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Elimination of Preanalytical Error in Cff-DNA Non Invasive Diagnosis Test

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Abstract

Objective: Cell-free fetal DNA (cff-DNA) is fetal DNA circulating in the maternal blood stream. The most studies have focused on how to extract this fetal source but what is less discussed in articles is determine the factors that affect the quality of cff-DNA. As this issue is so important in diagnosis fetal monogenic hereditary diseases, in this article the factors that can affect the quality of cff-DNA were evaluated. Method: From 6 pregnant women with gestational age of 11-15 weeks, after signing consent form, 12 ml blood was taken in k3 EDTA tube and the effect of storage time, temperature and centrifuge method, on the quality of cff-DNA were evaluated in 12 different modes according to Taguchi algorithm. Firstly DNA fraction was determined by Nanodrop then based on difference between length of fetal and maternal DNA base pairs, Real Time PCR was used to confirm the cff-DNA fraction. Results: The results demonstrated, in the samples that stored at 4ºc and their plasma was separated in two steps (5min 800g and 5min 1600g), maternal DNA didn't affect cff-DNA fraction quantity, up to 24h; but by increasing incubation time or/and temperature, maternal DNA degraded to fragments as same size as fetal DNA, therefor fetal fraction was increased falsely. **Conclusion:** To have a cff-DNA with the minimum maternal DNA interferes, plasma must be separated as quickly as possible and samples must be stored in 4ºc. In conditions, which, samples must be transferred to reference lab, instead of whole blood, plasma should be transferred.

Key Words: Circulating DNA, Prenatal Diagnosis, DNA

Introduction

Widespread use of different methods of prenatal diagnosis is more than three decades old. Prenatal diagnosis prevents birth of fetus with genetic diseases. Up to now, the ways to check fetal DNA are taking sample from amniotic fluid, cord blood and placental tissue, which will need amniocentesis, cordocentesis and CVS. They have a risk of miscarriage and other complications (Mujezinovic & Alfirevic, 2007a).

Amniocentesis is recognized as an invasive diagnostic test that poses potential risks. The risk of miscarriage is about 0/5-1%. Miscarriage can occur because of infection in the uterus, preterm rupture of membeans (Mujezinovic & Alfirevic, 2007b). Noninvasive prenatal testing (NIPT) is a simple blood screening test that analyzes cell free fetal DNA to pinpoint fetal's risk for a number of genetic disorders. NIPT can be performed after 9th weeks of pregnancy — earlier than any other prenatal screening or diagnostic test (Ekici, 2015). In comparison, nuchal translucency (NT) screening is done between weeks 10 to 14 (Spencer and et al., 1999); CVS is done at 10 to 12 weeks (Caughey and et al., 2006); quad screening is completed between weeks 15 and 20 (Lao and et al., 2009); and amniocentesis is usually performed between weeks 15 and 20 (Mujezinovic & Alfirevic, 2007b). The source that can be used in NIPT

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test is Cell-free fetal DNA (Cff-DNA). Cff-DNA is fetal genome that is released by the placenta and fetal cell circulates in a woman's blood during pregnancy (Alberry et al., 2007). It is present in small quantities starting in the first trimester and increases throughout pregnancy (8). Cff-DNA generally reflects the fetal genetic makeup. Various research have been done to evaluating cff-DNA extraction from maternal blood but what ignored is how to transport and storage blood sample till processing it to have a cff-DNA with the minimum maternal DNA interferes. The aim of this study is to finding the best condition to avoid contamination by maternal DNA. If we can optimize this and restrict entry maternal DNA in isolated Cff-DNA diagnosis monogenic disease can be determined more accurately.

Method and Materials

6 pregnant women with gestational age of 11-15 weeks (mean 13 weeks) and three pregnant women that have minor thalassemia enrolled in study and 12 ml blood was taken in k3 EDTA tube. The effect of storage time and temperature on the cff-DNA extraction were evaluated in 12 different modes according to Taguchi algorithm. Women enrolled in this test were informed both verbally and by written consent form about the aim of the project and only women that sign consent form were enrolled in this study.

Taguchi matrix

In order to evaluate the effect of plasma extraction method, storage temperature and time on the cff-DNA fraction, Taguchi test has been used. Taguchi method is statistical methods developed by Genichi Taguchi to improve the quality of manufactured goods, and more recently also applied to engineering, biotechnology, marketing and advertising (Rao and et al., 2008). The matrix provided by this method can be effectively analyzed the interaction of various factors at the same time therefor savings the time and cost. Taguchi tables can be seen in tables 1 and 2.

