CHAPTER III

MATERIALS AND METHODS

Research instruments

1. Pipette tip : 10 µl, 200 µl, 1000 µl (Axygen)
2. Fiter-tip : 10 µl, 100 µl,200 µl, 1000 µl (Axygen)
3. Microcentrifuge tube : 0.2 ml, 0.5 ml, 1.5 ml (Axygen)
4. Polypropylene tube : 15 ml (Sterilin)
5. Beaker : 50 ml, 100 ml, 500 ml, 1000 ml (Pyrex)
6. Flask : 100 ml, 200 ml (Pyrex)
7. Bottle : 100 ml, 500 ml, 1000 ml (Pyrex)
8. Cylinder : 50 ml, 100 ml, 500 ml (Pyrex)
9. Glass pipette : 5 ml, 10 ml (Pyrex)
10. Pipette rack (Axygen)
11. Cell line culture flask (Corning)
12. Stirring-magnetic bar
13. Stirring hot plate (Schott)
14. Fume hood (Captair)

15. Micropipette : 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl (Gilson)
16. Pipette boy (Brand)
17. Waterbath (Memmert)
18. Centrifuge (Haraeus)
19. Vortex (Scientific industries)
20. Microcentrifuge (Hettich)
21. Electrophoretic chamber (Amersham)
22. Electrophoretic power supply (Amersham)
23. Parafilm
24. Spectrophotometer (Thermo specific)
25. Cuvette (Starna)
26. Gel doc (Syngene)
27. Thermal cycler (MJ research)
28. Refrigerator (Sanyo)
29. Freezer : -20 °C (Sanyo)
30. Freezer: -80 °C (Thermo Forma)
31. Freezer: -196 °C (Forma Scientific)
32. Comb (Amersham)
33. Gel doc software (Syngene)
34. Safety cabinet (Nuaire)

35. Incubator (Forma Scientific)

36. Realtime-PCR machine (MJ research)

37. Microcentrifuge tube for realtime PCR machine (Bio-Rad)

38. Microcentrifuge for realtime PCR machine (Bio-Rad)

39. Cryogenic vial (Corning)

40. Beacon designer software for TaqMan assay

41. siRNA design software (Promega)

42. MJ Opticon Monitor Analysis software version 3.1 (Bio-Rad)

43. Punch Biopsy

44. Plastic culture dish

45. 24 well pate

46. Semi-dry blotter (Weltech)

47. Gel gradient former

48. Film cassette

49. Medical X-ray film (Kodak)

50. Fluorescent microscopy (Zeiss)

Reagents

A. General reagents

1. Double distilled water
2. Absolute ethanol (Merck)
3. Tris base (USB)
4. Sodium chloride (Merck)
5. Sodium Hydroxide (Merck)
6. EDTA (Fluka)
7. Hydrochloric acid (Merck)
8. Ammonium persulfate (USB)
9. Ammonium acetate (USB)
10. Ethidium Bromide (Sigma)
11. Sodium hydroxide (AnalaR)
12. Sodium dodecyl sulfate (USB)
13. TEMED
14. 100 base pair ladder (Sigma)
15. 1 kilo base pair ladder (Sigma)
16. 100% DMSO
17. DMEM (Dulbecco's modified Eagles's medium; Invitrogen)
18. 0.05% trypsin

19. 0.5% trypsin

20. β-mercapto-ethanol

B. Reagents for PCR

1. 10X PCR buffer (Qiagen)
2. Magnesium chloride (Qiagen)
3. Deoxynucleotide triphosphates (dNTP) (Qiagen)
4. Oligonucleotide primers (Proligo)
5. HotstarTaq DNA polymerase (Qiagen)

C. Reagents for Reverse transcription

1. RNA blood mini kit (Qiagen)
2. Improm-II™ reverse transcription system (Promega)
3. RNase-free DNase set (Qiagen)
4. DEPC-treated water (USB)

