# CHAPTER III

# METHODOLOGY



**Research Instruments**

1. Pipette tip : 10 µl, 100 µl, 200 µl, 1,000 µl (Elkay, USA)
2. Microcentrifuge tube : 0.2 ml, 0.5 ml, 1.5 ml (Bio-RAD, Elkay, USA)
3. Polypropylene conical tube : 15 ml (Elkay, USA)
4. Beaker : 50 ml, 100 ml, 200ml, 500 ml, 1,000 ml (Pyrex)
5. Flask : 250 ml, 500 ml, 1,000 ml (Pyrex)
6. Reagent bottle : 100 ml, 250 ml, 500 ml, 1,000 ml (Duran, USA)
7. Cylinder : 25 ml, 50 ml, 100 ml, 250 ml, 500 ml, 1,000 ml (Witeg, Germany)
8. Glass pipette : 5 ml, 10 ml (Witeg, Germany)
9. Pipette rack (Autopack, USA)
10. Thermometer (Precision, Germany)
11. Parafilm (American National Can, USA)
12. Plastic wrap
13. Stirring-magnetic bar
14. Combs
15. Automatic adjustable micropipette : P2 (0.1-2 µl), P10 (0.5-10 µl), P20 (5-20 µl), P100 (20-100 µl), P1000 (0.1-1 ml) (Gilson, France)
16. Pipette boy (Tecnomara, Switzerland)
17. Vortex (Scientific Industry, USA)
18. pH meter (Eutech Cybernatics)
19. Stirring hot plate (Bamstead/Thermolyne, USA)
20. Balance (Precisa, Switzerland)
21. Centrifuge (J.P.Selecta, Span)
22. Microcentrifuge (Eppendorf, Germany)

23. Mastercycler personal (Eppendorf, Germany)
24. Thermal cycler (Touch Down, Hybraid USA)
25. Power supply model 250 (Gibco BRL, Scothland)
26. Power poc 3000 (Bio-RAD)
27. Horizon 11-14 (Gibco BRL, Scothland)
28. Sequi-gen sequencing cell (Bio-RAD)
29. Heat block (Bockel)
30. Incubator (Memmert)
31. Thermostat shaking-water bath (Heto, Denmark)
32. Spectronic spectrophotometers (Genesys5, Milon Roy USA)
33. UV Transilluminator (Fotodyne USA)
34. UV-absorbing face shield (Spectronic, USA)
35. Gel doc 1000 (Bio-RAD)
36. Refrigerator 4 $^0$C (Misubishi, Japan)
37. Deep freeze -20 $^0$C, -80 $^0$C (Revco)
38. Water purification equipment (Water pro Ps, Labconco USA)
39. Water bath (J.P.Selecta, Span)
40. Storm 840 and ImageQuaNT solfware (Molecular dynamics)
41. 12-well culture plates (Corning, New York)
42. T-25 and T-75 Flasks (Corning, New York)
43. Costar® Stirpipette®: 0.2 ml, 10 ml, 25 ml (Corning, New York)
44. Haematocytometer counting chamber
45. Petridish (Sterilin limited, UK)
46. Cryotube vial 2.0 ml (Corning, New York)
47. Cryo 1°C Freezing container (Nalgene® Labware)
48. GloMax. 20/20 Luminometer (Promega)

**Reagents**

1. General reagents

1.1 Absolute ethanol (Merck)

1.2 Agarose, molecular glade (Promega)

1.3 Ammonium acetate (Merck)

1.4 Boric acid (Merck)

1.5 Bromphenol blue (Pharmacia)

1.6 Disodium ethylenediamine tetracetic acid : EDTA (Merck)

1.7 Ethidium bromide (Gibco BRL)

1.8 Ficoll 400 (Pharmacia)

1.9 Hydrochloric acid (Merck)

1.10 Mineral oil (Sigma)

1.11 Phenol (Sigma)

1.12 Chloroform (Merck)

1.13 Isoamyl alcohol (Merck)

1.14 Sodium chloride (Merck)

1.15 Sodium dodecyl sulfate (Sigma)

1.16 Sodium hydroxide (Merck)

1.17 Sucrose (BDH)

1.18 Tris base (USB)

1.19 Triton X-100 (Pharmacia)

1.20 100 base pair DNA ladder (Biolabs)

1.21 40%acrylamide/bis solution 19:1 (Bio-RAD)

1.22 GelStar (Camberx)

2. PCR reagents

2.1 10X PCR buffer (500 mM KCl, 200 mM Tris-HCl pH 8.4) (Promega)

2.2 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 8.8, 0.8% Nonidet P40) (Fermentas)

2.3 Magnesium chloride (Promega)

2.4 Magnesium chloride (Fermentas)

2.5 Deoxynucleotide triphosphates (dNTPs) (Promega)

2.6 Deoxynucleotide triphosphates (dNTPs) (Fermentas)

2.7 Oligonucleotide primers (BSU)

2.8 Oligonucleotide primers (Biogenomed)

2.9 *Taq* DNA polymerase (Promega)

2.10 *Taq* DNA polymerase (Fermentas)

2.11 100% DMSO

2.12 Genomic DNA sample

3. Restriction enzymes

3.1 *BamH*I (Biolabs)

3.2 *EcoR*I (Biolabs)

3.3 *Bgl*II (Biolabs)

4. Bacterial culture media

4.1 Yeast extract powder (Bio Basic Inc.)

4.2 Agar bacterial powder (Conda, Spain)

4.3 Tryptone powder (Bio Basic Inc., Canada)

4.4 Sodium chloride (BDH AnalaR®, Merk group)

5. Cell culture

5.1 Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone)

5.2 Fetal Bovine Serum (FBS) (GIBCO, Invitrogen)

5.3 PenStrep (GIBCO, Invitrogen)

5.4 Trypsin-EDTA (GIBCO, Invitrogen)

5.5 Phosphate-buffered saline (PBS)

5.6 Charcoal-stripped FBS (Hyclone)

5.7 3, 3', 5-triiodothyronine (Sigma)

5.8 Tryphan blue

6 Transfection reagents

6.1 Lipofectamine™ 2000 (Invitrogen)

6.2 Opti-MEMI Reduced Serum Medium (GIBCO, Invitrogen)

7. Dual-luciferase reporter assay (Promega)

**Experimental Procedure**

**1. Subjects and sample collection**

After informed consent, 3 ml. of peripheral blood were obtained from each individual.

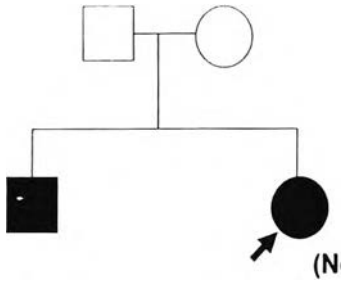
**Subjects**

Six patients from six unrelated families were clinically diagnosed with RTH at the Pediatric Clinic of the King Chulalongkorn Memorial Hospital and were included in the study. Selection criteria were based on clinical presentation and thyroid function tests as shown in figure 11.

**Controls**

Controls were healthy volunteers unaffected with RTH and had no family history of RTH. DNA from the controls was used for mutation screening in the *TRβ* gene identified in the RTH patients. DNA from the patients' family members who were at risk was also investigated.