Table 1. The level of effective factor on quality of Cff-DNA in Taguchi test

	Level 1	**Level 2**	**Level 3**
Centrifuge	2 step(5min 800g, 5 min 1600g)	1 step (10 min 2500-3000g)	-
Delivery time	2-4 h	24h	48 h
Delivery temperature	4c	RT	-

Table 2. Different test condition provided by Taguchi test. The numbers in the table shows different levels of experimental factors

Test number	centrifuge	time	temp	
1	1	1	1	2step centrifuge, delivery in 4c in 2-4h
2	1	2	1	2step centrifuge, delivery in 4c in 24 h
3	1	3	1	2step centrifuge, delivery in 4c in 48 h
4	1	1	2	2step centrifuge, delivery in 21c in 2-4h
5	1	2	2	2step centrifuge, delivery in 21c in 24h
6	1	3	2	2step centrifuge, delivery in 21c in 48 h
7	2	1	1	1step centrifuge, delivery in 4c in 2-4 h
8	2	2	1	1step centrifuge, delivery in 4c in 24h
9	2	3	1	1step centrifuge, delivery in 4c in 48h
10	2	1	2	1step centrifuge, delivery in 21c in 2-4 h
11	2	2	2	1step centrifuge, delivery in 21c in 24 h
12	2	3	2	1step centrifuge, delivery in 21c in 48h

Plasma separation

Plasma separation was done by two methods. One method had single step centrifuge that plasma was separated by centrifuge in 3000g for 10 min and the other was two step centrifuges, at first blood sample was centrifuged 5 min in 800g and separated plasma was transfered to a clean tube and recentrifuge at 1600g for 5min and was transfered the plasma to a new tube.

Cff-DNA Extraction

THP protocol (Triton- Heat- Phenol) was used to extract cff-DNA (Legler and et al., 2007). In this method, 5 µl Triton was added to 500 µl of plasma and incubated at 98ºC for 5 minutes then it were placed on ice for 5 minutes, An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v:v:v) was added to last mixture and was centrifuged for 10 minutes at 14,000 g. The aqueous phase was transferred to a clean tube and added an equal volume of chloroform-isoamyl alcohol (24:1, v: v) to eliminate phenol contamination then was centrifuged for 10 minutes at 14,000 g. aqueous phase was transferred to new clean tube then 1/10 volume of 3M NaOAc and 2.5 volume of 100% ethanol was added and incubation overnight at -20ºc. The DNA pellet was washed with ethanol 80, 70% respectively then air-dried and resuspended in 50 µl of ddH2O. Then OD280 nm of this sample was determined by Nano drop Spectrophotometers. This concentration is total maternal and Fetal DNA concentration and was defined by $OD_{BR\ (Before\ Recovery)}$.

Isolation Cff-DNA from maternal DNA by size fraction

The DNA extraction yield from last step consist of maternal and fetal cell free DNA. But as fetal DNA has smaller size (under 200bp) can be separated from maternal DNA by runing in 2% agar gel and recovering the bands smaller than 200bp according to DNA ladder. Recovery from gel was done according to manufacture recommendation. Then OD280 nm of this sample was determined by Nano drop Spectrophotometers. This concentration is likely related to cell free Fetal DNA concentration and define by $OD_{AR\ (After\ Recovery)}$.

Real Time PCR

Based on difference between length of fetal and maternal DNA base pairs, Real Time PCR was used to confirm the cff-DNA fraction. Samples were collected from 5 pregnant women with male fetus. Each sample was divided in 5 new clean tubes. To confirm the effect of storage time and temperature and centrifuge mode the samples were incubate in 5 Taguchi test modes (Mujezinovic & Alfirevic, 2007a; Mujezinovic & Alfirevic, 2007b; Ekici, 2015; Sayres & Cho, 2011). The samples which prepare according to Taguchi test were amplified by SRY primer (150bp) as fetal DNA source and b-actin (400bp) primer as maternal source. SRY Ct can demonstrate fetal DNA concentration and b-actin shows maternal DNA concentration.

Table 3- primer sequnces

Primer	Sequence	Tm	Length
SRY F	TATCGACCTCGTCGGAAG	56	150bp
SRY R	TTGAGTGTGTGGCTTTCG	56	
B-actin F	TGGCACCACACCTTCTACAATG	56	400bp
B-actin R	GCACAGCTTCTCCTTAATG	56	
GAPDH F	CCTGCACCACCAACTGCTTA	56	120bp
GAPDH R	CCTGCACCACCAACTGCTTA	56	

Data analysis

For all samples in all 16 mod of tests offered by Taguchi, the OD_{280} of samples before gel extraction ($OD_{BR)}$ and after gel extraction (OD_{AR}) were determined and compared by t- test between different experimental modes to find out how to keep blood samples to minimize errors and obtain the results with the highest sensitivity and accuracy. The first experimental modes in Taguchi test define as control condition (20 b-globin mutation in extracted cff-DNA in this mode tested by sequencing and all of their fetus mutation can be diagnosed accurately according to CVS results).