D. Reagent for quantitative realtime-PCR

1. QuantiTect Probe PCR Kit (Qiagen)
2. Oligonucleotide primer (Proligo)
3. TaqMan probes (Operon)

E. Reagent for cloning and transfection

1. siLentGene-2 U6 Hairpin Cloning Systems (Human) Kit (Promega)
2. DNA purification Kit (Qiagen)
3. LB plate
4. LB media
5. *E.coli* strain JM109
6. SOC media
7. X-gal
8. IPTG
9. DsRed II plasmid (Takara Bio)
10. Oligonucleotide primer (Proligo)
11. Restriction enzyme EcoRV (Biolab)
12. Restriction enzyme NheI (Biolab)
13. Restriction enzyme XhoI (Biolab)
14. Amplicilin
15. G-418 (Sigma)
16. Lipofectamine 2000 (Invitrogen)
17. Qiaquick® gel extraction kit (Qiagen)

F. Reagent for Western Blotting

1. Tween20

2. PIPES (Sigma)

3. Proteinase inhibitor

4. TEMED

5. APS

6. Goat Anti-Calcipressin 1 / DSCR1 Antibody (Santacruz)

7. Donkey anti-goat antibody (Santacruz)

Procedure

1. Cell lines and cell culture

Fibroblasts have a branched cytoplasm surrounding an elliptical, speckled nucleus having 1 or 2 nucleoli. Active fibroblasts can be recognized by their abundant rough endoplasmic reticulum. Inactive fibroblasts, which are also called fibrocytes, are smaller and spindle shaped. They have a reduced rough endoplasmic reticulum. Fibroblasts make collagens, glycosaminoglycans, reticular and elastic fibers, and glycoproteins found in the extracellular matrix. In growing individuals, fibroblasts are divided and synthesized ground substance. Tissue damage stimulates fibrocytes and induces the mitosis of fibroblasts. Fibroblasts can give rise to other cells, such as bone cells, fat cells, and smooth muscle cells. Note that all of these cells are of mesodermal origin. The fact that fibroblasts easily proliferate makes them a popular cell type for cell cultures in biological research.

Procedure

Ten normal human fibroblasts and ten Down syndrome human fibroblasts were taken from skin biopsies by punch biopsy, and scissor excision under local anaesthesia from the anterior surface of the forearm. Biopsy specimens were transferred to plastic culture dish, minced and stuck in grooves in the dish. DMEM (Dulbecco's modified Eagles's medium; Invitrogen) supplemented with 20% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin were floated in the dish and placed in 5% CO_2, 37°C incubator with humidity. Fibroblast cells were grown in 8-12 days with monolayer-spindle shape and changed media once to twice a week. Fibroblast cells were subcultured in 12-20 days with 80-90% confluence by trypsinization.

Trypsinization method: Growth medium were removed from the dish, placed with 2 ml of 0.05% trypsin to wash the cell surface and discarded immediately. 0.5% trypsin were placed to digest protein surface and incubated at 37°C for 1 minute to activate enzyme trypsin activity. Growth medium were added to inactivate enzyme activity. Monolayer fibroblast cells were became cell suspension and ready to subculture into new 25 cm^3 cultured flask or freeze with DMEM media + 20% fetal brovine serum + 8% DMSO or harvest for RNA/ protein extraction.

2. siRNA designing and cloning

A. siRNA primer design & amplification

siRNA is cloned by siLentGene-2 U6 Hairpin Cloning Systems-Neomycin (Human) Kit (Promega). siRNA downstream primer is designed by Promega siRNA designer program and prevented off-target by Blast program. The downstream primer contains the following: 5' phosphate group, partial EcoR V sequence to generate an EcoR V site when the amplified cassette is ligated into one of the psiLentGene vectors, U6 terminator sequence to allow the U6 polymerase to terminate with the 3' overhangs that are required for successful siRNAs , target sequence reverse complement-loop sequence-target sequence to create fold-back stem-loop structures and U6 cassette

matching sequence to allow the down-stream primers to bind to the U6 cassette during amplification.

1. Down stream primer is designed and used for generate siRNA as follow;

Table1 : siRNA sequence as down stream primer construct.

