

Segmental Duplications as a Complement Strategy to Short Tandem Repeats in the Prenatal Diagnosis of Down Syndrome

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What's Known

- For the prenatal diagnosis of aneuploidies, short tandem repeats (STRs) have already been used.
- Some STR markers can be uninformative in a portion of patients, especially in countries with high rates of consanguineous marriage.

What's New

- Designing and validating a new PCR-based diagnostic method based on the simultaneous amplification of segmental duplications and STRs for the prenatal diagnosis of Down syndrome.
- This new method is applicable, especially in countries with high rates of consanguineous marriage such as Iran.

Abstract

Background: Quantitative fluorescence-polymerase chain reaction (QF-PCR) is an inexpensive and accurate method for the prenatal diagnosis of aneuploidies that applies short tandem repeats (STRs) as a chromosome-specific marker. Despite its apparent advantages, QF-PCR is not applicable in all cases due to the presence of uninformative STRs. This study was carried out to investigate the efficiency of a method based on applying segmental duplications (SDs) in conjunction with STRs as an alternative to stand-alone STR-based QF-PCR for the diagnosis of Down syndrome.

Methods: Fifty amniotic fluid samples from pregnant women carrying Down syndrome fetuses, 9 amniotic fluid samples with 1 or without any informative STR marker (inconclusive), and 100 normal samples were selected from Shiraz, Iran, between October 2015 and December 2016. Analysis was done using an in-house STR-SD-based multiplex QF-PCR and the results were compared. Statistical analysis was performed using MedCalc, version 14.

Results: All the normal, Down syndrome, and inconclusive samples were accurately identified by the STR-SD-based multiplex QF-PCR, yielding 100% sensitivity and 100% specificity. Karyotype analysis confirmed all the cases with normal or trisomic results.

Conclusion: The STR-SD-based multiplex QF-PCR correctly identified all the normal and trisomy 21 samples regardless of the absence of informative STR markers. The STR-SD-based multiplex QF-PCR is a feasible and particularly useful assay in populations with a high prevalence of homozygote STR markers.

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Keywords • Multiplex polymerase chain reaction • Microsatellite repeats • Down syndrome • Segmental duplications

Introduction

All pregnant women considered to have increased risk factors or, less commonly, to be anxious about pregnancy should be offered a prenatal diagnostic test for the confirmation of fetal chromosome aneuploidy.¹ The most common numerical variations of chromosomes recognized in prenatal samples are 13, 18, or 21 trisomies and sex chromosome aneuploidies, representing approximately 70% of all chromosomal anomalies detected.²

Since these syndromes are incurable, prenatal diagnosis is imperative. Various methods have been introduced for the antenatal diagnosis of these syndromes such as karyotyping, a common diagnostic test for chromosomal abnormalities, which typically takes between 2 and 4 weeks.³ This conventional cytogenetic technique has been the gold standard for the prenatal diagnosis of chromosomal abnormalities for the past decades. Although some karyotyping barriers have been to some extent prevailed over with fluorescence in situ hybridization (FISH), both of these techniques have some drawbacks.⁴⁻⁷ Therefore, a rapid and accurate molecular test to exclude the most common chromosomal anomalies in all women undergoing amniocentesis or chorionic villus sampling is a necessity.² Several polymerase chain reaction (PCR)-based molecular methods such as quantitative fluorescence-PCR (QF-PCR) have been developed to address these problems. The QF-PCR approach is the most widely used, highly accurate, and robust PCR method and its results are available in less than 48 hours.⁵ Many studies have confirmed the effectiveness and accuracy of QF-PCR.^{2, 8-12} Since an authentic QF-PCR assay result can be achieved in the presence of at least 2 heterozygote (informative) short tandem repeat (STR) markers, in some cases the result remains inconclusive due to homozygote (uninformative) STR markers. As a major drawback of this technique, informative polymorphism sites observed in one human population may be uninformative in another,⁴ so a population-specific study of STR markers is needed for a certain population. To that end, successful studies using segmental duplication (SD) QF-PCR^{4, 5} and paralogous sequence quantification¹³ have been carried out. These approaches are associated with some drawbacks such as inability to specify the origin of the extra chromosome and failure to detect technician errors, low-level mosaicism, and maternal cell contamination.⁵ SDs are blocks of DNA with similar sequences and different fragment lengths that map 2 or more genomic locations.^{10, 14} These sequences, when co-amplified using a single pair of fluorescent primers, maintain the original ratio between the 2 different chromosomes. The PCR products are subsequently analyzed through capillary electrophoresis, and the aneuploidies are characterized based on the relative dosage between the 2 chromosomes. According to the mentioned descriptions, the simultaneous application of STRs and SDs can overcome the limitations of QF-PCR in the prenatal diagnosis of Down syndrome.

