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APPENDICES

APPENDIX A

FISH Applications in Characterization of Chromosome Rearrangement

Absent pulmonary valve syndrome in a *de novo* recombinant chromosome 9 with duplication of 9p13→9pter and subtelomeric deletion of 9q

Introduction

Common clinical manifestations in patients with trisomy 9p include mental retardation, wide fontanelles, microcephaly, down-slanting sunken eyes, prominent nasal root with a bulbous nasal tip, low-set abnormal ears, skeletal anomalies (hypoplastic phalanges, clinodactyly of the fifth finger and hypoplastic nails) and dermatophytic characteristics (single palmar crease, absence of fusion of palmar triradii d and c). Intrauterine growth retardation, cleft lip/palate and congenital heart malformations are seen infrequently, unless the trisomic segments extend through 9q22-9q32.

In some cases of partial trisomy, determination of the genotype-phenotype relationship is complicated by complex chromosome rearrangement, which cannot be defined using the conventional G-banding technique alone. Molecular cytogenetic methods, such as mFISH and mBAND analysis, are required to support the interpretation. As small rearrangements involving chromosome end are not well represented in mFISH and mBAND, the subtelomeric FISH probe set has been developed; this can be applied to identify cryptic aberrations within the subtelomeric region that might be the cause of unexplained dysmorphic features and idiopathic mental retardation in some patients.

In the current report, we present a patient with clinical features similar to those of trisomy 9p, who had an absent pulmonary valve syndrome with

double outlet right ventricle. The G-banded karyotype revealed an additional dark band at the 9qter. Therefore, mFISH, mBAND and subtelomeric FISH were applied to clarify the precise chromosomal rearrangements.

Clinical report

The proband was a female infant born at term, by vaginal delivery, to a 24-year-old, Gravida 0 Para 0 mother. Ultrasonography at 18 weeks gestational age revealed polyhydramnios, and at 32 weeks gestational age revealed ventricular septal defect, pulmonary valve stenosis and tricuspid regurgitation. Birth weight was 2560 g (P25), length was 46 cm (P25-50), and head circumference was 30.5 cm.

Multiple dysmorphic features were noted at birth, including overlapping cranial sutures, bitemporal narrowing, sunken eyes with short and down-slanting palpebral fissures, a right preauricular skin tag and over-folding of the helices, redundant nuchal skin and a soft tissue mass at the midline of the anterior chest wall (Fig. A1-3). Also present were bilateral clinodactyly and hypoplasia of the middle phalanges of the fifth fingers and ulnar deviation of both thumbs. The toes were overlapping. The external genitalia appeared normal and there was a pilonidal sinus at the coccyx. Neurologically, the infant was hypoactive with hypotonia and normal neonatal reflexes. Cardiac evaluation after birth revealed the absent pulmonary valve syndrome with double outlet right ventricle. Family history was negative for congenital heart defects and consanguinity.

Materials and methods used in molecular cytogenetics

1. Cytogenetics analysis

Metaphase chromosomes were obtained from phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes, and G-banding was performed using standard methods.

2. Multicolor fluorescence *in situ* hybridization (mFISH) and multicolor banding (mBAND)

mFISH was performed on metaphase chromosomes using a 24XCyte probe kit (MetaSystems, Germany) according to the manufacturer's instructions.

mBAND was performed on metaphase chromosomes using an XCyte9 chromosome 9-specific mBAND probe (MetaSystems). Both mFISH and mBAND images were captured and analyzed using a Zeiss Axioplan 2 imaging microscope (Carl Zeiss, Jena, Germany) and the Isis software (MetaSystems, Germany).

3. Subtelomeric FISH

FISH analysis using subtelomeric probes of 9p and 9q (BAC/PAC clones RP11-174M15 and RP11-885N19, respectively) was performed on metaphase chromosomes.

Results

The patient's karyotype, identified using the standard G-banding technique, was interpreted as 46,XX,der(9)t(9;?)(q34.3;?) at the 550-band level of resolution (Fig. B). The derivative chromosome 9 had extra material attached to the 9qter. Both the paternal and maternal karyotypes were normal, suggesting a *de novo* event.

mFISH confirmed that the der(9) extra material was of the chromosome 9 origin (Fig. C1-3). Subsequently, mBAND was performed to determine the precise regions involved in the derivative chromosome 9. The result revealed an additional copy of 9p13→9pter attached to the 9qter in a direct fashion, resulting in partial trisomy of 9p (Fig. C4 and D). To determine the presence of 9q subtelomeric region of the derivative chromosome, FISH using 9p and 9q subtelomeric probes was carried out. The results demonstrated a subtelomeric deletion at the insertion breakpoint on the 9qter (Fig. E).