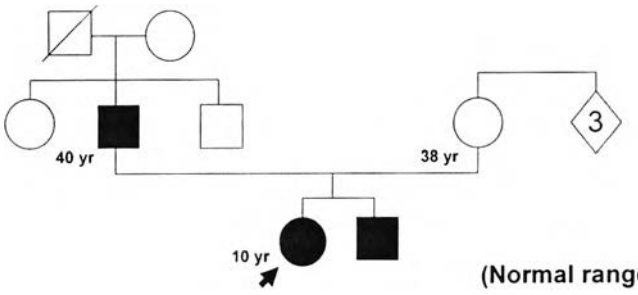
# Family 1



(Normal range)

| | | | |
|---|---|---|---|
| $FT_4$ | **2.49** | 1.29 | **2.11** (0.8-1.8 ng/dl) |
| $FT_3$ | **3.62** | 2.83 | **4.17** (1.6-4.0 pg/ml) |
| TSH | **4.11** | 2.52 | **4.56** (0.3-4.1 ulU/ml) |

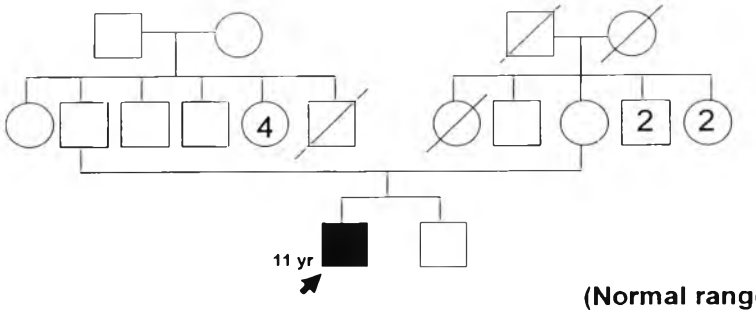
# Family 2




(Normal range)

| | | | | |
|---|---|---|---|---|
| $FT_4$ | **3.42** | **4.06** | **7.1** | 1.3 (0.8-1.8) |
| $FT_3$ | **4.68** | **14.6** | **14.4** | 1.57 (1.6-4.0) |
| TSH | **1.65** | **2.17** | **2.4** | 1.1 (0.3-4.1) |

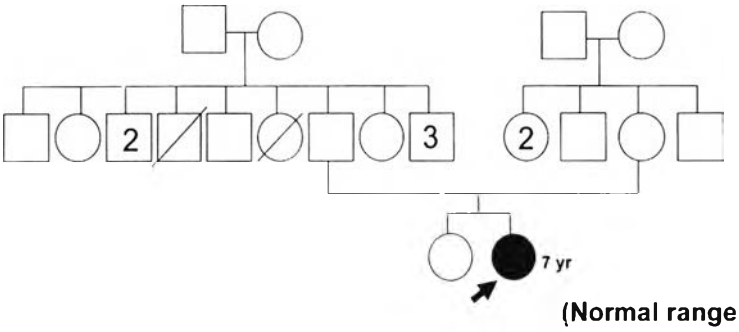
# Family 3




(Normal range)

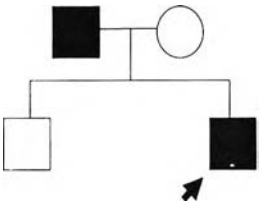
| | | |
|---|---|---|
| $FT_4$ | **2.2** | (0.8-1.8) |
| $FT_3$ | **5.25** | (1.6-4.0) |
| TSH | **1.96** | (0.3-4.1) |

## Family 4



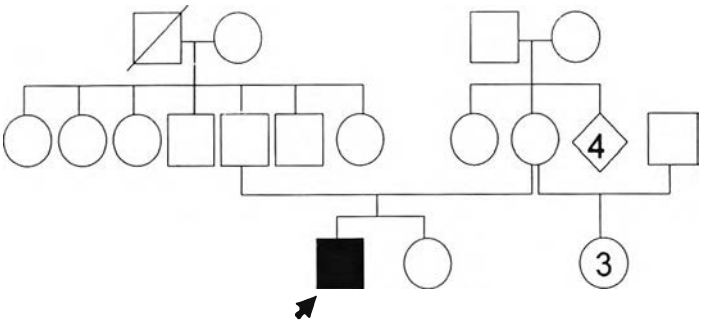

| | | | | (Normal range) |
|---|---|---|---|---|
| $FT_4$ | 1.37 | 1.5 | **4.52** 1.50 | (0.8-1.8) |
| $FT_3$ | 2.14 | 4.1 | **12.03** 2.70 | (1.6-4.0) |
| TSH | 1.24 | 3.1 | **1.41** 1.52 | (0.3-4.1) |

## Family 5



| | | | | (Normal range) |
|---|---|---|---|---|
| $FT_4$ | **5.30** | 1.14 | **6.67** | (0.8-1.8) |
| $FT_3$ | **7.26** | 2.12 | **7.60** | (1.6-4.0) |
| TSH | **2.40** | 1.85 | **3.01** | (0.3-4.1) |

## Family 6




| | | (Normal range) |
|---|---|---|
| $FT_4$ | **3.97** | (0.8-1.8) |
| $FT_3$ | **10.94** | (1.6-4.0) |
| TSH | **2.79** | (0.3-4.1) |

**Figure** 11 Pedigree and results of thyroid function tests in six families.

**2. Genetic analysis**

**2.1 DNA extraction**

After informed consent, genomic DNA was isolated from peripheral blood leukocytes. This procedure was performed as follows:

1. 3 ml. of whole blood were centrifuged for 10 minutes at 3,000 rpm.

2. Remove supernatant and transfer buffy coat to a new polypropylene tube. Then add 10 volumes of cold lysis buffer 1 (or 10 ml.), mix thoroughly and incubate at –20°C for 5 minutes.

3. Centrifuge for 8 minutes at 1,000 g, and remove supernatant.

4. Add 3 ml. of cold lysis buffer 1, mix thoroughly and centrifuge for 8 minutes at 1,000g,

5. Discard supernatant and add 900 µl of lysis buffer 2, 10µl of proteinase K solution (20 mg of proteinase K in 1.0 ml. of 1% SDS-2 mM EDTA), and 50µl of 10% SDS. Mix vigorously for 15 seconds.

6. Incubate the tube(s) in a 37°C shaking waterbath overnight for complete digestion.

7. Add 1 ml. of phenol-chloroform-isoamyl alcohol and shake vigorously for 15 seconds and centrifuge at 6,000 rpm for 5 minutes.

8. Transfer the supernatant from each tube (containing DNA) to a new microcentrifuge tube.

9. Add 0.5 volume of 7.5 M $CH_3COONH_4$ and 1 volume of 100% ethanol and mix by inversion. The DNA should immediately form a stringy precipitate. Recover the DNA by centrifugation at 14,000 rpm for 15 minutes. Then remove supernatant.

10. Rinse the pellet with 70% ethanol. Decant the ethanol and air-dry the pellet. (It is important to rinse well to remove any residual salt and phenol.)

11. Resuspend the DNA in 20-300 µl of the double distilled water at 37°C until dissolved.

### 2.2 DNA amplification

Genomic DNA was amplified by polymerase chain reaction (PCR) with the specific oligonucleotide primers for exon 1, exons 3-10 of the *TRβ* gene shown in figure 12. For details on the structure of the *TRβ* gene, see Frankton, S., *et al.* (2004) [11]. The PCR primer sets were designed by using the genomic DNA sequence of the *TRβ* gene from NCBI database. All primer sequences, PCR components and PCR condition were showed in table 3, table 4 and table 5 respectively.

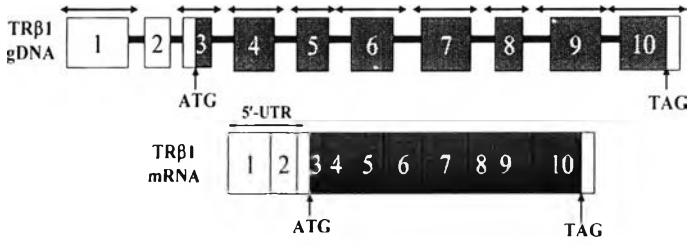



**Figure12** Strategy of sequencing the *TRβ1* coding areas and the flanking regions[11]. Exons are represented by the boxes (black indicates coding and white shows noncoding regions). The thick lines with double arrows indicate the sequenced areas.

