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TECHNOLOGICAL INNOVATIONS

Evaluation and statistical optimization of a method for methylated cell-free fetal DNA extraction from maternal plasma

Mostafa Akbariqomi[1] · Reza Heidari[1] · Soraya Saleh Gargari[2] · Mir Davood Omrani[3] · Garshasb Rigi[4,5] · Nafiseh Sadat Sanikhani[1] · Hamid Kooshki[6] · Fatemeh Mahmoudian[1] · Mohammad Ali Mazlomi[7] · Gholamreza Tavoosidana[1]

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Abstract

Purpose Methylated cell-free fetal DNA (cffDNA) in maternal plasma can potentially be used as a biomarker for accurate noninvasive prenatal testing (NIPT) of fetal disorders. Recovery and purification of cffDNA are key steps for downstream applications. In this study, we aimed to developed and evaluated different aspects of an optimized method and compared its efficiency with common methods used for extraction of methylated cffDNA.

Methods Single factor experiments, Plackett-Burman (PB) design, and response surface methodology (RSM) were conducted for conventional Triton/Heat/Phenol (cTHP) method optimization. The total cell-free DNA (cfDNA) was extracted from pooled maternal plasma using the optimized method called the Triton/Heat/Phenol/Glycogen (THPG), cTHP method, a column-based kit, and a magnetic bead-based kit. In the next step, methylated cfDNA from the extracted total cfDNA was enriched using a methylated DNA immunoprecipitation (MeDIP) kit. Real-time quantitative polymerase chain reaction was performed on the RASSF1 gene and hyper region to determine the genomic equivalents per milliliter (GEq/ml) values of the methylated cfDNA and cffDNA, respectively.

Results The optimum values of the significant factors affecting cfDNA extraction from 200 μl of plasma were 3% SDS, 1% Triton X-100, 0.9 μg/μl glycogen, and 0.3 M sodium acetate. The GEq/ml values of methylated cffDNA extracted using the THPG method were significantly higher than for the tested extraction methods ($p < 0.001$).

Mostafa Akbariqomi and Reza Heidari contributed equally to this work.

✉ Gholamreza Tavoosidana
g-tavoosi@tums.ac.ir

Mostafa Akbariqomi
m-akbariq@razi.tums.ac.ir

Reza Heidari
r-heidary@razi.tums.ac.ir

Soraya Saleh Gargari
soraya_saleh2000@yahoo.co.uk

Mir Davood Omrani
davood_omrani@sbmu.ac.ir

Garshasb Rigi
garshasbbiotech@gmail.com

Nafiseh Sadat Sanikhani
Sanikhani@yahoo.com

Hamid Kooshki
hmdkooshki@gmail.com

Fatemeh Mahmoudian
Fmahmoudian@razi.tums.ac.ir

Mohammad Ali Mazlomi
m-mazlomi@tums.ac.ir

[1] Department of Molecular Medicine, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran

[2] Department of Gynecology & Obstetric, Shohada Tajrish Educational Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[3] Department of Medical Genetics, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[4] Department of Genetics, Faculty of Basic Science, Shahrekord University, Shahrekord, Iran

[5] Research Institute of Biotechnology, Shahrekord University, Shahrekord, Iran

[6] Nanobiotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

[7] Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran

Conclusions Our results indicate that the THPG method is more efficient than the other tested methods for extraction of low copy number methylated cffDNA from a small volume of maternal plasma.

Keywords Methylated cell-free fetal DNA · cffDNA extraction · Epigenetic markers · THPG method · Noninvasive prenatal testing · Plasma

Introduction

Genomics-based noninvasive prenatal testing (gNIPT) using the cell-free fetal DNA (cffDNA) present in the plasma of pregnant women offers an innovative intermediate test for common fetal aneuploidies screening [1, 2]. In recent years, cffDNA as a prenatal screening option for clinical applications has been commercially launched in both developing and developed countries and has been shown to remarkably reduce the need for invasive tests [1, 3]. However, positive NIPT results should be confirmed by definitive invasive tests, including chorionic villus sampling and amniocentesis.

The cffDNA is comprised of short fragments of extracellular DNA in the maternal blood that are mainly below 313 bp and which originate from apoptotic and necrotic trophoblastic cells [4]. Despite the fact that cffDNA can be detected in maternal plasma after the first month of pregnancy, it is only trustworthy after 7 weeks [5]. However, it clears from maternal circulation in an average half-life of 16 min (range 4–30 min), so that 2 h after parturition, it becomes undetectable [5, 6]. Hence, cffDNA has been used as an authentic genetic source for noninvasive prenatal evaluation with tremendous potential for fetal gender, aneuploidies, rhesus D status, and genetic diseases including myotonic dystrophy, achondroplasia, congenital adrenal hyperplasia, B-thalassemia, and Huntington's disease [5, 7, 8]. The concentration of cffDNA varies among individuals, and overall, it constitutes 3 to 30% of total cell-free DNA (cfDNA) in the plasma of pregnant women [6, 9]. The limited amount of cffDNA in a huge pool of cfDNA in maternal plasma poses a major challenge for its extraction for use in NIPT of fetal disorders.