EcoRV Half site	U6 Terminator	Target	Loop	Target reverse complement	U6 Cassette Matching Sequence
5'-ATC	TAAAAA	GAGGACGCATTCCAAATCA	AGAGAACTT	TGATTTGGAATGCGTCCTC	GGTGTTTCGTCCTTTCCACAAGA

2. Prepare reaction mix as follow;

Table 2 : Component for siRNA amplification

No.	PCR Reagent	Stock concentration	Final concentration
1	Nuclease-Free Water	variable	-
2	siLentGene High Fidelity PCR Master Mix (Promega)	2 X	1 X
3	siLentGene U6 Cloning Upstream Primer (Promega)	20 µM	0.4 µM
4	siRNA downstream primer (Proligo)	20 µM	0.4 µM
5	siLentGene U6 Cassette DNA template (Promega)	1ng/µl	0.02ng

Table 3 : PCR condition

Step	Temperature and incubation time
1. Initial denaturation	95°C/ 15 min
2. 30 cycles of Denaturation Annealing Extension	 94°C/ 30 sec 65°C / 30 sec 72°C/ 45 sec
3. Final extension	72°C/ 10 min

3. The amplification product is analyzed on a 1% agarose gel.

B. Ligation of amplified siRNA into psiLentGene vector

1. PCR product is cleaned up by DNA purification Kit (Qiagen) and ligated into psiLentGene vector (Promega).

2. Set up ligation reactions as described below.

Table 4 : Component for ligation

No.	Ligation component	Standard reaction	Negative control (minus insert)
1	Nuclease-Free Water	variable	variable
2	2 X Rapid Ligation Buffer (Promega)	5 µl	5 µl
3	PCR product ~15ng	variable	-
4	3 units/ µl of T4 DNA Ligase (Proligo)	2 µl	2 µl
5	psiLentGene™ Vector (50ng)	1 µl	1 µl

3. Mix the reactions by pipetting and incubate the reactions overnight at 4°C.

E. Transformation of *E.coli*

E.coli str ain JM109 competent cells are used for transformation of ligated reactions to produce a number of siRNA constructions.

Procedure

1. Prepare 2 LB/ampicillin/IPTG/X-Gal plates for each ligation reaction and control transformation. Equilibrate the plates to room temperature before plating.

2. Remove the frozen high-efficiency competent cells from –70°C storage and place them in an ice bath until just thawed (about 5 minutes). Mix the cells by gently flicking the tube.

3. For each ligation reaction and transformation control, carefully transfer 50 µl of the cells to a sterile 15 ml tube.

4. Briefly centrifuge the tubes containing the ligation reactions. Add 10 µl of each ligation reaction to the tube prepared in Step 3. To perform a transformation control, add 0.1ng of supercoiled plasmid DNA (Promega) to 100 µl freshly thawed competent cells in a sterile 15ml tube.

5. Gently flick the tubes to mix and place them on ice for 20 minutes.

6. Heat-shock the cells for 45–50 seconds in a water bath at exactly 42°C. Do Not Shake.

7. Immediately return the tubes to ice for 2 minutes.

8. To the tubes containing cells transformed with the experimental ligations, add 950 µl of room temperature SOC medium. For the transformation controls, add 900 µl of room temperature SOC medium.

9. Incubate for at 37°C with shaking (approximately150rpm) for 1.5 hours.

10. Centrifuge cells at 1,000 × g for 10 minutes and resuspend in 200 µl of SOC medium.

11. Plate 100 µl of each transformation onto duplicate LB/ampicillin/ IPTG/X-Gal plates.

12. Incubate the plates overnight (16–24 hours) at 37°C.

F. Screening Transformants for Inserts

Successful cloning of an insert into the psiLentGen Vectors interrupts the coding sequence of ß-galactosidase; recombinant clones can usually be identified by color screening on indicator plates. The siLentGen U6 Cloning Upstream Primer includes stop codons in all possible reading frames to facilitate producing white colonies from inserts.

G. Purifying recombinant plasmid DNA

Standard miniprep procedure is used for isolating plasmid DNA.