**Table 3** Primer sequences and PCR products

| Exon | Primer sequences for PCR (5′ to 3′) | | Product size (bp) |
|---|---|---|---|
| | Forward | Reverse | |
| 1 | TGACATTTTGCAGGACTCGC | CAGGTAATCAGGCTGATGAC | 474 |
| 3 | GTCCCCAGTTCTGAGTTGAG | CGGTTGCTAAAACTCGCAAG | 298 |
| 4 | CCCGTCAAGTGGCATGTGAA | CATGGGACACCATACATTGG | 502 |
| 5 | GTTACCTCTTCCTGGCAAGC | CACCTGGATGATCACAGACC | 351 |
| 6 | TAGAGGCCTAGAAACAGGT | TTCAGGGCAAGCCTGGATGT | 443 |
| 7 | AATGGCAGTTTGCTGGTGAG | TCAGCCTCTCAGAGCTACGG | 480 |
| 8 | CATTGTCACTGAGGGCCTAT | CCCAGTATTCCTGGAAACTG | 425 |
| 9 | ATGGGCTCAAAGAATGCAGG | GGGGACTGAAAACTCAAGTG | 475 |
| 10 | GGCCTGGAATTGGACAAAGC | TCCATGTCTATGCCAAGCAG | 460 |

**Table** 4 Mixture of PCR reactions

| Components | Volume per reaction* (µl) | |
|---|---|---|
| | **Exon 1** | **Exons 3-10** |
| **1. 10X PCR buffer(+$KCl$,-$MgCl_2$)** | 2.0 (1X) | 2.0 (1X) |
| **2. 10% $DMSO_2$** | 2.0 (1%) | - |
| **3. 25 mM $MgCl_2$** | 1.2 (1.5 mM) | 1.5 (1.8 mM) |
| **4. 10 mM dNTP** | 0.4 (0.2 mM) | 0.4 (0.2 mM) |
| **5. 10 µM Forward primer** | 0.2 (0.1 µM) | 0.3 (0.15 µM) |
| **6. 10 µM Reverse primer** | 0.2 (0.1 µM) | 0.3 (0.15 µM) |
| **7. 5 U/µl Taq polymerase** | 0.1 (0.5 U) | 0.1 (0.5 U) |
| **8. Distilled water** | 14.9 | 11.3 |
| **9. 50 ng/µl of genomic DNA** | 1.0 (50 ng) | 2.0 (100 ng) |
| **Total volume (µl)** | 20 | 20 |

* Final concentration per reaction in each PCR reaction

**Table 5** PCR cycle and condition

| Step | Temperature and incubation time | |
|---|---|---|
| | **Exon 1** | **Exons 3-10** |
| **1. Initial denaturation** | 95 $^0$C / 5 min | 94 $^0$C / 3 min |
| **2. PCR cycle** | 30 cycles | 35 cycles |
| **Denature** | 95 $^0$C / 30 sec | 94 $^0$C / 30 sec |
| **Annealing** | 55 $^0$C / 30 sec | 60 $^0$C / 30 sec |
| **Extension** | 72 $^0$C / 30 sec | 72 $^0$C / 30 sec |
| **3. Final extension** | 72°C/ 7 min | 72 $^0$C / 7 min |

### 2.3 Agarose gel electrophoresis and DNA sequencing

The PCR products were verified for correct size on ethidium bromide-stained 1.5% agarose gel. The PCR products were then treated with ExoSAP-IT (USP Corporation, Cleveland, OH) according to the manufacturer's recommendations, and sent for direct sequencing at the Macrogen Inc., Seoul, Korea. The sequence was analyzed using Sequencher (version 4.2; Gene Codes Corporation, Ann Arbor, MI).

### 3. Functional analysis

This procedure was performed to investigate an effect of the mutation detected in patients with RTH. The luciferase reporter gene assay was used to determine the ligand-dependent transcriptional activity and dominant negative effect (DNE). The construction of plasmids, *in vitro* site-directed mutagenesis, and transient transfection procedures are described below.

#### 3.1 Construction of plasmids

##### 3.1.1 Construction of mammalian expression vectors

pcDNAI/Amp-TRβ1 was constructed by Refetoff's laboratory[42]. This expression construct containing the TRβ1 cDNA (1,463-bp fragment) was inserted between *BamH*I and *EcoR*I recognition sites of the pcDNAI/Amp (Invitrogen, San Diego, CA) under the transcriptional control of the T7 promoter (figure 13).

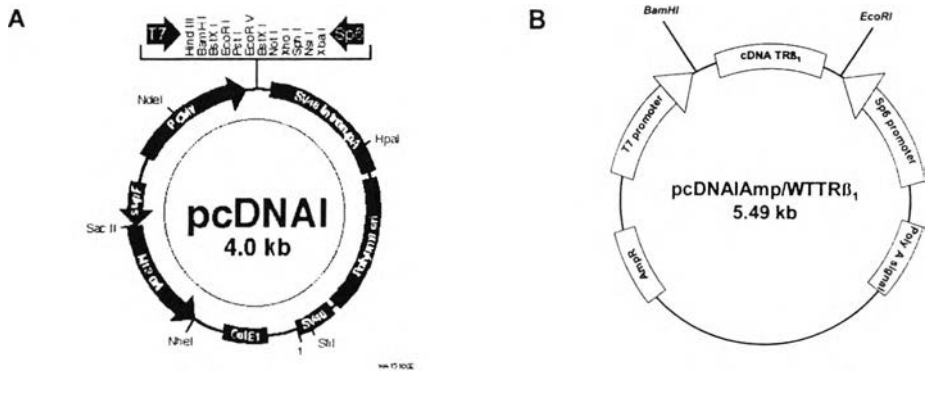



**Figure 13** pcDNAI/Amp-TRβ1 expression vector [42].
Panel A shows pcDNAI/Amp, a vector for pcDNAI/Amp-TRβ1 construction.
Panel B shows a construct of pcDNAI/Amp-TRβ1.

##### 3.1.2 Construction of reporter vectors

To investigate for transcriptional activity, the TREpalx3-Luc was used as a reporter vector. This vector was a kind gift from Refetoff's laboratory[42]. The TREpalx3-Luc was constructed by placing the respective TRE (AGGTCATGACCT) TK promoter elements, into the multiple cloning site of the pGL2-Basic Vector (Promega Corp.) upstream of the firefly luciferase (Luc) fusion gene. The TREpalx3 consists of three copies of two consensus TRE hexamer half motifs in palindromic arrangement spaced by 10 nucleotides (aagctttcAGGTCATGACCTga) between each TREpal repeat (Figure 14).

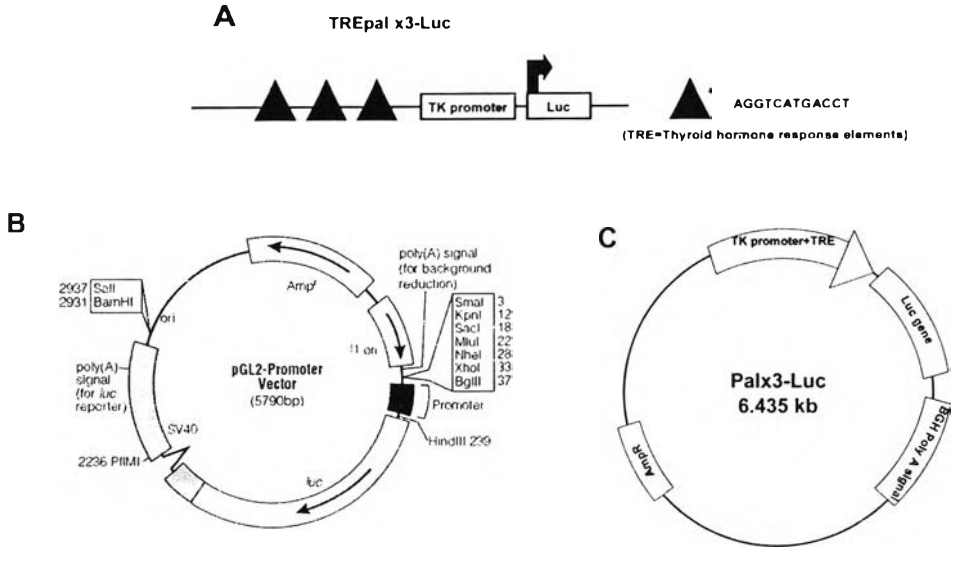



**Figure 14** The TREpal x3-Luc construct[42].

Panel A shows TRE orientation, TK promoter and Luc gene position on the TREpalx3-Luc construct.

