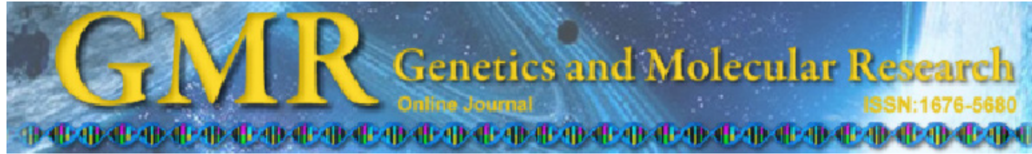

Validation of quantitative fluorescent-PCR for rapid prenatal diagnosis of common aneuploidies in the Chinese population

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ABSTRACT. Quantitative fluorescent polymerase chain reaction (QF-PCR) is an accurate and reliable method for rapid detection of aneuploidy; however, it is not routinely used in China. We aimed to validate QF-PCR as a means for prenatal common aneuploidy screening and to analyze the heterozygosities of short tandem repeat (STR) markers in the Chinese population. The sequences of 19 STR markers in chromosomes 21, 18, 13, X, and Y were designed; three kinds of fluoresceins were used to label the primers, and the QF-PCR detecting conditions were explored and optimized. The results of analysis of 210 prenatal samples by multiplex QF-PCR were compared with karyotyping analysis. All cases were successfully tested by QF-PCR and conventional cytogenetic analysis. QF-PCR

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results were consistent with the results of cytogenetic analyses, with the exception of two cases. The sensitivity and specificity of QF-PCR to diagnose common aneuploidies were 94.74 and 100%, respectively. The heterozygosities of most of the markers were lower than reported for Western populations, but relatively similar to those of other Asian populations. We conclude that QF-PCR is able to detect the common aneuploidies for prenatal diagnosis with high detection efficacy; therefore it is suitable for rapid prenatal diagnosis and for large-scale testing in laboratories. However, we need to add new STR markers or to find alternative STR markers with high heterozygosity in order to make this technique useful for routine diagnosis.

Key words: QF-PCR; Prenatal diagnosis; Aneuploidy

INTRODUCTION

Karyotyping is the gold standard in prenatal diagnosis. Chorionic villus samples (CVS), amniotic fluid, and fetal blood can be analyzed by this cytogenetic method. It can identify aneuploidy and unbalanced structural rearrangements in the fetus with high accuracy (Nicolini et al., 2004). However, the main disadvantage for karyotyping is the lengthy time it takes from sampling to getting the results. During this waiting period, patients often feel worried and anxious. In Europe, it takes 2 weeks to get the report, whereas in China most laboratories take more than 3 weeks to get the results.

Since the 1970s, great advances have been made to improve the efficiency of prenatal diagnoses, yet there are still many downsides and disadvantages to be addressed (Breuning, 2005; Shaffer and Bui, 2007). Due to the lack of efficient detection techniques, the demands for rapid aneuploidy diagnosis (RAD) are increasing in recent years. Among the wide spectrum of chromosomal abnormalities, 21, 18, 13, X and Y chromosomes are the most common sites where chromosome abnormalities occur among newborns. RAD targets abnormalities on the aforementioned chromosomes. The first technique for RAD is fluorescence *in situ* hybridization (FISH) (Klinger et al., 1992). Using FISH, rapid results may be obtained on interphase stage cells (Tepperberg et al., 2001; Leung et al., 2004; Caine et al., 2005; Wyandt et al., 2006). However, this technique is relatively expensive, and more labor-intensive (Cirigliano et al., 2002; Hulten et al., 2003; Donaghue et al., 2005; South et al., 2008).

Quantitative fluorescence polymerase chain reaction (QF-PCR) is an alternative choice for RAD. It was first introduced in rapid prenatal diagnosis in the early 1990s (Petersen et al., 1991; von Eggeling et al., 1993; Mansfield, 1993; Pertl et al., 1994). It is a rapid method for the detection of common aneuploidies by PCR amplicaion of short tandem repeats (STRs) in human chromosomes (Adinolfi et al., 1997; Cirigliano et al., 1999; Mann et al., 2001; Ogilvie et al., 2005). Compared with interphase FISH, the QF-PCR technique has some advantages; in particular, it is more economic and automated and can be used in large scale detection (Hulten et al., 2003; El Mouatassim et al., 2004; Shaffer and Bui, 2007).