Difference in the methylation status of fetal and maternal DNA that could be used to identify fetal-specific markers and quantify cffDNA is a promising epigenetic approach for NIPT [10]. Recently, differentially methylated regions (DMRs) between fetal and maternal DNA have been considered suitable epigenetic-based biomarkers [11]. This includes the RASSF1 gene on chromosome 3 (hypermethylated in fetal and hypomethylated in maternal DNA) and the tandem repeat hyper region on chromosome 22 (hypermethylated in fetal and maternal DNA) [11, 12].

Methylated DNA immunoprecipitation (MeDIP) is an antibody-based approach that uses fetal-specific DMRs to capture and enrich methylated fetal DNA fragments from maternal plasma [11, 13]. The efficiency of this approach can be improved by choosing a suitable extraction method. Although various methods with different efficacies have been introduced and manipulated for extraction of cffDNA from maternal plasma, the low DNA extraction yield is a major challenge [14–17]. The conventional Triton/Heat/Phenol (cTHP) method has been introduced as an efficient protocol for extracting cfDNA [18]. It has been hypothesized that the challenge of low yield may be overcome using effective statistical methods such as Plackett-Burman (PB) and response surface methodology (RSM) to optimize cTHP extraction.

The present study was undertaken to evaluate the factors and conditions of cTHP as the control for extracting cffDNA using the single factor method and the PB design. Optimization then was performed using RSM based on central composite design (CCD). The optimized extraction method was compared with two commonly used commercial extraction kits (column-based kit and magnetic bead-based kit) to determine the most efficient method for extraction of methylated cffDNA consequent enrichment that using the MeDIP approach and assessment of GEq/ml values of DMRs.

Materials and methods

Ethics approval

The study was approved by the ethical committee of the Tehran University of Medical Sciences, Iran (IR.TUMS.REC.1394.1156). All participant provided written informed consent prior to venipuncture.

Sample collection and processing

Peripheral blood samples of 35 normal pregnant women with singleton gestation were collected during the first and second trimester (10th–16th weeks of gestation) between February and November of 2017. The accurate gestational age and healthy singleton pregnancy were confirmed by ultrasound examination. Ten milliliters of maternal peripheral blood was collected in to K3EDTA tube, and immediately, the plasma was separated by centrifugation at 1600*g* for 12 min at 4 °C. The recovered plasma was recentrifuged at 16,000*g* for 12 min at 4 °C and then aliquoted into fresh tube and stored at − 80 °C until further processing.

Optimization of methylated cffDNA extraction

The extraction of methylated cffDNA from maternal plasma by the cTHP method was optimized and modeled in three steps: single factor experiments, PB design, and response surface methodology (RSM). The results of each experiment were evaluated using the methylated DNA immunoprecipitation real-time quantitative polymerase chain reaction (MeDIP-real-time qPCR) to obtain the GEq/ml values of the extracted methylated cffDNA.

Single-factor experiments

The effect of time and temperature on incubation of proteinase K (Prk) in methylated cffDNA extraction was investigated at 37 °C for 2 h (control), 37 °C for 30 min, 56 °C for 2 h, 56 °C for 30 min, and room temperature (RT) overnight. The following surfactants were used as extraction buffer: 1% Triton X-100 (control), TNES buffer (10 mM Tris (pH 7.5), 400 mM NaCl, 100 mM EDTA, 0.6% SDS), 2 M guanidinium thiocyanate, KCl extraction buffer (100 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 1 M KCl), 5 M sodium perchlorate, 6 M NaI, 1% Tween 80, 1% SDS-Triton X-100, and 7 M urea. The DNA purification was investigated using phenol-chloroform-isoamyl alcohol (control), phenol-chloroform-isoamyl alcohol+beta-mercaptoethanol (PCI + BME), TRIzol, and 2% CTAB protocols. The effect of ethanol-sodium acetate (control), ethanol-sodium citrate, ethanol-ammonium acetate, ethanol-glycogen-sodium acetate, ethanol-potassium acetate, PEG8000-Ethanol-NaCl, and PEG8000-NaCl-$MgCl_2$ was studied on nucleic acids precipitation (Table 1).

Experimental design

PB design

PB design is a two-level factorial design screening n variables in $n + 1$ experiments [19] that is used to identify factors affecting methylated cffDNA extraction. This statistical design allows evaluation of interactions between the independent variables to determine the optimal factors. In the present study, the vital variables affecting cfDNA extraction of the volume of plasma (μl), incubation temperature (°C), SDS (%), Triton X-100 (%), sodium acetate (M), volume of sodium acetate (μl), pH of sodium acetate, glycogen (μg/μl), and precipitation time (h) were optimized. Table 2 shows the variables tested in PB design at two widely spaced intervals.

