THESIS

MOLECULAR DIAGNOSIS OF DOWN SYNDROME AND SEX CHROMOSOME BY REAL-TIME PCR METHOD

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THESIS

MOLECULAR DIAGNOSIS OF DOWN SYNDROME AND SEX CHROMOSOME BY REAL-TIME PCR METHOD

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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In this study, the real-time PCR method was adapted to determine DS status in 3 particular regions of Down syndrome critical region (DSCR), i.e., *DSCR1*, *DSCR3* and *DSCAM*. Sex identification was performed using Sex-Determining Region (*SRY*) on Y chromosome.

The duplex and triplex real-time PCR assay were used to screen 50 DS patients and 50 normal controls by relative quantification using comparative Ct method, while singleplex real-time PCR assay was used to screen 10 DS patients and 10 normal controls by relative quantification using standard curve method. The singleplex, duplex and triplex real-time PCR assays for the detection of DS gave 100 %, 82 % and 74 % accurate results, repectively. As for sex identification, 100% accuracy was achieved. These data suggested that the real-time PCR method can not be totally used to replace the conventional cytogenetic analysis for DS detection. Therefore, further improvement for real-time PCR conditions is needed to get 100 % accurate results for clinical implementation.

However, real-time PCR method is still considered practical for rapid preliminary screening of DS but cytogenetic analysis is also needed for confirmation of a positive result and a double check is required for a negative result.

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Student's signature Thesis Advisor's signature

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LIST OF ABBREVIATIONS

ABI	=	Applied Biosystems
AD	=	Alzheimer's disease
bp	=	base pairs
cDNA	=	complementary DNA
CFTR	=	cystic fibrosis transmembrane regulator
Ct	=	cycle threshold
CV	=	coefficienct of variation
CVS	=	chorionic villi sampling
°C	=	degree celcius
der	=	derivative
DNA	=	deoxyribonucleic acid
dNTP	=	deoxyribonucleoside triphosphate
DS	=	Down syndrome
DSCAM	=	Down syndrome cell adhesion molecule
DSCR	=	Down syndrome critical region
DSCR1	=	Down syndrome critical region gene 1
DSCR2	=	Down syndrome critical region gene 2
DSCR3	=	Down syndrome critical region gene 3
DSCR4	=	Down syndrome critical region gene 4
DSCR5	=	Down syndrome critical region gene 5
DSCR6	=	Down syndrome critical region gene 6
DSCR8	=	Down syndrome critical region gene 8
DSCR9	=	Down syndrome critical region gene 9
DSCR10	=	Down syndrome critical region gene 10
dsDNA	=	double-stranded DNA
E.coli	=	*Escherichia coli*
EDTA	=	ethylenediaminetetraacetic acid
FAM	=	6-carboxy-fluorescein, a blue fluorescent dye
FISH	=	fluorescence *in situ* hybridization

FRET	=	fluorescence resonance energy transfer
g	=	gram
g	=	relative centrifugal force
GAPDH	=	glyceraldehyde 3-phosphate dehydrogenase
GlcNAc	=	N-acetylglucosamine
GPI	=	glycosylphosphatidylinositol
HCG	=	human chorionic gonadotrophin
Ig	=	immunoglobulin
IGF-1	=	insulin-like growth factor-1
KCl	=	potassium chloride
LB medium	=	Luria-Bertani medium
M	=	molar
MGB	=	minor groove binding
MGBNFQ	=	minor groove binder/non-fluorescent quencher
$MgCl_2$	=	magnesium chloride
$MgSO_4$	=	magnesium sulfate
min	=	minute
ml	=	milliliter
mM	=	millimolar
mos	=	mosaic
MSAFP	=	maternal serum alpha-fetoprotein
µg	=	microgram
µl	=	microliter
µM	=	micromolar
NaCl	=	sodium chloride
NaOAc	=	sodium acetate
NCBI	=	National Center for Biotechnology Information
NED	=	2,7',8'-benzo-5'-fluoro-2',4,7-trichloro-5-carboxyfluorescein, a yellow fluorescent dye
NFQ	=	non-fluorescent quencher
ng	=	nanogram
nM	=	nanomolar

NPV	=	negative predictive value
NT	=	nuchal translucency
PAPP-A	=	pregnancy-associated plasma protein A
PCR	=	polymerase chain reaction
PI	=	phosphatidylinositol
PPV	=	positive predictive value
PUBS	=	percutaneous umbilical blood sampling
QF-PCR	=	quantitative fluorescent polymerase chain reaction
RQ	=	relative quantity
R^2	=	coefficient of determination
sec	=	second
SD	=	standard deviation
SRY	=	sex-determining region
Tm	=	melting temperature
U	=	enzyme unit
uE3	=	unconjugated estriol
UNG	=	uracil-N-glycosylase
VIC	=	green fluorescent dye
ΔCt	=	difference of cycle number

MOLECULAR DIAGNOSIS OF DOWN SYNDROME AND SEX CHROMOSOME BY REAL-TIME PCR METHOD

INTRODUCTION

Down syndrome (DS) is a genetic disorder that causes mental retardation and other developmental problems ranging from mild to serious symptoms. The incidence of DS is one in every 800 infants as reported by Mayo Foundation for Medical Education and Research.

In 2004 to 2005, the record of Human Genetics Unit, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University showed that among 2,229 cases of suspected chromosomal abnormalities investigated by conventional cytogenetic method, 84 cases of prenatal and postnatal samples were DS (3.77 %) while 112 cases showed other chromosomal abnormalities (5.03 %). Therefore, to reduce the incidence of DS in children a rapid diagnosis method is needed.

The cause of DS is the presence of the extra copy of chromosome number 21. Down syndrome critical region (DSCR) is a region on the long arm of chromosome 21 known to be responsible for DS features such as mental retardation, a distinct facial appearance and protruding tongue. A total number of DSCR gene on human chromosome 21 consists of 10 regions and the regions focused in this study are 3 regions of Down syndrome critical region gene 1 (*DSCR1*), Down syndrome critical region gene 3 (*DSCR3*) and Down syndrome cell adhesion molecule (*DSCAM*) because the effect from these three regions may involve mental retardation which is a major phenotype of DS. *DSCR1* affects central nervous system development and DSCR1 protein is overexpressed in the brain of DS fetuses, *DSCR3* involves in embryogenesis and is expressed in most tissues including fetal and adult brain, heart, lung and liver, while *DSCAM* involves in the development of nervous system and congenital heart disease.

Cytogenetic analysis is a routine method for DS diagnosis that provides reliable results but it is a difficult and time consuming technique which requires a well-trained person. FISH (fluorescence *in situ* hybridization), on the other hand, has been used to analyze uncultured fetal cells for screening of chromosomal aneuploidies that provides correct diagnosis for trisomy 21 at the interphase stage within 24 to 48 hours but it is also an intensive method in time and labor. QF-PCR (quantitative fluorescent PCR), however, has been developed for rapid prenatal diagnosis of sex and chromosomal aneuploidies including trisomy 21. Results from QF-PCR technique can be obtained within 24 hours and only a small amount of sample is needed but the procedure of polymerase chain reaction and detection are separately performed and it also faces the problem of allele drop out. Recently, real-time PCR has been developed to establish a rapid prenatal diagnosis of trisomy 21. The procedure of polymerase chain reaction and detection are simultaneously used which turns out to be faster than QF-PCR technique and a large number of samples can be screened at one time.

Because the incidence of the X-linked genetic diseases is different between male and female children especially X-linked recessive gene, for example, if the mother is homozygous for the disease allele and the father is normal, their daughters will be carriers while the sons will be abnormal. Therefore, sex identification is an alternative assay to detect the disorder in such cases.

In this study, real-time PCR method is used to simultaneously screen DS in 3 particular regions, *DSCR1*, *DSCR3* and *DSCAM* and sex identification on Y chromosome is done by Sex-Determining Region (*SRY*) detection.

OBJECTIVES

1. To develop real-time PCR technique for rapid screening of DS and sex identification.

2. To test the feasibility of real-time PCR technique for routine service of DS screening and sex identification in clinical laboratory.

LITERATURE REVIEW

John Langdon Down had first identified Down syndrome (DS) in 1866. It is the most common genetic cause of severe learning disabilities in children and there is by far no medical cure for this condition. Children with DS have a distinct facial appearance but not necessarily the same features. The more common features are flattened facial features, protruding tongue, small head, upward slanting eyes and unusual shaped ears. Children with DS may also have poor muscle tone, broad, short hands with a single crease in the palm, relatively short fingers and excessive flexibility (Mayo Clinic Staff, 2005). Conditions that may be associated with DS include heart defects, hearing difficulties, eye problems, seizure disorders, stomach or intestinal problems, trouble swallowing, thyroid problems, bone and joint problems (Pace, 2001). People with DS often develop leukemia and Alzheimer's disease (AD) (Wisniewski *et al*., 1985; Shen *et al*., 1995; Rajantie, 1996).

Causes of DS relate with three types of abnormal cell division involving the chromosome 21. All three abnormalities result in extra genetic material from chromosome 21, which is responsible for the characteristic features and developmental problems of DS. These causes of DS include first, trisomy 21, which contributes to more than 90 percent of cases of DS. A child with trisomy 21 has three copies of chromosome 21 instead of the usual two copies in all of his or her cells. This form of DS is caused by abnormal cell division during the development of the sperm cell or the egg cell. The second is mosaic DS, a rare form of DS, characterized by an extra copy of chromosome 21 in some cells. This mosaic of normal and abnormal cells is caused by abnormal cell division after fertilization. The third is translocation DS**,** a part of chromosome 21 becomes attached onto another chromosome. Children with translocation DS have the usual two copies of chromosome 21, but they also have an additional material from chromosome 21 stuck to the translocated chromosome. Although this form of DS is uncommon but it can be passed from parent to child while most cases of DS are not inherited. The mother or father is a balanced carrier of the translocation and he or she can pass the

translocation to children. The chance of passing on the translocation depends on the sex of the parent who carries the rearranged chromosome 21. If the father is the carrier, the risk is about 3 percent while the mother is responsible for the risk of about 12 percent. However, there are no known behavioral or environmental factors that cause DS (Mayo Clinic Staff, 2005). In fact, there are three types of risk factors, i. e., 1) mothers age 35 or older, but most children with DS are actually born to women under age 35 because this younger group of women has far more babies, 2) mothers who have already had one child with DS, 3) parents who are carriers of the genetic translocation for DS (Mayo Clinic staff, 2005).

Chromosome 21 is the smallest human autosome consisted of 127 known genes, 98 predicted genes and 59 pseudogenes (Hattori *et al*., 2000). It has been presumed that several dosage-sensitive genes in a section of human chromosome 21 called the Down syndrome critical region (DSCR) are responsible for many of the features of this disease (Nelson and Gibbs**,** 2004).

From the mapview of *Homo sapiens* genome Build 35.1 statistics showed that the total number of DSCR genes on human chromosome contain 10 regions (NCBI, 2005a). These genes include *DSCR1*, *DSCR2*, *DSCR3*, *DSCR4*, *DSCR5*, *DSCR6*, *DSCR8*, *DSCR9*, *DSCR10* and *DSCAM*.