Discussion

This patient presented with multiple congenital anomalies and an abnormal karyotype involving chromosome 9. Although most of the clinical manifestations in this patient were typical of those of patients with trisomy 9p, the cardiac anomalies were less common phenotypes, unless the trisomic segments extend through 9q22-9q32. The cytogenetic G-banding analysis revealed a derivative chromosome 9 with an abnormally long arm. As the extra material of the long arm of the der(9) chromosome could not be interpreted, and the possibility of monosomy 9q34.3 could not be excluded, molecular cytogenetic methods were applied. mFISH and mBAND were initially performed to characterize the der(9) chromosome. mFISH was used to identify the origin of the extra material, and mBAND was used to identify the bands involved. The results revealed an unusual case of *de novo* trisomy 9p with an additional copy of the 9p13→9pter inserted at the 9qter. Although the distal half of the short arm of chromosome 9 (9p13→9pter) is responsible for the major clinical features of trisomy 9p, cardiac anomalies, such as absent pulmonary valve syndrome or conotruncal anomalies, are less common. Subtelomeric FISH demonstrated that there was a cryptic subtelomeric

deletion at the insertion breakpoint (9q34.3) on the 9qter, which might have accounted for the congenital heart defect in this patient. A review of the literature showed that the phenotypic findings of subtelomeric deletions of chromosome 9q34.3 generally consist of mental retardation, distinct facial features and congenital heart defects (primarily conotruncal defects). The cardiac abnormalities in this patient were more compatible with the phenotypic finding of 9q34.3 deletion. It is possible that the subtelomeric region of 9q might be critical for heart development, especially for conotruncal development.

In most previously reported cases, the partial trisomy 9p was the result of a parental reciprocal translocation between chromosome 9 and another autosome; in a few cases, it was due to a nonfamilial aberration, such as tandem duplication. In the case described here, the duplicated 9p segment (9p13→9pter) was inserted at the terminal end of the long arm, and subtelomeric region of 9q was found to be deleted. One possible mechanism to explain how this unusual cytogenetic finding occurred is the telomere capture, through which a terminally deleted chromosome is stabilized by acquiring a new telomeric sequence from another chromosomal location, which results in a derivative chromosome. However, such a chromosome abnormality was not typical of cases of *de novo* trisomy 9p and could not be confirmed by the G-banding technique alone. The current study verified that mFISH and mBAND are useful techniques for characterization of the chromosomal aberrations, and are necessary for genotype-phenotype correlations in cases of partial trisomy.