Optimization of Plackett–Burman design variables by RSM design

Further optimization and modeling of affective variables determined by PB design in the last phase were conducted by the RSM of the central composite design (CCD). In this study, five levels were used to optimize the three affective variables of sodium citrate, SDS, and glycogen in 20 experiments (Table 3). The mean GEq/ml value of the extracted methylated cffDNA was considered to be the response.

cfDNA extraction

The total cfDNA was extracted from 35 pooled plasma samples with the optimized method called the Triton/Heat/Phenol/Glycogen (THPG), cTHP, and two commercially available kits. The cfDNA was extracted using column-based kit (QIAamp DSP Virus Kit, Qiagen, Hilden, Germany; DSP) and magnetic bead-based kit (NextPrep-Mag™ cfDNA Isolation Kit, Bioo Scientific, Austin, USA; NPM) according to the manufacturer's instructions, and the purified DNA was eluted in 50 μL of supplied elution buffer. For the cTHP method, cfDNA was extracted as previously described [18]. For the THPG method, 200 μl of plasma was incubated with 20 μl of PrK (100 mg/ml) at 56 °C for 2 h. Then, 3% SDS + 1 Triton X-100 (Sigma-Aldrich, UK) was added, and the mixture was heated at 90 °C for 5 min. After cooling, an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma-Aldrich, UK) was added and centrifuged for 15 min at 15,000g. The upper phase was precipitated with 0.3 M of sodium acetate (Sigma-Aldrich, UK), 0.9 μg/μl of glycogen (Thermo Fisher Scientific, Waltham, MA), and 2.5 volume of 100% ethanol for 1 h incubation at − 20 °C. The cfDNA pellet was washed with 70% ethanol at − 20 °C, air-dried, and eluted in 50 μl of sterile DNase-free water. For all extraction methods, 2 μl of the extracted DNA was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDropTechnologies, Wilmington, DE) to determine the purity and concentration based on the A260/A280 ratios.

MeDIP-real-time qPCR

Methylated cfDNA enriched and captured from the total cfDNA using the MeDIP kit (ab117133; Abcam; USA) according to the manufacturer's instructions. Real-time qPCR was performed to evaluate the results of the experimental design and efficiency of applied methods in extraction of methylated cffDNA by targeting differentially methylated regions (DMR). RASSF1 gene and the hyper region were analyzed by SYBR Green fluorescence real-time qPCR with a Rotor-Gene Q instrument (Qiagen, Hilden, Germany).

The primer pairs were designed using AlleleID primer design software version 7.5 (Premier Biosoft, USA) (Table 4). The qPCR reactions were set up in a volume of 20 μl containing SYBR Green (RealQ plus Master Mix Green without ROX, Ampliqon, Denmark), specific primers, and methylated cfDNA. The amplification program consisted of an initial denaturation of 95 °C for 15 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s. All sample reactions were performed in triplicate, and melting curve

Table 1 Determination of the optimal factors and conditions for methylated cffDNA extraction using single factor experiments

	Variables	Mean GEq/ml ± SD	GEq 95% CI	GEq/ml relative (%)	*p* value
Digestion conditions	37 °C 2 h (control)	39.48 ± 3.79	[35.2, 43.75]	100	
	56 °C 2 h	45.3 ± 1.06	[44.1, 46.49]	114.74	0.049
	RT overnight	37.44 ± 1.61	[35.61, 39.26]	97.28	NS
	37 °C 30 min	6.13 ± 0.47	[5.59, 6.66]	15.94	< 0.001
	56 °C 30 min	6.23 ± 0.72	[5.41, 7.04]	16.21	< 0.001
Extraction buffers	1% Triton (control)	41.83 ± 3.14	[38.24, 45.35]	100.00	
	NaI	52.24 ± 2.62	[49.24, 55.15]	124.00	< 0.001
	1% Tween 80	43.41 ± 1.04	[42.22, 44.57]	103.00	NS
	KCl buffer	35.79 ± 2.11	[33.4, 38.17]	85.00	0.031
	1% Triton-SDS	58.65 ± 1.96	[56.4, 60.8]	140.00	< 0.001
	TNES buffer	26.52 ± 2.12	[24.1, 28.9]	63.00	< 0.001
	Guanidinium thiocyanate	48.77 ± 1.63	[46.93, 50.6]	116.00	0.0107
	Urea	6.34 ± 0.53	[5.73, 6.93]	15.00	< 0.001
	Sodium perchlorate	45.3 ± 3.42	[40.43, 50.16]	108.00	NS
Purification	PCI (control)	40.75 ± 5.3	[34.57, 46.56]	100.00	
	PCI + BME	5.38 ± 0.77	[4.50, 6.25]	13.20	< 0.001
	TRIzol	8.48 ± 1.03	[7.31, 9.64]	20.82	< 0.001
	CTAB	7.89 ± 0.72	[7.07, 8.70]	19.36	< 0.001
Precipitation agents	E-NaOAc (control)	42.71 ± 1.11	[41.45, 43.96]	100.00	
	PNM	16.24 ± 1.39	[14.66, 17.81]	38.04	< 0.001
	EGS	62.07 ± 7.4	[53.69, 70.44]	145.32	< 0.001
	E-NH_4OAc	21.74 ± 2.43	[18.99, 24.48]	50.90	< 0.001
	E-$Na_3C_6H_5O_7$	15.004 ± 1.36	[13.46, 16.54]	35.12	< 0.001
	E-KOAc	35.92 ± 4.43	[30.90, 40.93]	84.11	NS
	PEN	15.52 ± 1.86	[13.41, 17.62]	36.35	< 0.001