DSCR1 is also known *as CSP1, DSC1, RCN1, MCIP1 or ADAPT78*. The protein encoded by this gene interacts with calcineurin A and inhibits calcineurin-dependent signaling pathways, possibly affecting central nervous system development. This gene is overexpressed in the brain of DS fetuses. Chronic overexpression of this gene may lead to neurofibrillary tangles such as those associated with Alzheimer's Disease (AD). *DSCR1* is located on chromosome 21, at 21q22.1-q22.2 and it covers 102.40 kb region (NCBI, 2005b). Basic functions of *DSCR1* involve transcriptional regulation and signal transduction (GENATLAS, 2004a).

DSCR2 is also known as *C21LRP or LRPC21*. *DSCR2* is located on chromosome 21, and it covers 9.08 kb region (NCBI, 2005c). Basic function of DSCR2 is cell proliferation (GENATLAS, n.d.).

The synonym symbols of *DSCR3* are *DCRA, DSCRA* or *DSR3*. *DSCR3* is located on chromosome 21, at 21q22.13 with basic function of embryogenesis (GENATLAS, 2002). Nakamura *et al.* (1997) identified a novel gene and subsequently cloned cDNA that designated as *DCRA* (Down syndrome critical region gene A). It consists of eight exons of 3,252 bp in total and encodes a large open reading frame of 297 amino acid residues. *DCRA* is expressed in most tissues including fetal and adult brain, heart, lung, liver, and kidney.

DSCR4 is also known as *DCRB, DSCRB* or *LOC440780*. It contributes significantly to the pathogenesis of many characteristics of DS, including morphological features, hypotonia, and mental retardation. This gene is found in this region and multiple transcripts may exist. *DSCR4* is located on chromosome 21, at 21q22.13 and it covers 169.73 kb region (NCBI, 2005d). Nakamura *et al.* (1997) identified a novel gene and cloned cDNA that designated *DCRB* (Down syndrome critical region gene B). It consists of three exons of 1,095 bp in total and encodes a large open reading frame of 118 amino acid residues. The amino acid sequence of *DCRB* was detected in adult heart and skeletal muscle from Northern blot analysis.

DSCR5 is also known as *DCRC*, *DSRC*, *PIGP* or *DCRC-S*. This gene involves in the first step of glycosylphosphatidylinositol (GPI)-anchor biosynthesis. The GPI-anchor is a glycolipid found on many blood cells and serves to anchor proteins to the cell surface. This gene encodes a N-acetylglucosaminyl transferase component that is part of the complex that catalyzes transfer of N-acetylglucosamine (GlcNAc) from UDP-GlcNAc to phosphatidylinositol (PI). *DSCR5* is located on chromosome 21, at 21q22.2 and it covers 7.82 kb region (NCBI, 2005e). Togashi *et al*. (2000) reported the expression of *DSCR5* in several tissues including liver, skeletal muscle, heart, pancreas and testis. Choi *et al*. (2001) found the mouse homolog called

Dscr5 which expressed in tongue during development and suggested that it may be involved in the pathophysiology of the tongue malformation observed in DS.

DSCR6 is located on 21q22.22 (GENATLAS, 2001). It synthesized Down syndrome critical region protein 6 (AmiGo, 2007) of an unknown function, but expressed in fetal kidney and fetal brain at high levels, suggesting a role in early embryogenesis (Shibuya *et al.*, 2000).

DSCR8 is located on 21q22.22 having a synonym name of melanoma-testis-associated protein 2 or malignant melanoma associated 1A but its basic function is also unknown (GENATLAS, 2005).

DSCR9 and *DSCR10* are located on 21q22.13 with unknown basic functions (GENATLAS, 2003a; GENATLAS, 2003b).

DSCAM is located on 21q22.2-q22.3. Its synonym is *CHD2-42*, *CHD2-52*, having basic functions in mediating cation-independent homophilic binding activity and involves in nervous system development and congenital heart disease (GENATLAS, 2004b). Yamakawa *et al*. (1998) and Barlow *et al*. (2002) suggested that *DSCAM* involved in neural differentiation and the central and peripheral nervous system defects in DS. DSCAM protein is a novel member of the immunoglobulin (Ig) superfamily representing a new class of neural cell adhesion molecules.

Clinical screening tests for DS were performed during the first and second trimester of pregnancy. These tests help to identify fetus that has an increased possibility of DS. Screening methods such as the triple screen and quadruple screen are used (CIGNA, 2006).

If maternal serum alpha-fetoprotein (MSAFP) alone is tested, 20 % of DS fetuses will be detected with lower percentage, which means MSAFP testing is not always reliable. Therefore, additional biochemical tests should be used to make more accurate determinations. In cases where MSAFP and human chorionic gonadotrophin

(HCG) are determined together, 50-60 % of DS fetuses could be identified. When MSAFP, HCG and unconjugated estriol (uE3) are all tested (triple screen), 60-70 % of DS fetuses could be identified (Wilson, 1998). The triple screen positive means screening by low MSAFP level, high levels of human chorionic gonadotrophin (HCG) and unconjugated estriol (uE3) which are performed in the second trimester. Quadruple screen includes all the parameters of the triple screen combined with a high serum level of inhibin-A, which are performed in the second trimester. This test finds 5 % false-positive from 67-76 % of detection to be DS. Limitation of the triple and quadruple screens is the false-positive rate. It has been reported to result in as many as 60 amniocentesis being done for each case of DS identified (CIGNA, 2006). A nuchal translucency (NT) screening test are used to measure a specific region on the back of a baby's neck by the doctor (Mayo Clinic Bookstore, 2006). Measurements equal to or greater than 3 mm are considered abnormal (Agarwal, 2003). This test should be measured between 10 and 14 weeks' gestation. Measurement of NT, pregnancy-associated plasma protein A (PAPP-A) and HCG called "combined test" are performed in the first trimester. A low level of PAPP-A with a high level of HCG provides more effective screening than each individual test (CIGNA, 2006).

Prenatal diagnosis for DS includes amniocentesis, chorionic villi sampling (CVS) and percutaneous umbilical blood sampling (PUBS). Amniocentesis is done after 15 weeks of gestation. A risk of miscarriage of this test is one in 200. Chorionic villus sampling (CVS) is performed between the 9th and 14th week of pregnancy. This test has a risk of miscarriage about one in 100. Percutaneous umbilical blood sampling (PUBS) is performed after 18 weeks of gestation. This test carries a risk of miscarriage greater than amniocentesis or CVS. Each test has 98 % to 99 % accuracy for prenatal diagnosis (Mayo Clinic Bookstore, 2006). There are many types of molecular diagnosis for DS. van Opstal *et al.* (1995) used fluorescent *in situ* hybridization (FISH) technique to identify chromosomal abnormality of fetuses and DS patients. Several researchers used quantitative fluorescence polymerase chain reaction (QF-PCR) assay to diagnose aneuploidies of chromosomes 13, 18, 21 and X (Pertl *et al*., 1997; Findlay *et al.*, 1998; Samura *et al*., 2001; Cirigliano *et al*., 2002;

Lee *et al.*, 2004). Real-time quantitative polymerase chain reaction (PCR) was used for detection of fetal aneuploidies (Zhong *et al.*, 2000) and dosage measurement of *DSCR1* and *DSCR3* genes for detection of trisomy 21 (Solassol *et al.*, 2003; Hu *et al.*, 2004). Internal control gene is needed in real-time PCR assay. These internal control genes consist of *CFTR* (cystic fibrosis transmembrane regulator), insulin-like growth factor (*IGF*)-1 and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Zimmermann *et al.*, 2002; Hu *et al.*, 2004; Yang *et al*., 2005). Among these genes, *GAPDH* was used in several studies involving DS because it is a housekeeping gene, common to all genome (Zhong *et al*., 2000, Zhong *et al*., 2001, Zimmermann *et al*., 2002; Zhong *et al*., 2003; Hu *et al*., 2004).

For sex identification, Sex-Determining Region *(SRY*) gene on the Y chromosome was used in several studies (Zhong *et al*., 2000; Zhong *et al*., 2001; Hromadnikova *et a*l., 2002; Zhong *et al*., 2003; Birch *et al*.,2004). This gene provided 100 % accurate results (Charalsawad *et al*., 2000). Another gene for sex detection, amelogenin, has been reported to contain a deletion on Y chromosome (Chang *et al*., 2003) and a mutation on the X chromosome (Shewale *et al*., 2000; Shadrach *et al*., 2004).

PCR assay was developed by Kary Mullis in the 1980s and real-time PCR was first used by Higuchi *et al*. (1993) for construction of a system to detect PCR products during amplification process. The real-time PCR system is based on the detection and quantitation of a fluorescent reporter which increases in direct proportion to the amount of PCR products in a reaction that occurs during exponential phase (Dorak, 2006). During the exponential phase in real-time PCR, a fluorescence signal threshold is determined at the point where all samples can be compared. This threshold is calculated by the amount of background fluorescence and is plotted at a point where the signal generated from a sample is significantly greater than the background fluorescence. Thus, the fractional number of PCR cycles required to generate enough fluorescence signal to reach this threshold is defined as the cycle threshold or Ct (Ginzinger, 2002). When the fluorescent signal reporter increases to a

detectable level, it can be captured and displayed as an amplification plot (Applied Biosystems, n.d.b).

There are seven signal detection formats in real-time PCR such as DNA-binding fluorophores, 5'- nuclease hydrolysis oligonucleotide probes, fluorescence resonance energy transfer (FRET) oligonucleotide probes, hairpin oligonucleotide probes or molecular beacons, light-up probes, sunrise primer and scorpion primer. However, recently only four main types of fluorescent light are used to detect PCR amplification products in real-time PCR. The first, dsDNA-specific dye, SYBR Green I, is the most commonly used. When it binds the Minor Groove of dsDNA, the intensity of fluorescence emission increase. The second, a hydrolysis probe, is an oligonucleotide labeled with a 5'-terminal reporter dye and a 3'-terminal fluorescence quencher. The third, a hybridization probe, uses donor and receptor fluorophores. The last, molecular beacon probe, is a DNA hybridization probe which forms a stem-loop structure and having label on both ends while the loop portion is complementary to the target sequence (Kaltanboeck and Wang, 2005).

Hydrolysis probe, also called 5'-nuclease probe due to the 5'- exonuclease activity of DNA polymerase cleaves the probe, is likely the most widely used fluorogenic probe format, while TaqMan probe is a typical one. Its structure is sequence-specific dually fluorophore with labeled DNA oligonucleotides. One fluorophore is called the quencher and the other is the reporter. When the quencher and reporter are closed as they are both attached to the same short oligonucleotide, the quencher absorbs the signal from the reporter. During amplification, the oligonucleotide is broken by 5'- nuclease activity of DNA polymerase, so the reporter and quencher are separated. Reporter's energy and fluorescent signal are independent. Therefore, destruction of oligonucleotide results in an increase of reporter signal and corresponds with the specific amplification of DNA (Valasek and Repa, 2005). A novel type of TaqMan probe, a minor groove binding (MGB), is known to increase the effective melting temperature (Tm) of the probe (Ginzinger, 2002).