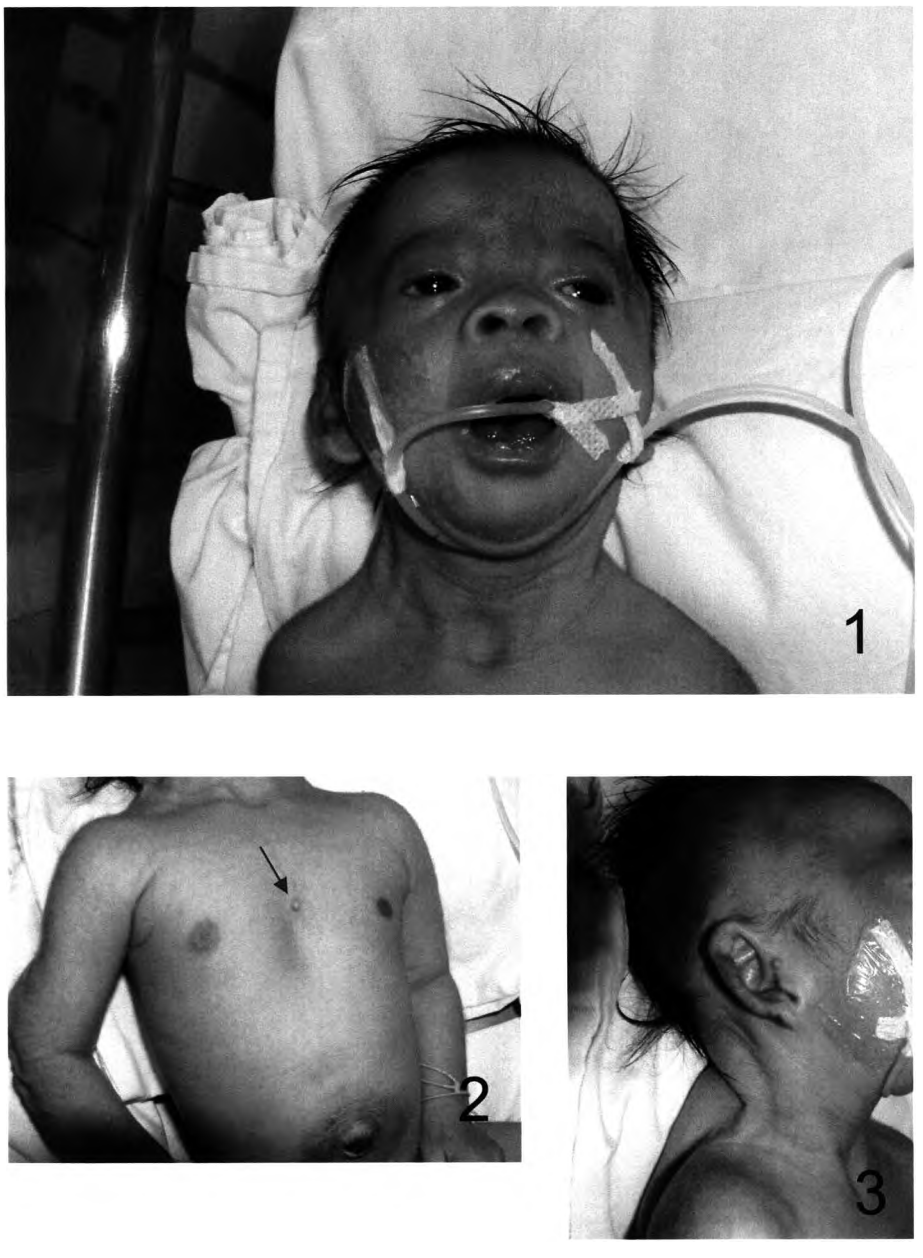

Fig. A. Photograph of the patient at age 1 month. (1) Facial dysmorphic features. (2) Skin tag at anterior chest wall (arrow). (3) Malformed helix and pre-auricular skin tag.

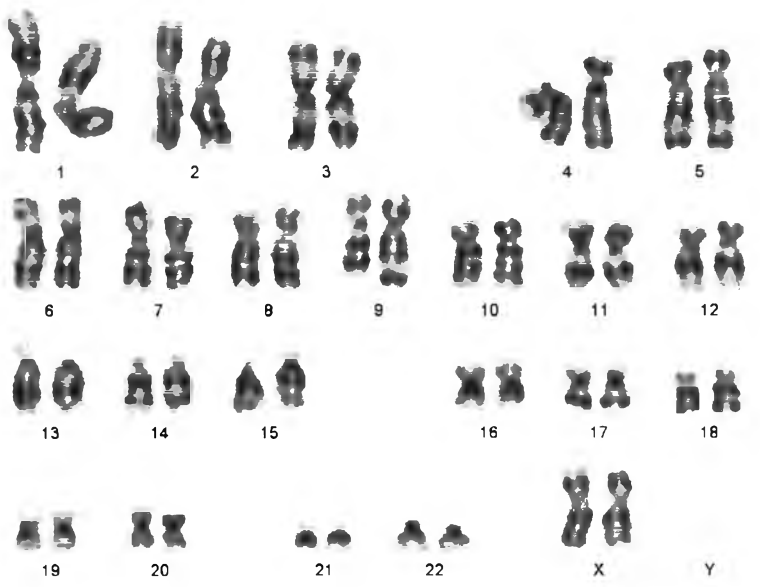

Fig. B. Karyotype of G-banded chromosomes, showing the normal chromosome 9 and the derivative chromosome 9.

