

Effective Method for Extraction of Cell-Free DNA from Maternal Plasma for Non-Invasive First-Trimester Fetal Gender Determination: A Preliminary Study

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Purpose: To find the most effective method for extraction of cell-free DNA (cf-DNA) from maternal plasma, we compared a blood DNA extraction system (blood kit) and a viral DNA extraction system (viral kit) for non-invasive first-trimester fetal gender determination.

Materials and Methods: A prospective cohort study was conducted with maternal plasma collected from 44 women in the first-trimester of pregnancy. The cf-DNA was extracted from maternal plasma using a blood kit and a viral kit. Quantitative fluorescent-polymerase chain reaction (QF-PCR) was used to detect the *SRY* gene and *AMEL* gene. The diagnostic accuracy of the QF-PCR results was determined based on comparison with the final delivery records.

Results: A total of 44 women were tested, but the final delivery record was only obtained in 36 cases which included 16 male-bearing and 20 female-bearing pregnancies. For the blood kit and viral kit, the diagnostic accuracies for fetal gender determination were 63.9% (23/36) and 97.2% (35/36), respectively.

Conclusion: In non-invasive first-trimester fetal gender determination by QF-PCR, using a viral kit for extraction of cf-DNA may result in a higher diagnostic accuracy.

Key Words: Cell free DNA (cf-DNA), Quantitative fluorescent-polymerase chain reaction (QF-PCR), Non-invasive fetal gender detection

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Introduction

Since the detection of fetal cell free-DNA (cf-DNA) in maternal plasma¹⁾, maternal plasma has emerged as a new material for non-invasive prenatal diagnostics research. Numerous groups have reported on fetal Rhesus D genotyping and fetal gender determination using the cf-DNA in maternal plasma²⁻⁶⁾. However, the small quantity of fetal cf-DNA in maternal plasma is still considered to be the most serious problem in non-inva-

sive prenatal diagnosis using maternal plasma. Therefore, the smallest improvement in fetal cf-DNA yield may have a great impact on the success of non-invasive prenatal diagnosis.

Recent findings have shown that the length of most fetal DNA fragments in maternal plasma is <300 bp^{7, 8)}. In prior reports, a size fractionation method was suggested for enrichment of small size fetal cf-DNA in maternal plasma^{7, 8)}. The use of this method resulted in a 28.4% increase in the amount of fetal DNA per <300 bp fraction in maternal plasma samples obtained early in pregnancy⁸⁾. However, this method required a large volume of maternal plasma and additional technical processes such as agarose gel electrophoresis and re-extraction of cf-DNA from the agarose section.

Recently, an extraction system optimized for viral DNA was suggested for increase of fetal cf-DNA in maternal plasma, because it favors small-size DNA fragments⁹⁻¹¹⁾. In a workshop report about cf-DNA, Legler et al. suggested that using the DSP Virus Kit may be an optimal method for extracting a high yield of fetal cf-DNA from total plasma¹¹⁾. We have thus performed a comparative analysis of the diagnostic accuracies of the two methods, a blood DNA extraction system (blood kit) and a viral DNA extraction system (viral kit), to determine which is more effective at extracting cf-DNA from maternal plasma for use in non-invasive first-trimester fetal gender determination.

Materials and Methods

1. Sample collection and processing

We performed a prospective study of women who enrolled in the Cheil General Hospital Non-invasive Prenatal Diagnosis Study (CNPD). In brief, the CNPD cohort was established in 2009 for the prospective study of non-invasive prenatal diagnosis of fetal rare and incurable diseases during the first trimester in pregnancy. Women who received antenatal care at Cheil

General Hospital were eligible for inclusion in the cohort. For the current study, women with singleton gestations between February, 2009, and May, 2009, who enrolled in the CNPD cohort at or before 12 weeks of gestation were eligible for inclusion. Maternal, fetal, and infant records were collected prospectively and were maintained in an electronic database. Under institutional review board approval from the Ethics Committee at Cheil General Hospital (#CGH-IRBGR-2008-07), 44 pregnant women gave informed consent to participate in the study. Gestational age was 5-12 weeks, calculated from last menstruation and confirmed by ultrasound. No subjects had history of preexisting hypertension, diabetes mellitus, liver disease, or chronic kidney disease and all were of Korean origin.