NaI sodium iodide, *PCI-BME* phenol-chloroform-isoamyl alcohol+beta-mercaptoethanol, *E-NaOAc* sodium acetate, *PNM* PEG8000-NaCl-$MgCl_2$, *EGS* ethanol-glycogen-sodium acetate, *E-NH_4OAc* ethanol-ammonium acetate, *E-$Na_3C_6H_5O_7$* ethanol-sodium citrate, *E-KOAc* ethanol-potassium acetate, *PEN* PEG8000-ethanol-NaCl, *NS* not significant

Table 2 The Plackett-Burman design for the methylated cffDNA extraction

RUN	A	B	C	D	E	F	G	H	GEq/ml value	Predicted	OD260/280
1	200	85	0	2	1	7	1	1	94.22 ± 13.49	90.55	1.41
2	1000	85	1	2	1	5	0	1	30.52 ± 4.7	26.2	1.31
3	1000	95	0	1	1	7	0	16	35.56 ± 2.65	39.5	1.19
4	1000	95	1	1	1	5	1	1	126.32 ± 14.25	112.66	1.52
5	1000	85	1	2	1	7	1	16	103.76 ± 17.64	112.66	1.35
6	200	85	0	1	1	5	0	1	33.13 ± 2.29	39.5	1.3
7	200	85	1	1	1	7	0	16	19.04 ± 2.04	26.2	0.9
8	200	95	1	2	1	5	0	16	63.6 ± 6.92	61.68	1.27
9	1000	85	0	1	1	5	1	16	48.86 ± 4.33	55.1	1.2
10	200	95	0	2	1	5	1	16	56.78 ± 4.65	55.1	1.35
11	1000	95	0	2	1	7	0	1	15.37 ± 1.67	4.09	0.87
12	200	95	1	1	1	7	1	1	73.32 ± 6.52	77.12	1.47

A volume of plasma (μl), *B* incubation temperature (°C), *C* SDS (%), *D* Triton (%), *E* sodium acetate (M), *F* pH, *G* glycogen (μg/μl), *H* precipitation time (h)

Table 3 The CCD optimization for the methylated cffDNA extraction

	Variables			GEq/ml value		OD 260/280
Run	A	B	C	Observed	Predicted	
1	2	0.5	1	137.69	144.35	1.66
2	3	0	0.75	114.2	95.51	1.6
3	3	0.3	0.75	266.7	241.84	1.78
4	2	0.1	0.5	31.382	38.97	1.24
5	3	0.3	0.33	58.24	61.1	1.43
6	3	0.3	0.75	201.81	241.86	1.79
7	3	0.3	0.75	179.18	168.75	1.73
8	2	0.5	0.5	77.14	72.25	1.51
9	2	0.1	1	84.93	90.63	1.8
10	4	0.1	1	126.41	144.35	1.8
11	4	0.5	0.5	36.92	40.26	1.45
12	3	0.3	1.2	212.53	201.27	1.8
13	3	0.3	0.75	252.32	241.84	1.79
14	3	0.3	0.75	245.16	246.62	1.73
15	3	0.3	0.75	233.9	241.84	1.81
16	4	0.5	1	142.91	144.35	1.75
17	4.68	0.3	0.75	120.69	110.03	1.61
18	3	0.64	0.75	63.68	64.69	1.45
19	4	0.1	0.5	48.52	53.8	1.59
20	1.32	0.3	0.75	80.56	77.49	1.55

A SDS (%), *B* sodium acetate (M), *C* glycogen (μg/μl)

analysis was carried out following each reaction to verify the specificity of the products and the absence of primer dimers.

Preparation of standard curve

The standard curves were established using serially diluted concentrations of the hyper region (150 bp) and RASSF1 (166 bp) for absolute quantification of methylated cfDNA and methylated cffDNA fragments, respectively. The nested PCR reaction was performed to amplify the selected regions using two specific primer sets (Table 4). Both PCR reaction solutions contained 5 μl of cfDNA, 12.5 μl of Taq DNA polymerase master mix RED (Ampliqon, Denmark.), 10 pM of each primer, and ddH_2O per 25 μl of total reaction volume.