Procedure

1. Pick up a single white colony and streak onto LB/ampicillin plate as a primary plate.
2. Incubate the plate overnignt (16-24 hours) at 37°C.
3. Pick up a single colony and streak onto LB/ampicillin plate as a secondary plate.
4. Incubate the plate overnignt (16-24 hours) at 37°C.
5. Pick up a single colony in 5 ml of LB/ampicillin broth and incubate on shaker with maximum speed at 37°C overnight (16-24 hours).
6. Pellet 1-1.5 ml aliquots of late log or stationary phase *E. coli* cultures for 30 minute in a microcentrifuge at 4°C maximum speed.
7. Remove the supernatant with a pipet tip.
8. Thoroughly resuspend the bacterial pellet in 100 µl of P1 buffer by pipetting the mixture up and down.
9. Add 200 µl of P2 buffer. Mix the contents by inverting the tube 4-6 times until the cell suspension "clears".
10. Add 150 µl of P3 buffer. Mix the contents by inverting the tube 4-6 times. A white precipitate consisting of cellular debris and chromosomal DNA will appear.
11. Incubate mixture on ice for 5 minutes.
12. Pellet cellular debris by spinning in a microcentrifuge at maximum speed for 10 minutes at 4°C.
13. Transfer the supernatant solution to a clean tube. Be careful not to take any white precipitate to the next tube.

14. Add 900 µl of 100% Ethanol for precipitation of plasmid DNA. Mix the contents by inverting the tube and incubate at room temperature for 2 minutes.
15. Spin the tube in a microcentrifuge at maximum speed for 10 minutes at 4°C.
16. Carefully remove the ethanol with a pipet tip.
17. Wash pellet twice with 70% Ethanol being careful not to lose the plasmid DNA and centrifuge at maximum speed for 2 minutes at 4°C.
18. Dry the pellet at room temperature and resuspend with 50 µl of sterile water.
19. Measure the DNA concentration by absorbance at 260 nm.
20. Aliquots purified recombinant plasmid DNA for siRNA sequencing.

3. siRNA transfection into fibroblast cell lines

Lipofectamine2000 reagent (Invitrogen) is used to transfect recombinant plasmid DNAs into fibroblast cells with cationic lipid of lipofectamine2000 condenses DNA to compact structure within DNA-lipid complexes. These positively charged complexes are binding to and are entering the cell by endocytosis. The DNA-lipid-complexes acts as a proton-sponge that buffers the endosomal pH, since the hydrophilic unit contains several sites which can be protonated. Continuous proton influx induces osmotic swelling and rupture. Nucleic acids are released simultaneously from the DNA-lipid complex by progressive proton-assisted lipid layer disintegration.

Procedure

1. One day before transfection, plate 0.5-2 x 10^5 cells in 500 µl of DMEM supplemented with 10% (v/v) FBS into 24-well plate so that cells will be 90-95% confluent at the time of transfection.

2. Dilute recombinant plasmid DNA in 50 µl of DMEM without serum using concentration as follows:

Table 5 : Plasmid DNA and lipofectamine2000 concentration.

DNA Concentration	DNA (μg) in media 50 μl	Lipofectamine2000 (μl) in media 50 μl
0.5-folds of DNA is recommend by manufactory	0.4	1
1-folds of DNA is recommend by manufactory	0.8	2
1.5-folds of DNA is recommend by manufactory	1.2	3

3. Mix Lipofectamine2000 gently before use, then dilute the appropriated amount in 50 μl of DMEM without serum.

4. Incubate for 5 minutes at room temperature.

5. After 5 minutes incubation, combine the diluted DNA with diluted Lipofectamine2000 (total volume 100 μl). Mix gently and incubate for 20 minutes at room temperature.

6. Add the 100 μl of complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth.

7. Incubate cells at 37°C in a CO_2 incubator and subculture into new 25 cm^3 cultured flask.

4. Selection of transfected cell lines

The psiLentGene-2 U6 hairpin cloning system-Neomycin (Human) kit (Promega) is consisted of neomycin antibiotic selection marker. Cell lines vary in the level of resistance to antibiotics, so the level of resistance of a particular cell line must be tested before attempting stable selection of the cells. A "kill curve" will determine the minimum concentration of the antibiotic needed to kill the untransfected cells. The antibiotic concentration for selection will vary depending on the cell type and growth rate. When cells are highly confluent, they are more resistant to antibiotic selection, so it is important

to maintain the cells at a subconfluent level. The typical effective ranges and lengths of time needed for selection are given in Table 6.