Panel B shows the pGL2-Basic vector which was used for TREpalx3-Luc construction.
Panel C shows the TREpal x3-Luc. The bovine growth hormone polyadenylation (*bgh*-PolyA) signal is a specialized termination sequence for protein expression in eukaryotic cells.

### 3.1.3 Preparation of internal control and empty vector

The pRL-TK is a commercially available vector containing the native *Renilla* luciferase gene under the transcriptional control of the herpes simplex virus thymidine kinase (TK) promoter region. It was used as an internal control for determination of transfection efficiency. Normalization of the levels of transcription of the experimental reporter gene (TREpalx3-Luc) to the internal control reporter gene minimizes the variability of the obtained results caused by differences in the transfection efficiency between different samples of transfected cells(Figure 15A).

For a control using the empty vector, the pcDNA$^{TM}$ 3.1/myc-His B (Figure 15B) was used the pcDNA$^{TM}$ 3.1/myc-His B is a commercially available vector (Invitrogen).

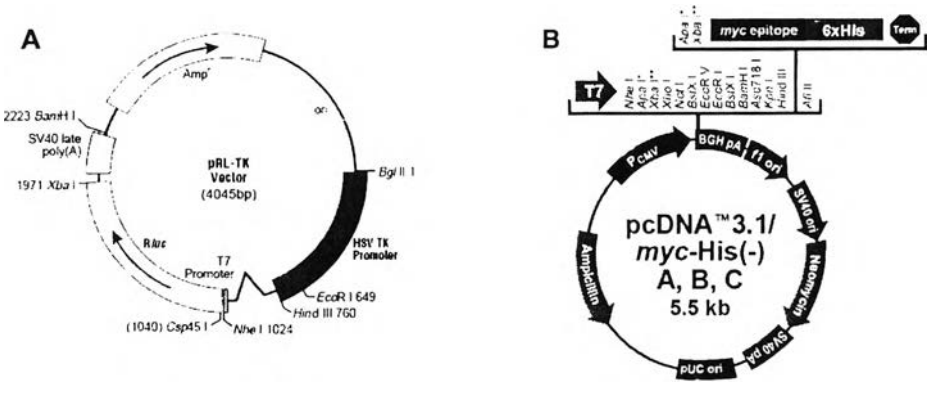



**Figure 15** Circle maps of the pRL-TK and the pcDNA™ 3.1/myc-His B.

Panel A shows the pRL-TK construct.

Panel B shows the pcDNA™ 3.1/myc-His B.

## 3.2 Amplification of expression vectors for transfection experiment

### 3.2.1 Preparation of competent cells

1. Grow bacteria (DH5α and XL-1 blue) from glycerol stock by streaking on the LB plate (without antibiotic) and incubate for 18 hours at 37°C.

2. Pick a single colony and grow them in 30 ml of LB broth without antibiotic (starter media) in a 100-ml flask. Incubate the culture for 18 hours at 37°C with shaking at 225 rpm.

3. Dilute the starter media at ratio 1:10 with fresh LB broth without antibiotic (original media). Incubate the culture for 90 minutes at 37°C with shaking at 225 rpm.

4. Transfer 10 ml of the culture to a pre-chilled sterile 15-ml centrifuge tube. Pellet the bacteria with a 4000 rpm spin for 10 minutes at 4°C. Discard supernatant and place the cell pellet on ice.

5. Resuspend cells in 10 ml of cold 0.1 M $CaCl_2$ solution. Pellet the bacteria with a 4000 rpm spin for 10 minutes at 4°C.

6. Discard supernatant and resuspend cells in 2 ml of cold 0.1 M $CaCl_2$ solution per original media and add 10% glycerol. Mix by slowly pipetting up and down and store cells at –80°C.

7. Test for cell competency by transformation with the control plasmid vector using heat shock (see 3.2.2).

### 3.2.2 Transformation

To make bacterial cells take up the plasmid/foreign DNA by using heat shock.

1. Take out competent (DH5α or XL-1 blue) cells from –80 $^0$C and thaw on ice for 5 minutes.

2. Add 5 µl of plasmid DNA into 50 µl of competent cells in a 1.5-ml microcentrifuge tube and gently stir with tip. Incubate for 30 min at –4 $^0$C.

3. Put tubes with DNA into heat block at 42 $^0$C for 45 seconds.

4. Put tubes back on ice for 2 minutes to reduce damage to the cells.

5. Add 1 ml of culture medium at 37 $^0$C containing SOC 96 µl, $Mg^{2+}$ 2 µl, and 10 M glucose 2 µl (without antibiotic added). Incubate tubes for 90 minutes at 37 $^0$C with shaking at 225 rpm.

6. Spread 20-50 µl of culture by the spreader on warmed LB plates (with 100mg/ µl of ampicillin). Grow them overnight at 37 $^0$C for 18 hours.

7. Pick a fresh single colony and place in 5 ml of LB broth (with 100mg/µl of ampicillin), and then incubate at 37 $^0$C with shaking at 225 rpm for 16 hours.

8. Extract and purify the plasmid DNA with mini prep.

### 3.2.3 Plasmid DNA extraction

1. Harvest bacteria from culture tubes into 1.5-ml microcentrifuge tubes. Centrifuge at 13,400 rpm for 3 minutes. Bacterial cells may be harvested in 15 ml tubes. Centrifuge at 5,400 x g for 10 minutes at 4 $^0$C.

2. Discard supernatant and add 250 µl of chilled complete Qiagen suspension solution (P1), vortex or pipette up and down until no cell clumps remain. Transfer suspension cells to a 1.5 ml-microcentrifuge tube.

3. Add 250 µl of Qiagen lysis solution (P2) and mix thoroughly by inverting 10 times and let stand for 2 minutes at room temperature or until the lysate solution is clear.

4. Add 350 µl of Qiagen neutralize solution (N3) and mix thoroughly by inverting 10 times. Centrifuge for 10 minutes at 13,400 rpm in a table-top microcentrifuge. A compact white pellet will form.

5. Transfer the supernatant from step 4 to spin column tubes and centrifuge for 1 minute. Discard the flow-through.

6. Add 500 µl of Qiagen wash buffer (PB) and centrifuge for 1 minute. Discard the flow-through.

7. Remove all residual buffer PB by adding 750 µl of Qiagen wash buffer (PE) and centrifuge for 1 minute. Discard the flow-through and centrifuge again for 1 minute.

8. Transfer spin columns to a new 1.5-ml microcentrifuge tube, add 30-50 µl of filtered $dH_2O$ or elution buffer (EB), and let stand for 5 minutes at room temperature. Centrifuge for 5 minutes at 13,400 rpm in a table-top microcentrifuge. Remove the column and store DNA at -20 $^0$C.

**3.2.4 DNA digestion**

1. Obtain a buffer that works for all of the enzymes being used in the digestion. Vortex well and keep on ice.

2. Set-up a master mix consisting the buffer (1, 2, 3, or 4) that works for all enzymes being used, filtered $dH_2O$, and the enzymes. The digestion reaction was summarized in table 4.