Currently, QF-PCR has been widely used in the most developed countries, but

has not been used in China. Because STRs have shown polymorphisms and variations were observed in different populatons, some healthcare researchers think it needs further validation before its wide use in clinical settings (Cho et al., 2009). For example, Americans have lower heterozygosity at the locus D18S391 (0.75), whereas they have higher heterozygosity at the two loci P39 and DXS8377 (0.87 and 0.95, respectively) (Brown et al., 2006). But, in a Southeast Asian population, Quaife et al. (2004) reported that the heterozygosity for D18S391 was as low as 0.61. Gole et al. (2008) reported that among the Asian ethnic groups, the Chinese showed a higher heterozygosity index for X22 (90%) as compared to the Indians (72.7%) and Malays (66.6%). These authors also found that the three ethnic groups showed similar heterozygosity for the XHPRT marker, i.e., 69.3, 72.7 and 72.2% for Chinese, Indians and Malaysians, respectively. In another study, Cirigliano et al. (2009) reported that the Caucasian population showed a similar heterozygosity index for X22 (91%) as the Chinese population, while a 75% heterozygosity index for XHPRT, which was higher than that for the three Asian ethnic groups. Thus, the heterozygosity for the various STR loci needs to be determined before QF-PCR can be used in rapid prenatal diagnosis.

To establish the molecular basis for using RAD to detect the common aneuploidies in prenatal specimens, we performed a prospective study to validate QF-PCR. We used multiple QF-PCR to test 210 fetal samples, including amniotic fluid and chorionic villi. The heterozygosity for each STR locus was investigated. This is the first multiple QF-PCR study carried out in prenatal diagnosis in China.

MATERIAL AND METHODS

Samples

Samples were collected between May 2008 and September 2009. A total of 210 prenatal samples were included in this study. The majority of prenatal samples were amniotic fluids (133) collected between 16 and 22 weeks of gestation and CVS (39) collected between 11 and 14 weeks of gestation; 20 fetal blood samples and 18 villous from aborted fetuses were also investigated. Both QF-PCR and conventional cytogenetic studies were performed on all samples.

Methods

Genomic DNA was extracted from 2 mL amniotic fluid, 200 μL fetal blood, and a small villous fragment using a QIAamp DNA Mini kit (Qiagen, Germany) according to the manufacturer instructions. The visible blood stained samples were excluded in this study. Six markers were used for chromosome 21, four for chromosomes 13 and 18, three for X chromosome and one for Y chromosome; the non-polymorphic sequence of the amelogenin (AMXY) gene was also included to determine fetal sex.

All forward primers were labeled with fluorescent molecules allowing for accurate sizing and quantification of QF-PCR products. Primers producing amplicons of similar sizes were labeled with different fluorochromes to be amplified and analyzed in the same multiplex QF-PCR.

In the first set of QF-PCR reactions, 8 markers were co-amplified in one multiplex QF-PCR assay, which included AMXY, DXS8377, D21S1435, IFNAR, D18S978, D18S535,

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D13S634, and D13S305. In the second set of QF-PCR reactions, another 8 markers were used, including DXS6803, DYS448, D21S11, D21S1270, D18S391, D18S386, D13S742, and D13S628. If definitive conclusions could not be drawn according to the results from these two sets of QF-PCR, a third set of 3 markers would be used, namely P39, D21S1411 and D21S226.

QF-PCR was performed in 25 μL containing genomic DNA, 200 μM dNTPs, 1.6-22 pmol each primer (Sangon Biological Engineering Technology & Services Co., Ltd.) (Tables 1, 2 and 3), 1.5 mM $MgCl_2$, 2 U *Taq* polymerase and 1X *Taq* polymerase buffer. Initial denaturation at 95°C for 5 min was followed by 25 cycles of 95°C for 25 s, 55°C for 45 s, and 72°C for 30 s. Final extension was for 20 min at 72°C.