There are two types of real-time quantitative PCR analysis, i.e., relative quantitation and absolute quantitation or standard curve quantitation (Ginzinger, 2002). Absolute quantification is the process that determines the absolute quantity of a single nucleic acid target sequence within an unknown sample (Applied Biosystems, 2004a) and is used to quantitate unknown samples by interpolating their quantity from a standard curve (Applied Biosystems, n.d.a.). This analysis required a sample of known quantity of interested gene that can be diluted to generate a standard curve to which unknown samples are compared (Valasek and Repa, 2005). Sources of known sample consist of plasmid DNA of interested gene, a synthetic oligonucleotide of all amplicon and a cell line with a known copy number or expression level of the interested gene (Ginzinger, 2002). Relative quantification describes the change in expression of target nucleic acid sequence in a test sample relative to the same sequence in a calibrator sample. A calibrator is the sample used as the basis for comparative results (Applied Biosystems, 2004b) and is used to compare within a sample which is performed with the interested gene and a control gene. This quantitation is done by subtracting the Ct of the control gene from the Ct of the interested gene and difference of cycle number (ΔCt) is the exponent of the base 2 that represents the fold difference of template for these two genes (Ginzinger, 2002).

The calculation methods used for relative quantitation are standard curve method and comparative Ct method. Standard curves for relative quantitation is easy to prepare because the quantity is expressed relative to some basis sample such as the calibrator. For all experimental samples, quantity is determined from the standard curve divided by the quantity of the calibrator. Thus all quantities are expressed as an n-fold difference relative to the calibrator. Comparative Ct method for relative quantitation is simlar to the standard curve method, except it uses the arithmetic formula, $2^{-\Delta\Delta Ct}$ to achieve the same result for relative quantitation. The standard curve method for absolute quantitation is similar to the standard curve method for relative quantitation but the absolute quantities of the standards must first be known by some independent methods. Plasmid DNA is commonly used to prepare absolute standards and concentration is measured by the spectrophotometer which subsequently

converted to the number of copies using the molecular weight of the DNA or RNA (Applied Biosystems, n.d.a).

In this study, real-time PCR method is used to simultaneously screen DS in three particular regions, *DSCR1*, *DSCR3* and *DSCAM*. Sex identification using *SRY* gene is also elucidated.

MATERIALS AND METHODS

Biological samples

For detection of DS and sex chromosome, a total of 100 Thai persons from two groups of patients were used in this study. Fifty blood samples of DS patients whose ages between 1 day to 40 years were obtained from two genetic centres, 33 samples were obtained from Rajanukul Institute, Department of Mental Health, Ministry of Plublic Health. The remaining 17 samples were obtained from Human Genetics Unit, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University. Fifty blood samples of normal healthy persons whose ages between 10 to 60 years old who submitted the DNA samples at Human Genetic Unit, Department of Pathology, Ramathibodi Hospital, Mahidol University were collected. The documents for sample collection were submitted to Ramathibodi Hospital Ethics Committee.

DNA extraction

Genomic DNA was extracted from blood samples using the DNA IQ^{TM}Systems kit (Promega, USA) according to the manufacturer's protocol and stored at 4°C until used.

Primers and MGB Taqman probes (Applied Biosystems, Foster City, CA)

Primers and probes were designed to span within *DSCR1*, *DSCR3*, *DSCAM*, *SRY* and *GAPDH* genes. The sequences and detailed information are shown in Table 1.

Table 1 The sequences of primers and probes designed to span within *DSCR1*, *DSCR3*, *DSCAM*, *SRY* and *GAPDH* genes for the detection of DS and sex chromosome.

Gene	Primer	Sequence	Tm (°C)	%GC	Amplicon length (bp)
DSCR1	Forward	5' CCGGGTGCCATGAACAGTA 3'	59	58	
	Reverse	5' TCCCAGTTCATGTTGCTCAGAA 3'	59	45	85
	Probe	5' FAM TGTGCAGCATTAGAAC 3' MGBNFQ	69	44	
DSCR3	Forward	5' CTCTGCCTTGGCGAGTATCTG 3'	59	57	
	Reverse	5' GGTCTGTGGCCAGTTGACATC 3'	59	57	81
	Probe	5' FAM AACTTCCTGACCATCAAG 3' MGBNFQ	69	44	
DSCAM	Forward	5' CTTCGCACGCATTTCAGACTAT 3'	58	45	
	Reverse	5' TTACGCCTGCATTTAGGTGATG 3'	59	45	92
	Probe	5' VIC CAGTCACTAGCCAAATC 3' MGBNFQ	69	47	
SRY	Forward	5' TGACTCTTTGGTTCACCATGTTG 3'	59	43	
	Reverse	5' TGCACGGTTTCTTTTGAGGAT 3'	58	43	86
	Probe	5' VIC TCACCTCTCTCTAGTTCCA 3' MGBNFQ	69	47	
GAPDH	Forward	5' CTCCCCACACACATGCACTTA 3'	58	52	
	Reverse	5' AGGGCTTTGATTTGCCAAGTT 3'	59	43	90
	Probe	5' NED CCACTCCTGATTTCT 3' MGBNFQ	69	47	

Real-time PCR analysis

1. DS screening and sex identification by relative quantification using comparative Ct method

The real-time PCR analysis was performed on an Applied Biosystems 7500 Real-Time PCR System. This quantitation was performed in duplicate assays in 96-well plates; each 25 µl reaction consisted of 12.5 µl of 2X Taqman universal PCR Mastermix (Applied Biosystems, USA),150 nM forward and reverse primers of *DSCR1,* 250 nM forward and reverse primers of *DSCR3*, 300 nM forward and reverse primers of *DSCAM*, 100 nM forward and reverse primers of *SRY* and 150 nM forward and reverse primers of *GAPDH*, 100 nM MGB Taqman probes of *DSCR1*, 230 nM MGB Taqman probes of *DSCR3*, 270 nM MGB Taqman probes of *DSCAM*, 100 nM MGB Taqman probes of *SRY* and 100 nM MGB Taqman probes of *GAPDH*, 1ng genomic DNA. Thermocycling was done at 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min.

The experiment of duplex assay for three reactions were performed on each pair of *DSCR1* and *GAPDH*, *DSCR3* and *GAPDH*, *DSCAM* and *GAPDH* genes, at which *DSCR1*, *DSCR3* and *DSCAM* acted as target genes and *GAPDH* as an endogenous control gene. Three duplex assays were used to simultaneously determine these three target genes. At least two target genes in each sample was used as an indicator for normal healthy persons or DS patients. The target genes and endogenous control gene were amplified in the same wells. The normal healthy control was used as a calibrator. The SDS software (Applied Biosystems, USA) was used to set gene dosage for the calibrator samples to 1. Therefore, gene dosages of *DSCR1*, *DSCR3* and *DSCAM* of normal healthy persons were equal to 1, and those of DS patients were equal to 1.5.

Triplex assay was performed only on *DSCR1*, *SRY* and *GAPDH* genes. In this case, *DSCR1* and *SRY* genes functioned as target genes and *GAPDH* as an endogenous control gene. The target genes and endogenous control genes were also

amplified in the same wells. Calibration was done as previously mentioned. Sex identification was determined by the presence or absence of *SRY* gene sequence.

2. DS screening by relative quantification using standard curve method

Real-time PCR analysis was performed on an Applied Biosystems 7500 Real-Time PCR System. This quantitation was done in duplicate assays in 96-well plates. Each 25 µl reaction mixture consisted of 12.5 µl of 2X Taqman universal PCR Mastermix (Applied Biosystems, USA), 270 nM forward and reverse primers of *DSCR3* and 150 nM forward and reverse primers of *GAPDH*, 270 nM MGB Taqman probes of *DSCR3* genes and 100 nM MGB Taqman probes of *GAPDH*, 1ng genomic DNA. Each gene was performed in singleplex assay. Thermocycling was done at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. Each of the 96-well real-time quantitative PCR plate included serial dilutions (0.000001, 0.00001, 0.0001, 0.001, 0.01, 0.1 and 1 ng) of plasmid DNA, which were used to generate standard curves for *DSCR3* and *GAPDH*. The dosage of the *DSCR3* was obtained by calculating the ratio of *DSCR3* to *GAPDH* (*DSCR3/GAPDH*).

3. Statistical analysis

Mean, standard deviation (SD) and coefficiency of variation (% CV) from relative quantity (RQ) and relative ratio of each assay were calculated and used to determine the repeatability or precision of the assay. Sensitivity, specificity, accuracy, positive predictive value (PPV), negative predictive value (NPV), false positive and false negative were used to evaluate real-time PCR efficiency for clinical sample analysis.

4. Producing a plasmid DNA for standard curve generation by PCR cloning

4.1 Producing PCR product

PCR reaction was performed in a final volume of 25 µl containing 1.5 U *Taq* polymerase, 200 µM of each dNTP, 2.5 µl of 10X PCR buffer, 2.0 mM $MgCl_2$, and 300 nM of each primer. PCR was set for the initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec and final extension was done at 72°C for 7 min and held amplification products at 4°C until used.

4.2 TOPO cloning reaction and chemical transformation

TOPO cloning reaction consisted of 1.0 µl fresh PCR product, 1.0 µl sterile water, 0.5 µl salt solution (1.2 M NaCl and 0.06 M $MgCl_2$) and 0.5 µl TOPO vector. The solution was mixed and incubated for 5 min at room temperature. Then the reaction mixture was placed on ice for 30 min and proceeded to the chemical transformation step. The 1 µl of TOPO cloning reaction was added into a 25 µl vial of Mach1 chemically competent *E.coli* (Invitrogen, USA) and mixed. The reaction mixture was incubated on ice for 30 min. The cells were heat-shocked for 1 min and 30 sec at 42 °C and immediately transferred to ice. The samples were mixed with 250 µl of SOC medium (20 g of Tryptone, 5 g of yeast extract, 0.5 g of NaCl, 2.5 ml of 1M KCl, 1000 ml of distilled water and 20 ml of 1M glucose) and shaken at 37 °C for 1 hour. The 10-50 µl of each transformation was spreaded on a pre-warmed LB agar plate (10 g of Tryptone, 5 g of yeast extract, 10 g of NaCl, 15 g of agar and 1000 ml of distilled water) containing 100 µg/ml spectinomycin and incubated overnight at 37 °C. The white colonies were brought for analysis of positive clones.

4.3 Analysis of positive clones

All the white colony was cultured in 2 µl of LB medium (10 g of Tryptone, 5 g of yeast extract, 10 g of NaCl and 1000 ml of distilled water) containing

100 µg/ml spectinomycin at 37°C and shook for overnight. An overnight culture was harvested by centrifugation. 330 µl of lysis buffer (80 g of sucrose powder, 200 ml of 250 mM EDTA, 10 ml of 1M Tris-HCl pH 8.0), 740 ml of distilled water and 50 ml of 10% Triton-X) was added to the pellet, mixed by vortex. Approximately, 25 µl of lysozyme was added, mixed and boiled for 1 min, immediately centrifuged at 16,000 x g at room temperature for 10 min, and the pellet was removed using tooth pick. Finally, 33 µl of 3 M sodium acetate (NaOAc) and 0.5 ml of isopropanol were added, mixed and incubated at room temperature for 15 min. After the incubation, the reaction mixture was centrifuged at 16,000 x g at room temperature for 13 min. The supernatant was discarded and the pellet was rinsed with 1 ml of 80% ethanol, mixed, and centrifuged at 16,000 x g at room temperature for 2 min. This process was repeated twice. The supernatant was discarded, while the pellet was vacuum-dried for 10-15 min and resuspended with 50 µl distilled water. The plasmids DNA were sequenced.

4.4 Cycle sequencing

The ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit was used with the ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA). The fluorescence-based cycle sequencing reaction was performed on a double-stranded DNA template as described by the manufacturer.

RESULTS

1. Karyotype analysis

The karyotypes of 50 DS patients were tested using a conventional G-banding analysis based on standard procedures. Trisomy 21 was the most frequent abnormality found, i.e., 96 % with 34 % female and 62 % male patients. The other abnormal chromosomes were mosaicism DS (2 %) and translocation DS (2 %) (Table 2).