Results

Table 4 and table 5 demonstrate OD_{BR} and OD_{AR} respectively in 12 different experimental conditions. In order to compare the effects of the examined factors, the modes that only one factor was different compare together by t-test.

Table 4- OD_{BR} samples in different Taguchi experimental condition

	1	2	3	4	5	6	7	8	9	10	11	12
mean	113.8	126.6	157.5	133.3	168.8	251.1	123.1	141.1	179.1	136.6	184.3	314.5

Table 5- OD $_{AR}$ samples in different Taguchi experimental condition

mean	1	2	3	4	5	6	7	8	9	10	11	12
mean	12.1	12.3	20	12.1	15.1	41	12.3	16.1	25.1	12.5	14.8	42.5

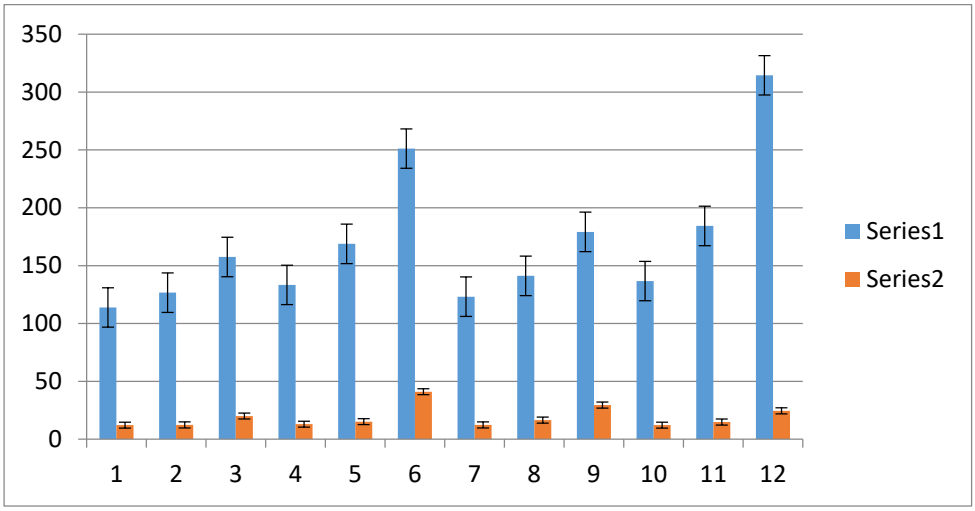

Chart 1. Compression chart between OD$_{BR}$ (series 1) and OD $_{AR}$ (series 2) in 12 different experimental conditions

These data show, by increasing storage time and temperature, maternal cell lyse and their DNA release in to plasma. In 4c storage temperature, up to 24h As only OD$_{BR}$ increase (p-value=0.04) and OD$_{AR}$ didn't changes significantly (p-value=0.6) we can conclude that only concentrations of large fragment size DNA increased due to lysing of maternal cells without any Fragmentation. These finding were confirmed by real time PCR. Its data show the fetal DNA concentration will be constant up to 48 hours incubation in 4ºc but Maternal DNA were significantly increased in plasma even after 24 hour(figure 1).

Analysis of OD showed, after 48h sample incubation in 4c, OD$_{AR}$ increased significantly (p-value $_{BR\ (Pair1-3)}$ =0.004 & p-value $_{AR\ (pair1-3)}$ = 0.02) as well as increasing OD$_{BR}$. In 21c, after 24h OD$_{BR}$ & OD$_{AR}$ increase significantly (p-value $_{BR\ (Pair1-5)}$ =0.004 & p-value $_{AR\ (pair1-5)}$ = 0.002) (table4).

To evaluating the centrifuge method effect we compare the situation that show minimum contaminated with maternal genetic resource due to storage time and temperature (time 2-4h, temperature 4ºc). The analyzed data can be seen in table 8. as concentration DNA before recovery was increase significantly in experimental condition 7 compared to 1 (p-value=0.008), it can be concluded one step centrifuge with higher speed compare to two step with lower speed can damage the blood maternal cell and release their DNA in plasma but when samples store at 4ºc for 2-4h, DNA concentration after recovery wasn't increase significantly (p-value=0.7) but after 24h, difference will be significant (p-value=0.001).

