Extraction of Cell-Free DNA and Subsequent Preparation of NGS Libraries in a High-Throughput, Cost-Effective Manner

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1 Abstract

An efficient workflow from sample to sequencer is critical to maintaining integrity of circulating, cell-free DNA (cfDNA) from liquid biopsy samples. Each step in the process: blood collection, DNA extraction, and library creation, can affect the quality of sequencing results. Here we discuss cfDNA isolation on the PerkinElmer chemagic™ 360 system, followed by use of the PerkinElmer Sciclone® G3 NGSx to generate next generation sequencing (NGS) libraries from cfDNA using the Accel-NGS® 2S PCR-Free Library kit from Swift Biosciences. cfDNA was extracted from plasma collected from whole blood samples utilizing the chemagic 360 instrument and chemistry, enabling automated isolation of high quality samples.

Multiple methods were applied to check for contamination of high molecular weight DNA and integrity of the cfDNA. Sequencing on the HiSeq 2500 instrument, performed by PerkinElmer NGS Services, demonstrated reproducibility and quality. Highly efficient library preparation driven by end repair of both the 3’ and 5’ cfDNA termini, delivers a more complex library requiring less sequencing, enabling comprehensive analysis of DNA samples such as cfDNA. Swift’s automated protocol has an easy-to-use interface, requiring no adapter titration, while producing libraries of quality comparable to that of manually prepared libraries.

The library preparation minimizes consumable use and sample loss typically observed on automated platforms, and can produce up to 96 PCR-Free libraries in 3 hours. As there is no need to pause the program to re-load a robotic deck, the automated Accel-NGS 2S library prep allows a complete walk-away solution. The combined technologies provide the opportunity for a completely automated method for extraction and NGS library preparation of cfDNA.

Automated Processing

Simple Liquid Biopsy Processing for NGS

- Automated extraction of cfDNA on chemagic 360 system
- NGS prep from 10 ng cfDNA with Accel-NGS 2S PCR-Free Library Kit
- Automated DNA library preparation on Sciclone Liquid Handler

Circulating cell-free DNA was obtained using the chemagic 360 extraction system equipped with the 24 rod head. The chemagic circulating NA 4k protocol was run as described per the manufacturer’s instructions. Samples were eluted in 10 mM Tris-HCl, pH 8.0.

Accel-NGS®

Enables preparation of high complexity NGS libraries from dsDNA, and PCR-free libraries from as low as 100 ng of high quality genomic DNA or 10ng of cfDNA.

NGS libraries were generated using an automated library preparation method for the Accel-NGS 2S PCR-Free Library Kit on the Sciclone G3 NGSx instrument.

2 Liquid Biopsy Workflow

SAMPLE COLLECTION
- Blood was collected in Streck Cell-Free DNA BCT® tubes.

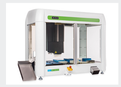
PLASMA SEPARATION
- Plasma was separated from the other blood components.
- Then the plasma was centrifuged to remove cell debris.

AUTOMATED LIBRARY PREPARATION
- Automated cfDNA extraction from 4 mL of plasma was performed on the chemagic 360 instrument.
- cfDNA was eluted in 10 mM Tris-HCl, pH 8.0.

AUTOMATED cfDNA EXTRACTION
- cfDNA was quantified using qPCR with ALU repeat primers.
- NGS libraries were constructed using the Accel-NGS 2S PCR-Free Library Kit on the Sciclone G3 NGSx instrument.
- Library concentration and size distribution were assessed with qPCR and LabChip® GX Touch instrument.

NEXT-GENERATION SEQUENCING
- The Illumina® HiSeq 2500 system was used to analyze the cfDNA libraries.

chemagic 360



Sciclone G3 NGSx

Accel-NGS®

Accel-NGS 2S PCR-Free DNA Library Kit

3 Concentration and Integrity of Extracted cfDNA

To assess cfDNA quantity and integrity, a qPCR assay was performed using two ALU repeat assays: 115 bp and 247 bp. cfDNA has an average length of ~170 bp. The 115 bp assay determines concentration while the 247/115 ratio of products is used to rate the quality of the cfDNA and detect contamination of high molecular weight cellular DNA. A ratio of 0.3-0.5 is considered ideal for cfDNA, where cellular DNA has a ratio of 1.

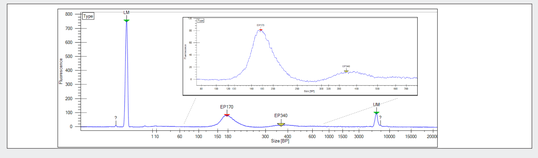
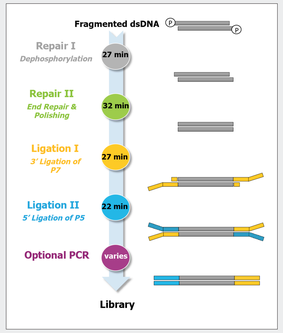


Fig. 1: Representative trace of input cfDNA from the chemagic 360 instrument. This shows the key cfDNA sizes as analysed by the ALU qPCR.