3. Incubate the reaction (see in table 4) in the 37°C water bath for at least one hour.

4. Check the cut sizes by electrophoresis of 20 µl of the digestion products on a 0.8% agarose gel.

**Table 6** Mixture of DNA digestion reactions for plasmid detection.

| Components | Volume per reaction* (µl) | | | |
|---|---|---|---|---|
| | pcDNAI/Amp-TRβ1 | Palx3-luc | Renilla-TK | pcDNA™ 3.1/myc-His B |
| 1.10X NEBuffer3 | 2 (1X) | 2 (1X) | 2 (1X) | 2 (1X) |
| 2.100X BSA | 0.2 (1X) | - | 0.2 (1X) | 0.2 (1X) |
| 3.Plasmids | 5 | 5 | 5 | 5 |
| 4.Distilled water | 10.8 | 12 | 10.8 | 11.8 |
| 5.20U/µl *BamH*I | 1 (20 U) | - | 1 (20U) | 1 (20U) |
| 6.20U/µl *EcoR*I | 1 (20 U) | - | - | - |
| 7.10U/µl *Bgl*II | - | 1 (10U) | 1 (10U) | - |
| Total volume (µl) | 20 | 20 | 20 | 20 |
| Incubation time | 1 hr. or O/N | O/N | O/N | 1 hr. or O/N |

*Final concentration per reaction in each DNA digestion reaction
Note: Keep the enzymes in the cold block and add last to the master mix.

### 3.2.5 DNA precipitation

1. Add a half volume of 10 M $NH_4OAc$ to 1 volume of the DNA sample.

2. Add an equal volume of chilled complete 100% EtOH to the DNA sample, add 5 µl of glycogen, and mix thoroughly by gently inverting.

3. Incubate at –20 °C overnight or at –80 °C for 1 hour.

4. Centrifuge at 14,000 rpm for 15 minutes at 4 $^0$C and remove the ethanol and salt.

5. Add 1 ml of chilled complete 70% EtOH, mix thoroughly by gently inverting, and centrifuge at 14,000 rpm for 5 minutes at 4 $^0$C.

6. Remove the ethanol with care and dry the pellet in 50 °C oven for 5 minutes or dry the pellet at room temperature overnight.

7. Resuspend the dried DNA in appropriate amount of sterile TE (pH 8.0), or water, and store at 4 °C for further manipulation or at –20 °C for long-term storage.

### 3.3 Mutant strand synthesis

Eight uncharacterized TR$\beta$1 mutants including the one identified in our patient were selected for functional analysis as shown in figure 16. The mutant constructs were generated by *in vitro* site-directed mutagenesis (Stratagene's QuickChange site directed mutagenesis kit) using the pcDNAI/Amp-WT TR$\beta$1 as a template.

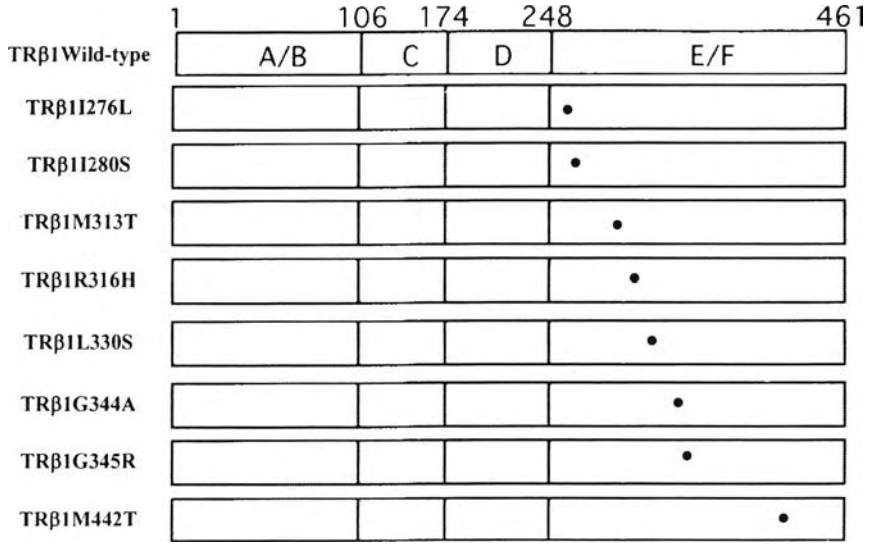



**Figure 16** Structures of the mutant TR$\beta$1 used in the study[43-49].
● Amino acid substitutions

#### 3.3.1 Mutant strand synthesis reaction (thermal cycling)

1. Synthesize two complimentary oligonucleotide primers containing the desired point mutation. The mutagenesis primer sets were designed by using *Stratagene's web-based QuikChange® Primer Design Program* available online at http://www.stratagene.com/qcprimerdesign. All mutagenesis primer sequences were shown in table 7.

**Table 7** Mutagenesis primer sequences for site directed mutagenesis by using PCR.

| Mutant Types | Mutagenesis primer sequences for PCR (5' to 3') |
|---|---|
| **1. I276L** | **I276L MF:**5'-ATTTTACAAAAATCCTCACACCAGCAATT-3'<br>**I276L MR:**5'-AATTGCTGGTGTGAGGATTTTTGTAAAAT-3' |
| **2. I280S** | **I280S MF:**5'-CATCACACCAGCAAGTACCAGAGTGGTGG-3'<br>**I280S MR:**5'-CCACCACTCTGGTACTTGCTGGTGTGATG-3' |
| **3. M313T** | **M313T MF:** 5'-CTGCATGGAGATCACGTCCCTTCGCGCTG-3'<br>**M313T MR:**5'-CAGCGCGAAGGGACGTGATCTCCATGCAG-3' |
| **4. R316H** | **R316H MF:**5'-GATCATGTCCCTTCACGCTGCTGTGCGCT-3'<br>**R316H MR:**5'-AGCGCACAGCAGCGTGAAGGGACATGATC-3' |
| **5. L330S** | **L330S MF:** 5'- TGAGACTTTAACCTCGAATGGGGAAATGG-3'<br>**L330S MR:** 5'- CCATTTCCCCATTCGAGGTTAAAGTCTCA -3' |
| **6. G344A** | **G344A MF:** 5'- CCAGCTGAAAAATGCGGGTCTTGGGGTGG-3'<br>**G344A MR:** 5'-CCACCCCAAGACCCGCATTTTTCAGCTGG-3' |
| **7. G345R** | **G345R MF:**5'-CAGCTGAAAAATGGGCGTCTTGGGGTGGTGT-3'<br>**G345R MR:**5'-ACACCACCCCAAGACGCCCATTTTTCAGCTG-3' |
| **8. M442T** | **M442T MF:** 5'-CCGCTTCCTGCACACGAAGGTGGAATGCC-3'<br>**M442T MR:** 5'-GGCATTCCACCTTCGTGTGCAGGAAGCGG-3' |

**2.** Prepare the sample reaction(s) as indicated in table 8.

**3.** Cycle each reaction using the cycling outlined in table 9.

**Table** 8 Mixture of PCR reactions for site directed mutagenesis.

| Components | Volume per reaction (µl) |
|---|---|
| **1. 10X PCR reaction buffer** | 5.0 (1X) |
| **2. 50 ng/µl pcDNAI/Amp-TRβ1** | 5.0 (250 ng) |
| **3. 125 ng Forward primer** | 1.25 |
| **4. 125 ng Reverse primer** | 1.25 |
| **5. dNTP mix** | 1 |
| **6. Distilled water** | 36.5 |
| **7. 5U/µl Pfu.Turbo polymerase** | 1 |
| **Total volume (µl)** | 51 |

*Final concentration per reaction in each PCR reaction

**Table 9** PCR cycle for site directed mutagenesis.

| Step | Temperature and incubation time |
|---|---|
| **1. Initial denaturation** | 95°C/ 30 sec |
| **2. PCR cycle** | 12 cycles |
| **Denature** | 95°C/ 30 sec |
| **Annealing** | 55°C/ 1 minute |
| **Extension** | 68°C/ 6 minutes |
| **3. Final extension** | 72°C/ 7 minutes |

### 3.3.2 *Dpn*I digestion of the amplification products

This step was performed for digestion of the nonmutated parental DNA template with *Dpn*I.