Table 2 Karyotypes of DS patients in this thesis (n=50)

Chromosome abnormality	**Karyotype**	**n*** (%)
Trisomy21	47, XX, +21 (female) 47, XY, +21 (male)	17 (34 %) 31 (62 %)
Mosaic	mos47, XY, +21[22]/46, XY[53]	1 (2 %)
Translocation	46, XY, der(14;21)(q10;q10), +21	1 (2 %)

Note: n=number of cases

2. Real-time PCR

2.1 DS screening and sex identification by relative quantification using comparative Ct method

DNA samples collected from 100 Thai subjects identified as 50 DS patients and 50 normal healthy persons were tested for relative quantification using a comparative Ct method.

Three tubes of duplex assays using primers and probes for (i) *DSCR1-GAPDH*, (ii) *DSCR3-GAPDH* and (iii) *DSCAM-GAPDH* genes were performed in each DNA sample. The relative quantity (RQ) comparing target gene with *GAPDH* gene quantity was calculated from each sample in the same tube. Theoretically, the RQ value in DS patient should be 1.5 times higher than the normal control. From our system the sample is diagnosed as DS when RQ values are ≥1.250 and as normal when RQ are less than 1.250. In the normal controls and DS patients, the data of duplex assay for *DSCR1* gene showed the threshold cycle (Ct) ranging from 27.24 to 32.57 while the Ct of *GAPDH* gene were 28.27-33.27. The RQ for *DSCR1* and *GAPDH* in normal healthy persons were 1.19±0.35 (mean ± SD) while DS patients were 1.76±0.70 (mean ± SD). In both groups, the data of duplex assay for *DSCR3* gene showed Ct in the range of 27.30 to 35.00, while the Ct of *GAPDH* gene were 28.34-34.35. The RQ for *DSCR3* and *GAPDH* in normal healthy persons were 1.16±0.33 (mean ± SD) but those of DS patients were 1.91±0.58 (mean ± SD). In both groups, the data of duplex assay for *DSCAM* gene, however, showed the Ct in the range of 28.03 to 33.88, while the Ct of *GAPDH* were 28.14-33.03. The RQ for *DSCAM* and *GAPDH* in normal healthy persons were 1.20±0.65 (mean ± SD) and DS patients were 1.95±1.41 (mean ± SD).

For RQ of *DSCR1* in the normal control, 31 samples (62 %) were in the range of 0.703-1.246 (samples No. 1, 3-9, 15, 18-24, 20-28, 30-33, 34-35, 37-38, 40-41, 43, 47, 49) that were assumed to be normal person while the other 19 samples (38 %) gave the RQ of 1.250-2.768, which considered to be DS patient. Among 50 DS patients, 34 samples (68%) had RQ of 1.252-1.976 (samples No. 1-7, 9-11, 13, 16, 18-27, 30-32, 34-35, 38, 42-45, 49-50) and 8 (16 %) samples had RQ as high as 2.077-4.474 (samples No. 8, 12, 28-29, 33, 36, 47). Both groups were considered to be DS patients. The remaining 7 samples (14 %) of DS patients had RQ of 1.117-1.209 (samples No. 14-15, 17, 37, 39, 41, 48) which were the ratio of normal person.

For RQ of *DSCR3* in the normal control, 34 samples (68 %) were in the range of 0.663-1.241 (samples No. 1-10, 13-15, 17-22, 24, 26, 29-31, 34-41, 48-49) which assumed to be normal person. The remaining 16 samples (32 %) had RQ of

1.268-2.289 and assumed to be DS patient. In DS patient group, 34 samples (68 %) had RQ in the range of 1.255-1.993 (samples No. 1-2, 4-7, 12-18, 20-27, 30, 32, 34-35, 38-44, 49-50) and 13 samples (26 %) had RQ in the high range of 2.026-4.395 (samples No. 3, 8-11, 19, 28-29, 31, 33, 36, 47-48) and considered to be DS patient. The other 2 samples (4 %) had RQ of 1.016-1.121 (samples No. 37, 45) which were in the range of normal person.

For RQ of *DSCAM* in the normal control, 34 samples (68 %) were in the range of 0.583-1.154 (samples No. 7-10, 14-15, 19-38, 40-41, 43, 45-46, 48-50) assuming to be normal, while the remaining 16 samples (32 %) had RQ of 1.263-3.789 and could be assigned as DS patient. In the DS group, 30 samples (60 %) had RQ in the range of 1.316-1.964 (samples No. 1-3, 5-7, 13-14, 16, 19, 21-27, 30, 32, 34-35, 37-41, 43, 48-50), and 13 samples (26 %) had RQ in the high range of 2.105-10.585 (samples No. 4, 8, 11-12, 15, 17, 28-29, 31, 33, 36, 42, 47) designated as DS patient. However, 6 samples (12 %) of this group had RQ in the low range of 0.748-1.244 (samples No. 9-10, 18, 20, 44-45) and could be considered as normal.

For one mosaic DS sample (sample No. 46), it was found that RQ of *DSCR1* was 1.213 and RQ of *DSCR3* was 0.607 which were the ratio of normal person but RQ of *DSCAM* gave the high value of 3.344. The results are demonstrated in Tables 3 and 4.

In these duplex assays, the RQ value of the three genes were determined in each DNA sample. If the RQ values of two genes are equal to or higher than 1.250, it would likely be a DS person but those lower than 1.250 would be normal persons. In the normal control group, 35 samples (70 %) gave RQ in the range of normal person (samples No. 1, 3-10, 14-15, 18-24, 26-32, 34-38, 40-41, 43, 48-49), while the remaining 15 samples (30 %) were detected in DS range. In DS patient group, the RQ of 47 samples (94 %) were detected as DS (samples No. 1-36, 38-44, 47-50) whereas the remaining 3 samples (6 %) including one mosaic DS sample (sample No. 46) were determined as normal.

Table 3 RQ of *DSCR1*, *DSCR3* and *DSCAM* genes from duplex assays in 50 normal healthy persons.

Normal healthy persons (No.)	**RQ (DSCR1/GAPDH)**	**RQ (DSCR3/GAPDH)**	**RQ (DSCAM/GAPDH)**
1	0.794	0.894	2.810
2	1.316	1.125	1.277
3	1.131	0.966	1.289
4	1.132	1.023	3.789
5	1.236	1.195	1.263
6	1.167	0.720	3.542
7	0.939	1.169	0.698
8	0.886	0.849	1.096
9	0.942	1.032	0.788
10	1.360	1.241	1.134
11	1.761	1.649	1.742
12	1.313	1.303	1.384
13	1.260	1.098	1.390
14	1.595	1.053	0.986
15	1.190	0.963	1.039
16	1.285	1.880	1.662
17	1.416	1.200	1.317
18	0.703	0.663	1.371
19	0.713	0.840	0.984
20	1.195	0.961	1.018
21	1.242	0.879	1.102
22	1.130	0.806	0.981
23	0.950	1.493	0.588
24	1.006	0.960	0.589
25	1.513	1.286	0.748

Table 3 (Continued)

Normal healthy persons (No.)	RQ (DSCR1/GAPDH)	RQ (DSCR3/GAPDH)	RQ (DSCAM/GAPDH)
26	0.817	0.996	0.786
27	0.973	1.336	1.073
28	1.178	1.693	0.811
29	1.301	0.847	0.982
30	1.080	1.148	1.045
31	0.868	1.030	0.797
32	1.102	1.551	0.976
33	1.274	1.268	0.600
34	0.918	0.986	0.583
35	1.122	0.859	0.789
36	1.374	1.237	1.154
37	0.752	0.770	0.804
38	0.968	0.945	0.861
39	2.040	1.057	1.451
40	0.830	1.073	0.655
41	1.041	1.143	1.138
42	2.768	2.289	2.285
43	1.138	1.724	1.118
44	1.328	1.574	1.263
45	1.342	1.402	1.153
46	1.276	1.426	1.050
47	0.923	1.415	1.423
48	1.250	0.791	0.876
49	1.246	0.831	0.917
50	1.545	1.610	0.982

Table 4 RQ of *DSCR1*, *DSCR3* and *DSCAM* genes from duplex assays in 50 DS patients.

DS patients (No.)	RQ (DSCR1/GAPDH)	RQ (DSCR3/GAPDH)	RQ (DSCAM/GAPDH)
1	1.252	1.766	1.490
2	1.714	1.280	1.462
3	1.312	2.026	1.329
4	1.504	1.332	2.239
5	1.374	1.610	1.733
6	1.976	1.406	1.620
7	1.502	1.575	1.352
8	2.471	2.871	2.598
9	1.469	2.379	0.748
10	1.610	2.479	0.780
11	1.667	2.208	2.216
12	3.368	1.825	10.585
13	1.430	1.909	1.556
14	1.162	1.658	1.735
15	1.204	1.255	3.025
16	1.498	1.863	1.735
17	1.209	1.616	3.663
18	1.673	1.980	1.047
19	1.841	2.496	1.964
20	1.564	1.840	1.136
21	1.485	1.705	1.356
22	1.440	1.883	1.694
23	1.424	1.575	1.692
24	1.460	1.894	1.545
25	1.389	1.675	1.583

Table 4 (Continued)

DS patients (No.)	RQ (DSCR1/GAPDH)	RQ (DSCR3/GAPDH)	RQ (DSCAM/GAPDH)
26	1.701	1.864	1.544
27	1.851	1.993	1.822
28	2.772	2.560	2.292
29	3.542	4.395	3.790
30	1.776	1.824	1.674
31	1.927	2.569	2.105
32	1.267	1.894	1.464
33	2.560	2.219	2.170
34	1.639	1.585	1.570
35	1.774	1.544	1.645
36	3.581	3.020	2.930
37	1.162	1.016	1.431
38	1.552	1.568	1.555
39	1.117	1.548	1.367
40	2.077	1.657	1.404
41	1.145	1.526	1.316
42	1.309	1.503	2.171
43	1.566	1.903	1.616
44	1.276	1.672	1.066
45	1.593	1.121	1.244
47	4.474	3.010	3.103
48	1.149	2.155	1.421
49	1.687	1.423	1.449
50	1.771	1.773	1.662

Note: DS patient No.46 (mosaicism) was not included.

The triplex assays using primers and probes for *DSCR1*, *SRY* and *GAPDH* genes were performed. The data of triplex assays in all samples tested for *DSCR1*, *SRY* and *GAPDH* genes showed Ct values in the range of 26.59 to 34.31, 28.86 to 32.86 and 28.29 to 34.76, respectively. RQs of *DSCR1* in normal healthy persons were 1.08±0.31 (mean ± SD) but those of DS patients were 1.66±0.61 (mean ± SD). *SRY* gene was present in all males while females were undetected. As for the RQ of *DSCR1* and *GAPDH* in the normal control, 40 samples (80 %) gave the values in the range of 0.506-1.233 (samples No.1-3, 5, 7-10, 11, 13-25, 27-35, 39-41, 43, 45-47, 49-50) which were assumed to be normal while the remaining 10 samples (20 %) had RQ of 1.278-2.500. In DS patient group, 25 samples (50 %) had RQ in the moderate range of 1.303-1.997 (samples No.5-7, 9-11, 13, 15, 17, 19, 28-29, 31-35, 37-41, 43-45) which assumed to be DS patient, and 9 samples (18%) had high RQ values of 2.044-3.516 (samples No.8, 12, 14, 16, 18, 36, 47-49) but 15 samples (30 %) had RQ values of only 0.780-1.204 (samples No. 1-4, 20-27, 30, 42, 50) which were the ratio of normal person. For one mosaic DS sample (sample No. 46), however, the RQ was found to be 0.952 which is considered a normal person. For *SRY* gene, the Ct value higher than 35 cycles was scored as negative and all female samples were not detected as expected but all male samples were detected within the Ct value of 33 (Tables 5 and 6). Since sex identification was determined by the presence or absence of *SRY* gene, results were completely concordant with sex identification, i. e., all 59 males gave positive results for *SRY* gene and 41 females gave negative results.

