Clean Circulating LV DNA Kit: automated cfDNA extraction for NIPT

Abstract

With emerging technologies like Non-Invasive Prenatal Testing (NIPT)1,3 and so called "liquid biopsies"2, there is an increasing need for circulating free DNA isolation methods. To meet this demand, CleanNA6 offers the Clean Circulating LV DNA Kit, designed for high throughput and reliable isolation of cell free DNA (cfDNA) from up to 4 mL of plasma or serum samples based on CleanNA's proprietary magnetic particles. With this application note it will be demonstrated how a scalable automated cfDNA extraction solution on a Hamilton Microlab® STAR™ system7 using the CleanNA Circulating kit was be developed. Up to 24 samples can be processed simultaneously providing high quality cfDNA that allows for direct input in the Illumina Truseq™ library preparation protocol5 and subsequent sequencing on the NextSeq 500 system. After Wisecondor data interpretation of chromosomal aberrations the CleanNA prepared samples are shown to provide comparable sequencing results to manually isolated samples. In addition, the automated CleanNA isolation method requires less sample input while reducing hands-on time, risks of sample swaps and enabling full sample tracking and tracing.

Introduction

With emerging technologies like NIPT and so called "liquid biopsies", there is an increasing need for circulating free DNA isolation methods. This fragmented type of DNA is found within the cell free fraction of whole blood and originates from apoptotic and necrotic cells. Although total concentrations of cfDNA differ per individual, it is estimated that for expecting women around the 10th week of pregnancy, 3-6% of cfDNA[3] comes from trophoblasts[1] shedding placental particles into the maternal bloodstream. Combining the extraction of total cfDNA from maternal blood with the power of Next Generation Sequencing technology, has enabled clinical laboratories to offer a non-invasive screening method for fetal aneuploidies. As this technique offers many advantages over traditional invasive methods like amniotic fluid punctures for the welfare of the patient, this has resulted in an increased request for NIPT in these laboratories.

To meet this demand, we offer the CleanNA Clean Circulating LV DNA Kit, designed for high throughput and reliable isolation of cfDNA from up to 4 mL of

plasma or serum samples. Based on CleanNA's proprietary magnetic particles the unique binding system targets DNA fragments smaller than 300bp, with minimal binding of larger fragments. Thanks to the use of magnetic particles the need for funnels or vacuum steps has been eliminated, enabling easy automation on most liquid handlers.

This application note shows the scalable automated cfDNA extraction solution on a Hamilton Microlab® STAR™ system using the Clean Circulating LV DNA kit. This has allowed the clinical genetics laboratory to move away from their current manual procedure reducing hands-on time, and lowering risk of sample swaps by implementing full sample track and tracing. We also show that after libraries were generated using the Truseq™ Nano DNA library preparation kit[5] are sequenced on the Illumina NextSeq 500. The results of the Clean Circulating LV DNA Kit are comparable to manual extracted samples in data analysis.

Materials & Methods

Equipment

- Hamilton Star system, configured with 8 1ml channels, core grippers and an autoload scanner. The installed vacuum station was used as an active waste system. Microlab Venus Two® operating software installed.
- Permagen™ 24-Well Magnetic Separation Plate (P/N MSP24)

Chemicals

- Clean Circulating LV DNA Kit, 96 preparations. Contains all necessary buffers, reagents and magnetic particles. (P/N CCLV-D0096)
- Nuclease free water

Labware

- Whatman™ 24 well deepwell plate, round bottom
- 15 mL Greiner tubes
- 5 mL Eppendorf tubes
- Nalgene 5 mL tubes

Protocol Overview

Sample Preparation

Blood (10 mL) was drawn from 24 patients and collected in Cell-Free Streck tubes which prevent release of genomic DNA from nucleated cells. When the tubes arrive in the lab, 20 µL SASI-seq[4] (Sample Assurance Spike-In sequences) barcodes with a concentration of 0.003ng/ul are added immediately. These unique barcodes undergo the same isolation and library preparation as the rest of the sample material to which it is added. After sequencing this allows for detection of cross contamination and sample swaps during the procedure. The tubes are centrifuged for 20 minutes at 2000g to separate de plasma fraction from the blood cells. On average 4 ml of plasma is transferred to a barcoded 5 ml Eppendorf tube, this tube is again centrifuged, this time 5 minutes at 13000g, to pellet any remaining cells or debris and the supernatant is transferred to a new 5 ml barcoded Eppendorf tube. Samples are stored at -80°C and thawed when the isolation protocol can be started.