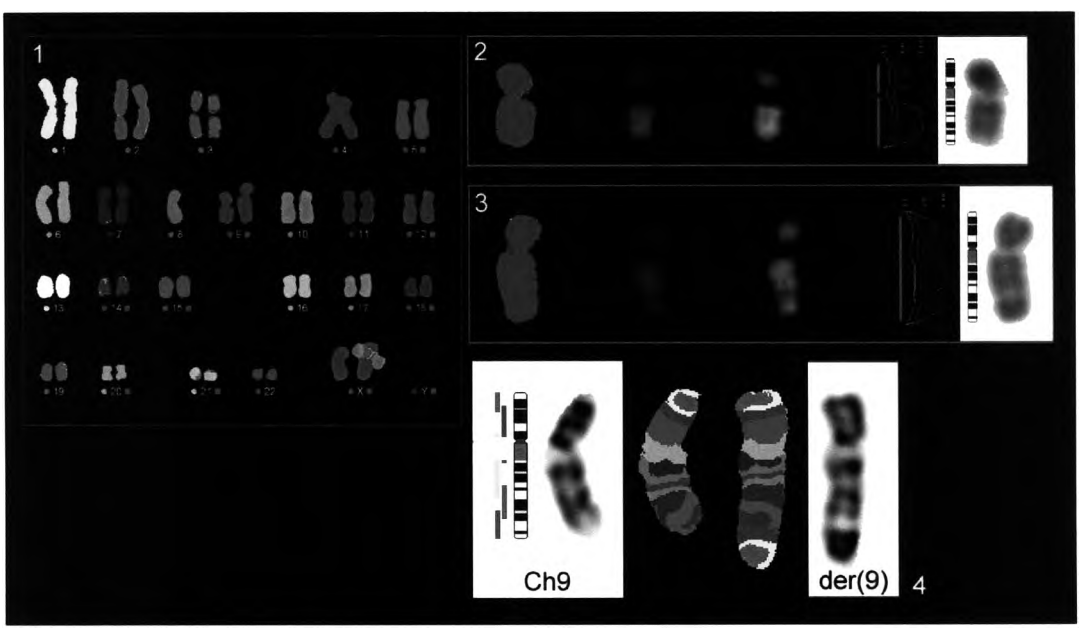

Fig. C. (1) Karyotype of mFISH confirmed that the extra material of the derivative chromosome 9 was all of chromosome 9 origin (2 and 3). (4) mBAND showed the duplicated 9p13→pter inserted to the 9qter.

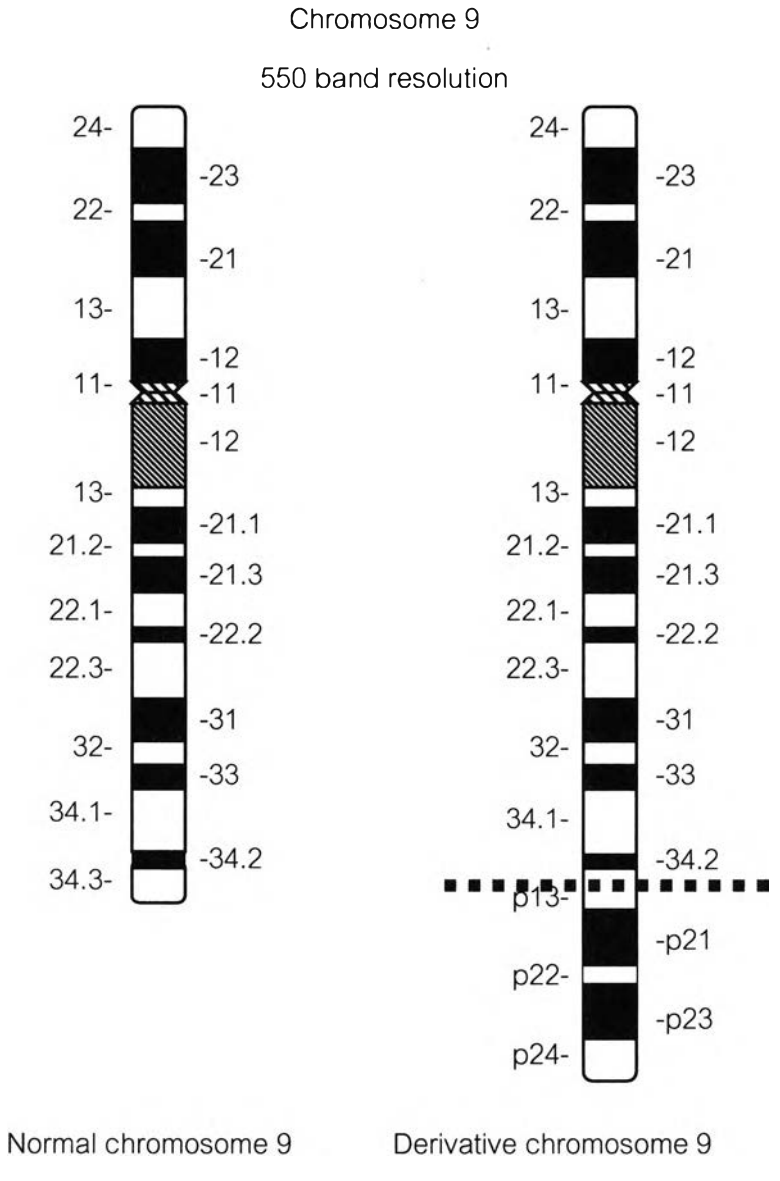

Fig. D. Ideogram illustrates the duplicated segment of short arm and insertion breakpoint at the long arm of the derivative chromosome 9.

Fig. E. Metaphase spreads were analyzed for the presence of subtelomeric region of the long arm of the derivative chromosome at the insertion breakpoint by FISH using subtelomeric probe. The green signals indicated the subtelomeric region of the short arm of chromosome 9 and the red signals were the subtelomeric region of the long arm of chromosome 9. The 9q subtelomeric region of derivative chromosome 9 was found to be deleted at insertion breakpoint.