Table 6 : Conditions for selection of stable transfectants.

Vector	Antibiotic	Effective antibiotic concentration	Time needed for selection
psiLentGene™-Neomycin	G-418	100 - 1,000 µg/ml	4 - 14 days

Procedure

1. Vary G-418 at concentrations of 0, 100, 200, 400, 600, 800 and 1,000 µg/ml in DMEM+ 10% (v/v) FBS.

2. Subculture nontransfected fibroblast cells at low concentration (30-50% confluent) in 500 µl of DMEM with antibiotics from step 1 into 24-well plate.

3. Change the medium every 2-3 days until all nontransfected cells are dead.

4. Use the appropriate drug concentration and time for selection of transfected cell lines.

5. Uptake rate of siRNA into cell lines

Coding region of red florescence protein (RFP), 700 based pairs, are cloned into recombinant plasmid DNA and expressed under SV40 promoter by restriction site of enzyme NheI (Biolabs) and XhoI (Biolabs) at the position of 841 and 1658, respectively. RFP is amplified using DsRed2 plasmid (Takara Bio) as a template with recognition sequence of enzyme NheI (Biolabs) and XhoI (Biolabs) for the restriction endonuclease included at the 5' end of the primer. The primer sets, PCR components and PCR condition are shown as follow;

Table 7 : Primer sets for RFP cloning

Name	Restrinction enzyme	recognition site	Sequence (5' - 3')
RFP-NheI-F	NheI	5′...G▼CTAGC...3′ 3′...CGATC▲G...5′	ATCGGCTAGCCGCCACCATGGCCTCCT
RFP-XhoI-R	XhoI	5′...C▼TCGAG...3′ 3′...GAGCT▲C...5′	ATCGCTCGAGGTTCCTGTAGCGGCCGC

Table 8 : Reaction components for PCR/ 20 µl final volume

No.	Components	Stock concentration	Final concentration
1	Sterile dd H_2O	variable	-
2	Buffer(QIAGEN)	10 X	1 X
3	MgCl2(Qiagen)	-	-
4	dNTP(Biolabs)	25 mM	0.2 mM
5	Forward primer (Proligo)	10 µM	0.5 µM
6	Reverse primer(Proligo)	10 µM	0.5 µM
7	HotstarTaq(Qiagen)	5 unit/µl	0.625 unit
8	pDsRed 2 (Takara Bio)	100 pg-100 ng	

Recombinant plasmid vector and RFP PCR product are cleaved with enzyme NheI (Biolabs) and XhoI (Biolabs). After digestion, recombinant plasmid and PCR product are detected by 1% agarose gel electrophoresis and purified by Qiaquick® gel extraction kit (Qiagen).

Table 9 : Reaction component for digestion/ 50 µl final volume.

Digestion component	Stock concentration	Final concentration
NEBuffer 2 (Biolabs)	10X	1X
Bovine Serum Albumin-BSA (Biolabs)	100X	1X
XhoI (Biolabs)	20,000 U/ml	1 U/DNA 1 µg
NheI (Biolabs)	10,000 U/ml	1 U/DNA 1 µg
DNA	variable	
ddH2O	variable	

The digested PCR product and recombinant plasmid are ligated with enzyme T4 ligase. Ligated condition and component are shown as follow;

Table 10 : Reaction components for ligation/ 20 µl final volume

No.	Ligation component	Standard reaction	Negative control (minus insert)
1	Nuclease-Free Water	variable	variable
2	10 X Rapid Ligation Buffer (Promega)	2 µl	2 µl
3	PCR product ~15ng	variable	-
4	3 units/ µl of T4 DNA Ligase (Proligo)	1 µl	1 µl
5	psiLentGene™ Vector (50ng)	variable	variable

Recombinant RFP plasmids are selected by PCR amplification of RFP product using RFP-NheI forward primer and RFP-XhoI reverse primer and confirmed by sequencing.

6. RNA extraction

RNA extractions were performed using QIAamp RNA miniprotocol for isolation of total RNA from cultured cells QiAamp® RNA blood mini Kit.

