A novel method for efficient and hands-free purification of circulating DNA from human plasma

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1. Abstract

Circulating cell-free DNA (ccfDNA) in plasma can be used to detect biomarkers that show great promise for diagnosis and monitoring of cancer, and is already being used as a non-invasive method to detect trisomy in fetuses. There is currently great interest in the discovery of new cancer biomarkers and their potential clinical application. However, reproducible and efficient purification of these highly fragmented and low-concentration species represents a major challenge.

Here we present a novel method which is completely automated and allows parallel purification of circulating nucleic acid from plasma and serum using a medium-throughput robot. Sixteen samples can be processed simultaneously. The method was optimized to produce high-quality DNA that is suitable for use in quantitative PCR and next generation sequencing. In addition, the absence of any pre-processing steps improves reproducibility and lowers the risk of contamination.

Initial characterization on plasma from pregnant women showed that fetal DNA could be detected as early as 4 weeks into gestation and could be tracked throughout pregnancy. Subsequent work on plasma from patients with colorectal cancer showed that the system could purify enough DNA to allow next generation sequencing and detection of mutations that are biomarkers for cancer.

2. Areas of interest for ccfDNA

- Fetal Applications
  - ccfDNA analysis is filling many of the roles once filled by amniocentesis and chorionic villus sampling (CVS)
  - Quantitative levels of ccfDNA
    - Determine gender through Y chromosome detection
    - Marker for various conditions
  - SNP Analysis
    - Paternity
    - Epigenetic fetal markers
- Cancer Applications
  - ccfDNA often exhibits the same alterations as DNA from tumor tissues.
  - Quantitative changes in ccfDNA levels
    - Normal levels of ccfDNA are 10-30ng/ml plasma; in cancer patients levels can be significantly higher
  - Biomarkers for cancer
    - Mutations in oncogenes such as KRAS are detected in various cancers
    - Mutagenic KRAS can be detected in plasma of cancer patients
  - Microsatellite alterations
    - Microsatellite Instability and Loss of Heterozygosity are suggested to play a role in carcinogenesis
    - MSI and LOH are seen in ccfDNA from cancer patients
  - Epigenetic alterations
    - Changes in methylation patterns can be seen in ccfDNA

3. Materials and Methods

Promega's Maxwell® 16 robot was used to purify ccfDNA from both plasma and serum.

Three sources of plasma were used. Bioreclamations plasma (Westbury NY), spiked with various amplicons, was used for general studies. For fetal studies, plasma was collected from 35 pregnant women on the Promega campus. For cancer studies, plasma from patients with stage 1 and 2 colorectal cancer were purchased from Proteogenex (Culver City CA).

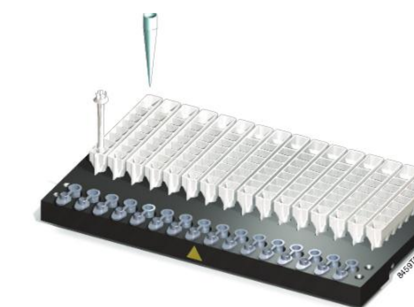
Concentration of DNA was typically determined using qPCR. Promega's Plexor® HY system and Life Technologies TaqMan™ system. Fluorescence quantitation was done using Promega's QuantiFluor® dsDNA system.

NGS: Next generation sequencing was performed at the **University of Wisconsin Biotechnology Center** using an Ion Torrent™ sequencer. Mutations were detected using the Ion AmpliSeq™ Cancer Hotspot Panel v2 from Life Technologies.