One microliter of PCR products was mixed with 10 μL formamide and 0.5 μL size standard in a MicroAmp (Applied Biosystems) optical 96-well reaction plate. After being denatured for 2 min at 95°C and cooled for 3 min at -20°C, this mix was run on an ABI model 3130 capillary electrophoresis instrument using POP-7 polymer. For fragment analysis, Genescan Analysis version 3.0 was used on a 3130 Genetic Analyzer.

Table 1. QF-PCR primer mix No. 1.

Marker	Location	Size	Sequence and label	pmol/reaction
AMXY	Xp22.1-22.31 Yp11.2	X 104 Y 110	FAM- CCCTGGGCTCTGTAAAGAATAGTG(F) ATCAGAGCTTAAACTGGGAAGCTG(R)	1.6
D21S1435	21q21.3	160-222	HEX- CCCTCTCAATTGTTTGTCTACC(F) ACAAAAGGAAAGCAAGAGATTTCA(R)	14
D18S978	18q12.3	180-220	TAMRA- GTAGATCTTGGGACTTGTCAGA(F) GTCTCCCATGGTCACAATGCT(R)	10
DXS8377	Xq28	203-245	FAM- CACTTCATGGCTTACCACAG(F) GACCTTTGGAAAGCTAGTGT(R)	8
IFNAR	21q22.1	370-410	FAM- CATTTGATCTTAGCCATCTATTGC(F) ACTATGCAGCCATTTGAAAGACTA(R)	18
D13S634	13q14.3-q22	385-440	TAMRA- GGCAGATTCAATAGGATAAATAGA(F) GTAACCCCTCAGGTTCTCAAGTCT(R)	18
D13S305	13q12.1-q14.1	430-465	HEX- GCCTGTTTGAGGACCTGTCGTTA(F) TGGTTATAGAGCAGTTAAGGCAC(R)	10
D18S535	18q12.3	455-550	TAMRA- CAGCAAACTTCATGTGACAAAAGC(F) CAATGGTAACCTACTATTTACGTC(R)	20

FAM, HEX and TAMRA are standard fluorescent dyes.

Table 2. QF-PCR primer mix No. 2.

Marker	Location	Size	Sequence and label	pmol/reaction
DXS6803	Xq12-q21.33	106-125	HEX- GAAATGTGCTTTGACAGGAA(F) CAAAAAGGGACATATGCTACTT(R)	10
D18S391	18pter-p11.22	140-180	FAM- GGACTTACCACAGGCAATGTGACT(F) CTGGCTAATTGAGTTAGATTACAA(R)	6
D21S11	21q21.1	225-280	TAMRA- TTTCTCAGTCTCCATAAATATGTG(F) GATGTTGTATTAGTCAATGTTCTC(R)	16
D13S742	13q11-q21.1	235-315	HEX- ATAACTGGGCTAGGAATGGAAATA(F) GACTTCCCAATTCAGGAGGACT(R)	8
D21S1270	21q21-q22.1	285-340	TAMRA- CTATCCCACTGTATTATTCAGGGC(F) TGAGTCTCCAGGTTGCAGGTGACA(R)	22
D18S386	18q22.1	330-400	FAM- TGAGTCAGGAGAATCACTTGGAAC(F) CTCTTCCATGAAGTAGCTAAGCAG(R)	12
DYS448	Yq11.2	350-380	TAMRA- CAAGGATCCAAATAAAGAACAGAGA(F) GGTTATTTCTTGATTCCCTGTG(R)	10
D13S628	13q31-q32	425-470	FAM- TAACATTCATTGTCCCTTACAGAT(F) GCAAGGCTATCTAACGATAATTCA(R)	15

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Table 3. Extra markers.