Sample #	Final conc (ng/ul)	Total Yield (ng)	Quality Score (Alu 247/115)
1	0.72	21.5	0.38
2	0.67	20.1	0.23
3	0.25	7.6	0.45
4	0.21	6.4	0.28
5	0.23	7.0	0.22
6	0.38	11.5	0.30
7	0.23	10.3	0.45
8	0.17	7.8	0.36
9	0.16	7.4	0.45
10	0.32	14.4	0.32

Tab. 1: Ten samples of 10 mL blood were collected in Streck BCT® tubes and plasma was separated within 24 hours. cfDNA was isolated on chemagic 360 system with final concentration and quality score determined by qPCR. All 10 samples had a quality score in the ideal range, indicating no cellular DNA contamination.

4 PCR-free Workflow



- Simple with-bead protocol
- Broad input range: 10 pg - 1µg
- Sequential repair steps enable use of damaged DNA
- Compatible with cfDNA and FFPE samples
- Increased library complexity
- Balanced coverage of AT-/GC-rich regions

Fig. 2: Accel-NGS 2S PCR-Free workflow. Accel-NGS 2S PCR-Free has 4 steps: Repair I dephosphorylates the 5’ ends of the DNA, Repair II repairs and polishes DNA ends, Ligation I adds the P7 adapter to the 3’ terminus, Ligation II adds the P5 adapter to the 5’ terminus. Optional PCR amplifies the library when required.

5 Library Quality Control

Library concentration was measured at Swift Biosciences using an inhouse qPCR assay and commercial standards. Size distribution of library molecules was determined by PerkinElmer on a LabChip GX Touch instrument. A predominant peak was present at 300 bp representing the 170 bp adapted cfDNA fragments with a minor secondary peak at 470 showing a cfDNA digestion intermediate.

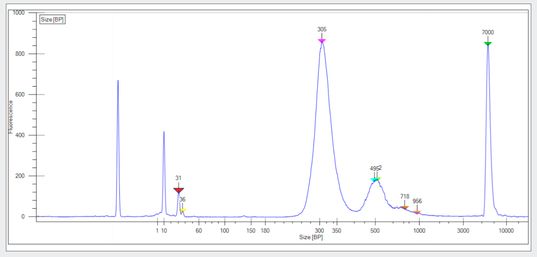


Fig. 3: Representative trace of completed NGS libraries. Analyzed with DNA NGS 3k assay on the LabChip GX Touch platform to confirm distribution of insert sizes within library molecules.

Sample #	Input (ng)	Library Concentration (nM)
1	10	1.58
2	10	3.07
7	15	1.80
10	15	3.83

Tab. 2: Final library concentration of select samples. Four of the highest quality samples were selected for NGS library preparation with the Accel-NGS 2S PCR-Free DNA Library Kit using either 10 or 15 ng of DNA. Final libraries quantified with an in-house qPCR assay.

6 NGS Analysis of cfDNA

Two samples were selected from the set of 10 for NGS analysis on the Illumina HiSeq 2500 using a rapid run and v3 chemistry to confirm quality of the extracted cfDNA. Uniform genome coverage was obtained from a mean 15X depth.

Sequencing Metrics	Sample 7	Sample 10
Total Reads	238,230,712	254,136,768
% Aligned	99.4%	99.4%
Average Genome Coverage	14.6 x	15.6 x
% Genome Missing	1.9%	2.0%
% Genome Covered ≥ 5X	99.6%	99.7%
% Genome Covered ≥ 10X	94.9%	95.8%
% Genome Covered ≥ 14X	92.6%	93.2%
% Duplication	0.04%	0.08%
Median Insert Size	172 bp	168 bp

Tab. 3: Sequencing metrics from Illumina HiSeq 2500. cfDNA samples number 7 and 10 were selected for sequencing on HiSeq 2500 Rapid Run with v3 chemistry. Analysis was performed using a custom pipeline using BWA as aligner and enrichment metrics were collected using Picard tools.

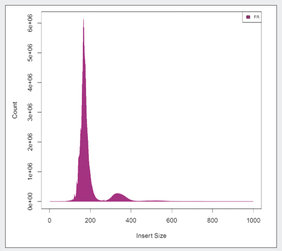


Fig. 4: Aligned insert size of NGS libraries. The curve reflects the library size profile, where both peaks represent properly paired alignment. Chimeric reads were detected at 1.7 and 1.6% for samples 7 and 10.

6 Conclusions

Herein, we have demonstrated an efficient automated workflow for cfDNA samples from nucleic acid extraction to NGS library preparation without compromising sample quality or sequencing data integrity. The Accel-NGS 2S PCR-Free DNA Library Kit was automated on the Sciclone G3 NGSx instrument offering an easy-to-use graphical interface to set-up and run the protocol. The kit provides rapid generation of libraries (96 cfDNA libraries in < 3 hours) of higher complexity without the need for adapter titration. Numerous sample quality control checks enabled us to verify optimal sample output at each step.

- qPCR assay post-nucleic acid extraction on the chemagic 360 indicated high cfDNA sample integrity.
- Library concentration and correct size distribution were assessed using in-house qPCR assay and LabChip GX Touch instrument, respectively.
- Sequencing results demonstrated reproducibility and quality as indicated by ~15x genome coverage with greater than 99.39% alignment, and < 0.08% duplication rate.

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