Procedure

1. Centrifuge 10-ml cultured cells at 300 g for 5 minutes, remove supernatant.
2. Wash pellets two times with 1X PBS by pipette up and down, centrifuge at 300 x g for 10 minutes.
3. In final wash, rest 1 ml PBS for pellet resuspension.
4. Transfer suspension into new sterile 1.5-ml centrifuge tube.
5. Disrupt cells by add 600 µl of buffer RLT+ β mercapto-ethanol, mix by pipet
6. Pipet lysate into QIAshredder spin column sitting in a 2-ml collection tube, centrifuge at 13,000 rpm. Discard QIAshredder spin column and save homogenized lysate.
7. Add 1 volume of 70% ethanol to the homogenized lysate and mix well by pipetting.
8. Pipet sample in to a new spin column sitting in a 2-ml collection tube. Centrifuge for 15 seconds at 13,000 rpm.
9. Transfer the QIAamp spin column into a new 2-ml collection tube. Add 700 µl Buffer RW1 into spin column, centrifuge for 15 second at 13,000 rpm.

10. Transfer the spin column into a new collection tube. Pipet 500 µl buffer RPE in to the spin column and centrifuge for 15 seconds at 13,000 rpm.

11. Add 500 µl Buffer RPE, centrifuge at 13,000 rpm for 3 minutes.

12. Transfer the spin column into a new 1.5-ml collection tube and pipet 50 µl of RNase-free water directly on to the membrane, centrifuge for 1 minute at 13,000 rpm for elution.

13. Use immediately or store at -80°C until use.

7. DNase-I treatment

The mRNA samples are treated with DNase-I to eliminate the residue genomic DNA which might interfere with subsequent processes.

8. Reverse transcription

The DNase-I treated mRNA samples are transcribed to cDNA by reverse transcriptase using oligo dT primer. To prevent RNase contamination, DEPC-treated water and RNase inhibitor are applied.

Procedure

A. Target RNA and primer combination and denaturation

1. Place sterile thin-walled dilution tubes and reaction tubes on ice.
2. Prepare the reaction as follow

Table 11 : Components for RNA-Primer combination and denaturation

No.	Component	Volume
1	RNA template	Up to 1 μg
2	Oligo-dT Primer	20pmol or 0.5 μg
3	Nuclease free water	Adjust to 5 μl

3. Incubate at 70 ° C for 5 minutes.
4. Chill on ice immediately at 4 ° C for 5 minutes.

B. Reverse transcription

1. Prepare reaction mix (table 12)

Table 12 : Components for reverse transcription/ 15 μl total volume

No.	Component	Stock concentration	Final concentration
1	Nuclease-free water	variable	-
2	ImProm-II™ reaction buffer	5X	1X
3	$MgCl_2$	25mM	3mM(1.5-8)
4	dNTP mix	25mM	0.5mM
5	Recombinant RNasin®Ribonuclease inhibitor		20 unit
6	Vortex the mixture, then add Improm-II™Reverse Transcriptase		

2. Aliquot reaction mix to each reaction tube on ice. Add 5 µl of RNA and primer mix to each reaction.
3. Anneal by incubate at 25 ° C for 5 minutes.
4. Extend by incubate at 42 ° C for one hour.
5. Inactivate enzyme by incubate at 70 ° C for 15 minutes
6. Proceed next step or store at -20 ° C until use.

9. RT-PCR

The primer sets were design to cover *DSCR1* mRNA exon 3 and 4.

Table 13 : Components for RT-PCR

No.	Components	Stock concentration	Final concentration
1	Sterile ddH2O	variable	-
2	Buffer(QIAGEN)+15 mM MgCl2	10 X	1 X
3	MgCl2 (Qiagen)	25mM	0.5 mM
4	dNTP(Biolabs)	25 mM	0.2 mM
5	Forward primer (Proligo)	10 µM	1 µM
6	Reverse primer(Proligo)	10 µM	1 µM
7	HotstarTaq(Qiagen)	5 unit/ µl	2.5-5 unit
8	cDNA	variable	< 500 ng/ reaction

Table 14 : RT-PCR condition

Step	Temperature and incubation time
1. Initial denaturation	95°C/ 15 min
2. 35 cycles of Denaturation Annealing Extension	 94°C/ 1 min 62°C / 1 min 72°C/ 1 min
3. Final extension	72°C/ 10 min

10. Quantitative real-time PCR

Since relative quantification is used for determine the expression level of interested gene compare with the house-keeping gene, the PCR efficiency must be controlled. Before proceed to a relative quantification, the validation of multiplexed PCR must be completed.