Marker	Location	Size	Sequence and label	pmol/reaction
P39	Xq28	140-166	FAM- AGCACATGGTATAATGAACCTCCACG(F) CAGTGTGAGTAGCATGCTAGCATTTG(R)	2
D21S1411	21q22.3	256-340	FAM- ATAGGTAGATACATAAATATGATGA(F) TATTAATGTGTGTCCTTCCAGGC(R)	6
D21S226	21q22.1	440-470	FAM- GCAAATTTGTGGATGGGATTAACAG (F) AAGCTAAATGTCTGTAGTTATTCT(R)	2

Interpretation of results

For interpretation of results, the following criteria were used: allele dosage ratios (shorter allele/longer allele) between 0.8 and 1.4 were defined as normal, and an allele ratio greater than 1.8, less than 0.65, or the presence of three alleles of equal areas indicated trisomy. A single peak was described as uninformative. A minimum of two informative markers for each chromosome (21, 18, 13, and X) was required for confident interpretations.

RESULTS

Comparison with conventional cytogenetic analysis

All cases were successfully tested by QF-PCR and conventional cytogenetic analysis. The results of these two methods are presented in Table 4. Eight cases of trisomy 21, 3 cases of trisomy 18, 2 cases of triploidy, 1 case of Turner's syndrome, 2 cases of Klinefelter's syndrome, 1 case of 47,XXX, and 1 case of 47,XYY were detected by QF-PCR (Figures 1-4). There were no false positives. QF-PCR results were consistent with the results of cytogenetic analyses, with the exception of two cases. The undetected cases were an inherited balanced structural abnormality with karyotype 46,XX,t(11;20). Another case of 46,XX produced an uninformative result for all the markers of X chromosome by QF-PCR, and it was confirmed by interphase FISH.

Table 4. Results of testing 210 fetal samples by QF-PCR and conventional cytogenetic analysis.

Karytotype	Cytogenetics	QF-PCR
46,XX; 46,XY	191	192
47,XX +21;47,XY+21	8	8
47,XX +18;47,XY+18	3	3
69,XXX	2	2
45,X	1	1
47,XXY	2	2
47,XXX	1	1
47,XYY	1	1
Structural balanced	1	0
Total abnormalities	19	18
Sensitivity (%)		94.74
Specificity (%)		100
Positive predictive value (%)		100
Negative predictive value (%)		99.48