Up to 24 samples can be processed simultaneously on the Hamilton Star system. When the protocol is initiated the system will instruct the user how to prepare the deck. The system then compares the sample worklist template with all the prepared sample source, lysis and elution tubes using the autoload barcode reader provided on the Hamilton Star system. Next, the CleanNA automated procedure starts as illustrated in figure 1, which can be divided in 2 parts.

Clean Circulating LV DNA Kit Isolation Process

First, 2 mL of plasma is transferred from the sample source tube to the 15 mL Greiner Lysis tube. Then 30 µL protK and 135 µL of CCLV Lysis buffer are added and mixed thoroughly. The system will unload the Greiner tubes and prompt the user to cap the tubes and incubate them at 60°C for 25 minutes in a water bath. This step ensures any remaining interfering proteins will be digested and cfDNA is not attached to any proteins or cell particles for the second part of the protocol. The user will reload the uncapped Greiner tubes into the Hamilton Carrier after incubation. The autoload again compares all the barcodes of the Greiner tubes so no swaps will occur in the meantime. Next the system will transfer 2 mL of CCLV Binding buffer to the samples, together with 20 µL of CleanNA particles CCLV. After mixing these components vigorously by pipetting, the samples are incubated at room temperature for 10 minutes to allow the cfDNA to bind to the magnetic CleanNA particles.

After the incubation, samples are automatically transferred to the Whatman™ 24 deepwell plate, which together with the Permagen™ magnet allows for separating the magnetic particles from the solution and discarding the supernatant. The cfDNA which is bound to the magnetic particles can then

be washed with the provided wash buffers as illustrated in figure 2.

Instead of a lengthy drying step after the washes to evaporate traces of ethanol, which is common in most magnetic bead-based protocols, the specially coated CleanNA particles allow for a rapid water wash step to facilitate this, without eluting the cfDNA of the particles. This is done by adding 500 µL of water to the samples while the plate is on the magnet, and then removing the water within 20 seconds. After this final wash the samples are eluted in 100 µL in provided elution buffer and transferred to the barcoded elution tubes.

Yield of the isolated cfDNA samples is then determined using the Qubit dsDNA High Sensitivity assay kit. From each sample, 80 µL is inserted into the Illumina Truseq™ Nano DNA library preparation kit where samples are genetically barcoded. After library preparation samples are again quantified using the Qubit High Sensitivity assay. Equimolar amounts of libraries are then pooled together and deposited on flow cells for the Illumina NextSeq 500 system. The NextSeq 500 facilitates cluster generation and sequencing of the generated libraries. Data analysis is performed using a custom pipeline and Wisecondor V1.0.0 (WIthin SamplE COpy Number aberration DetectOR[3]) to check for aneuploidies.

Figure 1: Schematic overview of the CleanNA Clean Circulating LV DNA Kit Isolation Process.