Table 5 RQ of *DSCR1* and the result of *SRY* detection from triplex assays in 50 normal healthy persons.

Normal healthy persons (No.)	**RQ of *DSCR1*/*GAPDH***	**SRY gene detection (Ct values)**	**Score for SRY gene detection**
1-M	0.702	29.983	positive
2-M	0.707	29.557	positive
3-M	0.506	30.006	positive
4-F	1.287	not detected	negative
5-M	1.155	30.613	positive
6- M	1.278	31.108	positive
7-F	0.985	not detected	negative
8- M	1.019	30.441	positive
9-F	0.995	not detected	negative
10-F	0.489	not detected	negative
11-M	1.153	31.509	positive
12-F	1.441	39.862*	negative
13-M	1.170	30.757	positive
14-F	0.601	not detected	negative
15-F	0.825	not detected	negative
16-M	0.871	31.945	positive
17-F	0.662	39.953*	negative
18-M	0.995	32.723	positive
19-F	0.86	not detected	negative
20-M	1.132	32.807	positive
21-M	1.173	31.545	positive
22-M	1.112	31.326	positive
23-F	0.963	not detected	negative
24-F	1.223	36.613*	negative
25-F	1.017	37.691*	negative

Table 5 (Continued)

Normal healthy persons (No.)	**RQ of *DSCR1*/ *GAPDH***	**SRY gene detection (Ct values)**	**Score for SRY gene detection**
26-M	1.322	32.394	positive
27-F	1.011	not detected	negative
28-M	1.020	31.629	positive
29-F	0.933	not detected	negative
30-M	0.923	30.605	positive
31-F	0.941	not detected	negative
32-F	0.898	not detected	negative
33-M	0.749	32.379	positive
34-F	0.890	not detected	negative
35-F	1.152	not detected	negative
36-F	1.295	not detected	negative
37-M	1.424	30.956	positive
38-F	1.495	37.935*	negative
39-M	1.051	32.354	positive
40-F	1.224	not detected	negative
41-F	0.985	not detected	negative
42-F	2.500	not detected	negative
43-F	1.222	not detected	negative
44-M	1.409	31.446	positive
45-M	1.233	30.798	positive
46-M	1.164	30.901	positive
47-M	0.899	30.813	positive
48-M	1.403	30.480	positive
49-M	1.132	30.337	positive
50-M	1.208	30.219	positive

Table 6 RQ of *DSCR1* and the result of *SRY* detection from triplex assays in 50 DS patients.

DS patients (No.)	RQ of *DSCR1*/*GAPDH*	SRY gene detection (Ct values)	Score for SRY gene detection
1-M	0.902	29.512	positive
2-M	1.078	29.947	positive
3-F	1.120	35.234*	negative
4-F	1.172	not detected	negative
5-M	1.705	29.312	positive
6-F	1.850	37.421*	negative
7-M	1.769	30.660	positive
8-F	3.516	37.437*	negative
9-M	1.597	31.545	positive
10-F	1.923	not detected	negative
11-M	1.738	31.327	positive
12-M	3.445	31.372	positive
13-M	1.852	31.325	positive
14-M	2.096	31.515	positive
15-F	1.626	not detected	negative
16-M	2.044	31.253	positive
17-F	1.997	37.011*	negative
18-M	2.149	30.220	positive
19-M	1.854	32.028	positive
20-M	1.124	31.297	positive
21-F	1.039	not detected	negative
22-F	0.958	not detected	negative
23-F	1.126	36.311*	negative
24-F	1.140	not detected	negative
25-M	1.149	32.834	positive

Table 6 (Continued)

DS patients (No.)	RQ of *DSCR1*/*GAPDH*	SRY gene detection (Ct values)	Score for SRY gene detection
26-M	0.958	31.685	positive
27-F	1.164	not detected	negative
28-F	1.993	not detected	negative
29-M	1.723	31.508	positive
30-M	0.780	31.828	positive
31-M	1.855	31.634	positive
32-M	1.572	32.300	positive
33-F	1.323	38.808*	negative
34-M	1.831	32.223	positive
35-M	1.308	32.490	positive
36-M	3.209	32.307	positive
37-M	1.726	32.185	positive
38-M	1.719	32.001	positive
39-M	1.335	32.211	positive
40-M	1.538	32.857	positive
41-F	1.386	not detected	negative
42-F	1.061	not detected	negative
43-M	1.303	31.290	positive
44-M	1.584	31.394	positive
45-M	1.586	30.834	positive
46-M	-	31.973	positive
47-M	2.828	31.236	positive
48-F	2.205	not detected	negative
49-M	2.145	30.859	positive
50-M	1.204	28.854	positive

Table 6 (Continued)

Note: F=Female, M=Male, *=Ct >35 and

DS patient No.46 (mosaicism) was not included for *DSCR1*/*GAPDH*.

2.2 DS screening by relative quantification using a standard curve method

Amplified products of *DSCR3* and *GAPDH* genes were cloned into pCR8/GW/TOPO plasmid vector and sequenced to confirm their identity using the dideoxy nucleotide chain termination method. These plasmid DNA of the two genes were used as a source of known samples for construction of the standard curve of each gene. There was a high linear correlation between Ct values and the log values of the concentrations of the genes from the standard curve of plasmid DNA for *DSCR3* and *GAPDH* gene (R^2= 0.999, 0.990) using serial dilutions of plasmid DNA from 0.00001 to 1 ng (Figures 1 and 2)

Twenty DNA samples, i.e., 10 DS patients and 10 normal healthy persons, were subjected to relative quantification using the standard curve method. The singleplex assays using primers and probes for *DSCR3* and *GAPDH* genes were performed. Theoretically, the ratio of *DSCR3*/*GAPDH* in DS patients should be 1.5 and 1.0 in the normal control. However, from our system to determine the potential DS patients, *DSCR3*/*GAPDH* value equal to or higher than 1.250 was used while *DSCR3*/*GAPDH* lower than 1.250 was designated as normal. Subsequently, the dosage of *DSCR3* and *GAPDH* genes were calculated from the *DSCR3*/*GAPDH* ratio, showing 0.712±0.196 (mean ± SD) for healthy persons and 2.302±0.739 (mean ± SD) for DS patients.

From the normal persons, all 10 samples gave *DSCR3*/*GAPDH* ratio in the range of 0.485-1.095. As for DS patients, five samples gave *DSCR3*/*GAPDH* ratio in the range of 1.393-1.986 (samples No. 9, 30, 31, 44, 49) and five samples were as high as 2.211-3.692 (samples No. 5, 6, 34, 40, 47) (Tables 7 and 8).

Table 7 *DSCR3*/*GAPDH* ratio from singleplex assays in 10 normal healthy persons.

Normal healthy persons (No.)	***DSCR3/GAPDH* ratios**
12	0.572
13	0.547
15	1.095
17	0.646
25	0.519
43	0.749
44	0.485
45	0.904
46	0.851
47	0.748

Table 8 *DSCR3*/*GAPDH* ratio from singleplex assays in 10 DS patients.

DS patients (No.)	***DSCR3/GAPDH* ratios**
5	2.211
6	2.856
9	1.986
30	1.692
31	1.618
34	2.790
40	2.957
44	1.822
47	3.692
49	1.393

Slope = -3.458

R^2 = 0.999

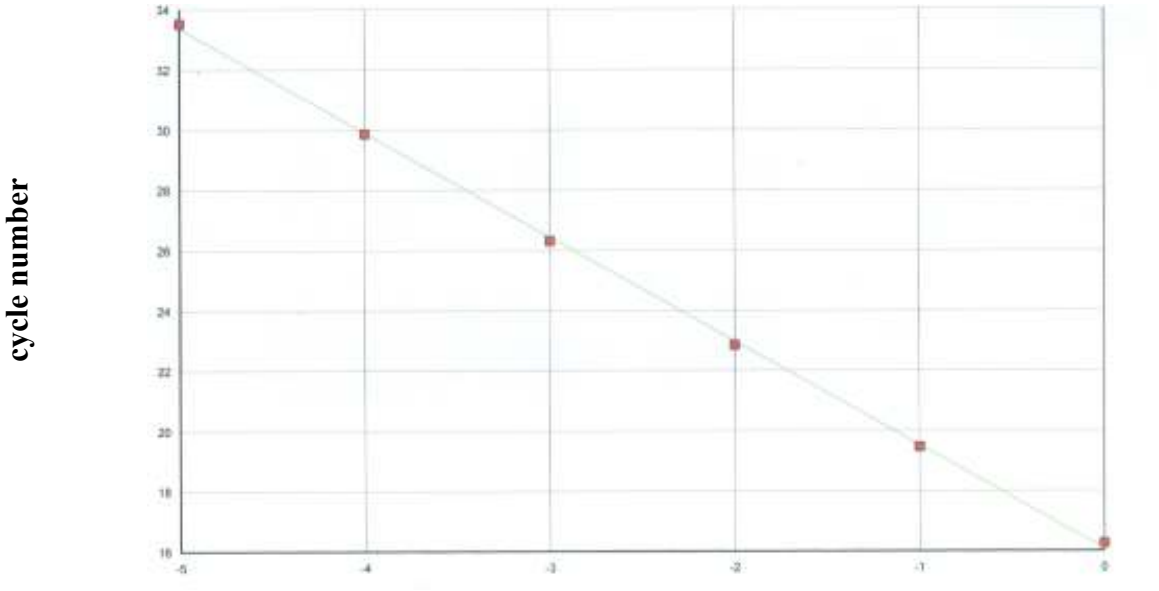

Figure 1 A standard curve of plasmid DNA real-time PCR for *DSCR3* gene showing a correlation between real-time PCR cycle number and plasmid DNA amount (log ng).