Each PCR reaction was carried out using the thermal cycler gradient PCR system (Eppendorf, Germany). The first amplification program included predenaturation at 95 °C for 10 min followed by 40 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min, and final extension 72 °C for 10 min. The second amplification program was predenaturation at 95 °C for 10 min followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and a final extension 72 °C for 10 min. The concentration of RASSF1 and hyper region in the stock solution were analyzed using a fluorospectrometric technique. The Finnzymes program (https://www.thermofisher.com/us/en/…/dna-copy-number-calculator.html) was used to calculate the initial copy number of the calibrator dilution in the standard series.

Statistical analysis

The experiments were performed in triplicate with the mean values obtained for analysis. Statistical software package Design-Expert 10 (Stat-Ease Inc., USA) was used for multiple regression analysis of the model data. The analysis of variance (ANOVA) employed to analysis the predicted response obtained from CCD to fit the following second-order polynomial Eq. 1:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_1\beta_1 A^2 + \beta_2\beta_2 B^2 + \beta_3\beta_3 C^2 + \beta_1\beta_2 AB + \beta_1\beta_3 AC + \beta_2\beta_3 BC \quad (1)$$

where Y is the response variable; β_0 is the constant coefficient; β_1, β_2, and β_3 are the linear coefficients; $\beta_{1,1}$ $\beta_{2,2}$, and $\beta_{3,3}$ are the squared coefficients; $\beta_{1,2}$, $\beta_{1,3}$, and $\beta_{2,3}$ are the interaction coefficients; and A, B, C, A^2, B^2, C^2, AB, AC, and BC are the level of independent variables.

Fisher's test value (F value) was used for determination of statistical significance of the quadratic polynomial. Analysis of contour and response surface plots obtained was used to determine the regression coefficients and to fit the

Table 4 Primer pairs used to amplify specific methylated sequences

Gene	GenBank accession no.[a]	Primer sequence	Primer position (hg18)[b]	Amplicon size (bp)
RASSF1	NG_023270	F: 5′-GGCGACTTCATCTGG-3′	50352784–50353036	253
		R: 5′-CTCTGCTCATCTGTGG-3′	50352803–50352968	166
		F: 5′-TACGAGAGCGGAAG-3′		
		R: 5′-AGATACGAGTGG-3′		
Hyper	AL590096.16	F: 5′-TGTAGAGATGTGGTATT-3′	19991227–191991445	219
		R: 5′-TCAGTTCAAGTGTTCA-3′	19991297–19991445	150
		F: 5′-CCAAGGATTCAGCC-3′		
		R: 5′-TCAGTTCAAGTGTTCA-3′		

[a] Accession numbers are for GenBank (http://www.ncbi.nlm.nih.gov/genbank)

[b] Human reference genome

mathematical models of the experimental results aiming to optimize the response variable. The determination coefficient R^2, the adjusted determination coefficient R^2, and the lack of fit were calculated to evaluate the adequacy of the model and the performance of the regression equation.

Data analysis of single factor experiments and qPCR was performed with Prism, version 8.0.1 (GraphPad Software, Inc., La Jolla, CA, USA) using one- and two-way ANOVA, respectively, followed by post hoc Tukey's multiple comparison or Bonferroni tests when appropriated. Data are presented as means GEq/ml ± standard deviation (SD), and p values ≤ 0.05 were considered statistically significant. All data represented by copy number are expressed as GEq/ml value (1 GEq/ml = 2 copies/ml = 6.6 pg/ml).

Results

The pooled plasma samples were from 20 male-bearing and 15 female-bearing pregnancies. At blood sampling, the median maternal age was 35 years (range 25 to 45 years).

The first study step focused on optimization of the cTHP method. Table 1 shows the single-factor experiment results to investigate the effects of digestion conditions, extraction buffers, chemical purifications, and precipitators on cfDNA extraction. As the results showed, digestion with PrK at 56 °C for 2 h was the optimum incubation time and temperature condition to improve the efficiency of methylated cffDNA extraction. Evaluation of the effect of alternative buffers on the of methylated cffDNA extraction showed that the use of the 1% SDS + Triton X-100 produced a greater methylated cffDNA concentration. By changing in chemical purifications, the results obtained were as follows: PCI > TRIzol > CTAB > PCI + BME. The effect of alternative precipitators showed that the maximum methylated cffDNA yield was achieved by ethanol-glycogen-sodium acetate.

The PB experimental design for interactive effects of eight variables on the methylated cffDNA extraction is shown in Table 2. The results showed broad variation in the methylated cffDNA concentration from 15.37 to 126.32 GEq/ml, reflecting the necessity to optimize the methylated cffDNA extraction condition from maternal plasma. In the PB step, the greatest concentration of cffDNA was attained from 200 μl maternal plasma using 1% SDS + Triton X-100 incubation at 90 °C for 5 min and precipitation with 0.5 μg/μl of glycogen (20 mg/ml), 0.5 M sodium acetate (pH 5.2), and 2.5 volumes of 100% ethanol for 1 h incubation at − 20 °C.

