapter tube at a time and change gloves between pipetting the different barcode adapters to avoid cross-contamination.

    IMPORTANT: Only a single adapter should be used per ligation reaction.

Table 3. Reaction mix for adapter ligation

Component	Volume/reaction (µl)
DNA from step 8 (has undergone end-repair and A-addition)	31
Ligation Buffer, 2x	45
1:10 Diluted GeneRead Adapter (from step 9)*	2.5*
T4 DNA Ligase	4
RNase-free water	7.5
Total reaction volume	**90**

* If adapters from other suppliers are used, follow the supplier's instructions and add the correct amount of adapter to a final concentration of 0.1 µM.

11. Mix thoroughly.
12. Program a thermocycler to incubate for 10 min at 25°C.

    IMPORTANT: Do not use a thermocycler with a heated lid.

Reaction cleanup and removal of adapter dimers

13. Add 72 µl Agencourt AMPure XP Beads to each ligation reaction (90 µl) and pipet up and down thoroughly to mix the beads and ligation reaction mix.
14. Incubate the mixture at room temperature for 5–15 min to bind DNA to the beads.
15. Place the tube on a magnetic rack and wait until the liquid is clear to capture the beads. Carefully remove and discard the supernatant.
16. Keep the tube on the magnetic rack and add 200 µl of 80% ethanol to wash the beads.
17. Incubate the tube at room temperature and wait for 30–60 s.
18. Carefully remove and discard the ethanol.
19. Repeat steps 16–18 again.

    Try to remove the residual ethanol as much as possible without disturbing the beads.

20. Dry the beads at room temperature. To avoid over-drying the beads, drying time should be no longer than 15 min.
21. Remove the tube from the magnetic rack.
22. Resuspend the beads in 25 µl Buffer EB.
23. Incubate the tube at room temperature for 2 min to elute DNA from the beads.
24. Place the tube back on the magnetic rack to capture the beads. Incubate until the liquid is clear.
25. Use 20 µl of the eluate in the library amplification procedure.

Library amplification

26. Prepare a reaction mix according to Table 4.

Table 4. Reaction mix for library enrichment

Component	Volume/reaction (µl)
HiFi PCR Master Mix, 2x	25
Primer Mix (10 µM each)	1.5
Library DNA (from step 25)	20
RNase-free water	3.5
Total reaction volume	**50**

27. Program a thermocycler according to Table 5.

Table 5. Cycling conditions

Time	Temperature	Number of cycles
2 min	98°C	1
20 s	98°C	
30 s	60°C	12
30 s	72°C	
1 min	72°C	1
∞	4°C	Hold

PCR cleanup

28. Add 50 µl Agencourt AMPure XP Beads to each reaction (50 µl) and pipet up and down thoroughly to mix the beads and PCR mix.
29. Repeat steps 14–24 for reaction cleanup.

30. Elute amplified, purified library in 25 µl Buffer EB.

31. Assess the quality of the library using a capillary electrophoresis device such as the Agilent BioAnalyzer. Check for the correct size distribution (see Figure 1) of library fragments and for the absence of adapters or adapter-dimers.

    Note: The library should show a distinct ladder pattern with the most dominant peak at about 300 bp (see Figure 1). This reflects the size of the circulating DNA plus sequencing adapter length, which is around 120 bp.

32. Quantify the library using the GeneRead Library Quant Kit (QIAGEN, cat. no. 180612) or a comparable method.

    Note: The library DNA can be stored at –20°C until ready to use for sequencing. DNA LoBind tubes should be used to store the library.

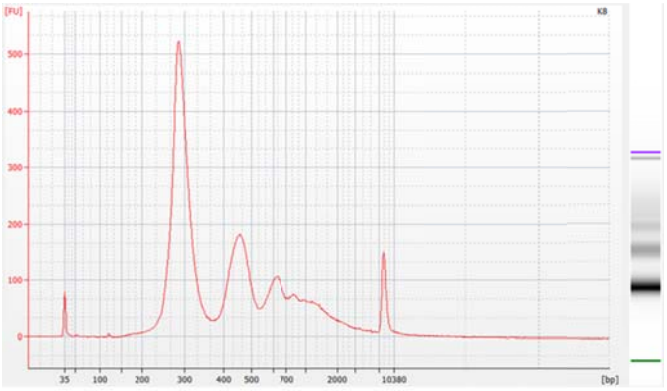

Figure1. Agilent BioAnalyzer trace data showing the correct size distribution of the library.

QIAGEN handbooks can be requested from QIAGEN Technical Service or your local QIAGEN distributor. Selected handbooks can be downloaded from **www.qiagen.com/literature**.

Safety data sheets (SDS) for any QIAGEN product can be downloaded from **www.qiagen.com/safety**.

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