APPENDIX B

PROTOCOLS USED IN THE STUDY

A. Protocol for Transforming B lymphocytes by EBV (abbreviated):

1) Collect 5-10 ml of peripheral human blood in collection tubes containing either acid-citrate dextrose or K-EDTA as an anticoagulant.
2) Separate peripheral blood mononuclear cells (PBMC) by centrifugation on a Ficoll gradient.
3) Wash PBMC two times with RPMI-1640 with 25mM HEPES, pH 7.4.
4) Resuspend PBMC in RPMI 1640 with 20% FBS, 2mM L-glutamine, 2.0 g/L glucose, 2.0 g/L sodium bicarbonate and no antibiotics in a T25 tissue culture flask or other cultureware. The volume of medium to be used can vary with the initial volume of blood used to isolate the PBMC. A full 8 ml of blood requires 8 ml of medium. If very few cells are isolated (2 ml or less of blood), 2 ml of medium can be used.
5) Add 1 ml EBV, 0.3 to 1.0 ml of 1% phytohemaglutinin, depending on volume of cell suspension. Incubate at 37°C, 5% CO_2.
6) Twice weekly, examine flask for a change to an acidic pH and the appearance of "clumps" of cells growing in suspension. Adjust the volume of medium in the flask by removing spent medium and adding more or less fresh medium to maintain a slightly acidic pH. Be sure to let the cells settle to the bottom of the flask before adjusting the volume of the medium.
7) By 21 to 35 days in culture, the volume should have increased to approximately 20 mls. Cells should be growing in loose aggregates that can be broken apart by gentle trituration. At this time the lymphocytes

can be subcultured at a seeding density of not less than 2x105 viable cells per ml.

8) When cell density reaches 8x105 to 1x106 cells per ml, the culture should be split at not less than 2x105 cells per ml or cell stocks should be cryopreserved.

B. Fluorescence in situ hybridization (FISH) analysis

Using BAC or PAC DNA as FISH probes to physical map the breakpoint on the chromosome.

Plasmid purification

1) Grow a 10 ml overnight bacterial/plasmid culture in selective media. Freeze half as a bacterial stock and use the other half in this protocol.
2) Prepare in advance per 5 ml culture: Two 1.5 ml microcentrifuge tubes with 0.5 ml isopropanol in one; Solution I (180 µl); Solution II (400 µl, not more than one week old); Solution III (300 µl, keep cold in a refrigerator); Lysozyme solution (20 µl, 5 mg/ml in solution I).
3) Centrifuge the cells at 2500 rpm for 12 minutes at 4°C and carefully pour off the supernatant.
4) Resuspend the cells in 180 µl of solution I and transfer to an empty microcentrifuge tube. Add 20 µl of lysozyme solution and give it a quick vortex. Incubate for 5 minutes at room temperature.
5) Add 400 µl of solution II. Mix by inversion a few times - do not vortex. Incubate on ice for 5 min.
6) Add 300 µl pre-cooled solution III. The tube contents should be very viscous. Mix with a quick vortex. Incubate on ice for 10 minutes.
7) Centrifuge the sample for two minutes in a microcentrifuge at room temperature, then carefully transfer the supernatant to a second

microcentrifuge tube containing 500 µl (0.6 volumes) of isopropanol to precipitate the DNA (avoid transferring the white material). (Optional: To increase the yield - centrifuge the tube containing the residual white crud for another two minutes, collect any supernatant, and add it to the above supernatant.)

8) Vortex and centrifuge for 3 minutes in a microcentrifuge at room temperature. Pour off as much supernatant as possible. At this point, you should be able to see a small white pellet in the bottom of the tube.
9) Resuspend the pellet in 196 µl of TE. Optional phenol and ether extractions should be performed at this point if you intend to save the DNA for more than about a week. Dissolve pellet by vortexing, then add 4 µl of 5M NaCl and 0.6 ml of cold 95% (or absolute) ethanol. Precipitate as usual. Spin, dry, and dissolve pellet in 50 µl TE.

FISH Analysis

FISH analysis was performed on fixed cultured EBV transformed lymphoblastoid cell line. Slides were stored for 24 hours at room temperature. After being dehydrated in ethanol series and air-dried, slides were denatured in 70% formamide solution at 72°C for 2 minutes. Probes were also denatured at 72°C for 5 to 10 minutes. Five µl of the mixture of probe solution were added to each slide and covered by a coverslip. The preparations were hybridized at 37°C overnight in a moist chamber. Posthybridization washing consisted of three changes of 10 minutes each with 50% formamide solution at 45°C and one change of 10 minutes with 2**X** standard saline citrate at 45°C and one last change of 5 minutes with 2**X** standard saline citrate/0.1 Nonidet P-40. FISH was performed with chromosome BAC DNA probe.

Nick Translation Assay procedure

1) Place a microcentrifuge tube on ice and allow the tube to cool.
2) Add these components to the tube in the order listed. Briefly centrifuge and vortex the tube before adding the enzyme (last component).