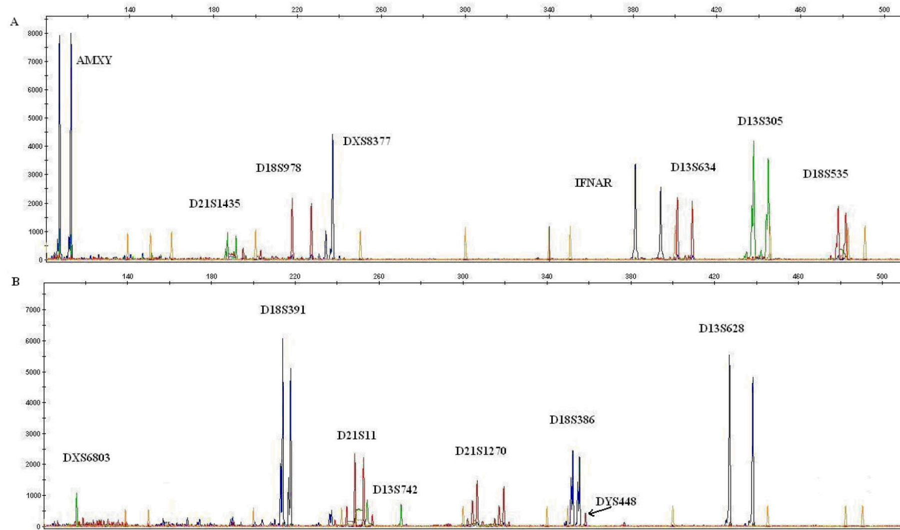

Figure 1. Genotype profiles of a normal diploid sample amplified with the multiplex QF-PCR and analyzed on a 3130 Genetic Analyzer. Fragment size in base pairs shown on the horizontal axis, arbitrary fluorescence units on the vertical axis. **A.** First set of QF-PCR: 1) AMXY (blue), 2) D21S1435 (green), 3) D18S978 (red), 4) DXS8377 (blue), 5) IFNAR (blue), 6) D13S634 (red), 7) D13S305 (green), 8) D18S535 (red); **B.** Second set of QF-PCR: 1) DXS6803 (green), 2) D18S391 (blue), 3) D21S11 (red), 4) D13S742 (green), 5) D21S1270 (red), 6) D18S386 (blue), 7) DYS448 (red), 8) D13S628 (blue). All markers are heterozygous and exhibit 2 alleles with peak areas in a 1:1 ratio except DXS8377, DXS6803, D13S742, and DYS448, which are homozygous. The karyotyping result are 46,XY.

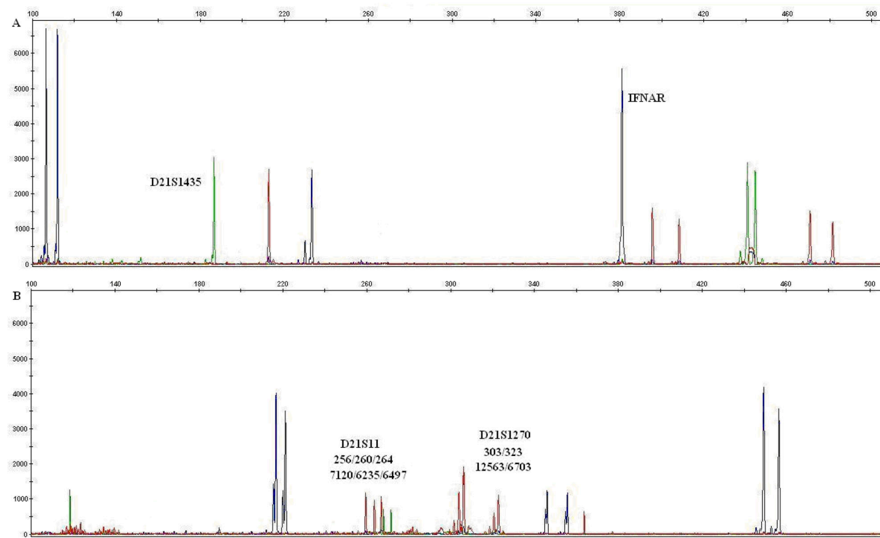

Figure 2. Genotype profiles of a trisomy 21 sample amplified with the multiplex QF-PCR and analyzed on a 3130 Genetic Analyzer. Chromosome 21 STR markers exhibit 3 (D21S11) or 2 alleles in 2:1 peak ratio (D21S1270); D21S1435 and IFNAR are homozygous and therefore uninformative. The karyotyping result is 47,XY+21.