Ten milliliters of peripheral blood were obtained from each participant using ethylenediaminetetraacetic acid (EDTA) as an anti-coagulant. Immediately after blood sampling, the plasma was separated from the whole blood by centrifugation at 2,500 g for 10 min. Recovered plasma was then centrifuged for an additional 10 min at 16,000 g. The cf-DNA extraction and fetal gender detection were performed within four hours of sampling.

2. Cf-DNA extraction and fetal gender detection

The cf-DNA from 500 µL of maternal plasma was extracted using a DSP Virus Kit (Qiagen, Germany) and a blood DNA kit (Qiagen, USA) according to the manufacturer's instructions. The DNA was eluted into 50 µL sterile, DNase-free water. Extracted DNA was stored at 4°C until quantitative fluorescent-polymerase chain reaction (QF-PCR) analysis.

QF-PCR was used to detect the *SRY* gene and the *AMEL* gene. Each forward primer was labeled with 6-carboxyfluorescein, and the primers for PCR were as follows: for the *SRY* gene-forward: 5'-TGGCGATTAA GTCAAATTCGC-3', reverse: 5'-CCCCCTAGTACCCT GACAATGTATT-3'; for the *AMEL* gene - forward: 5'-CCCTGGGCTCTGTAAAGAATAGT-3', reverse: 5'-AT CAGAGCTTAAACTGGGAAGCTG-3'. To amplify the

target regions in the two genes, the PCR reaction solution contained 10 pM primers, 0.25 mM dNTPs, 1.5 mM MgCl₂, 10X buffer, 0.25 U Taq polymerase, and 3 μL cf-DNA per 10 μL total reaction volume. For the *SRY* gene, PCR conditions included predenaturation at 94°C for 15 min, 35 cycles of 94°C for 30 sec, 60°C for 10 sec, 72°C for 30 sec, and a final extension at 60°C for 30 min. For the *AMEL* gene, PCR conditions included predenaturation at 94°C for 5 min, 30 cycles of 94°C for 30 sec, 60°C for 60 sec, 72°C for 30 sec, and final extension at 60°C for 30 min. After PCR amplification, the fluorescently labeled DNA fragments were analyzed on an Applied Biosystems 3100 Avant genetic analyzer (Applied Biosystems Inc., CA) using the Pop-7 polymer and GeneScan-500 LIZ size standard (Applied Biosystems Inc., CA). All tests were performed in triplicate.

The *SRY* gene in Y chromosome was used as specific marker to detect male fetus and represented a 136 bp fragment. The *AMEL* gene presents in both X and Y chromosome. Primer set used in this study was designed to produce PCR products of different lengths for each chromosome. Therefore, the *AMEL* gene in X and Y chromosome showed as a 105 bp and a 110 bp fragment,

respectively. The *AMEL* gene in Y chromosome (*AMY* gene) was used to confirm male fetus in company with the *SRY* gene, while the *AMEL* gene in X chromosome (*AMX* gene) was used to detect total cf-DNA in maternal plasma.

3. Statistical analysis

Results of QF-PCR were compared with the final delivery records in an electronic database. The diagnostic accuracy of fetal gender determination was calculated by dividing the number of correct results from QF-PCR by the total number of women with a final delivery record, using the Microsoft Excel program.

Results

A total of 44 women were tested. The median gestational age was 10^{0/7} weeks (range 5^{6/7} to 12^{3/7} weeks). Test results for both genes were obtained from all subjects and with both extraction systems. The final delivery records were obtained in only 36 cases, the other eight cases did not give birth in our hospital. The 36 cases included 16 male-bearing and 20 female-