Slope = -3.444

R^2 = 0.990

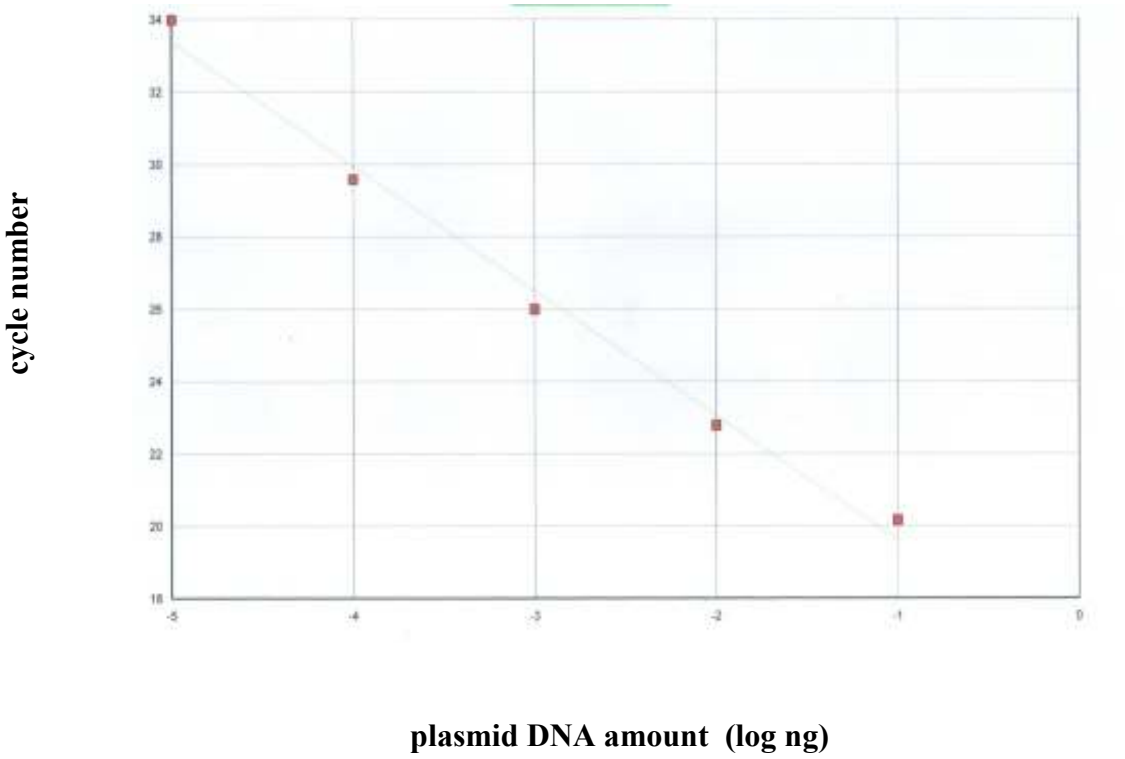

Figure 2 A standard curve of plasmid DNA real-time PCR for *GAPDH* gene showing a correlation between real-time PCR cycle number and plasmid DNA amount (log ng).

2.3 Statistical analysis

2.3.1 Repeatability test

The repeatability of the singleplex, duplex and triplex assays was performed using one DNA sample from a normal male. For within-run repeatability, the DNA sample was subjected to five replicate analyses in a single run of each assay. The same sample was used for between-run repeatability by replicating four times for singleplex assay, ten times for triplex assay, twelve times for duplex assay of *DSCR1* and *GAPDH*, *DSCAM* and *GAPDH* and eleven times for duplex assay of *DSCR3* and

GAPDH. The mean, standard deviation (SD) and coefficient of variation (%CV) of RQ and relative ratio are shown in Tables 9 and 10.

Table 9 The results of within-run repeatability testing in each gene by real-time PCR method.

No.	**Within-run (n=5)**					
	Singleplex assay	**Duplex assay**			**Triplex assay**	
	DSCR3/GAPDH	***DSCR1/GAPDH***	***DSCR3/GAPDH***	***DSCAM/GAPDH***	***DSCR1/GAPDH***	***SRY***
1	1.138	0.918	1.189	1.534	1.137	positive
2	1.107	1.138	0.804	1.430	1.586	positive
3	1.273	1.272	0.806	0.819	1.501	positive
4	1.092	1.358	1.128	1.163	1.154	positive
5	0.955	1.272	1.351	0.758	1.253	positive
Mean	1.113	1.192	1.056	1.141	1.326	-
SD	0.114	0.172	0.243	0.350	0.205	-
% CV	10.211	14.435	23.006	30.643	15.493	-

Table 10 The results of between-run repeatability testing in each gene by real-time PCR method.

No.	**Between-run**					
	Singleplex assay (n=4)	**Duplex assay**			**Triplex assay (n=10)**	
	DSCR3/GAPDH	***DSCR1/GAPDH* (n=12)**	***DSCR3/GAPDH* (n=11)**	***DSCAM/GAPDH* (n=12)**	***DSCR1/GAPDH***	*SRY*
1	1.053	1.028	1.017	0.816	1.019	positive
2	0.884	1.019	1.054	0.932	1.155	positive
3	0.979	0.989	0.910	0.851	1.170	positive
4	0.698	0.893	0.711	0.961	0.871	positive
5	-	0.769	1.137	1.126	1.112	positive
6	-	1.049	0.754	1.047	1.132	positive
7	-	1.008	1.072	1.050	0.749	positive
6	-	1.153	1.004	0.959	0.837	positive
9	-	1.029	0.970	0.992	0.774	positive
10	-	1.138	1.074	1.169	0.721	positive
11	-	1.214	0.956	0.984	-	-
12	-	1.217	-	0.801	-	-
Mean	0.904	1.042	0.969	0.974	0.954	-
SD	0.153	0.129	0.133	0.115	0.182	-
% CV	16.986	12.389	13.732	11.781	19.049	-

2.3.2 Evaluation of real-time PCR efficiency for clinical sample analysis

The values of relative ratio using standard curve and RQ obtained by comparative Ct of each assay were compared. First, evaluation of *DSCR3/GAPDH* ratio from a singleplex assay using standard curve method, showed no false negative or false positive in the experimental data. Therefore, there was a 100 % accuracy of relative ratios in the normal healthy persons and the patients. Second, duplex assay to determine three genes (*DSCR1*, *DSCR3* and *DSCAM*) by RQ was considered together. In the patients No. 37, 45 and 46 (mosaicism) (Table 4) the RQ values of two from three genes were lower than 1.250 while in the normal healthy persons No. 2, 11-13, 16-17, 25, 33, 39, 42, 44-47, 50 (Table 3)) the RQ values of at least two from three genes were higher than 1.250. Therefore, there were false negative results in the patients No. 37, 45,46 and false positive results in the normal healthy persons No. 2, 11-13, 16-17, 25, 33, 39, 42, 44-47, 50. Finally, the RQ of *DSCR1* and *GAPDH* from triplex assay gave false negative results in the patients No. 1-4, 20-27, 30, 42, 46, 50 (Table 6) and false positive results in the normal persons No. 4, 6, 12, 26, 36-38, 42, 44 and 48 (Table 5). However, sex identification using primers and probe for *SRY* gene was totally agreed with the known phenotype of the samples.

Cross-tabs relative to the ratio between real-time PCR result and true result described the methods in terms of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) had been used to show the efficiency of the test (Table 11).

Table 11 Cross-tabs of statistical analysis

Known result	**Real-time PCR result**	
	Positive	**Negative**
Positive	True Positive	False Negative
Negative	False Positive	True Negative

Source: Everitte, 1994

The results from 50 DS and 50 normal persons were compared with the known results (Tables 12-15). The sensitivity and specificity of singleplex real-time PCR assay for the detection of DS were both 100 %. The predictive values of the positive and negative test were also 100 %, while the false positive and negative rates were all 0 % (Table 12). As for duplex real-time PCR assay for the detection of DS, the sensitivity and specificity were 94 % and 70 %, respectively. The predictive values of the positive and negative test were 75.81 % and 92.11 %, whereas the false positive and negative rates were 30 % and 6 %, respectively (Table 13). The sensitivity and specificity were 68 % and 80 % for triplex real-time PCR assay for the detection of DS, the predictive values of the positive and negative test were 77.27 % and 71.43 %, the false positive and negative rates were 20 % and 32 %, respectively (Table 14). The sensitivity and specificity for triplex real-time PCR assay for the detection of sex were both 100 % as anticipated. The predictive values of the positive and negative test were also 100 % while the false positive and negative rates were 0 % (Table 15).

Table 12 Comparison between the known and real-time PCR results by singleplex assay.

Known result	**Singleplex real-time PCR assay**		**Total (n)**
	DS (n)	**Normal (n)**	
Positive (10)	10	0	10
Negative (10)	0	10	10
Total (20)	10	10	20

Sensitivity	= (10/(10+0))x100	= 100 %
Specificity	= (10/(10+0))x100	= 100 %
Accuracy	= ((10+10)/20)x100	= 100 %
Positive predictive value	= 10/(10+0))x100	= 100 %
Negative predictive value	= (10/(10+0))x100	= 100 %
False positive	= (0/10)x100	= 0 %
False negative	= (0/10)x100	= 0 %

Table 13 Comparison between the known and real-time PCR results by duplex assay.

Known result	**Duplex real-time PCR assay**		**Total (n)**
	DS (n)	**Normal (n)**	
Positive (50)	47	3*	50
Negative (50)	15	35	50
Total (100)	62	38	100

Sensitivity	= (47/(47+3))x100	= 94 %
Specificity	= (35/(35+15))x100	= 70 %
Accuracy	= ((47+35)/100)x100	= 82 %
Positive predictive value	= (47/(47+15))x100	= 75.81 %
Negative predictive value	= (35/(35+3))x100	= 92.11 %
False positive	= (15/50)x100	= 30 %
False negative	= (3/50)x100	= 6 %

Note: * = One case was Mosaic DS with mos47,XY,+21[22]/46,XY[53] karyotype.

Table 14 Comparison between the known and real-time PCR results by triplex assay for detection of DS.

Known result	**Triplex real-time PCR assay (Detection of DS)**		**Total (n)**
	DS (n)	**Normal (n)**	
Positive (50)	34	16*	50
Negative (50)	10	40	50
Total (100)	44	56	100

Sensitivity	= (34/(34+16))x100	= 68 %
Specificity	= (40/(40+10))x100	= 80 %
Accuracy	= ((34+40)/100)x100	= 74 %
Positive predictive value	= (34(34+10))x100	= 77.27 %
Negative predictive value	= (40/(40+16))x100	= 71.43 %
False positive	= (10/50)x100	= 20 %
False negative	= (16/50)x100	= 32 %

Note: * = One case was Mosaic DS with mos47,XY,+21[22]/46,XY[53] karyotype.

Table 15 Comparison between the known and real-time PCR results by triplex assay for sex detection.

Known result	**Triplex real-time PCR assay (Sex detection)**		**Total (n)**
	Male (n)	**Female (n)**	
Positive (59)	59	0	59
Negative (41)	0	41	41
Total (100)	59	41	100

Sensitivity	= (59/(59+0))x100	= 100 %
Specificity	= (41/(41+0))x100	= 100 %
Accuracy	= ((59+41)/100)x100	= 100 %
Positive predictive value	= (59/(59+0))x100	= 100 %
Negative predictive value	= (41/(41+0))x100	= 100 %
False positive	= (0/59)x100	= 0 %
False negative	= (0/41)x100	= 0 %

DISCUSSION

The standard method used for chromosome abnormality detection is karyotypic analysis. This method gives accurate results but the disadvantages of this technique are the requirement for a large number of cells, technical expertise, labor intensive and time consuming. In the last decade, real-time PCR has been applied to use for the detection of trisomy 21 (Zimmermann *et al*., 2002; Pont-Kingdon and Lyon, 2003; Yang *et al*., 2003; Hu *et al*., 2004; Tsujie *et al*., 2006). This method permits analysis of multiple samples and target genes in an automated manner which also results in rapid determination.

In the present study, real-time PCR assay was developed for the detection of DS from three particular regions, *DSCR1, DSCR3* and *DSCAM*, and sex identification from *SRY* on Y chromosome. These three genes were selected because they were part of the DSCR of chromosome 21 especially *DSCR3* gene, which had been used for the detection of trisomy 21 (Hu *et al*., 2004). In this assay, singleplex identification for *DSCR3* and *GAPDH* in relative standard curve method, duplex identification for *DSCR1* and *GAPDH*, *DSCR3* and *GAPDH*, *DSCAM* and *GAPDH* in comparative Ct method, as well as triplex identification for *DSCR1*, *SRY* and *GAPDH* in comparative Ct method were used.