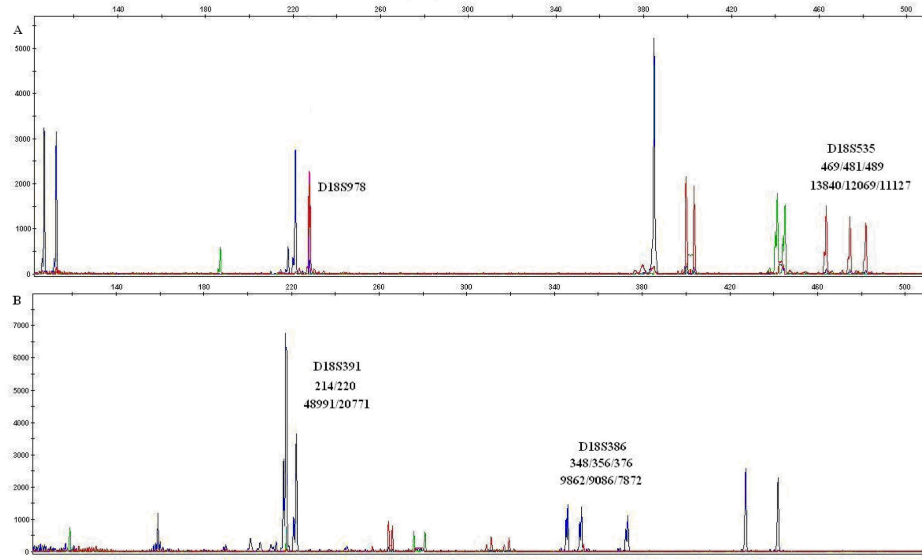

Figure 3. Genotype profiles of a trisomy 18 sample amplified with the multiplex QF-PCR and analyzed on a 3130 Genetic Analyzer. Chromosome 18 STR markers exhibit 3 (D18S535 and D18S386) or 2 alleles in 2:1 peak ratio (D18S391); D18S978 is homozygous and therefore uninformative. The karyotyping result is 47,XY+18.

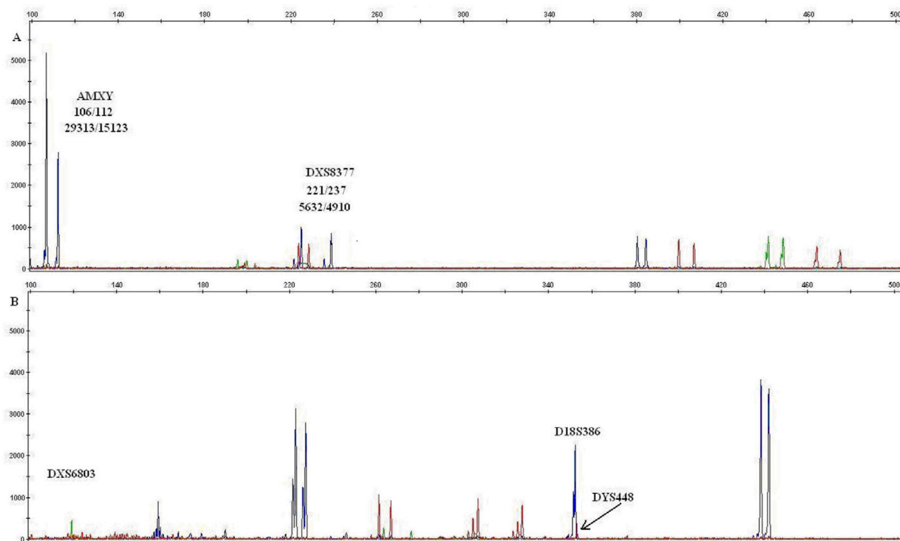

Figure 4. Genotype profiles of a Klinefelter's syndrome sample amplified with the multiplex QF-PCR and analyzed on a 3130 Genetic Analyzer. Sex chromosome STR markers exhibit 2 alleles in 2:1 peak ratio (AMXY); DXS8377, DXS6803 and DYS448 are homozygous. Chromosome 13, 18 and 21 STR markers exhibit 2 alleles with a normal 1:1 peak ratio except D18S386. The karyotyping result is 47,XXY.

Analysis of the heterozygosities of STR markers

The heterozygosities of markers selected for QF-PCR are given in Table 5. We compared our results of STR markers in the Chinese population with Western populations (Cirigliano et al., 2004) and other Asian populations (Cho et al., 2009). The heterozygosities of all markers in chromosome 21 were markedly lower than in Western populations, but relatively similar with other Asian populations. The heterozygosities for chromosome 18 from our study samples were lower than in Western populations, with the exception of D18S535, in which heterozygosity was higher than in the Korean population and Southeast Asian population. Among the markers of chromosome 13, D13S634 showed a lower heterozygosity than in Western populations and other Asian populations, while D13S305 was higher than in Western populations. As for the sex chromosome, DXS6803 and DXS8377 showed higher a heterozygosity in Western populations than Chinese populations.

Table 5. List of STR markers analyzed in Chinese population.