(17.5-X) µl	nuclease-free water
X µl	For 1 µg extracted DNA
2.5 µl	0.2 mM labeled d-UTP
5 µl	0.1 mM dTTP
10 µl	dNTP mix
5 µl	10X Nick translation buffer
10 µl	Nick translation enzyme
50 µl	Total volume

3) Briefly centrifuge and vortex the tube.
4) Incubate 6-16 hours at 15°C.
5) Stop the reaction by heating in a 70°C water bath for 10 minutes.
6) Chill on ice.

Precipitating the probe

1) Pipet 5 µl (~100 ng of probe) of the Nick translation reaction mixture into a microcentrifuge tube.
2) Add 1 µg COT-1 DNA, 2 µg human placental DNA and 4 µl purified water to the tube
3) Add 1.2 µl (0.1 volume) 3 M sodium acetate, then add 30 µl (2.5 volumes) of 100% EtOH to precipitate the DNA. Vortex briefly and place on dry ice for 15 minutes.
4) Centrifuge at 12,000 rpm for 30 minutes at 4°C to pellet the DNA.
5) Remove the supernatant and dry the pellet for 10-15 minutes under a vacuum at ambient temperature.

6) Resuspend the pellet in 3 µl purified water and 7 µl hybridization buffer.
7) Denature the probe by heating the probe mix for 5 minutes in a 73°C water bath.

Slide Denaturation

1) Denature metaphase chromosome slides
2) Incubate slides in denaturing solution (in water-bath) for 2 minutes at 75°C.
3) Denature solution: 70% (v/v) formamide, 2X SSC (pH7.0), 0.1mM EDTA, pH7.0
4) Add 175mL formamide, 25mL 20X SSC (pH7.0), 50mL 0.5 M EDTA, pH7.0 and 50mL purified H_2O to make 250mL solution and mix thoroughly. Verify that the pH is 7.0-7.5 by measuring the pH at ambient temperature. Between use, store covered at 4°C. Discard after 7 days.
5) Dehydrate the slide through a **cold** alcohol series (70%, 90%, and 100% ethanol for 1 min each).
6) Air dry the slides and place on a hot plate at 45-50°C.
7) Apply 10 µl of denatured probe mix to the slide.
8) Immediately apply a coverslip and seal with rubber cement. Keep the slide in a moist chamber at 37°C overnight.

Washing the slide

1) Remove the rubber cement seal and the coverslip and immediately place the slide into the 0.4X SSC/0.3% NP-40 wash solution at 73±1°C. Agitate the slide for 1-3 seconds.
2) Place the slide in the 2X SSC/0.1% NP-40 wash solution at ambient temperature. Agitate the slide for 1-3 seconds and then let stand for 5 second to 1 minute.
3) Air dry slide in the darkness.

4) Slides were counterstained with diaminophenylindole and were mounted with Vectashield antifade solution (Vector).

C. Ethanol precipitation of nucleic acids

To ethanol precipitate nucleic acids, add salt, mix, add ethanol, mix, then freeze. Centrifuge to pellet the material.

Choice of salts:

- Sodium acetate - estimate aqueous volume of DNA
  Add 1/10 of this volume from a 3M sodium acetate stock
- Ammonium acetate - estimate aqueous volume of DNA
  Add 1/2 of this volume from a 7.5 M ammonium acetate stock.
  Ammonium ions help keep free nucleotides in solution.

Ethanol:

Use absolute ethanol (i.e. 200 proof = 100%) only.

Add 2.5 - 3 times the volume of the DNA/salt mixture. Mix well before freezing.

Freezing Conditions:

Freeze in a dry ice /ethanol bath for 20 minutes, a $-70^{\circ}C$ freezer for a minimum of 40 minutes, or a $-20^{\circ}C$ freezer overnight.

D. Long PCR

Protocol

1. Prepare a reaction mixture for the appropriate number of samples to be amplified. The following table provides an example of a reaction mixture for the amplification of a target $\geq$20 kb. The recipe listed in the table is for one reaction and can be adjusted for multiple samples. Add the components in order and mix gently.

Component	Quantity per reaction
Distilled water	X µl to final volume of 50.0 µl
10×EXL reaction buffer	5.0 µl
dNTP mix (25 mM of each dNTP)	1.0 µl
DNA template: Genomic DNA	250–1000 ng
Primer #1	200 ng
Primer #2	200 ng
EXL DNA polymerase (5 U/µl)	1.0 µl
DMSO	Either 0–3%, 3–6%, or 5–7%
Stabilizing solution	1 µl
Total reaction volume	50.0 µl

2. Before thermal cycling, aliquot 50 µl of the master mixture into sterile thin-walled PCR tubes.
3. Overlay each reaction with ~50 µl of DNase-, RNase-, and protease-free mineral oil even if the temperature-cycler is equipped with a heated cover.