1. Add 1 µl of the *Dpn*I restriction enzyme (10 U/µl) directly to each amplification reaction.

2. Gently and thoroughly mix each reaction mixture by pipetting the solution up and down several times. Spin down the reaction mixtures in a microcentrifuge for 1 minute and immediately incubate each reaction at 37°C for 1 hour to digest the parental DNA.

### 3.3.3 Transformation of competent cells

This step was performed for repairing the nicks in the mutated plasmid and for colony selection.

1. Gently thaw the XL1-Blue (or DH5α) from -80$^0$C on ice for 5 minutes. For each sample reaction, aliquot 50 µl of the supercompetent cells to a prechilled 1.5-ml microcentrifuge round-bottom tube.

2. Transfer 5 µl of the *Dpn*I-treated DNA from each sample reaction to the 50-µl supercompetent cells and gently stir with tip. Incubate at –4 $^0$C for 30 minutes.

3. Put tubes with DNA into heat block at 42 $^0$C for 45 seconds.

4. Put tubes back on ice for 2 minutes to reduce damage to the cells.

5. Add 1 ml of medium at 37 $^0$C containing SOC 96 uL, $Mg^{2+}$ 2 µl, and 10M glucose (without antibiotic added). Incubate tubes for 90 minutes at 37 $^0$C with shaking at 225 rpm.

6. Plate the appropriate volume of each transformation reaction on agar plates containing the 100mg/μl of ampicillin for colony selection.

7. Incubate the transformation plates at 37°C for 18 hours.

8. Pick a single colony and grow the cells in 5 ml LB broth (with 100mg/ul of ampicillin), and then incubate at 37 $^{0}$C with shaking at 225 rpm for 16 hours.

9. Extract the plasmid DNA with Qiagen miniprep (See plasmid DNA extraction protocol at 3.3.3).

10. Digest the plasmid DNA with appropriate enzymes. For the pcDNAI/TRβ1Amp vector, digest the plasmid with *BamH*I and *EcoR*I and check for the correct size on ethidium bromide-stained 1.0 % agarose gel.

11. To check the products of *in vitro* site-directed mutagenesis for desired mutations, the plasmids were sent for direct sequencing.

### 3.4 Transfection assay

To test for an effect of mutations on receptor-mediated transcriptional activity (or $T_3$-dependent transcriptional activity) and dominant negative effect (DNE), co-transient transfection-based luciferase reporter gene assays were used as described below.

#### 3.4.1 $T_3$-dependent transcriptional activity

**Day 1: Plating cells**

This step was prepared for seeding cells in 12-well culture plates with DMEM containing 10%FBS (GIBCO) without antibiotics for 24 hours before transfection by Lipofectamine$^{TM}$ 2000 (Invitrogen). The number of cells for seeding were $10^5$ cells/well.

1. Inspect COS7 cells (simian virus 40-transformed African green monkey kidney fibroblasts) in the T-75 flask by using an inverted microscope to ensure that they were 80-100% confluent.

2. Remove the growth medium from T-75 flask, cells were washed with 10 ml of 1X phosphate buffer saline (PBS), and remove the rinse solution.

3. Add 2 ml of trypsin-EDTA and incubate cells in a 5% $CO_2$ incubator for 5 minutes.

4. Inhibit trypsinization with growth medium (DMEM+10% FCS) and subculture cells at ratio 1:3. Adjust solution volume to 10 ml of growth medium (DMEM+10% FCS).

5. Pipette up and down to mix cells, then transfer cells to a 15-ml centrifuge tube.

6. Using micropipette (size 1,000 µl) to mix cells again and sampling cells by pipetting 1 ml of suspension cells to a 1.5-ml microcentrifuge tube. Stain cells 50 µl with trypan blue 450 µl (Ratio cells:dye = 1:10).

7. Count cells using the hemocytometer by transferring cell solution into a counting chamber.

8. Calculate the number of cells by using formula as described below:

N (cell number per ml) = the average count per square x dilution factor x $10^4$

9. Seed cells with the number calculated in DMEM containing 10% FBS (Hyclone) and incubate cells for 24 h in the $CO_2$ incubator.

**Note:** In this step, don't add antibiotics to growth medium as this causes cell death.

**Day 2: Transfection**

1. Prepare DNA for transfection

| | | |
|---|---|---|
| pcDNAIAmp/TRβ1 (wild-type or mutants ) | 30 | ng/well |
| TREpal x3-Luc | 1 | µg/well |
| pRL-TK | 0.1 | µg/well |
| pcDNA$^{TM}$ 3.1/myc-His B | 0.47 | µg/ well |

2. Dilute the plasmid DNA into 100 µl of Opti-MEM I reduced serum Medium without serum (GIBCO; Invitrogen). See table 10

**Table 10** Mixture of transfection reaction for $T_3$-dependent transcriptional activity

| Constructs | Final concentration | Volume per reaction (µl) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Negative control | | Wild-type | | Mutants | |
| | | No $10^{-7}$ M $T_3$ | $10^{-7}$ M $T_3$ | No $10^{-7}$ M $T_3$ | $10^{-7}$ M $T_3$ | No $10^{-7}$ M $T_3$ | $10^{-7}$ M $T_3$ |
| pcDNAI/Amp-wt TRβ1 | 0.03µg/ µl | - | - | 1 µl | 1 µl | - | - |
| pcDNAI/Amp-mt TRβ1 | 0.03µg/ µl | - | - | - | - | 1 µl | 1 µl |
| TREpalx3 luc | 1µg/ µl | 1 µl | 1 µl | 1 µl | 1 µl | 1 µl | 1 µl |
| pRL-TK | 0.1µg/ µl | 1 µl | 1 µl | 1 µl | 1 µl | 1 µl | 1 µl |
| pcDNA3.1Ver.B | 0.47µg/ µl | 1 µl | 1 µl | 1 µl | 1 µl | 1 µl | 1 µl |
| Opti-MEMI | - | 97 µl | 97 µl | 96 µl | 96 µl | 96 µl | 96 µl |
| Total Volume | - | 100 µl | 100 µl | 100 µl | 100 µl | 100 µl | 100 µl |

3. Mix Lipofectamine$^{TM}$2000 gently before use, then dilute 4.0 µl of Lipofectamine$^{TM}$ 2000 in 100 µl of Opti-MEM I reduced serum medium. Incubate for 5 minutes at room temperature (table 11).

**Table 11** Dilute LF$^{TM}$ 2000 in OptimemI-Reduced Serum Medium without Serum for $T_3$-dependent transcriptional activity

| Components | Volume |
|---|---|
| **LF$^{TM}$ 2000 reagent** | 4 µl |
| **Opti-MEMI** | 96 µl |
| **Total Volume** | 100 µl |

4. Combine the diluted DNA (from step 2) with the diluted LF $^{TM}$2000 reagent (from step 3) (total volume = 200 µl). Incubate at room temperature for 20 minutes to allow DNA- LF $^{TM}$2000 reagent complexes to form.