Marker	Heterozygosities	Allele No.	Allele length (bp)
D21S1435	0.70	8	174, 178, 182, 186, 190, 194, 198, 202
IFNAR	0.65	7	376, 380, 384, 388, 392, 396, 400
D21S226	0.46	6	442, 446, 450, 454, 458, 462
D21S1411	0.79	13	260, 276, 284, 292, 296, 300, 304, 308, 312, 316, 320, 324, 328
D21S1270	0.80	17	275, 279, 287, 291, 295, 299, 303, 307, 311, 315, 319, 323, 327, 331, 335, 347, 355
D21S11	0.75	8	232, 248, 252, 256, 260, 264, 268, 272
D18S535	0.84	14	437, 445, 449, 453, 457, 461, 465, 469, 473, 477, 481, 485, 489, 493
D18S391	0.63	13	154, 158, 174, 178, 190, 206, 210, 214, 218, 222, 226, 230, 234
D18S386	0.82	13	340, 344, 348, 352, 356, 360, 364, 368, 372, 376, 380, 384, 388
D18S978	0.82	10	199, 203, 207, 211, 215, 219, 223, 227, 231, 239
D13S742	0.77	16	248, 252, 256, 260, 264, 268, 272, 276, 280, 284, 288, 292, 300, 304, 308, 320
D13S305	0.77	13	380, 396, 404, 424, 428, 432, 436, 440, 444, 448, 452, 456, 460
D13S634	0.71	11	383, 391, 395, 399, 403, 407, 408, 411, 415, 419, 423
D13S628	0.66	10	423, 427, 431, 435, 439, 443, 447, 451, 455, 459
DXS8377	0.79	13	213, 217, 221, 225, 229, 233, 237, 241, 245, 249, 253, 257, 261
DXS6803	0.52	7	102, 106, 110, 114, 118, 122, 126
P39	0.50	4	146, 154, 158, 162
DYS448			340,348,352, 356, 360, 364, 368
AMXY	-		X:104-106; Y:110-112

DISCUSSION

QF-PCR has been applied in rapid aneuploidy diagnosis for almost twenty years. This technique has been improved and widely used nowadays. It has many advantages, such as accurate, easy to manipulate, quick to generate results, high throughput to test samples, cost effective, etc. In this study, we adapted a single-tube QF-PCR technique in which 8 markers (2 STR markers specific for each chromosome 13, 18, 21 or sex chromosome) were co-amplified in one multiplex PCR assay. Apart from one specimen of inherited balanced structural abnormality with karyotype 46,XX,t(11;20), all the aneuploid abnormalities involving chromosomes 13, 18, 21, X and Y were detected, and there was no discordance between the results with QF-PCR and conventional cytogenetics. However, one of the main disadvantages for QF-PCR is that it cannot easily differentiate 46,XX from 45,XO (Cirigliano et al., 2002). Therefore, interphase FISH should be considered in detecting 45,XO. In this study, one case with 46,XX gave an uninformative result for X chromosome because all the STR markers in X chromosome showed

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a single peak which meant to be homozygous. We then performed interphase FISH and confirmed 46,XX. Another case with 46,XX,t(11;20) belonged to paternal balanced translocation. This abnormality is beyond the five chromosomes that QF-PCR was designed to target, and therefore, this case was not considered a misdiagnosis (Mann et al., 2004).

As Brown suggested, each laboratory that uses QF-PCR needs to perform an independent validation test. This will demonstrate the heterozygosities of various STR loci and the efficiency of multiplex combinations. We have done extensive literature reviews for the choice of the STR markers, and selected the most popular STR markers involving 13, 18, 21, X and Y chromosome to be used in our laboratory. From this study, we found that the heterozygosities of most of STR markers in Chinese populations were lower than those in Western populations, where especially all markers in chromosome 21 were markedly lower than in Western populations. The reasonable explanation for this phenomenon may be that the Chinese population was more conservative culturally and historically, and marriage between different ethnic groups is not commonly seen (Quaife et al., 2004). From this study, the STR markers for chromosome 21 show lower heterozygosities, so we need to add new STR markers or alternative STR markers with high heterozygosity in the future studies. In addition, we plan to choose new markers in X chromosome or autosomal markers as internal control for quantification. The data from this assay help assessing the feasibility of applying QF-PCR kits to Chinese populations.

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