The aim of the present study was to design and

validate a new PCR-based diagnostic method based on the simultaneous amplification of SDs and STRs on different autosomal chromosomes for the detection of trisomy 21.

Materials and Methods

Sample Collection

From October 2015 to December 2016, a total of 3167 amniotic fluid samples were collected in the Comprehensive Genetic Center, Shiraz, Iran. These samples were taken from pregnant women who were at high risk for Down syndrome according to the first or second trimester screening tests, which showed that the risk of trisomy was greater than the screening cutoff point. Among them, 50 samples affected with trisomy 21, 100 normal samples, and 9 samples with inconclusive results according to the Devyser Resolution 21 v2 Kit were selected for further investigations. All the samples (N=159) were numbered randomly and tested blindly.

DNA Extraction

Genomic DNA was extracted from the amniotic fluid samples using the QIAamp DNA Mini Kit (QIAGEN, GmbH, Germany) according to the manufacturer's instructions. The concentration of the extracted genomic DNA was determined by measuring the ultraviolet absorbance at 260 nm with the NanoDrop Lite spectrophotometer (Thermo Scientific, USA). About 30 to 50 ng of the genomic DNA was used for QF-PCR.

Assay Design

The most appropriate SDs were found by BLASTing the sequence of chromosome 21 against the human genome based on the following criteria: (a) the segments of chromosome 21 and reference chromosomes were amplified simultaneously with a single primer set, and the amplicons were different in sizes due to size variation and (b) sex chromosomes and some autosomal chromosomes such as 13 and 18 were not selected as reference chromosomes since they have a higher chance of numerical abnormalities than other autosomes. The most appropriate markers for our population were selected through an analysis of 1000 amniotic fluid QF-PCR results and calculation of the heterozygosity of 12 markers using the PowerStats, version 12 (Promega Corp., Madison, WI, USA), in a previous study.^{11, 15} Seven STR markers with the highest heterozygosity were selected covering the Down syndrome critical region, 21q21.22. After different pairs of primers for the simultaneous amplification of

STRs and SDs were designed, the best primers for capillary electrophoresis were selected via the trial-and-error strategy. The sensitivity, specificity, positive predictive value, and negative predictive value were calculated using MedCalc, version 14 (MedCalc Software, Belgium).

STR-SD-Based Multiplex QF-PCR

We developed an in-house STR-SD-based multiplex QF-PCR comprising 3 segmental duplicates and 8 STR markers located on chromosome 21 (table 1). For each marker, one of the forward or reverse primers was labeled with fluorescent dyes: 6-FAM, VIC, PET, and NED (ABI, USA). The reaction mixture was premixed 2x solution TEMPase Hot Start DNA Polymerase (Ampliqon, Denmark). The PCR cycling conditions were as follows: initial denaturation at 95 °C for 15 minutes, followed by 27 cycles at 94 °C for 50 seconds, 58 °C for 1 minute 30 seconds, and 72 °C for 1 minute 30 seconds, with final synthesis at 72 °C for 30 minutes.

Electrophoretic Analysis

The PCR products were separated and visualized using the ABI 3500 genetic analyzer with a 50-cm capillary array length and the Performance Optimizing Polymer 7 (POP 7). The run time and sizing standard were 1500 seconds and ABI LIZ 500, respectively. The detected peaks were analyzed using the Gene mapper ID, version 4.2. The relative dosage of the chromosomes was calculated based on (a) height and area of the SD peaks and (b) the number, height, and area of the peaks of the STR markers.

Ethics

Sample collection and genetic analysis were

performed after obtaining written informed consent from each contributor according to the Helsinki Protocol. The study was approved by the Medical Ethics Committee of Shiraz University of Medical Sciences.