**Note:** Complexes are stable for 6 hours at room temperature.

5. Remove the growth medium from the cells and add 1 ml of DMEM (without serum) and 200 µl of DNA-LF$^{TM}$ 2000 complexes to each well. Add the DNA- LF$^{TM}$ 2000 reagent complexes (200 µl) directly to each well (total volume = 1.2 ml/well) and mix gently by rocking the plate back and forth.

6. Incubate the cells at 37°C in a $CO_2$ incubator for 5 hours. Remove the transfection solution and replace with 1 ml of DMEM containing **10% charcoal-stripped FBS**[50] (Hyclone), 1% PenStrep, and incubate for 24 hours in a $CO_2$ incubator.

**Day 3-5: Cells were treated with $10^{-7}$ M $T_3$ and harvest cell**

1. Cells were treated with $10^{-7}$ M $T_3$ or without $10^{-7}$ M $T_3$ (Sigma) for an additional incubation for 48 hours.

2. On day 4, cells were harvested and lysed to assay for Firefly and Renilla-TK luciferase activities by using the standard protocol of Promega dual-luciferase reporter assay system. The cell lysate procedure was described as follows:

1. Remove the growth medium from the 12-culture well plates, and wash with 1 ml per well of phosphate buffer saline (PBS). Completely remove the rinse solution before applying the passive lysis buffer (PLB) reagent.
2. Add 250 µl of 1X PLB to each well.
3. Place plates on the orbital shaker and gently shake for 15 minutes at room temperature.
4. Inspect cells to ensure that they are lysed and freeze the samples overnight at -80 °C.

**Day 6: Detection of dual luciferase activities by using luminometer.**

1. Thaw the frozen lysate (from day 4) on ice.

2. Transfer the lysate to a labeled 1.5-ml microcentrifuge tube and vortex for 15 seconds.

3. Centrifuge for 30 seconds at 4 °C and transfer supernatant into a new labeled 1.5-ml microcentrifuge tube.

4. Aliquot 20 µl of cell lysate and mix with 100 µl of LARII reagent in 1.5-ml microcentrifuge tube and measure the activity by GloMax 20/20 Luminometer with Single Auto-Injector. The number represents the firefly luciferase activity.

5. Add 100 µl of Stop and Glo reagent and read the luciferase activity again. This number represents the renilla luciferase activity.

6. Record all readings.

7. Data analysis (using excel on the lab bench computers)

a. Calculate the ratio of firely to renilla luciferase activities

b. Relative luciferase activity was calculated and shown as fold induction relative to the luciferase activity of the control vector pcDNA™ 3.1/myc-His B. All experiments were performed in triplicate and repeated two times. The results were reported as fold induction ± SD. Statistical analyses were performed using ANOVA.

**3.4.2 Dominant negative activity**

**Day 1: Plating cells**

This step was prepared for seeding cells in 12-well culture plates with DMEM containing 10% FBS (GIBCO) without antibiotics for 24 hours before transfection by Lipofectamine™ 2000 (Invitrogen). The cell number for seeding was $10^5$ cells/well.

1. Inspect COS7cells (simian virus 40-transformed African green monkey kidney fibroblasts) in the T-75 flask by using an inverted microscope to ensure that they were 80-100% confluent.

2. Remove the growth medium from T-75 flask, cells were washed with 10 ml of 1X phosphate buffer saline (PBS), and remove the rinse solution.

3. Add 2 ml of trypsin-EDTA and incubate cells in a 5% $CO_2$ incubator for 5 minutes.

4. Inhibit trypsinization with growth medium (DMEM+10%FCS) and subculture cells at ratio 1:3. Adjust solution volume to 10 ml of growth medium (DMEM+10% FCS).

5. Pipette up and down to mix cells, then transfer cells to a 15-ml centrifuge tube.

6. Using micropipette (size 1,000 µl) to mix cells again and sampling cells by pipetting 1 ml of suspension cells to a 1.5-ml microcentrifuge tube. Stain cells 50 µl with trypan blue 450 µl (Ratio cells:dye = 1:10).

7. Count cells using the hemocytometer by transferring cell solution into a counting chamber.

8. Calculate the number of cells by using formula as described below:

N (cell number per ml) = the average count per square x dilution factor x $10^4$

9. Seed cells with the number calculated in DMEM containing 10% FBS (Hyclone) and incubate cells for 24 h in the $CO_2$ incubator.

**Note:** In this step, do not add antibiotics to growth medium as this causes cell death.

**Day 2: Transfection**

1. Dilute DNA into 100 µl of Opti-MEM I reduced serum medium **without serum** (GIBCO; Invitrogen). See table 12.

**Table 12** Mixture of transfection reaction for dominant negative activity

| Constructs | Final Concentration | Volume per reaction (µl) | | | |
|---|---|---|---|---|---|
| | | Negative Control | WT:WT (Normal control) 1:1 | WT: MT | |
| | | | | 1:1 | 1:4 |
| pcDNAI/Amp-wt TRβ1 | 0.03µg/ µl | - | 2 µl | 1 µl | 1 µl |
| pcDNAI/Amp-mt TRβ1 | 0.03µg/ µl | - | - | 1 µl | 4 µl |
| TREpalx3luc | 1µg/ µl | 1 µl | 1 µl | 1 µl | 1 µl |
| pRL-TK | 0.1µg/ µl | 1 µl | 1 µl | 1 µl | 1 µl |
| pcDNA3.1Ver.B | 0.47µg/ µl | 1 µl | - | - | - |
| pcDNA3.1Ver.B(1:1) | 0.44µg/ µl | - | 1 µl | 1 µl | - |
| pcDNA3.1Ver.B(1:4) | 0.35µg/ µl | - | - | - | 1 µl |
| Opti-MEMI | - | 97 µl | 95 µl | 95 µl | 92 µl |
| Total volume | - | 100 µl | 100 µl | 100 µl | 100 µl |

2. Mix Lipofectamine$^{TM}$2000 gently before use, then dilute 4.0 µl of LF $^{TM}$ 2000 in 100 µl of Opti-MEM I reduced serum medium. Incubate for 5 minutes at room temperature (table 13).

**Table 13** Dilute LF$^{TM}$ 2000 in OptimemI-Reduced serum medium without serum for dominant negative activity testing

| Components | Volume |
|---|---|
| **LF$^{TM}$ 2000 reagent** | 4 µl |
| **Opti-MEM I** | 96 µl |
| **Total volume** | 100 µl |

3. Combine the diluted DNA (from step 1) with the diluted LF ™ 2000 reagent (from step 2) (total volume = 200 µl). Incubate at room temperature for 20 minutes to allow DNA- LF ™ 2000 reagent complexes to form.

**Note:** Complexes are stable for 6 hours at room temperature.

4. Remove the growth medium from the cell cultures and add 1 ml of DMEM (without serum) and 200 µl of DNA- LF ™ 2000 complexes to each well. Add the DNA- LF ™ 2000 reagent complexes (200 µl) directly to each well (total volume = 1.2 ml/well) and mix gently by rocking the plate back and forth.

5. Incubate the cell cultures at 37°C in a $CO_2$ incubator for 5 hours. Remove the transfection solution and replace with 1 ml of DMEM containing **10% charcoal-stripped FBS**[50] (Hyclone), 1% PenStrep, and incubate for 24 hours in a $CO_2$ incubator.

**Day 3-5: Cells were treated with $10^{-7}$ M $T_3$ and harvest cells**

1. Cells were treated with $10^{-7}$ M $T_3$ (Sigma) for an additional incubation for 48 hours.

2. On day 4, cells were harvested and lysed. The Firefly and Renilla-TK luciferase activities were measured by a standard protocol of Promega dual-luciferase reporter assay system. To make cell lysate, the passive lysis buffer (PLB) was used as follows:

1. Remove the growth medium from the 12-culture well plates, and wash with 1 ml per well of phosphate buffer saline (PBS). Completely remove the rinse solution before applying the PLB reagent.
2. Add 250 µl of 1X PLB to each well.
3. Place the plates on the orbital shaker and gently shake for 15 minutes at room temperature.
4. Inspect cells to ensure that they are lysed and freeze cell lysate overnight at -80 °C.