The results of ANOVA for each variable are shown in Table 5. The variables were screened at a confidence level of greater than 95%. Accordingly, SDS (C), glycogen (G), and sodium acetate (E) were found to be significant factors and were used for further optimization. Based on the experimental results, a first-order polynomial equation was derived to represent methylated cffDNA extraction as a function of the significant variables:

$$Y = 58.38 + 11.05\ \mathrm{C} - 17.73\ \mathrm{E} + 25.5\ \mathrm{G} \quad (2)$$

Table 5 Statistical analysis of the Plackett-Burman design

Source	Sum of squares	*df*	Mean square	*F* value	*p* value Prob > *F*
Model	13,042.14	3	4347.38	58.54	< 0.0001
C	1466.14	1	1466.14	19.74	0.0022
E	3770.29	1	3770.29	50.77	< 0.0001
G	7805.71	1	7805.71	105.12	< 0.0001
Residual	594.07	8	74.26		
Cor total	13,636.21	11			

$R^2 = 0.956$, $R^2_{\mathrm{Adj}} = 0.9401$, Pred $R^2 = 0.9020$

C SDS (%), *E* sodium acetate (M), *G* glycogen (μg/μl)

The "*F* value" and "Prob > *F*" of the model were 58.29 and < 0.0001, respectively, which imply that the model was highly significant. There was only a 0.01% chance that a model *F* value this large could occur due to noise. The signal-to-noise ratio of the model was measured by adequate precision, and a ratio greater than 4 was considered desirable. In this study, the ratio was found to be 14.76, indicating an adequate signal and suggesting that the model can be used to navigate the design space for attaining optimum conditions.

The CCD was used to evaluate the mathematical relationship between the significant variables and determine their optimal levels after 20 experiments. The experimental matrix and results are shown in Table 3. Multiple regression analysis of the experimental data was used to determine the second-order polynomial equation as:

$$\begin{aligned} Y = {} & 241.84 + 4.43A + 9.32B + 38.94C - 11.7AB \\ & + 8.72\,AC + 4.39\,BC \\ & + 54.26A^2 - 63.17B^2 - 34.15C^2 \end{aligned} \quad (3)$$

where Y is the predicted methylated cffDNA concentration (GEq/ml), and A, B, and C are coded values for SDS, glycogen, and sodium acetate concentration, respectively.

The detailed results of statistical analysis are shown in Table 6. The model *F* value of 35.27 indicates that the model is highly significant. A prob. > *F* of less than 0.05 indicates that the model terms are significant. In this case, C, A^2, B^2, and C^2 are significant model terms. The "Lack of Fit *F* value" of 0.2 indicates that the Lack of Fit is not significant relative to the pure error. There is a 96.15% chance that the lack of fit *F* value this large could occur due to noise. The coefficient of determination (R^2) was found to be 0.9695, supporting a strong correlation between experimental and predicted values. The predicted R^2 (0.911) and adjusted R^2 (0.942) values were

Table 6 Statistical analysis of the CCD optimization

Source	Sum of squares	*df*	Mean square	*F* value	*p* value Prob > *F*
Model	1.104E+005	9	12,265.07	35.27	< 0.0001
A	263.56	1	263.56	0.76	0.4044
B	1113.27	1	1113.27	3.20	0.1039
C	24,985.00	1	24,985.00	71.84	< 0.0001
AB	1095.84	1	1095.84	3.15	0.1063
AC	608.69	1	608.69	1.75	0.2153
BC	153.84	1	153.84	0.44	0.5210
A^2	38,656.32	1	38,656.32	111.16	< 0.0001
B^2	48,723.73	1	48,723.73	140.11	< 0.0001
C^2	18,685.95	1	18,685.95	53.73	< 0.0001
Residual	3477.64	10	347.76		
Lack of fit	1122.44	7	160.35	0.20	0.9615
Pure error	2355.20	3	785.07		
Cor total	1.139E+005	19			

$R^2 = 0.9696$, $R^2_{Adj} = 0.942$, Pred $R^2 = 0.911$

A SDS (%), *B* sodium acetate (M), *C* glycogen (μg/μl)

closely correlated as well. The coefficient of variation (CV) of the model was 13.7% revealing the precision and reliability of the experiments.

The results of statistical analysis showed that glycogen (C) was significant, but the values of SDS (A) and sodium acetate concentration (B) were not significant. It also was shown that the interaction between A^2, B^2, and C^2 significantly affected the methylated cffDNA extraction by THPG method, but interactions AB, AC, and BC had no significant influence.