Table 6- estimated p-value obtained from T-test. A- Compression OD280 before recovery. -B compression OD280 after recovery.

A		p-value
compression	OD_{280} before recovery	
Pair 1	1 - 2	.043
Pair 2	1 - 3	.004
Pair 3	1 - 4	.024
Pair 4	1 - 5	.004
Pair 5	1 - 6	.002
Pair 6	1 - 7	.008
Pair 7	1 - 8	.004
Pair 8	1 - 9	.008

B		p-value
Pair 1	1-2	.695
Pair 2	1-3	.029
Pair 3	1-4	1.000
Pair 4	1-5	.002
Pair 5	1-6	.001
Pair 6	1-7	.793
Pair 7	1-8	.007
Pair 8	1-10	.530

Pair 9	1 - 10	.007	Pair 9	1-11	.010
Pair 10	1 - 11	.022	Pair 10	1-12	.008

Table 7- Estimated p-value obtained from T-test to evaluating centrifuge method. A- Compression OD280 before recovery. -B compression OD280 after recovery

A	p-value	B	p-value
Pair1 1-7	.008	Pair1 1-7	0.793
Pair2 2-8	.005	Pair2 2-8	.001

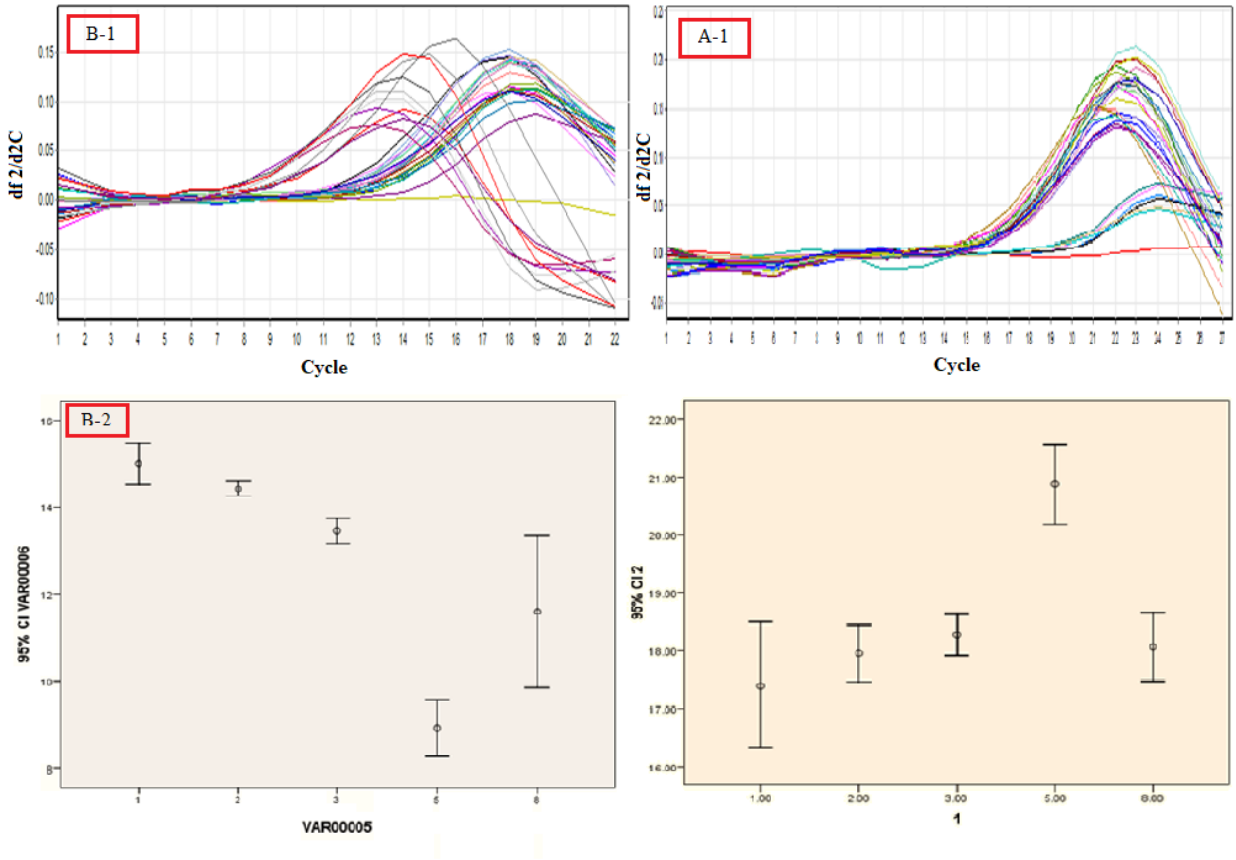

Fig. 1: Comparison ΔCt of amplification SRY and b-actin gens in samples prepare according to 1, 2,3,5,8 Taguchi test modes. A-1: Real time PCR result frome amplification SRY gen. A-2: graph show ΔCt of amplification SRY gen in samples prepare according to 1, 2,3,5,8 Taguchi test modes. B-1: Real time PCR result frome amplification b-actin gen. B-2: the graph shows ΔCt of amplification b-actin gen in samples prepare according to 1, 2,3,5,8 Taguchi test modes.

Discussion

Since the use of non-invasive prenatal diagnosis is expanding increasingly, more studies are required in the field of optimized sampling and preparation the samples for analyzing. One of the most common sources used in these tests is cell free fetal DNA (cff-DNA). Most studies have focused on different ways to isolate this source from maternal blood samples (Legler and et al., 2007; Legler, and et al., 2009). Research that examines how transfer and storage of samples are so rare, while control this step can affect accuracy of final results. In this study storage temperature and storage time as well as centrifuge method to separate plasma were evaluated. Interaction between these effective factors was evaluated by using different experimental condition offered by Taguchi test. Previous studies have

shown that true cff-DNA fragments are generally < 200 bp-long, likely because of cellular apoptosis (Smets and et al., 2008). An increase in the concentration of fragments that are > 300 bp-long may be an indication of a compromised blood sample wherein the maternal leukocytes have released their genomic DNA (Xue and et al, 2009; Müller and et al, 2011). In this study we use this different size to separate fetal DNA from maternal DNA and then compare the concentration of these DNA in different test condition offered by Taguchi to evaluate how we can use blood sample to have minimal maternal DNA. Based on our data it could be concluded if samples are stored at 4 ° C more than 24 hours or in 21c up to 24 hours, may not be reliable due to maternal blood cell lysing. In these experimental