4. Protocol

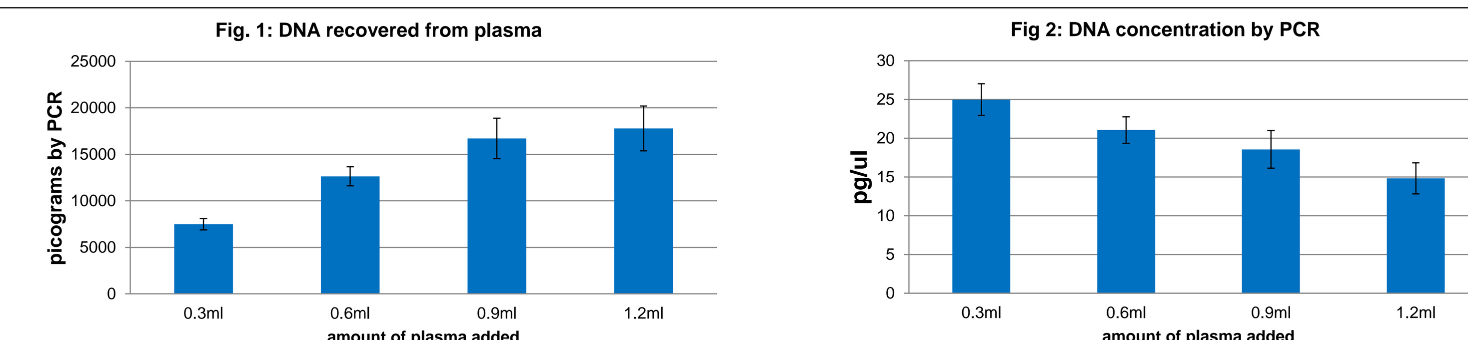
The Maxwell® 16 instrument is a small, magnetic particle-handling robot that allows efficient binding of ccfDNA to the paramagnetic particle in the first well of a prefilled cartridge and moves the sample through the wells of the cartridge, mixing during processing.

Promega's Maxwell CSC robot (above) and sample cartridges in deck (below)



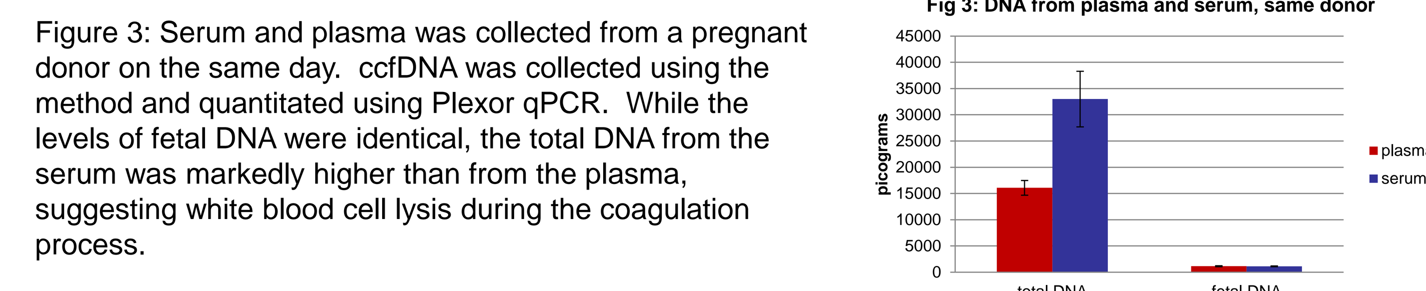
- **There is no pre-processing.** Plasma is added directly to well 1 of the cartridge.
- 16 samples can be processed simultaneously.
- Elution volume is 30 to 60 ul.
- Run time is 80 minutes.