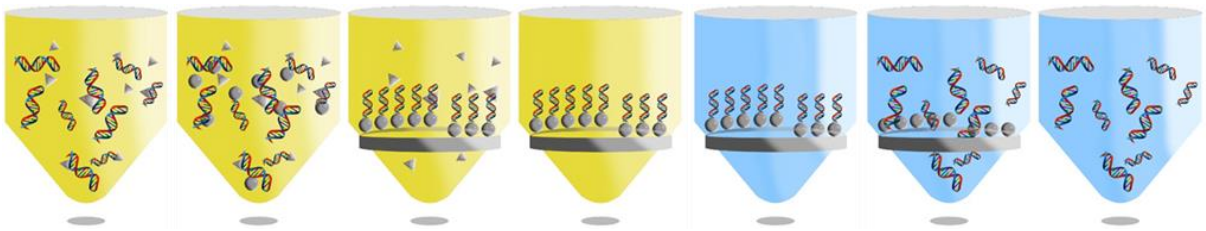

Figure 2: Visual representation of the magnetic particles based protocol. After lysis, magnetic particles and binding buffers are added to the sample and the cell free DNA is bound to the magnetic particles surfaces. Using a magnetic separation plate, the magnetic particles are separated from solution and the supernatant is discarded. The magnetic particles carrying the DNA are washed by resuspension off the magnet. After the washing steps have been completed, the DNA is eluted of the magnetic particles using the supplied elution buffer and transferred to a new sample container.

Results

Yield of Isolation

Yield of a random collection of samples was compared to customers manual method Q. To compensate for different starting volumes of plasma and different elution volumes the total yield per mL of starting material was calculated and compared in table 1. Average yield per mL plasma input for customer manual method Q was 6 ng/mL versus 7.8 ng/mL with our CleanNA automated method.

Yield of Truseq™ Nano DNA Library preparation

A collection of 22 CleanNA isolated samples were compared to the same samples isolated with manual method Q. Figure 3 shows the yield corrected for the amount of plasma starting material.

Table 1. Comparison of yield from isolation. Manual method refers to competitor Q.

Sample	Starting amount of plasma (mL)	Concentration (ng/µl)	Elution volume (µl)	Total yield (ng)	Total yield per mL starting material (ng/ul)
ManualQ1	4	0,40	50	20	5,00
ManualQ2	4	0,54	50	27	6,75
ManualQ3	4	0,48	50	24	6,00
ManualQ4	4	0,34	50	17	4,25
ManualQ5	4	0,64	50	32	8,00
CleanNA1	2	0,13	100	13	6,50
CleanNA2	2	0,17	100	17	8,50
CleanNA3	2	0,19	100	19	9,50
CleanNA4	2	0,08	100	8	4,00
CleanNA5	2	0,21	100	21	10,50