The 3D response surface plots and contour graphs of variables interacting on the methylated cffDNA extraction are depicted in Fig. 1. The interactions between the actual level of process variables and the response are clearly understood by these graphs. Figure 1a shows the 3D response surface and contour plots for methylated cffDNA extraction with varying concentration of sodium acetate and SDS at fixed glycogen concentration (0.75 μg/μl). The optimum condition for methylated cffDNA extraction was found to be at sodium acetate and SDS concentration of 0.3 M and 3%, respectively. In Fig. 1b, the 3D response surface and contour plots show the extraction yield of methylated cffDNA as a function of glycogen and SDS concentration at a fixed sodium acetate concentration. Higher methylated cffDNA yield was obtained with increasing glycogen concentrations ranging from 0.4 to 0.9 μg/μl. The extraction yield of methylated cffDNA affected by different glycogen and sodium acetate concentrations is given in Fig. 1c, when the SDS concentrations were maintained at its optimum. The response curves show that increased glycogen concentration resulted in increased extraction yield, within the chosen experimental range. Accordingly, the maximum concentration of extracted methylated cffDNA from maternal plasma was obtained using 3% SDS-1% Triton X-100, 0.9 μg/μl of glycogen (20 mg/ml), and 0.3 M sodium acetate, and the best OD ratio of 260/280 was 1.8.

In the second step, the extraction efficiencies of tested methods for both methylated cfDNA and cffDNA from maternal plasma were compared based on the GEq/ml values (Fig. 2). The methylated cfDNA was captured and followed by MeDIP protocol without fail. The presence of methylated cfDNA and methylated cffDNA was confirmed using the hyper region and RASSF1 gene, respectively, with real-time qPCR. The mean methylated cffDNA GEq/ml value extracted using the THPG method was 202.2 ± 7.04, using the cTHP method was 166.78 ± 7.8, using DSP kit was 89 ± 8.4, and using NPM kit was 65.3 ± 3.8.

Discussion

After the discovery of cffDNA in maternal plasma in 1997 [20], many efforts have been made to develop a new method for extracting high-quality fetal DNA to improve NIPT for fetal disorders. However, the low quantity of the cffDNA in maternal plasma and the high level of molecular similarity between it and maternal DNA are major technical challenges to ideal extraction [5]. Moreover, the amount of cfDNA and cffDNA extracted from maternal plasma are proportional [21]. Therefore, it is essential to use cffDNA enrichment methods to decrease false negative results and improve the accuracy of subsequent analysis. To our knowledge, this is the first study to optimize the methylated cffDNA extraction process from maternal plasma using statistical software "Design Expert" for use in NIPT.

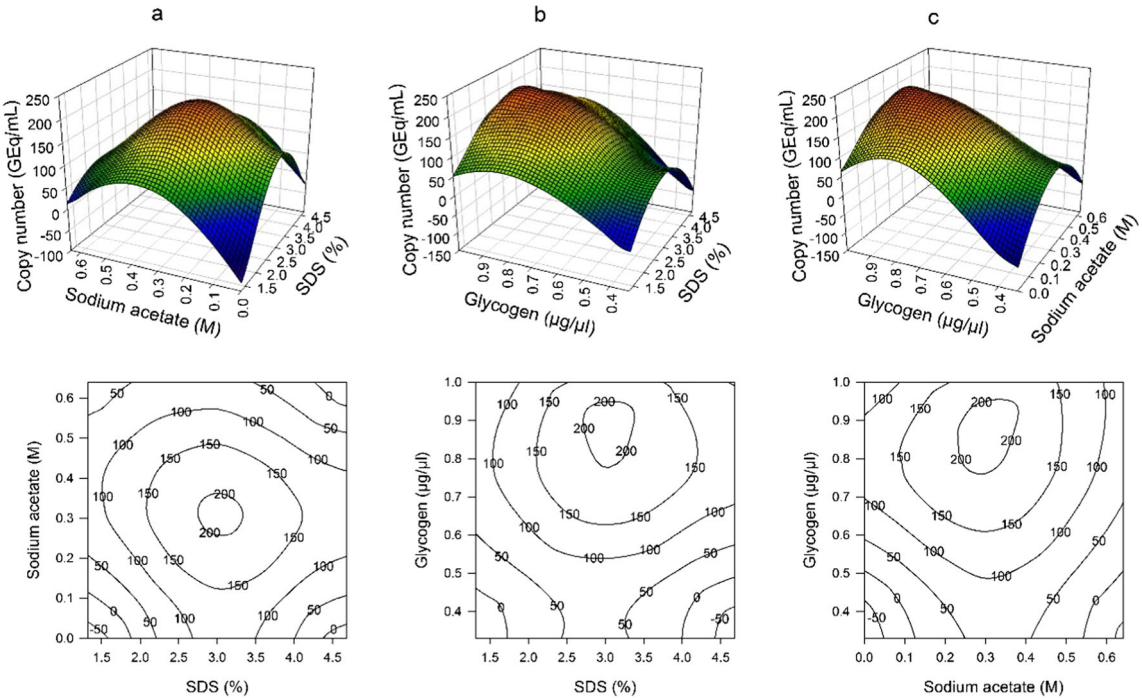

Fig. 1 Response surface and contour graphs of the effect of sodium acetate, SDS, and glycogen on methylated cffDNA extraction. **a** Sodium acetate and SDS. **b** Glycogen and SDS. **c** Glycogen and Sodium acetate

Given that cffDNA is highly fragmented and it has different size distributions [22], designing a method can affect DNA yield and purity. Most published comparison studies focused on various types of commercial kits for total cfDNA extraction from plasma. We studied the effect of different factors on cffDNA extraction using the cTHP method. The extraction efficiency of the THPG method compared with that of cTHP and the DSP and NPM kits was assessed by MeDIP real-time qPCR for extraction of methylated cffDNA.