TaqMan MGB probes and TaqMan Universal PCR Master Mix Reagent were used in this experiment. This system provided specific detection by Hot Start PCR property of the AmpliTaq Gold DNA Polymerase enzyme and prevention of reamplification of the carryover-PCR products by the AmpErase UNG enzyme (Applied Biosystems, 2002).

A total of 20 samples (10 normal samples and 10 DS patients) were analyzed by singleplex real-time PCR method for *DSCR3* and *GAPDH* in relative standard curve method. The results showed the mean ratio of *DSCR3*/*GAPDH* in normal healthy persons (0.712) and DS patients (2.302) at lower and higher levels than the

expected value, respectively. This high ratio result was also found in the report of Hu *et al*. (2004) showing *DSCR3*/*GAPDH* ratio of trisomy 21 patients and normal controls at slightly higher than the expected value. However, the ratios of our result were higher in DS patients but lower in the normal controls compared to the data obtained by Hu *et al*. (2004). In the DS samples, there were five samples having *DSCR3*/*GAPDH* ratio at high values of 2.211-3.692 which were concordant with the report of Yang *et al*. (2005) using insulin-like growth factor *(IGF)-1* gene as an internal control. They also found that the relative levels of D21S167 and S100B were 2.6 and 2.4 times higher in the blood of DS patients and 2.1 and 2.7 times higher in the amniotic fluid of DS fetuses than the control group.

For duplex real-time PCR assay, a total of 100 DNA samples (50 normal samples and 50 DS patients) were analyzed for *DSCR1* and *GAPDH*, *DSCR3* and *GAPDH*, *DSCAM* and *GAPDH* using a comparative Ct method. The results showed the mean RQ of *DSCR1*, *DSCR3* and *DSCAM* in normal healthy persons (1.19, 1.16 and 1.20, respectively) and DS patients (1.76, 1.91 and 1.95, respectively) higher than the expected values. A total of 100 DNA samples were also analyzed using triplex real-time PCR method for *DSCR1*, *SRY* and *GAPDH*. The results gave the mean RQ of *DSCR1* in normal healthy persons (1.20) and DS patients (1.95) which were also higher than the expected value, while the presence of *SRY* was perfectly matched with the true sex of the samples. These high mean RQ values from duplex and triplex real-time PCR assay probably resulted from competition of target and control genes, and PCR condition was more suitable for target gene.

For duplex real-time PCR assay of *DSCR1* and *GAPDH*, *DSCR3* and *GAPDH*, *DSCAM* and *GAPDH* using comparative Ct method, the criteria for making a decision of either normal or DS patient came from the RQ of at least two from the three genes. DS patients No. 37, 45 and 46 produced false negative results while normal persons No. 2, 11-13, 16-17, 25, 33, 39, 44-47 and 50 produced false positive results. For triplex real-time PCR assay of *DSCR1*, *SRY* and *GAPDH* using comparative Ct method, patients No. 1-4, 20-27, 30, 32, 46 and 50 produced false negative results while normal patients No. 4, 6, 12, 26, 36-38, 42, 44 and 48 produced false positive

results. These false results might be attributed to the use of unsuitable housekeeping genes (Garces, n.d.). Therefore, any single-copy gene located on different chromosome can be considered as a control gene (Tsujie *et al.*, 2006).

The relative changes in gene expression was analyzed by real-time PCR using comparative Ct method (Livak and Schmittgen, 2001) but there was no report on the detection of DS or other chromosome aneuploidies by this method. In this study, we found that this method correctly determined 35 of 50 normal samples (70 %) and 47 of 50 DS samples (94 %) from duplex assays when *DSCR1*, *DSCR3* and *DSCAM* genes were determined simultaneously. If each gene was considered separately, 31 (62 %), 34 (68 %) and 34 (68 %) of 50 normal samples were correctly determined for *DSCR1*, *DSCR3* and *DSCAM* genes, while 42 (84 %), 47 (94 %) and 43 (86 %) of 50 DS samples were correctly determined for *DSCR1*, *DSCR3* and *DSCAM* genes, respectively. For triplex assay, 40 of 50 normal samples (80 %) and 34 of 50 DS samples (68 %) were correctly determined for *DSCR1* gene.

For duplex identification in relative quantification using comparative Ct method, when *DSCR1*, *DSCR3* and *DSCAM* genes were considered together, 18 of 100 samples (18 %) produced false results. When each gene was considered to determine normal or DS patient, 27 (27 %), 19 (19 %), and 23 (23 %), of 100 samples for *DSCR1*, *DSCR3* and *DSCAM* genes, respectively, produced false results. For triplex assay, 26 of 100 samples (26 %), for *DSCR1* produced false results. The results of this method was not 100 % concordant with the true results. Therefore, the feasibility of these methods must be further evaluated before clinical implementation.

For singleplex real-time PCR assay of *DSCR3* and *GAPDH* using relative standard curve method, standard deviation (SD) of *DSCR3*/*GAPDH* ratio in normal healthy persons and DS patients were 0.196 and 0.739, respectively. For duplex real-time PCR assay of *DSCR1* and *GAPDH*, *DSCR3* and *GAPDH*, *DSCAM* and *GAPDH* using comparative Ct method, SD of RQ for *DSCR1*, *DSCR3* and *DSCAM* in normal healthy persons were 0.35, 0.33 and 0.65 and in DS patients were 0.70, 0.58 and 1.41, respectively. For triplex real-time PCR assay of *DSCR1*, *SRY* and *GAPDH* using

comparative Ct method, SD of RQ for *DSCR1* in normal healthy persons and DS patients were 0.31 and 0.61, respectively. These results showed that the SD value of each single amplicon was very high. The use of an endogenous control would increase variation because the total variation was the sum of the variations of both amplicons. It remained unknown as to which contributed more to variation between measuring DNA concentrations for an absolute standard curve approach or measuring Ct values of an endogenous control for a relative standard, ($2^{-\Delta\Delta Ct}$) approach (Bubner and Baldwin, 2004).

From singleplex identification of only *DSCR3* and *GAPDH* in relative standard curve method, no sample produced a false positive and negative results. In duplex identification for *DSCR1* and *GAPDH*, *DSCR3* and *GAPDH*, *DSCAM* and *GAPDH* using comparative Ct method by which *DSCR1*, *DSCR3* and *DSCAM* genes were determined together, 3 samples (6 %) of DS produced false negative results while15 samples (30 %) of normal persons produced false positive results. As for triplex identification of *DSCR1* and *GAPDH* in comparative Ct method, 16 samples 32 %) of DS patients gave false negative results while 10 samples (20 %) of normal patients gave false positive results. As for *SRY* identification, it produced accurate results in all samples. When the data of each assay were compared, the singleplex assay of *DSCR3* and *GAPDH* gave the most accurate result. On the other hand, duplex assay produced more reliable results when compared with triplex assay while the presence of *SRY* in triplex assay was concordant with the actual sex of all samples. This highly accurate of singleplex assay might be the results of using less samples than those of duplex and triplex assays and no competition of gene was present in the singleplex assay. For duplex and triplex assays, however, interferences and variation of PCR efficiency in each gene and primer could contribute to the less accurate outcome.

In this study, 1 ng of genomic DNA was used thus the concentration of template DNA was low as compared to 20 ng and 100 ng DNA for the detection of trisomy 21 reported by Hu *et al*. (2004) and Yang *et al*. (2005). Bubner and Baldwin (2004) reported that an important factor for a low SD is the size of the Ct values and

Ct values above 30 reflect poor primer binding or a low target molecule amount. They suggested that using sufficient template DNA would increase the Ct value to achieve Ct value of less than 25. Therefore, increasing the amount of input DNA may give better results. In this study, real-time PCR was done duplicately and in some cases, the results from each repeat were quite different (data not shown). Therefore, more replications are recommended for further investigation for compensation of the pipetting error. However, further improvement for real-time PCR conditions of target and control genes is needed to make practical implementation of the method.

For molecular sex determination on Y chromosome, *SRY* gene produced accurate outcome in all cases. Our result was perfectly concordant with the previous report showing 100 % specificity and sensitivity for sex detection using real-time PCR with SRY gene as a marker (Hromadnikova *et al*., 2003). However, using single-copy *SRY* gene was less reliable as compared with using a multi-copy sequence *DYS14* in maternal plasma with male cell circulating fetal DNA (Zimmermann *et al.*, 2005).

For a quantitative analysis, the repeatability is an important performance for the measurement. Good repeatability observed from the obtained result should be varied only a little from the previous one. % CV for within-run precision of *DSCR3*/*GAPDH* ratio using standard curve method in singleplex assay was 10.211. % CV for within-run precision of RQ of *DSCR1*, *DSCR3* and *DSCAM* in duplex assays were 14.435, 23.006 and 30.643, respectively. % CV for within-run precision of RQ of *DSCR1* in triplex assay was 15.493 while *SRY* produced positive results for all the samples tested. As for the total % CV comparison, it was suggested that singleplex assay using standard curve method might be the most accurate assay.

Since the real-time PCR assay is a very sensitive technique, the appropriate designation of the primers and probes are needed. A large-scale pilot study remains to be performed before this technique could be introduced for clinical application and further routine service establishment. Further optimization may help getting the proper ratios in the samples.

CONCLUSION

The real-time PCR method was used to determine DS patient using 3 loci of Down syndrome critical region (DSCR), i.e., *DSCR1*, *DSCR3* and *DSCAM*. Sex was also identified using Sex-Determining Region (*SRY*) on Y chromosome.

The duplex and triplex real-time PCR assay were used to screen 50 DS patients and 50 normal controls by relative quantification using comparative Ct method. The accuracy of the results were 82 % and 74 % for duplex and triplex assays repectively, while the sex identification was 100% accurate. The singleplex assay was used to screen 10 DS patients and 10 normal controls by relative quantification using standard curve method and 100 % accurate result was achieved.

The real-time PCR method can not be used to replace the conventional cytogenetic analysis for DS detection because the accuracy of determination from real-time PCR do not reach 100 % in duplex and triplex assay. Interestingly, singleplex real-time PCR assay gave 100 % accurate results but more samples are still needed for further study. In addition, specific primer and PCR reaction optimization are important steps for achieving accurate results. However, the real-time PCR assay can be used for rapidly preliminary screening of DS. If a result is positive, cytogenetic analysis will be done for further confirmation, while a negative result may requires double check.

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APPENDIX

Appendix Table 1 Cts of *DSCR1* and *GAPDH* in normal healthy persons from duplex assays.