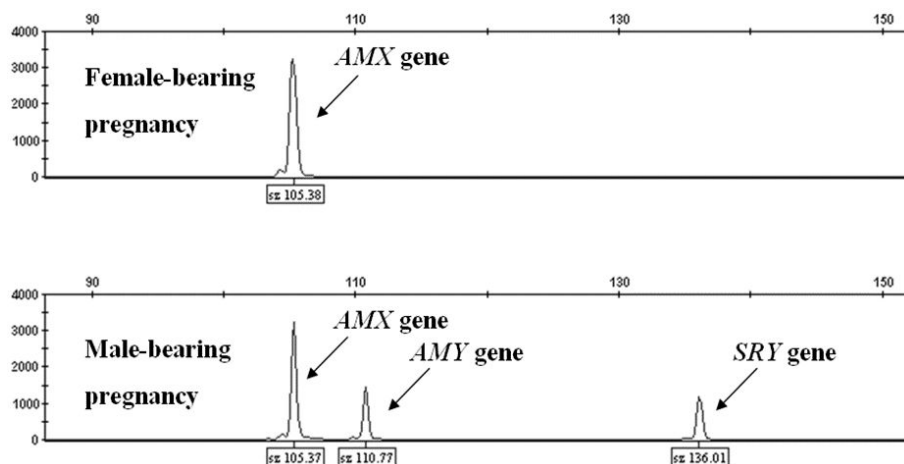


Fig. 1. QF-PCR results for non-invasive fetal gender determination. In male-bearing pregnancies, the cf-DNA showed three peaks including a 136 bp fragment representing the *SRY* gene, a 110 bp fragment representing the *AMY* gene, and a 105 bp fragment representing the *AMX* gene. In female-bearing pregnancies, the cf-DNA showed only one peak for the 105 bp fragment representing the *AMX* gene. Abbreviations: *AMX* gene, *AMEL* gene in X chromosome; *AMY* gene, *AMEL* gene in Y chromosome.

bearing pregnancies.

Fig. 1 shows the QF-PCR results of the two genes according to the fetal gender. In male-bearing pregnancies, the cf-DNA produced three peaks, including a 136 bp fragment representing the *SRY* gene, a 110 bp fragment representing the *AMY* gene, and a 105 bp fragment representing the *AMX* gene. In female-bearing pregnancies, the cf-DNA showed only one peak for the 105 bp fragment representing the *AMX* gene.

Using a blood kit and a viral kit, we observed fetal gender diagnostic accuracies according to gestational weeks and fetal gender. Table 1 shows the diagnostic accuracies of the two protocols according to gestational weeks. Using a blood kit showed various accuracies of the fetal gender detection according to gestational weeks, whereas using a viral kit showed high diagnostic accuracies regardless of gestational weeks. We observed final diagnostic accuracies of 63.9% (23/36) and 97.2% (35/36) in using a blood kit and a viral kit, respectively. In addition, the diagnostic accuracies of the two protocols were comparable according to fetal gender (Table 2).

Table 1. Diagnostic Accuracies of the Two Protocols According to Gestational Weeks

Gestational weeks	n	Diagnostic accuracy (%)	
		Blood kit	Viral kit
5	1	100.0 (1/1)	100.0 (1/1)
6	4	75.0 (3/4)	100.0 (4/4)
7	4	25.0 (1/4)	100.0 (4/4)
8	9	77.8 (2/9)	100.0 (9/9)
9	1	100.0 (1/1)	100.0 (1/1)
10	1	0.0 (1/0)	100.0 (1/1)
11	2	100.0 (2/2)	100.0 (2/2)
12	14	57.1 (8/14)	92.9 (13/14)
Total	36	63.9 (23/36)	97.2 (35/36)

Total : total women with the final delivery records

Using a blood kit, the diagnostic accuracy of female-bearing pregnancies was 70.0% (14/20) and the diagnostic accuracy of male-bearing pregnancies was 56.3% (9/16). A false positive finding of female-bearing pregnancies was observed in 30% (6/20) and a false negative finding of male-bearing pregnancies was observed in 43.7% (7/16). Using a viral kit, the diagnostic accuracy of female-bearing pregnancies was 100% (20/20) and the diagnostic accuracy of male-bearing pregnancies was 93.8% (15/16). A false negative finding of male-bearing pregnancies was observed only in one out of 16 cases (6.2%).

Discussion

Our goal of this study was to determine the most effective method for extraction of fetal cf-DNA from maternal plasma for use in non-invasive fetal gender determination. To our knowledge, this is the first study comparing these two extraction methods for use in detecting fetal gender using QF-PCR and cf-DNA from maternal plasma.

With regard to fetal gender detection, our data demonstrated that QF-PCR results using a viral kit showed a high diagnostic concordance (97.2%) with the results confirmed phenotypically at birth regardless of gestational weeks. Moreover, using a viral kit revealed 100% concordance in the non-invasive first-trimester fetal gender detection of female-bearing pregnancies. This result can be induced by only amplification of maternal DNA in maternal plasma. However, we could not detect differences in amplifications of maternal and fetal fragments in this study. Therefore, further studies using a viral kit are required to clarify accuracy in detection of