Table 15 : Probe-primer mixture preparation

No.	Components	Stock concentration	Final concentration
1	RNase-free water	-	-
2	Forward primer	100 µM	8 µM
3	Reverse primer	100 µM	8 µM
4	Probe	100 µM	4 µM

Table 16 Genes, primer sets and probes for quantitative real-time PCR

Gene	Region	Selected position	Name	Sequence	Annealing Temperature (°C)	Size (bp)
DSCR1	CDS	6, 7	DSCR1F	GCCAAATCCAGACAAGCAGTTT	56	149
			DSCR1R	CGTGCAATTCATACTTTTCCCCT	56	
			DSCR1P	CCCTCCCGCCTCTCCGCCAGT	69	
GAPDH		5'UTR-exon3	GAPDHF	CGACAGTCAGCCGCATCTTC	56	104
			GAPDHR	CGCCCAATACGACCAAATCCG	56	
			GAPDHP	CGTCGCCAGCCGAGCCACATCG	72	

Note The 5' terminal of *DSCR1* probe was labeled with FAM, while 3' was labeled with TAMRA.

The 5' terminal of GAPDH probe was labeled with Cy5, while 3' was labeled with BHQ (Black hole quencher).

Table 17 : Multiplexed-PCR components/ 20 µl total volume

No.	Components	Stock concentration	Final concentration
1	RNase-free water	-	-
2	Quantitect PCR master mix	2X	1X
3	Probe-primer mix (*GAPDH*)	8 µM	0.4 µM
4	Probe-primer mix (*DSCR1*)	8 µM	0.4 µM
5	cDNA	variable	< 500 ng/ reaction

Table 18 : Multiplexed PCR condition

Step	Temperature and incubation time
1. Initial denaturation	95°C/ 15 min
2. 40 cycles of	
Denaturation	94°C/ 15 sec
Annealing/extension	60°C / 30 sec
Read plate	

11. Agarose gel electrophoresis

1% agarose gel is used for observe the PCR products from RT-PCR and multiplexed PCR.

12. Protein Extraction

Protein extractions were performed the using miniprotocol for Isolation of cytoplasmic and nuclear protein from cultured cells.

Procedure

1. Centrifuge 10-ml cultured cells at 300 x g for 5 minutes, remove supernatant.

2. Wash pellets two times with ice cold 1X PBS by pipette up and down, centrifuge at 300 x g for 10 minutes.

3. The final washing, rest 1 ml PBS for pellet resuspension.

4. Transfer suspension into new sterile 1.5-ml centrifuge tube.

5. Disrupt cells by add 200 µl of freshly prepared lysed buffer.

6. Briefly mix and incubate on ice for 20 minutes.

7. Centrifuge at 5000 rpm at 4 °C for 5 minutes.

8. Save supernatant for cytoplasmic compartment on ice.

9. Wash nuclear cell pellet twice with 400-600 µl of ice cold 1X PBS and centrifuge at 5000 rpm at 4 °C for 5 minutes.

10. Resuspend cell pellet in 200 µl of freshly prepared lysis buffer.

11. Mix by vortex and incubate on ice for 10 minutes.

12. Save product for nuclear compartment on ice.

13. Incubate product from step 8 and 12 for 10 seconds with 1 not sonicator 3 times and chill on ice immediately.

13. Protein assay

Bradford method is used to measure protein by BIORAD kit.

Procedure

1. Prepare standard protein solution with BSA at concentration 1.5mg/ml, 1.2 mg/ml, 1 mg/ml, 0.8 mg/ml, 0.4 mg/ml, and 0.2 mg/ml.