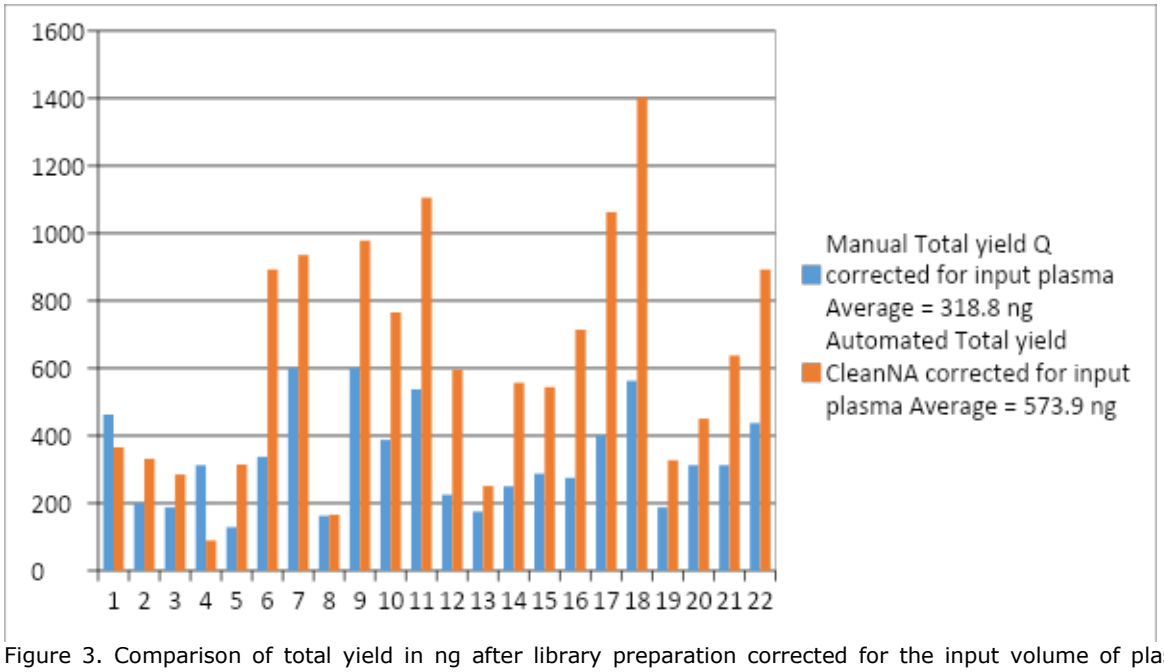

Figure 3. Comparison of total yield in ng after library preparation corrected for the input volume of plasma material. Blue represents competitor Q and Red represents CleanNA isolated samples.

Analysis of sequence data

Wisecondor analysis of CleanNA prepared samples showed an average of 17349016 mapped reads corresponding to 0.434 X total coverage, with an average allowed deviation of 5.8. These values are well within the criteria (mapped reads > 10M, Coverage > 0.175 for bins of 1Mb, Average allowed deviation <= 8) for trustworthy analysis results. SASI-seq barcode reads were above minimum criteria (>200 reads and 85% of these reads indicating the same barcode) for each sample and clearly indicated no samples were swapped or contaminated during the process prior to sequencing. 12 of the 22 samples showed no aberrations for both the manual and the CleanNA automated isolation procedure. The rest of the aberrations are summarized and compared in table 2. This data shows that within CleanNA prepared samples the same aberrations are found as in the corresponding manually prepared samples.

Figure 4 is an example of the Wisecondor bin calling of chromosome 21 for sample 5, where the highly deviating Z-scores of reads mapping to parts of chromosome 21 indicate an aneuploidy to be present. Figure 5 shows the bin calling plot for chromosome 18 of the same patient where no deviations are called. Figure 6 and figure 7 show the same patient plots for samples isolated with method Q

Table 2. Comparison summary of detected aberrations in samples pool.

Sample	**Manual prepared samples**	**CleanNA prepared samples**
Number	**NIPT report**	**NIPT report**
1	tri7 94% 52Mb and 85Mb	tri7 81% 46Mb and 68Mb
2	12p+ 27Mb	12p+ 11MB
3	tri 21 100%	tri 21 100%
4	tri 21 100%	tri 21 64% 10Mb and 11Mb
5	tri 21 100%	tri 21 100%
6	tri 22 95%	tri 22 100%
7	tri 21 100%	tri 21 92%
8	tri 18 98%	tri 18 11Mb and 20Mb
17	tri 22 96%	tri 22 96%
22	10MB chr21+	10MB chr21+

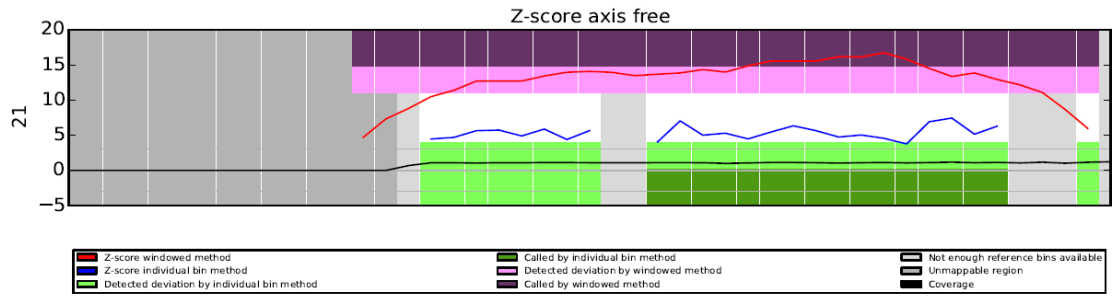

Figure 4. Sample 5 Wisecondor results of bincalling for chromosome 21, isolated using the CleanNA Clean Circulating LV DNA Kit