Normal healthy persons (No.)	Cts of DSCR1	Cts of GAPDH
1	28.415	28.268
2	28.517	30.005
3	28.972	29.175
4	29.930	30.388
5	29.415	30.398
6	29.049	29.553
7	30.199	30.727
8	29.370	30.169
9	28.423	28.954
10	28.507	28.667
11	29.774	30.206
12	30.259	31.330
13	30.042	31.054
14	32.569	33.268
15	28.203	29.428
16	29.379	30.358
17	29.780	31.226
18	29.250	29.359
19	28.525	29.569
20	28.326	29.481
21	29.197	30.408
22	29.357	30.430
23	30.285	30.831
24	30.306	30.934
25	29.145	30.686

Appendix Table 1 (Continued)

Normal healthy persons (No.)	Cts of DSCR1	Cts of GAPDH
26	30.552	31.158
27	28.106	28.964
28	30.346	31.480
29	30.649	31.520
30	29.133	30.336
31	29.906	29.728
32	28.285	29.324
33	29.077	30.045
34	29.156	29.059
35	29.368	30.153
36	28.053	28.642
37	27.866	28.987
38	29.255	30.301
39	28.359	29.255
40	30.357	30.113
41	29.178	30.210
42	29.387	30.986
43	29.120	29.747
44	29.108	29.957
45	28.829	29.693
46	28.752	29.544
47	29.589	29.912
48	29.447	30.323
49	29.334	30.207
50	29.763	30.007

Appendix Table 2 Cts of *DSCR1* and *GAPDH* in DS patients from duplex assays.

DS patients (No.)	Cts of DSCR1	Cts of GAPDH
1	29.355	30.298
2	28.571	30.246
3	29.699	30.710
4	29.234	30.500
5	28.835	29.971
6	28.254	29.914
7	29.339	31.018
8	27.236	29.456
9	29.193	30.367
10	29.361	30.667
11	29.424	29.777
12	27.623	29.655
13	28.916	30.524
14	29.860	29.692
15	29.292	29.840
16	28.845	30.520
17	28.653	29.208
18	28.912	30.274
19	28.440	30.413
20	29.072	30.336
21	29.308	29.904
22	29.088	29.230
23	28.034	29.160
24	28.757	29.433
25	28.530	28.721

Appendix Table 2 (Continued)

DS patients (No.)	Cts of DSCR1	Cts of GAPDH
26	28.199	30.058
27	29.432	29.936
28	28.851	30.348
29	29.256	30.696
30	29.399	30.906
31	28.884	30.775
32	29.195	29.667
33	29.921	29.893
34	29.544	31.201
35	29.017	30.742
36	28.294	31.032
37	18.109	28.942
38	27.986	29.518
39	28.212	28.988
40	28.658	30.657
41	27.649	28.461
42	28.336	29.623
43	28.338	29.883
44	28.678	29.648
45	28.146	29.373
47	27.277	30.117
48	27.616	28.714
49	27.874	29.572
50	27.955	28.497

Appendix Table 3 Cts of *DSCR3* and *GAPDH* in normal healthy persons from duplex assays.

Normal healthy persons (No.)	**Cts of DSCR3**	**Cts of GAPDH**
1	29.162	28.338
2	29.389	29.825
3	31.323	30.424
4	30.893	30.265
5	29.970	30.278
6	30.747	29.612
7	30.089	30.692
8	30.571	29.712
9	28.153	30.165
10	28.973	29.529
11	29.896	29.793
12	30.819	31.252
13	31.238	31.424
14	35.055	34.350
15	29.936	29.263
16	29.078	31.955
17	30.530	30.911
18	29.492	30.866
19	28.991	28.652
20	28.870	29.432
21	30.692	30.125
22	30.764	30.071
23	29.778	30.734
24	29.986	30.305
25	30.485	30.966

Appendix Table 3 (Continued)

Normal healthy persons (No.)	Cts of DSCR3	Cts of GAPDH
26	31.641	31.113
27	29.428	29.323
28	30.804	31.041
29	31.307	31.334
30	30.035	30.500
31	31.316	30.578
32	29.070	29.180
33	29.242	29.963
34	30.850	30.049
35	29.274	29.433
36	29.112	28.896
37	29.037	28.571
38	29.858	30.038
39	28.185	29.510
40	31.508	30.830
41	30.332	29.902
42	30.510	31.183
43	29.279	30.423
44	30.229	31.243
45	29.614	30.460
46	29.118	29.989
47	30.136	30.995
48	29.557	29.521
49	29.601	29.636
50	29.637	29.500

Appendix Table 4 Cts of *DSCR3* and *GAPDH* in DS patients from duplex assays.

DS patients (No.)	Cts of DSCR3	Cts of GAPDH
1	28.311	29.509
2	29.667	29.642
3	28.316	29.712
4	30.062	30.526
5	29.785	30.522
6	29.385	29.254
7	29.362	30.283
8	28.846	30.611
9	28.158	29.786
10	28.278	29.965
11	28.876	29.194
12	28.810	30.016
13	29.419	30.617
14	29.351	29.256
15	30.201	29.867
16	29.423	30.585
17	29.225	29.256
18	28.276	29.639
19	29.361	30.946
20	28.454	29.712
21	30.342	30.332
22	28.270	28.359
23	27.293	29.915
24	29.067	29.466
25	28.149	29.138

Appendix Table 4 (Continued)

DS patients (No.)	Cts of DSCR3	Cts of GAPDH
26	28.472	29.636
27	28.877	29.047
28	30.602	31.177
29	29.001	30.313
30	29.969	30.888
31	29.554	30.967
32	29.532	29.931
33	28.955	29.281
34	29.741	30.523
35	30.090	30.334
36	29.374	30.587
37	28.407	30.397
38	29.470	29.596
39	27.825	30.422
40	29.179	30.108
41	27.293	29.915
42	29.300	29.365
43	29.810	30.217
44	28.243	29.362
45	28.316	28.784
47	29.233	30.200
48	28.403	28.988
49	29.382	30.015
50	28.112	29.182

Appendix Table 5 Cts of *DSCAM* and *GAPDH* in normal healthy persons from duplex assays.

Normal healthy persons (No.)	Cts of DSCAM	Cts of GAPDH
1	28.803	29.392
2	29.975	30.333
3	30.123	29.661
4	29.885	30.906
5	30.547	29.698
6	28.883	29.806
7	30.447	30.354
8	31.650	31.267
9	29.020	28.663
10	28.984	28.374
11	30.377	30.229
12	31.075	30.359
13	31.553	30.842
14	33.875	33.026
15	30.224	29.764
16	29.902	30.623
17	31.920	31.676
18	29.258	29.700
19	29.471	29.211
20	30.084	29.399
21	30.557	29.986
22	30.051	30.313
23	30.779	30.440
24	30.762	30.424
25	31.624	30.565

Appendix Table 5 (Continued)

Normal healthy persons (No.)	Cts of DSCAM	Cts of GAPDH
26	31.551	30.494
27	29.432	28.823
28	32.066	30.875
29	32.001	31.680
30	30.699	30.467
31	30.770	29.615
32	30.217	29.293
33	30.103	29.793
34	29.981	28.374
35	29.933	30.017
36	29.414	28.733
37	28.918	28.376
38	30.833	30.321
39	28.397	28.142
40	31.783	30.344
41	30.854	30.525
42	30.349	30.653
43	29.900	30.148
44	30.495	30.919
45	30.215	30.509
46	29.931	30.089
47	30.554	31.151
48	30.235	30.182
49	29.985	29.847
50	30.494	29.519

Appendix Table 6 Cts of *DSCAM* and *GAPDH* in DS patients from duplex assays.

DS patients (No.)	Cts of DSCAM	Cts of GAPDH
1	28.582	29.583
2	29.328	29.165
3	29.094	29.930
4	29.416	29.394
5	30.221	29.829
6	29.948	30.129
7	30.447	30.587
8	28.281	28.866
9	29.654	29.662
10	30.005	30.072
11	29.606	29.805
12	28.500	31.003
13	29.922	30.265
14	29.706	29.553
15	29.697	30.393
16	30.188	30.688
17	28.528	29.500
18	28.915	29.407
19	31.837	30.838
20	29.160	29.770
21	30.269	29.880
22	29.618	29.429
23	28.053	28.799
24	30.040	29.780
25	28.396	28.267

Appendix Table 6 (Continued)

DS patients (No.)	Cts of DSCAM	Cts of GAPDH
26	31.680	30.857
27	29.952	29.869
28	29.805	30.172
29	30.200	31.173
30	30.866	30.424
31	30.685	30.573
32	30.072	29.733
33	29.779	29.948
34	31.928	31.937
35	29.729	29.737
36	29.021	29.862
37	28.573	28.862
38	28.680	28.605
39	28.327	28.765
40	30.974	30.322
41	28.030	28.579
42	28.695	28.925
43	29.644	29.448
44	28.851	29.710
45	28.656	28.958
47	29.781	30.899
48	28.949	28.567
49	30.216	30.110
50	28.452	28.393

Appendix Table 7 Cts and of *DSCR1* and *GAPDH* in normal healthy persons from triplex assays (this table would not show *SRY* data).

Normal healthy persons (No.)	Cts of DSCR1	Cts of GAPDH
1	29.730	30.481
2	29.785	60.547
3	30.107	30.387
4	29.586	29.619
5	29.170	29.048
6	29.417	29.441
7	29.313	29.437
8	29.183	29.356
9	30.516	30.992
10	31.895	32.126
11	30.829	31.519
12	29.739	30.749
13	29.631	30.342
14	31.755	32.126
15	29.279	30.085
16	30.601	30.939
17	30.139	30.650
18	31.224	31.490
19	33.531	33.583
20	31.634	32.065
21	30.273	30.505
22	29.957	30.755
23	30.432	31.023
24	31.391	31.242
25	30.799	30.324

Appendix Table 7 (Continued)

Normal healthy persons (No.)	Cts of DSCR1	Cts of GAPDH
26	31.755	31.719
27	31.101	30.667
28	30.125	30.912
29	34.312	33.885
30	30.955	30.659
31	31.574	31.158
32	31.418	30.935
33	30.930	30.462
34	30.408	30.190
35	30.257	30.910
36	31.902	32.151
37	31.328	31.715
38	30.465	30.922
39	31.569	32.062
40	34.047	34.759
41	32.161	32.126
42	29.832	31.141
43	28.904	29.972
44	29.288	30.561
45	28.483	29.564
46	28.788	29.786
47	29.079	29.705
48	28.309	29.576
49	28.504	29.461
50	27.755	28.806

Appendix Table 8 Cts and *of DSCR1* and *GAPDH* in DS patients from triplex assays (this table would not show *SRY* data).

DS patients (No.)	Cts of DSCR1	Cts of GAPDH
1	29.170	30.284
2	28.566	29.385
3	29.240	30.667
4	29.264	30.755
5	28.004	28.920
6	28.499	29.532
7	29.046	29.539
8	29.020	30.504
9	29.602	29.948
10	29.037	29.650
11	29.264	29.732
12	29.334	30.789
13	29.270	29.829
14	29.537	30.275
15	29.433	30.618
16	29.616	31.131
17	29.459	30.940
18	28.679	30.266
19	29.766	31.141
20	29.488	30.764
21	30.520	31.681
22	29.828	30.873
23	29.468	30.746
24	29.367	30.662
25	30.528	31.835

Appendix Table 8 (Continued)

DS patients (No.)	Cts of DSCR1	Cts of GAPDH
26	29.824	30.868
27	30.202	31.526
28	29.722	31.823
29	29.649	31.540
30	29.745	30.494
31	29.995	31.531
32	30.322	31.620
33	29.936	31.310
34	30.489	31.616
35	30.663	31.306
36	30.282	32.256
37	30.352	31.431
38	29.828	30.901
39	31.125	31.103
40	31.594	31.758
41	30.819	30.962
42	30.778	30.761
43	30.881	30.934
44	29.138	29.752
45	31.000	31.542
46	31.230	31.578
47	29.968	31.887
48	30.079	31.207
49	29.984	31.072
50	26.592	28.286

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