5. Purification of ccfDNA from Plasma and Serum

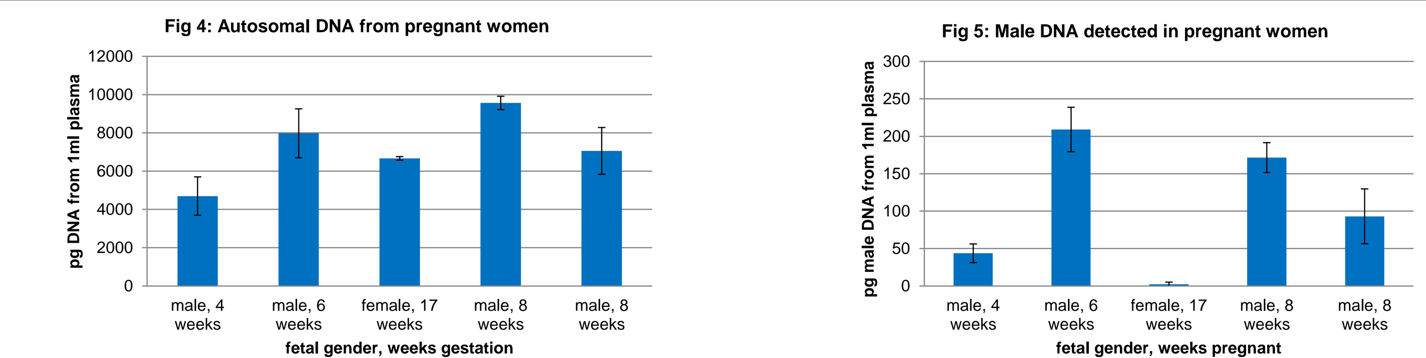


Figures 1&2: Increasing amounts of plasma were added to the cartridges and processed; DNA levels were quantitated using qPCR. The results show the method will recover DNA efficiently from small samples.

Figure 3: Serum and plasma was collected from a pregnant donor on the same day. ccfDNA was collected using the method and quantitated using Plexor qPCR. While the levels of fetal DNA were identical, the total DNA from the serum was markedly higher than from the plasma, suggesting white blood cell lysis during the coagulation process.

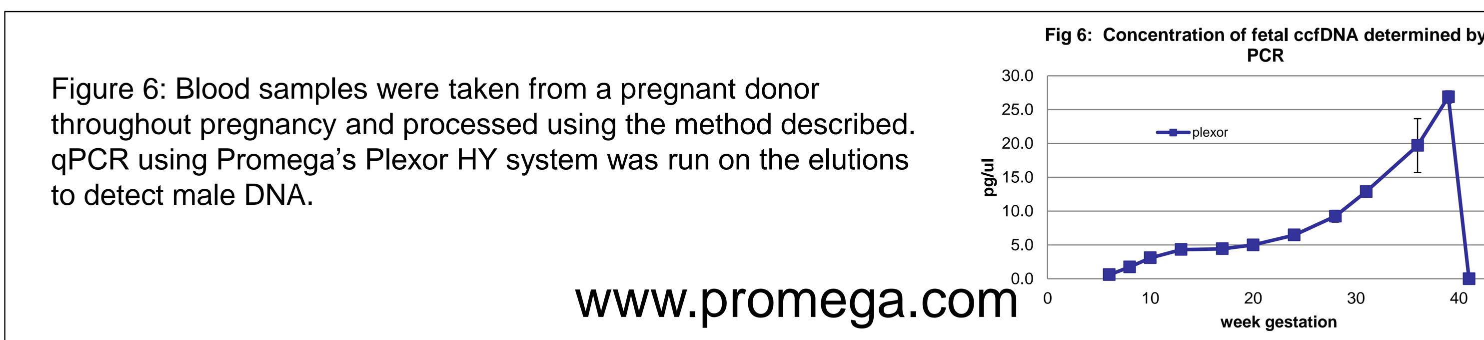


6. Purification of Fetal and Maternal ccfDNA



Figures 4&5: Plasma was drawn from 5 women in the first trimester of pregnancy and processed using the protocol, followed by Plexor HY qPCR to detect total and male ccfDNA. Four of the five samples showed the presence of male ccfDNA, indicating a male fetus. Results for all pregnancies were confirmed by ultrasound and live births.