Table 2. Diagnostic Accuracies of the Two Protocols According to Fetal Gender

	Diagnostic accuracy (%)			
	Male (n=16)	Female (n=20)	False negative (n=16)	False positive (n=20)
Blood kit	56.3 (9/16)	70.0 (14/20)	43.7 (7/16)	30.0 (6/20)
Viral kit	93.8 (15/16)	100.0 (20/20)	6.2 (1/16)	0.0 (0/20)

fetal fragments. In addition, QF-PCR using a blood kit showed a low diagnostic concordance (63.9%) for non-invasive fetal gender determination in this study, whereas QF-PCR using a blood kit led to the correct diagnosis of fetal gender in 92.0% of the 81 cases in a prior study¹²⁾. This discrepancy may be based on the difference in gestational weeks at blood sampling and the use of other Y chromosome-specific genes such as DYS390. Therefore, the diagnostic accuracy with the blood kit needs to be further addressed via QF-PCR analysis for various Y chromosome-specific markers in the larger cohort at the first trimester of pregnancy as our study.

The analysis of fetal genetic loci in maternal plasma is still problematic because fetal cf-DNA is only present in small amounts in maternal plasma. Therefore, various molecular methods have been applied to enrich fetal cf-DNA in maternal plasma and to detect a small quantity of fetal cf-DNA.

In recent reports, Rijnders et al. were able to detect fetal DNA at five weeks of gestation using the Ultrasens Virus Kit, and Clausen et al. reported the DSP Virus Kit gave a higher yield of fetal cf-DNA than of total cf-DNA from maternal plasma^{9,10)}. Moreover, in a workshop report about cf-DNA, Legler et al. reported that the DSP Virus Kit was an optimal method for extracting a high yield of fetal cf-DNA from total plasma¹¹⁾. These investigations indicate that an extraction system optimized for viral DNA may selectively enrich for the circulatory fetal DNA sequences in maternal plasma. Our data also showed that using a viral kit is more effective than using a blood kit in early non-invasive fetal gender determination via QF-PCR.

The introduction of fluorescence-based PCR such as real-time PCR and QF-PCR has increased the number of potential applications in non-invasive prenatal diagnostic research. QF-PCR has proven to be a rapid, highly reliable and highly accurate screening strategy, capable of detecting chromosomal abnormalities in the fetus¹³⁻¹⁶⁾. Although this technique is now commonly employed in

routine clinical practice and is often employed as an initial diagnostic method for aneuploidy detection, it is required for fetal genomic DNA, which is typically isolated by invasive procedures such as amniocentesis and chorionic villus samples¹³⁻¹⁶⁾. In the non-invasive prenatal diagnostic studies using QF-PCR, González-González et al. reported that QF-PCR using cf-DNA from maternal plasma was used for the detection of fetal gender and to make a diagnosis of Huntington's disease¹²⁾. We were also able to detect a high diagnostic concordance of fetal gender via QF-PCR using cf-DNA in maternal plasma during the first trimester of pregnancy. These data indicate that the high sensitivity of this technique may provide the ability to detect a small quantity of fetal cf-DNA in maternal plasma. However, we could not confirm whether peak height or area of each fragment in QF-PCR may represent a quantity of cf-DNA in maternal plasma. Therefore, quantification of cf-DNA by QF-PCR is needed to confirm via other molecular method as real-time PCR.

Our data indicate that fetal gender may be successfully detected early using cf-DNA from circulating maternal plasma; in addition, QF-PCR using cf-DNA extracted using a viral kit may be an effective method for non-invasive first-trimester fetal gender determination. Therefore, we suggest that the use of this technique may prove useful in non-invasive first-trimester fetal gender determination. Furthermore, this technique may allow for the implementation of more effective targeted therapeutic strategies via early non-invasive prenatal diagnosis of genetic disorders related with fetal gender.

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국문초록

목적: 모체 혈장으로부터 가장 효과적으로 세포 유리 DNA

(cell free DNA, cf-DNA)를 추출하는 방법을 찾기 위해 우리는 viral DNA 추출 방법과 일반 혈액 DNA 추출 방법을 이용하여 비침습적 임신 초기 태아 성별 확인 결과를 비교하였다.

대상 및 방법: 임신 초기 44명의 임신부로부터 모여진 모체 혈장을 통한 전향적 연구가 구성되었다. Cf-DNA는 viral DNA 추출 방법과 일반 혈액 DNA 추출 방법을 이용하여 각각 추출되었다. 정량 형광-중합효소 연쇄 반응(QF-PCR)을 이용하여 *SRY*와 *AMXY* 유전자를 검출하였다. QF-PCR의 진단 정확도는 최종 분만 기록을 토대로 결정하였다.