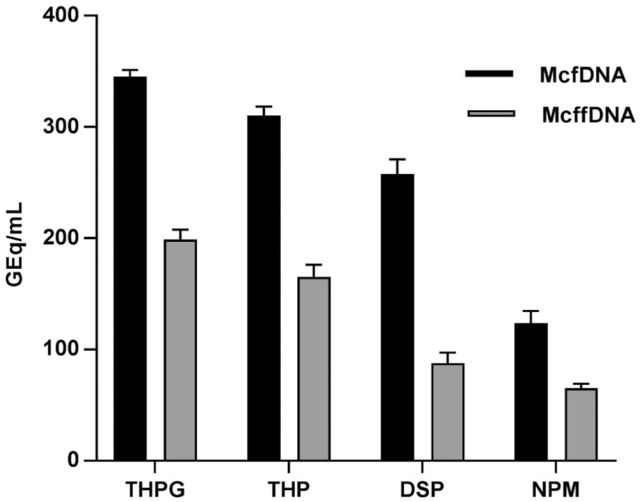

Fig. 2 Intramethod comparison of the performance of methylated cffDNA and cfDNA extraction methods. McfDNA methylated cell-free DNA, McffDNA methylated cell-free fetal DNA, THPG Triton/Heat/Phenol/Glycogen, THP Triton/Heat/Phenol, DSP QIAamp DSP Virus Kit, NPM NextPrep-Mag™ cfDNA Isolation Kit

Recent studies have tested the performance of cTHP method, the DSP, and NPM kits for extracting cffDNA from plasma [14, 18, 23, 24]. Xue et al. showed that the cTHP method has high efficiency to extract the short DNA fragments smaller than 100 bp [18]. Moreover, Keshavarz et al. evaluated the efficiency of cTHP method in extraction of cffDNA and cfDNA from plasma samples of pregnant and non-pregnant women, respectively [16]. They have demonstrated that DNA quantity was improved using cTHP method which is consistent with our results.

Comparison of the results of fetal DNA extraction methods in an international workshop indicates that the DSP kit provided higher cffDNA yield from maternal plasma [23]. In fact, the use of carrier RNA in the DSP kit enhances the affinity of the short DNA fragments for the membrane column to further improve the DNA recovery [14, 25]. Accordingly, in THPG method, glycogen was used as carrier RNA replacement for precipitation of cffDNA in the presence of salt and alcohol [26]. We found that the use of this co-precipitant in extraction significantly increased DNA yield. During the optimization of extraction, it was noticed that sodium acetate (pH 5.2) and glycogen play a significant role in obtaining high-quantity methylated cfDNA and methylated cffDNA from maternal plasma.

In an early study carried out by Jorgez et al., magnetic-beads kit has shown most efficient in cffDNA extraction from maternal blood without false-positive results in detection of total and fetal sequences by real-time qPCR [17]. In contrast, in this study, the methylated DNA quantity extracted by NPM

kit was significantly lower than other tested protocol. This is consistent with findings of Lim et al., who showed that although the magnetic bead kit is cheaper and quicker to use than the DSP kit, it is not suitable for extracting low copies of methylated cffDNA from maternal plasma for downstream analyses [24].

The statistical results show that incubation of PrK at 57 °C for 2 h improved the DNA yield, probably due to an increase in activity at higher temperatures. This contradicts the observations of Xue et al., who reported that incubation at 37 °C for 1 h was better in the DNA recovery [18]. As in past study, the use of PrK combined with 3% SDS achieved about 50% higher DNA yields than did 1% SDS [27].

Intramethod comparison revealed that the GEq/ml values of methylated cffDNA and methylated cfDNA isolated by THPG were significantly higher than other tested protocol ($p < 0.001$). As the results demonstrated, enrichment of methylated cfDNA following extraction using THPG method from maternal plasma can be improved, which could be potentially useful for further analysis of fetal DNA to increase accuracy of NIPT results. The THPG method also is simple, quick, and inexpensive to use so that the estimated total cost per case was $2.5 and several-fold lower than the tested commercial kits.

In summary, the data indicates that THPG is a more efficient extraction method than the other tested protocols to extract low copy number methylated cfDNA and methylated cffDNA from a small volume of maternal plasma. It also can reduce the costs of noninvasive prenatal diagnosis significantly. Furthermore, the reproducible and reliable results of qPCR using methylated cffDNA extracted using the THPG method may prove its suitability for use in clinical routines.

Compliance with ethical standards

The study was approved by the ethical committee of the Tehran University of Medical Sciences, Iran (IR.TUMS.REC.1394.1156). All participant provided written informed consent prior to venipuncture.

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