Optimization Parameters and Suggested Reaction Conditions (50 µl reaction)

Parameter	Genomic Targets (High-Complexity DNA)
Input template	250–1000 ng genomic DNA
EXL® DNA polymerase	5.0 U
DMSO concentration	0–3% for targets <23 kb 3–6% for targets >23 kb
dNTP concentration	500 µM each dNTP (2 mM total)
Primers (each)	~200 ng (0.5 µM)

Extension time	1 min per kb
Extension temperature	68 °C
Denaturing temperature	92 °C

4. Perform PCR using optimized cycling conditions.

Single-block temperature cycles

Segment	Number of cycles	Temperature	Duration
1	1	92 °C	2 minutes
2	10	92 °C	10 seconds
		Primer Tm-5 °C	30 seconds
		68 °C	1 minute/kb of PCR target
3	20	92 °C	10 seconds
		Primer Tm-5 °C	30 seconds
		68 °C	1 minute/kb of PCR target plus 10 seconds/cycle

5. Analyze the PCR amplification products by electrophoresis using an appropriate percentage acrylamide or agarose gel. Long PCR products greater than 20 kb in length may be separated on a 0.6% agarose gel, however a 0.8% agarose gel may be used if higher resolution with less separation is desired. For maximum separation and resolution, pulse field gel electrophoresis with a 1.0% gel is recommended.

E. Real-time quantitative RT -PCR (TaqMan)

This is performed as a 2-step reaction:

1. cDNA synthesis from DNase 1-treated total RNA

2. PCR

1. cDNA synthesis (Advantage™ RT-for-PCR Kit - Clontech)

All reagents listed are provided with the kit

a) Quickly thaw each tube in kit and place on ice

b) Spin each tube briefly in a tabletop microcentrifuge and return to ice

c) In a sterile 0.5ml microcentrifuge tube, add purified DNase 1 -treated RNA preparation to a volume of DEPC-treated H_2O that will give a total volume of 12.5 ul. (Can use 0.2-1ug of total RNA, but recommend 1ug where possible). Use the same amount of RNA for each sample.

d) Add 1ul of either the random hexamer or the oligo(dT)18 primer (both are provided with the kit). (Oligo(dT) priming is the method of choice; however, if the 5¢ gene-specific TaqMan primer is located greater than about 2-3 kb from the poly-A tail would recommend use of random hexamer)

e) Heat total RNA at 70°C for 2 min and rapidly place heated RNA in ice

f) Add the components listed in the table below according to the volumes given

Reagent	Volume
5X reaction buffer	4.0 ul
dNTP mix (10 mM) each	1.0 ul
Recombinant RNase inhibitor	0.5 ul
MMLV reverse transcriptase	1.0 ul

g) Mix the contents of the tube by pipetting up and down

h) Incubate the reaction at 42°C for 1 hour

i) Heat at 94°C for 5 min to stop the cDNA synthesis reaction and to destroy any DNase activity; then spin down the contents of the tube

j) Dilute the reaction to a final volume of 100ul by adding 80ul of DEPCtreated H_20. Vortex and spin again.

2. PCR protocol (multiplex reaction)

Before the PCR can be performed on the newly-synthesised cDNA in 1., a number of steps must be carried out:

a) Selection of a suitable internal standard

b) Design of primers and probes to target genes

c) Validation of equal efficiency of amplification of target gene and internal standard

d) Optimisation of primers

a) Internal standard selection

An appropriate gene for use as an internal standard and normalisation of data must be selected (this may be already designed and available from Applied Biosystems or can design own using PrimerExpress software)

N.B. For accurate quantitation, the internal standard must:

- be amplified simultaneously with the target
- be expressed at a constant level and be unaffected by the experimental treatment
- should be expressed at roughly the same level (preferably slightly more abundant – see primer optimisation below) as the RNA under

investigation, to avoid competition of the more abundant target for PCR reagents

b) Primer and probe design

Primers and probes may be designed for selected target sequence using Applied Biosystems PrimerExpress software.

The following must be considered:

- primer and probe selection is based on estimated Tm, the desire for small amplicon size and location
- must be designed to fit many specifications– Primer Express software
- primers should bind to separate exons to avoid false positive results arising from amplification of contaminating genomic DNA
- if intron/exon boundaries are unknown, or when targeting an intron-less gene, RNA should be treated with DNase I
- When ordering target gene and internal standard probes to be used in multiplex reactions, ensure they are labelled with different reporter dyes
- on arrival, primers should be resuspended in DEPC-H_2O to a concentration of 100 pmol/ul; probes arrive in solution at 100 pmol/ul

c) Amplification efficiency validation

A validation experiment must be performed to ensure that amplification efficiencies of the target gene and internal standard are approximately equal. This is necessary in order to determine if any competition may occur within the multiplex reaction.