conditions concentration of total DNA and fetal DNA increase significantly (p-value $_{AR\ (pair1\text{-}3)}$ = 0.02, p-value $_{AR\ (pair1\text{-}5)}$ = 0.002).Real time PCR data confirmed these results too. In the study have been done by Muller and his colleagues in 2011 determined the relation between the transport time of samples for NIPD and the concentration of fetal DNA in maternal plasma (Müller and et al, 2011). They show No decrease of cell-free fetal DNA was observed in samples with less than 6 days transport time. As they evaluating SRY gen copy number they didn't have any mention about contaminated cff-DNA by maternal DNA. Several studies have also shown that cff-DNA is stable within the first 24 hours after sample collection (Jung and et al., 2003; Xue and et al, 2009). Norton and his colleagues conducted a similar study to our study. In their study, Real time PCR was used to amplify a *β-actin* gene fragment to evaluate the total plasma cell-free DNA concentration, while an *SRY* gene fragment was used to quantify the cff-DNA. Our results are consistent with theirs. They observed a statistically significant increase in the amount of total DNA in the shipped K3 EDTA blood samples ($P < 0.01$) compared to the control values after 2 days of transportation under extreme outdoor temperatures, indicating the release of a greater quantity of DNA from the maternal cells (Norton and et al., 2013).

We analyzed the effect of centrifuge method as well as storage time and temperature. The data showed after incubation the sample 24h in 4c, one step centrifuge with higher speed compare to two steps with lower speed can damage the blood maternal cell and release their DNA in plasma. Data demonstrated, by using one step centrifuge in experimental condition have longer storage time and temperature, DNA concentration after recovery increased significantly (p-value=0.001). It can be concluded; the cells become more fragile against mechanical pressure by increasing the storage time and temperature. The results obtained by Barrett and her colleagues were consistence with ours. They demonstrated that fetal fraction can be preserved using a single centrifugation step followed by postage of plasma to the laboratory for further processing (Barrett and et al., 2014). We successfully demonstrated how long blood samples can be used confidentially to use in diagnosis monogenic hereditary diseases. Based on obtained data we can conclude the best storage temperature is lower temperature ($4^{o}c$) and in this temperature, blood samples are reliable up to 24 hours and then there will be the possibility of contamination of samples with maternal DNA due to blood cell lysing. To eliminate cff-DNA contamination by maternal DNA; it is better plasma be separated at first and then transferred to reference genetic lab.

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