2. Prepare reagent A' = reagent A 1ml + reagent S 20 μl

3. Prepare solution with reagent B 400 μl + reagent A' 50 μl + sample 10 μl

4. Measure standard and sample protein at O.D. 750nm

14. Calcineurin activity assay

The calcineurin activity assay is a kit that used for measurement of calcineurin activity by incubation with RII phophopeptide, a known substrate for calcineurin, and the free phosphate released is detected based on the malachite green assay. In cellular extracts RII phosphopeptide is also cleaved by other phosphatases; i.e. PP1, PP2A and PP2C. Using calcineurin activity assay may not a good choice for the measurement of calcipressin quantification.

15. Western blot

A western blot (alternately, immunoblot) is a method in molecular biology/biochemistry/immunogenetics to detect protein in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native proteins by shape and size. The proteins are then transferred out of the gel and onto a membrane (typically nitrocellulose), where they are "probed" using antibodies specific to the protein. As a result, researchers can examine the size, processing, or amount of protein in a given sample and compare several groups.

A. Polyacrylamide gel preparation

Procedure

1. Prepare 6% and 18 % polyacrylmide gel and pour into gel gradient former chamber A and B.

2. Set glass plate cassette and open valve to pour gel into the cassette.

3. 100 µg of protein samples and 1X loading dye solution are loaded into each well.

4. Proteins are separated by size at 200V for 50 minutes.

B. Transfer and blocking

Proteins are transferred from gel to nitrocellulose membrane by semi-dry blotter. The other space of nitrocellulose membrane is filled with protein in blocking solution.

Procedure

1. Gel is washed with transfer buffer on shaker for 5 minutes and placed on saturated membrane and filter paper. Roll out all air bubbles.

2. Transfer protein from gel to membrane at 250 mA for 30 minutes.

3. Membrane is washed with 1XTBST for 5 minutes two times.

4. Incubate membrane with blocking solution for 1 hour at room temperature.

C. Antibody blotting

DSCR1 protein is attached with DSCR1 antibody as primary antibody and then bound to Donkey anti-goat antibody as secondary antibody.

Procedure

1. Goat *DSCR1* antibody is used as primary antibody and diluted with blocking solution at 1:200.

2. Donkey anti-goat antibody is used as secondary antibody and diluted with blocking solution at 1:2000.

3. Wash membrane with 1XTBST for 5 minutes 3 times on shaker.

4. Seal membrane in plastic bag and fill with 5ml-diluted goat *DSCR1* antibody.

5. Incubate over night at 4°C on shaker.

6. After incubation, wash membrane with 1XTBST for 5 minutes 3 times.

7. Seal membrane in plastic bag, fill with 3 ml-diluted Donkey anti-goat antibody and incubate at room temperature for 1 hour.

D. Detection

The secondary antibody is conjugated with horse radish peroxidase (HRP) enzyme and detected by western blotting luminol reagent (SantaCruz), a non-radioactive, light-emitting system for detection proteins on a membrane. HRP mediates oxidation of luminol in the presence of hydrogen peroxide and this reaction produces an iridescent light, is detected by X-ray film.

Procedure

1. Wash membrane with 1XTBST for 5 minutes 3 times.

2. Mix 4ml per each of western blotting luminol reagent A and reagent B (Santa cruz).

3. Incubate membrane with homogeneous solution for 5 minutes at room temperature.

4. Remove the exceed reagent and enfold membrane with plastic wrap.

5. Medical X-ray film is placed on the membrane in dark room and contained by film cassette for 1 Hour.

6. Film is washed with developer solution for 10 second, washed with water 2 times and fixed by fixer solution for 10 minutes.

16. Coomasie blue gel staining

Coomassie (also known as Brilliant Blue, Brilliant Blue G, Acid Blue 90, C.I. 42655, or Brilliant Blue G 250) is a blue dye commonly used in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel is soaked in dye and then destained. This treatment allows the visualization of bands indicating the protein content of the gel. The visualization on the gel usually contains a set of molecular weight marker so that protein molecular weight can be determined in an unknown solution.

Procedure

1. Fix gel with fixing solution for 30 minutes.

2. Gel is stained by coomasie working solution for 25 minutes.

3. Destain gel with destaining solution on shaker until background is cleared.