Results

Validation of the STR-SD-Based Multiplex QF-PCR

In the current study, besides the utilization of microsatellite markers, 3 sets of SDs were designed to quantify the ratio between chromosome 21 and other autosomes. The

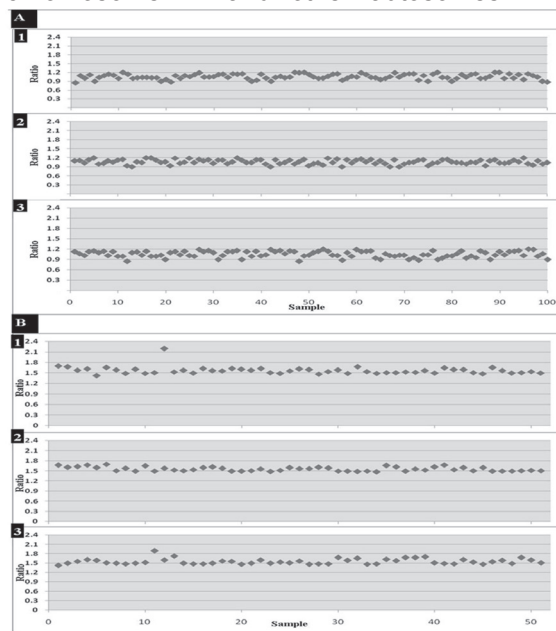


Figure 1: Ratios between the allele peak values of the segmental duplications in the normal and Down syndrome samples are shown. A) The ratio between the peak values of chromosomes 21/6 (A1), 21/7 (A2), and 21/11 (A3) in the 100 normal samples. B) The ratio between the peak values of chromosomes 21/6 (B1), 21/7 (B2), and 21/11 (B3) in the 51 Down syndrome cases.

Table 1: Details of STRs and SDs in the STR-SD-based multiplex QF-PCR

	Marker	Location	Marker Size Range (bp)	Label
STRs	D21S11	21q21.1	220-300	PET
	D21S1442	21q21.3	250-325	6-FAM
	D21S1409	21q21	175-245	6-FAM
	D21S2055	21q22.2	360-470	NED
	D21S1435	21q21.3	156-208	NED
	IFNAR	21q21.2	360-432	6-FAM
	D21S1444	21q22.13	265-340	VIC
	SDs	SD 21/11	21q22.1	chr 21: 159
11q13.2			chr 11: 139	
SD 21/6		21q22.1	chr 21: 256	VIC
		6q25.1	chr 6: 245	
SD 21/7		21q21	chr 21: 120	VIC
		7q11.21	chr 7: 117	

STR: Short tandem repeat; SD: Segmental duplication; QF-PCR: Quantitative fluorescence polymerase chain reaction; Chr: Chromosome

3 sets contained SDs to compare the number of chromosome 21 with chromosomes 6, 7, and 11. For all 3 sets, the ratio was calculated to range from 0.8 to 1.2 in the normal samples (figure 1). As was expected, the ratio range of all the sets in the Down syndrome cases was different (>1.4) (figure 1). For the STR markers, the pattern of the height and area of the peaks in the normal samples was 1:1 in the heterozygous STR markers (informative STR markers) and 2:1 or 1:1:1 in the heterozygous STR markers (informative STR markers) in the individuals with Down syndrome.

STR-SD-Based Multiplex QF-PCR

The aneuploidy of chromosome 21 was detected via the application of the SDs located on chromosomes 21/6, 21/11, and 21/7. These primer pairs were able to simultaneously amplify all the SDs on the chromosome of choice and the reference chromosomes. The STRs and SD-based-multiplex QF-PCR accurately detected all the normal and affected samples with informative or uninformative STR markers regardless of the informativeness of the STR markers (figure 2). Based on the samples used

in this experiment, 100% specificity and 100% sensitivity were achieved for the molecular diagnosis of Down syndrome using the STR-SD-multiplex QF-PCR (table 2). The 100 normal and 50 affected samples were correctly diagnosed, and among the 9 samples with inconclusive results based on Devyser Resolution 21 v2, trisomy 21 was diagnosed in 1 sample and 8 samples were normal (figure 2). All the obtained results were confirmed by karyotyping. Thus, this method can accurately diagnose the presence of an extra chromosome by determining the relative dosage between the chromosome of choice and the reference chromosome.