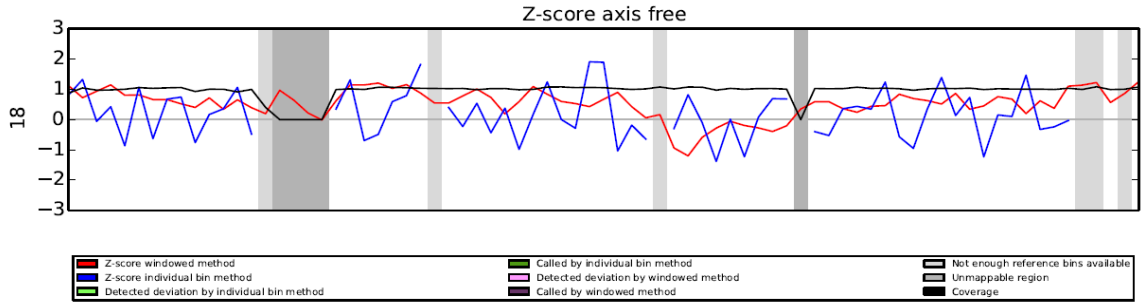

Figure 5. Sample 5 Wisecondor results of bincalling for chromosome 18, isolated using the CleanNA Clean Circulating LV DNA Kit

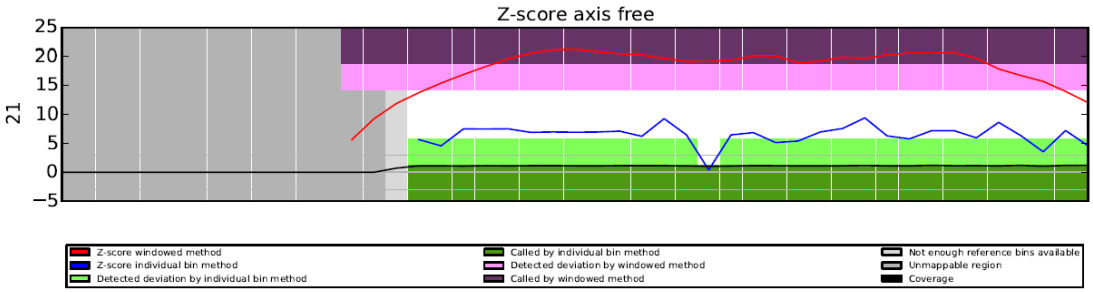

Figure 6. Sample 5 Wisecondor results of bincalling for chromosome 21, isolated using the Competitor Q Kit

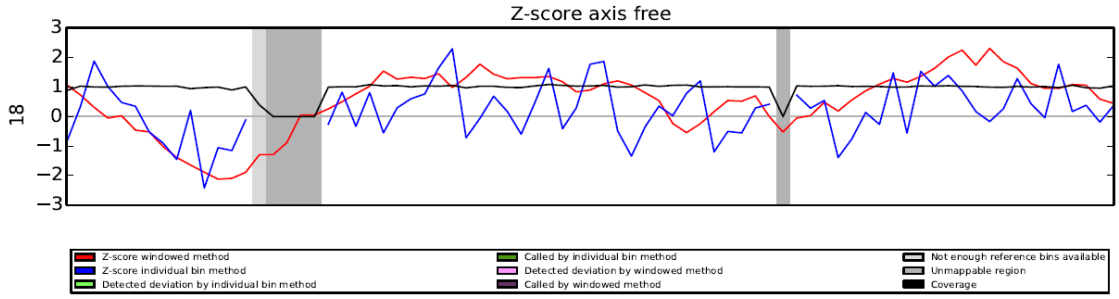

Figure 7. Sample 5 Wisecondor results of bincalling for chromosome 18, isolated using the Competitor Q Kit

Conclusion & Discussion

This application note shows that automation of the Clean Circulating LV DNA Kit provides a solution to isolate cfDNA with less hands-on time, decreased risk of sample swaps occurring in the process and full sample track and traceability. This method provides comparable down-stream performance in NGS analysis compared to the current manual isolation. In addition to this, less input is required for the isolation process, saving valuable patient material and providing the option to perform a second isolation if the sample is not performing well downstream.

References

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