결 과: 전체 44명의 여성이 실험에 참여하였지만, 최종 분만 기록은 단지 36명의 여성에서 획득하였다. 이들 중 16명은 남아를 20명은 여아를 임신하였다. 두 추출 방법에서 태아 성별의 진단적 정확도는 일반 혈액 DNA 추출 방법에 경우 63.9% (23/26)였으며 viral DNA 추출 방법에 경우 97.2% (35/36)였다.

결 론: QF-PCR을 이용한 비침습적 임신 초기 태아 성별 확인에 있어 viral kit를 사용하는 것이 높은 진단적 정확도를 이끌 수 있을 것으로 사료된다.

References

- Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. *Lancet* 1997;350:485-7.
- Lo YM, Hjelm NM, Fidler C, Sargent IL, Murphy MF, Chamberlain PF, et al. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. *N Engl J Med* 1998;339:1734-38.
- Rijnders RJ, van der Schoot CE, Bossers B, de Vroede MA, Christiaens GC. Fetal sex determination from maternal plasma in pregnancies at risk for congenital adrenal hyperplasia. *Obstet Gynecol* 2001;98:374-8.
- Bianchi DW, Avent ND, Costa JM, van der Schoot CE. Noninvasive prenatal diagnosis of fetal Rhesus D: ready for Prime(r) Time. *Obstet Gynecol* 2005;106:841-4.
- Geifman-Holtzman O, Grotegut CA, Gaughan JP. Diagnostic accuracy of noninvasive fetal Rh genotyping from maternal blood: a meta-analysis. *Am J Obstet Gynecol* 2006;195:1163-73.
- Scheffer PG, van der Schoot CE, Page-Christiaens GC, Bossers B, van Erp F, de Haas M. Reliability of fetal gender determination using maternal plasma. *Obstet Gynecol* 2010;115:117-26.
- Chan KC, Zhang J, Hui AB, Wong N, Lau TK, Leung TN, et al. Size distributions of maternal and fetal DNA in maternal plasma. *Clin Chem* 2004;50:88-92.
- Li Y, Zimmermann B, Rusterholz C, Kang A, Holzgreve W, Hahn S. Size separation of circulatory DNA in maternal plasma permits ready detection of fetal DNA polymorphisms. *Clin Chem* 2004;50:1002-11.
- Rijnders RJP, Van Der Luijt RB, Peters ED, Goeree JK, Van Der Schoot CE, Ploos Van Amstel JK, et al. Earliest gestational age for fetal gendering in cell-free maternal plasma. *Prenat Diagn* 2003;23:1042-4.
- Clausen FB, Krog GR, Rienck K, Dziegiel MH. Improvement in fetal DNA extraction from maternal plasma. Evaluation of the NucliSens Magnetic Extraction system and the QIAamp DSP Virus Kit in comparison with the QIAamp DNA Blood Mini Kit. *Prenat Diagn* 2007;27:6-10.
- Legler TJ, Liu Z, Mavrou A, Hromadnikova I, Galbiati S, Meaney C, et al. Workshop report on the extraction of foetal DNA from maternal plasma. *Prenat Diagn* 2007;27:824-829.
- González-González C, García-Hoyos M, Trujillo-Tiebas MJ, Lorda-Sánchez I, de Alba MR, Infantes F, et al. Application of fetal DNA detection in maternal plasma: a prenatal diagnosis unit experience. *J Histochem Cytochem* 2005;53:307-14.
- Pertl B, Weitgasser U, Kopp S, Kroisel PM, Sherlock J, Adinolfi M. Rapid detection of trisomies 21 and 18 and gendering by quantitative fluorescent multiplex PCR. *Hum Genet* 1996;98:55-9.
- Pertl B, Kopp S, Kroisel PM, Tului L, Brambati B, Adinolfi M. Rapid detection of chromosome aneuploidies by quantitative fluorescence PCR: First application on 247 chorionic villus samples. *J Med Genet* 1999;36:300-3.
- Schmidt W, Jenderny J, Hecher K, Hackelö er BJ, Kerber S, Kochhan L, et al. Detection of aneuploidy in chromosomes X, Y, 13, 18 and 21 by QF-PCR in 662 selected pregnancies at risk. *Mol Hum Reprod* 2000;6:855-60.
- Cirigliano V, Ejarque M, Canadas MP, Lloveras E, Plaja A, Perez MM, et al. Clinical application of multiplex quantitative fluorescent polymerase chain reaction (QF-PCR) for the rapid prenatal detection of common chromosome aneuploidies. *Mol Hum Reprod* 2001;7:1001-6.