Discussion

In recent years, successful alternative methods to karyotyping analysis have been introduced not only to increase test rapidity and chance of automation but also to lessen the workload. Currently, 3 famous molecular tests are applied to detect the most common aneuploidies: multiplex ligation-dependent probe amplification (MLPA), interphase FISH, and QF-PCR. The rapidity of these tests has reduced the reporting time to

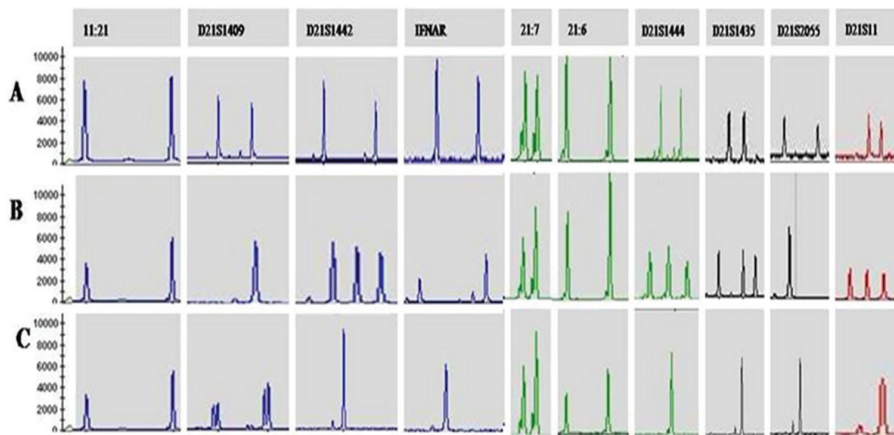


Figure 2: STR-SD-based QF-PCR was carried out and the results are shown in electrophoretograms. A) Control normal sample: all STRs (D21S1442, D21S1444, D21S1435, D21S11, D21S2055, IFNAR, and D21S1409) showed a normal heterozygous pattern with a peak ratio of 1:1 and SDs (21:11, 21:7 and 21:6) showed a normal pattern with a peak ratio of 1:1. B) Down syndrome samples (D21S1442, D21S1444, D21S1435, and D21S11) showed a trisomic tri-allelic pattern with a peak ratio of 1:1:1, IFNAR showed atrisomic di-allelic pattern with a peak ratio of 2:1, D21S1409 and D21S2055 showed 1 peak (homozygote pattern) and SDs (21:11, 21:7, and 21:6) showed a trisomic pattern with a peak ratio of 2:1. C) Inconclusive samples (D21S1442, D21S1444, D21S1435, D21S2055, D21S11, and IFNAR) showed 1 peak (homozygote pattern), D21S1409 showed a trisomic di-allelic pattern with a peak ratio of 2:1 and all SDs (21:11, 21:7, and 21:6) showed a trisomic patterns with a peak ratio of 3:2. STR: Short tandem repeat; SD: Segmental duplication; QF-PCR: Quantitative fluorescence polymerase chain reaction

Table 2: Performance characteristics of multiplex QF-PCR			
Characteristics	%	Number	CI
Positive predictive value	100	51/51	96.4 to 100%
Negative predictive value	100	108/108	93.02 to 100%
Sensitivity	100	51/51	96.4 to 100%
Specificity	100	108/108	93.02 to 100%
False negative rate	0	0/51	-
False positive rate	0	0/108	-

QF-PCR: Quantitative fluorescence-polymerase chain reaction

between 24 and 36 hours, thus relieving parents' anxiety.¹⁶ At present, QF-PCR is considered the technique of choice thanks to its cost/utility ratio. Previous research has confirmed that QF-PCR is a quick, straightforward, and accurate diagnostic test with a high degree of automation for the majority of prenatal cases.¹⁷ Since QF-PCR is an STR-based technique, one of its main disadvantages is the possibility that some STR markers can be uninformative in a portion of patients, especially in countries with high rates of consanguineous marriage. To resolve the abovementioned problems, investigators have tried various approaches over the years. For the first time in 1990s, Lee et al.¹⁸ used a single primer pair in homologous gene quantitative PCR for the detection of trisomy 21; nonetheless, a major drawback of this method was that the authors failed to achieve the original ratio due to a mismatch between the primers and the target sequences. In 2014, Kong et al.⁵ designed an SD-based multiplex QF-PCR containing 2 SDs for each intended chromosome. Although this technique was capable of simultaneously detecting aneuploidies in chromosomes 21, 18, 13, X, and Y, this idea was far from perfect. In another study, Long et al.³ devised rapid diagnosis of common aneuploidies based on the STR-SD-based multiplex QF-PCR using 2 fluorescent-labeled primer pairs for SDs, with 1 primer pair located on chromosomes 13, 18, and 21 and the other pair located on chromosomes 18 and X. Their study could have yielded more reliable results if: (a) the suspected chromosomes for common aneuploidies (chromosomes 18, 13, and 21) had not been selected as the reference chromosome and (b) a greater number of SDs had been selected. With respect to the results of previous studies, we conclude that the simultaneous application of SDs and STRs can overcome the limitations of either STR-QF-PCR or SD-QF-PCR. In the current study, we applied 3 SDs considering the previously mentioned criteria and selected 7 STR markers from tetranucleotide repeats to inhibit slippage strand and reduce stutter band production. Our designed STR-SD-based multiplex QF-PCR panel was able to accurately diagnose all cases of Down syndrome irrespective of whether informative microsatellite markers were present or not. The results of the present study are concordant with previous investigations that reported 100% specificity¹⁹ and 100% sensitivity of the technique for the diagnosis of Down syndrome.^{20, 21} Although this new approach successfully diagnosed normal and Down syndrome cases, more inconclusive samples are needed to confirm its efficiency. In