1. Prepare a mastermix of the reaction mix below so that there is enough for all samples to be investigated.

	Reagent	Volume (for 1 sample)
	TaqMan universal PCR mix	10.0 ul
Target gene	Forward primer	0.2 ul
	Reverse primer	0.2 ul
	Probe	0.05 ul
Internal Standard	Forward primer	0.2 ul
	Reverse primer	0.2 ul
	Probe	0.05 ul
	H_20	4.1 ul

2. Prepare serial 5-fold dilutions of original cDNA template

3. Add 15 ul of reaction mix above to each well in a 96-well plate.

4. Add 5 ul of each cDNA sample to separate wells in the plate.

5. Subject plate to the following cycling on the ABI Prism 7700 sequence detector:

50°C, 2 mins

95°C, 10 mins

40 cycles of the following:

95°C, 15 sec

60°C, 1 min

4. Following amplification, record CT values for target gene and internal standard.

d) Primer optimisation

Limiting primer concentrations may be defined by running a matrix of forward and reverse primer concentrations:

1. Make up enough mastermix for 76 samples as follows:

Reagent	Volume (for 1 sample)
TaqMan universal PCR mix	10.0 ul
Probe	0.05 ul
cDNA	5.0 ul
H_20	0.95 ul

2. Prepare a separate dilution series for each of the forward and reverse primers.

First dilute primers (as resuspended, see 2b) to 10 uM (i.e. a 1:10 dilution)

32ul 10uM primer + 368 ul H_20 $\Rightarrow$ 800nM

75ul 800nM primer + 25 ul H_2O $\Rightarrow$ 600nM

62.5ul 800nM primer + 37.5 ul H_20 $\Rightarrow$ 500nM

50ul 800nM primer + 50 ul H_20 $\Rightarrow$ 400nM

37.5ul 800nM primer + 62.5 ul H_20 $\Rightarrow$ 300nM

25ul 800nM primer + 75 ul H_20 $\Rightarrow$ 200nM

3. Add to wells in the PCR plate

- 2ul of 800nM forward primer to A1-12
- 2ul of 600nM forward primer to B1-12
- 2ul of 500nM forward primer to C1-12
- 2ul of 400nM forward primer to D1-12
- 2ul of 300nM forward primer to E1-12
- 2ul of 200nM forward primer to F1-12

4. Add to wells in the PCR plate

- 2ul of 800nM reverse primer to 1, 2 A-F
- 2ul of 600nM reverse primer to 3,4 A-F
- 2ul of 500nM reverse primer to 5,6 A-F
- 2ul of 400nM reverse primer to 7,8 A-F
- 2ul of 300nM reverse primer to 9, 10 A-F
- 2ul of 200nM reverse primer to 11, 12 A-F

5. Add 16 ul of master mix to each tube

6. Set up the thermal cycling conditions for the ABI Prism 7700 sequence detector as described previously:

50°C, 2 mins

95°C, 10 mins

40 cycles of the following:

95°C, 15 sec

60°C, 1 min

7. Following amplification, select the concentration of primers that show a reduction in DRn, but little effect on CT.

e) Real-time quantitative PCR

Once each of the steps a-d has been carried out, the quantitative PCR reaction may then be performed.

1. Prepare a mastermix of the reaction mix below so that there is enough for all samples to be investigated.

	Reagent	Volume (for 1 sample)
	TaqMan universal PCR mix	10.0 ul
Target gene	Forward primer (optimised conc.)	0.2 ul
	Reverse primer (optimised conc.)	0.2 ul
	Probe	0.05 ul
Internal Standard	Forward primer (optimised conc.)	0.2 ul
	Reverse primer (optimised conc.)	0.2 ul
	Probe	0.05 ul
	H_20	4.1 ul

2. Pipette 15 ul of the PCR mix above into individual wells on a 96-well plate

3. Add 5ul of each cDNA sample to separate wells in the plate.

4. Subject the plate to the following cycling on the ABI Prism 7700 sequence detector:

50°C, 2 mins

95°C, 10 mins

40 cycles of the following:

95°C, 15 sec

60°C, 1 min

5. Following amplification, compare CT values of samples (normalised to internal standard) in order to assess fold differences in mRNA levels of the target genes.

BIOGRAPHY

Mrs. Montakarn Tansatit was born on May 26, 1967 in Bangkok, Thailand. She received her degree of Doctor of Medicine (M.D., second class honors) in 1991 from the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. She has enrolled in graduate program for the Degree of Doctor of Philosophy Program in Biomedical Science at Graduate school, Chulalongkorn University since 2002.