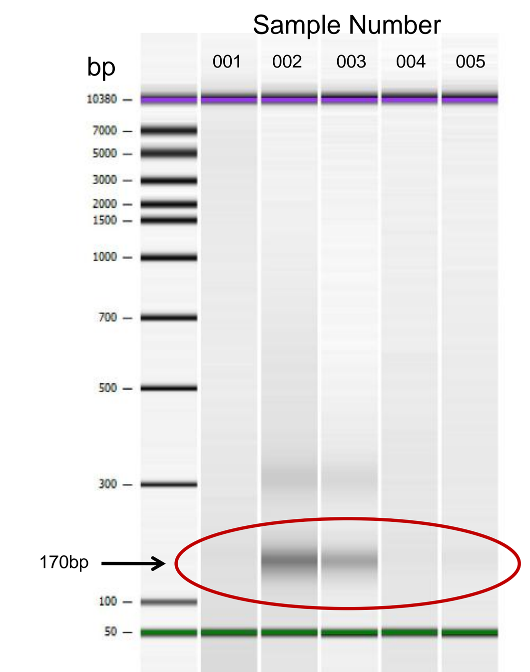
Figure 6: Blood samples were taken from a pregnant donor throughout pregnancy and processed using the method described. qPCR using Promega's Plexor HY system was run on the elutions to detect male DNA.



7. Purification of ccfDNA from Cancer Patients

DNA was purified from plasma samples from 5 patients with stage 1 or 2 colorectal cancer, and concentrated when necessary. DNA levels were measured by fluorescence and sized on a Bioanalyzer using a DNA 7500 chip. The 170bp size of the DNA matches values reported in the literature.

sample	pg/ul	ng yield
Pro14-001	50.8	5.1
Pro14-002	4163.9	416.4
Pro14-003	775.7	77.6
Pro14-004	77.5	7.8
Pro14-005	72.9	7.3



8. Detection of Mutations

Samples were sequenced using an Ion Torrent™ sequencer utilizing the AmpliSeq™ Cancer Hotspot Panel v2. Results were analyzed using Torrent Suite version 4.0.2. All 5 samples showed good coverage and sequenced well.

Sample	Bases	≥ Q20	Reads	Mean read length
Pro14-001	60,733,148	56,130,634	538,695	112 bp
Pro14-002	49,918,647	46,215,631	457,360	109 bp
Pro14-003	52,724,081	48,855,852	478,049	110 bp
Pro14-004	77,947,879	72,178,295	687,505	113 bp
Pro14-005	76,426,343	70,832,700	678,050	112 bp

Gene	Pro14-001	Pro14-002	Pro14-003	Pro14-004	Pro14-005
ALK		1			
APC	2	1	1	1	1
CSF1R	1		2	1	1
EGFR		1	1	1	1
ERBB4	1		1	1	1
FBXW7				1	
FGFR3	1	1	1	1	1
FLT3	1	1	1	1	1
HRAS		1	1		1
IDH1		1			
KDR	1	1	1	1	1
KIT					1
KRAS	1		1		
MPL		1			
NOTCH1	1	2		1	
PDGFRA	1	1	1	1	1
PIK3CA	1		3	2	
RB1	1				
RET	1	1	1		2
SMAD4		1			1
SMARCB1		1	1		
STK11	2	3			
TP53	2	2	2	1	1

9. Conclusion

The method described allows the rapid purification of ccfDNA from human plasma or serum. The protocol is completely automated, decreasing the chance of user error or contamination. Sixteen samples of up to 1ml can be processed in 80 minutes. As measured by qPCR, yields from plasma and serum from standard (non-cancerous) patients are typically between 10 to 30ng per ml plasma.

Using this method on plasma collected from pregnant women allows the collection of fetal ccfDNA. Samples are pure enough for qPCR allowing non-invasive analysis.

ccfDNA was purified from plasma from patients with early stage colorectal cancer. Next generation sequencing of the DNA detected mutations commonly associated with cancer, suggesting that this method could be used to screen plasma in a form of "liquid biopsy".

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