addition, further research is needed to determine whether the STR-SD-based multiplex QF-PCR can replace the STR-based QF-PCR in high-risk pregnancies.

Conclusion

The present investigation is the first study of its kind to simultaneously apply STRs and SDs to the prenatal diagnosis of Down syndrome. Relative to the known previous STR-based or SD-based methods, the STR-SD-based technique is feasible and useful in populations with a high prevalence of homozygote STR markers.

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Conflict of Interest: None declared.

References

- 1 de Jong A, Dondorp WJ, Krumeich A, Boonekamp J, van Lith JM, de Wert GM. The scope of prenatal diagnosis for women at increased risk for aneuploidies: views and preferences of professionals and potential users. *J Community Genet.* 2013;4:125-35. doi: 10.1007/s12687-012-0126-9. PubMed PMID: 23138342; PubMed Central PMCID: PMC3537971.
- 2 Allingham-Hawkins DJ, Chitayat D, Ciriigliano V, Summers A, Tokunaga J, Winsor E, et al. Prospective validation of quantitative fluorescent polymerase chain reaction for rapid detection of common aneuploidies. *Genet Med.* 2011;13:140-7. doi: 10.1097/GIM.0b013e3182036763. PubMed PMID: 21233717.
- 3 Long J, Ye X, Weng X, Fu K, Sun L, Pang W. Rapid diagnosis of aneuploidy in chromosomes 13, 18, 21, X and Y by quantitative fluorescence-PCR combined with short tandem repeat and fluorescence-labeled homologous gene quantitative PCR using 4-color fluorescently labeled universal primers. *Mol Med Rep.* 2013;8:1601-5. doi: 10.3892/mmr.2013.1678. PubMed PMID: 24042581.

- 4 Guo Q, Xiao L, Zhou Y. Rapid diagnosis of aneuploidy by high-resolution melting analysis of segmental duplications. *Clin Chem.* 2012;58:1019-25. doi: 10.1373/clinchem.2011.178475. PubMed PMID: 22431896.
- 5 Kong X, Li L, Sun L, Fu K, Long J, Weng X, et al. Rapid diagnosis of aneuploidy using segmental duplication quantitative fluorescent PCR. *PLoS One.* 2014;9:e88932. doi: 10.1371/journal.pone.0088932. PubMed PMID: 24625828; PubMed Central PMCID: PMC3953018.
- 6 Caine A, Maltby AE, Parkin CA, Waters JJ, Crolla JA, Cytogeneticists UKAOC. Prenatal detection of Down's syndrome by rapid aneuploidy testing for chromosomes 13, 18, and 21 by FISH or PCR without a full karyotype: a cytogenetic risk assessment. *Lancet.* 2005;366:123-8. doi: 10.1016/S0140-6736(05)66790-6. PubMed PMID: 16005334.
- 7 Ho SS, Chua C, Gole L, Biswas A, Koay E, Choolani M. Same-day prenatal diagnosis of common chromosomal aneuploidies using microfluidics-fluorescence in situ hybridization. *Prenat Diagn.* 2012;32:321-8. doi: 10.1002/pd.2946. PubMed PMID: 22467162.
- 8 Brown L, Abigania M, Warburton D, Brown S. Validation of QF-PCR for prenatal aneuploidy screening in the United States. *Prenat Diagn.* 2006;26:1068-74. doi: 10.1002/pd.1558. PubMed PMID: 16952193.
- 9 Mann K, Donaghue C, Fox SP, Docherty Z, Ogilvie CM. Strategies for the rapid prenatal diagnosis of chromosome aneuploidy. *Eur J Hum Genet.* 2004;12:907-15. doi: 10.1038/sj.ejhg.5201224. PubMed PMID: 15292918.
- 10 Ogilvie CM, Donaghue C, Fox SP, Docherty Z, Mann K. Rapid prenatal diagnosis of aneuploidy using quantitative fluorescence-PCR (QF-PCR). *J Histochem Cytochem.* 2005;53:285-8. doi: 10.1369/jhc.4B6409.2005. PubMed PMID: 15750003.
- 11 Muthuswamy S, Bhalla P, Agarwal S, Phadke SR. Performance of QF-PCR in targeted prenatal aneuploidy diagnosis: Indian scenario. *Gene.* 2015;562:55-61. doi: 10.1016/j.gene.2015.02.028. PubMed PMID: 25724394.
- 12 Onay H, Ugurlu T, Aykut A, Pehlivan S, Inal M, Tinar S, et al. Rapid prenatal diagnosis of common aneuploidies in amniotic fluid using quantitative fluorescent polymerase chain reaction. *Gynecol Obstet Invest.* 2008;66:104-10. doi: 10.1159/000128598. PubMed PMID: 18446039.
- 13 Deutsch S, Choudhury U, Merla G, Howald C, Sylvan A, Antonarakis SE. Detection of aneuploidies by paralogous sequence quantification. *J Med Genet.* 2004;41:908-15. doi: 10.1136/jmg.2004.023184. PubMed PMID: 15591276; PubMed Central PMCID: PMC1735643.
- 14 Sharp AJ, Locke DP, McGrath SD, Cheng Z, Bailey JA, Vallente RU, et al. Segmental duplications and copy-number variation in the human genome. *Am J Hum Genet.* 2005;77:78-88. doi: 10.1086/431652. PubMed PMID: 15918152; PubMed Central PMCID: PMC1226196.
- 15 Saberzadeh J, Miri M, Tabei M, Dianatpour M, Fardaei M. Genetic variations of 21 STR markers on chromosomes 13, 18, 21, X, and Y in the south Iranian population. *Genetics and molecular research: GMR.* 2016;15. doi: 10.4238/gmr15049065.
- 16 Badenas C, Rodriguez-Revenge L, Morales C, Mediano C, Plaja A, Perez-Iribarne MM, et al. Assessment of QF-PCR as the first approach in prenatal diagnosis. *J Mol Diagn.* 2010;12:828-34. doi: 10.2353/jmoldx.2010.090224. PubMed PMID: 20889556; PubMed Central PMCID: PMC2963915.
- 17 Donaghue C, Mann K, Docherty Z, Ogilvie CM. Detection of mosaicism for primary trisomies in prenatal samples by QF-PCR and karyotype analysis. *Prenat Diagn.* 2005;25:65-72. doi: 10.1002/pd.1086. PubMed PMID: 15662691.
- 18 Lee HH, Chang JG, Lin SP, Chao HT, Yang ML, Ng HT. Rapid detection of trisomy 21 by homologous gene quantitative PCR (HGQ-PCR). *Hum Genet.* 1997;99:364-7. PubMed PMID: 9050924.
- 19 Shin YJ, Chung JH, Kim DJ, Ryu HM, Kim MY, Han JY, et al. Quantitative fluorescent polymerase chain reaction for rapid prenatal diagnosis of fetal aneuploidies in chorionic villus sampling in a single institution. *Obstet Gynecol Sci.* 2016;59:444-53. doi: 10.5468/ogs.2016.59.6.444. PubMed PMID: 27896246; PubMed Central PMCID: PMC5120063.
- 20 Jain S, Agarwal S, Panigrahi I, Tamhankar P, Phadke S. Diagnosis of Down syndrome and detection of origin of nondisjunction by short tandem repeat analysis. *Genet Test Mol Biomarkers.* 2010;14:489-91. doi: 10.1089/gtmb.2009.0191. PubMed PMID: 20722466.
- 21 Jain S, Panigrahi I, Gupta R, Phadke SR, Agarwal S. Multiplex quantitative fluorescent polymerase chain reaction for detection of aneuploidies. *Genet Test Mol Biomarkers.* 2012;16:624-7. doi: 10.1089/gtmb.2011.0243. PubMed PMID: 22313045.