**Day 6: Detection of dual luciferase activities by using luminometer**

1. Thaw the frozen lysate (from day 4) on ice .

2. Transfer the lysate to a labeled 1.5-ml microcentrifuge tube and vortex for 15 seconds.

3. Centrifuge for 30 seconds at 4 °C and transfer supernatant into a new labeled 1.5-ml microcentrifuge tube.

4. Aliquot 20 µl of cell lysate and mix with 100 µl of LARII reagent in 1.5-ml microcentrifuge tube and measure the activity by GloMax 20/20 Luminometer with Single Auto-Injector. The number represents the firefly luciferase activity.

5. Add 100 µl of Stop and Glo reagent and read the luciferase activity again. This number represents the renilla luciferase activity.

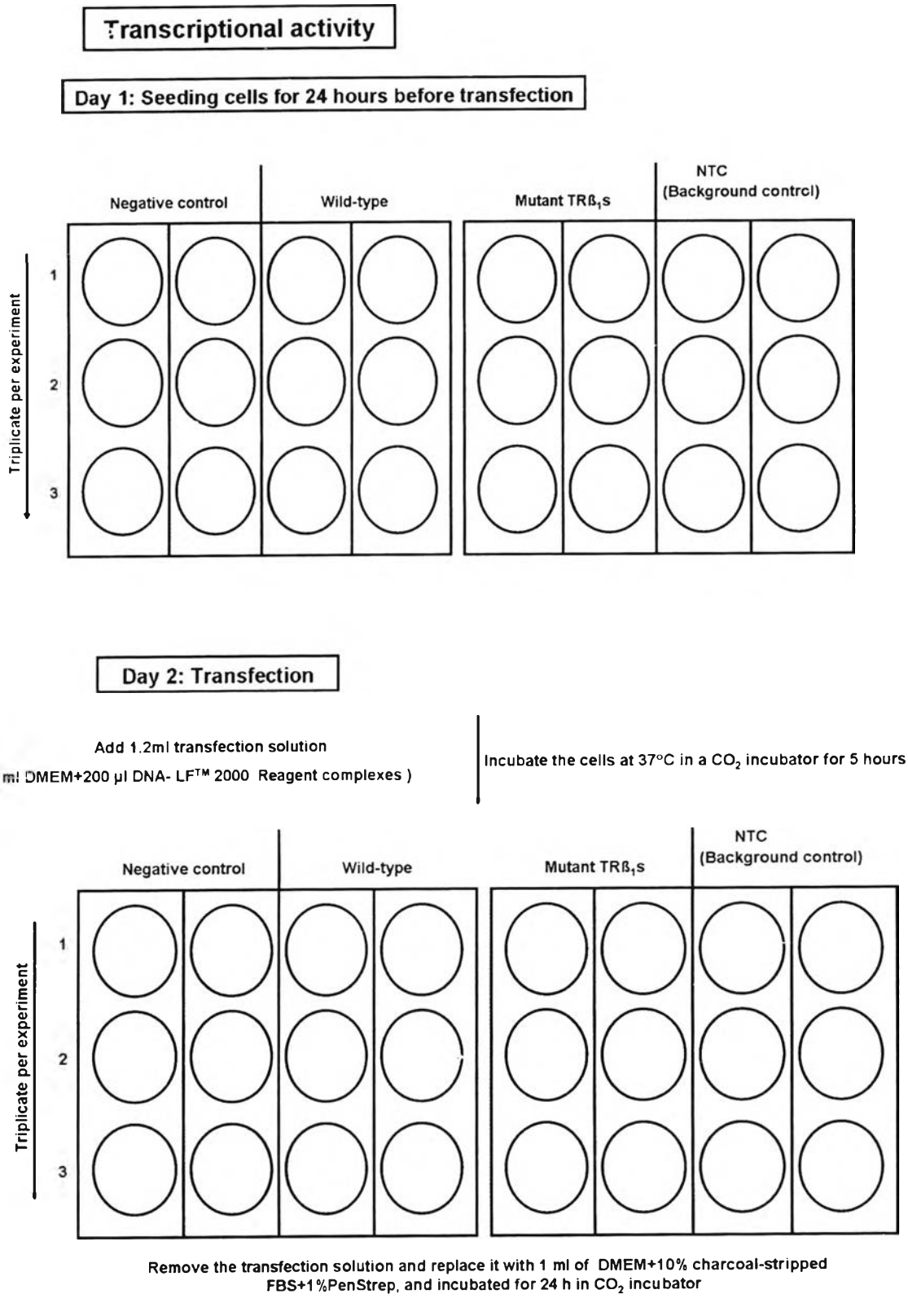
6. Record all readings.

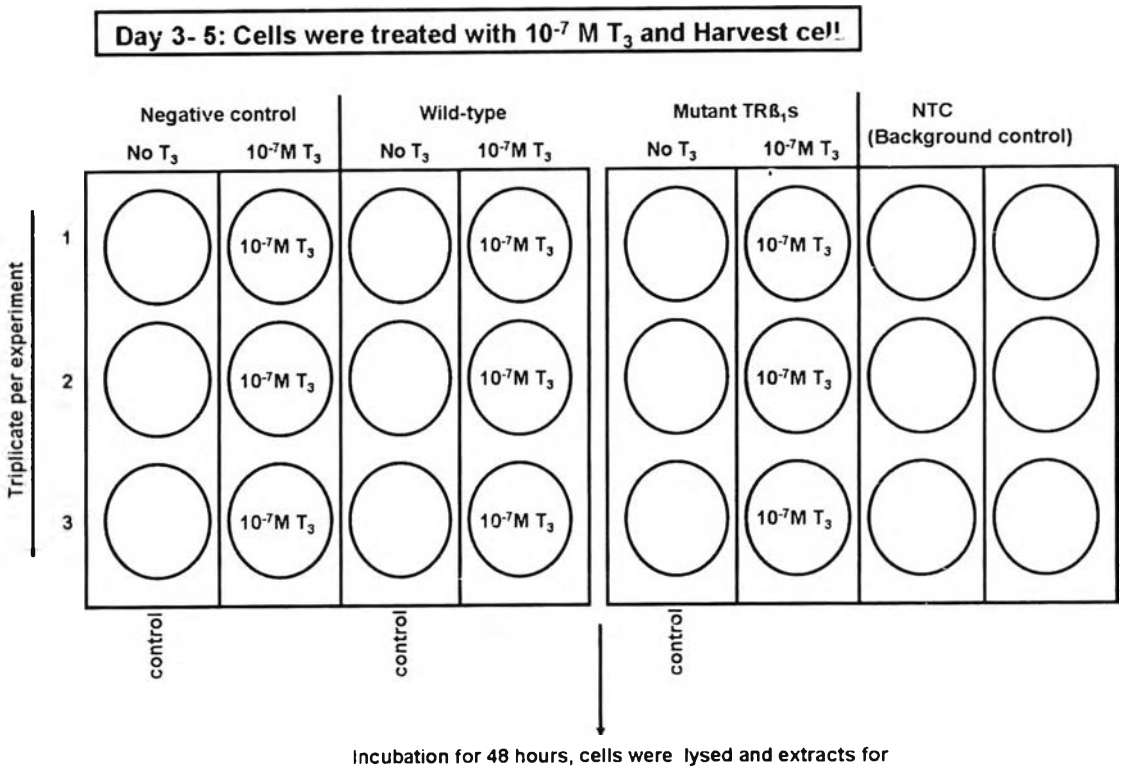
7. Data analysis (using excel on the lab bench computers)

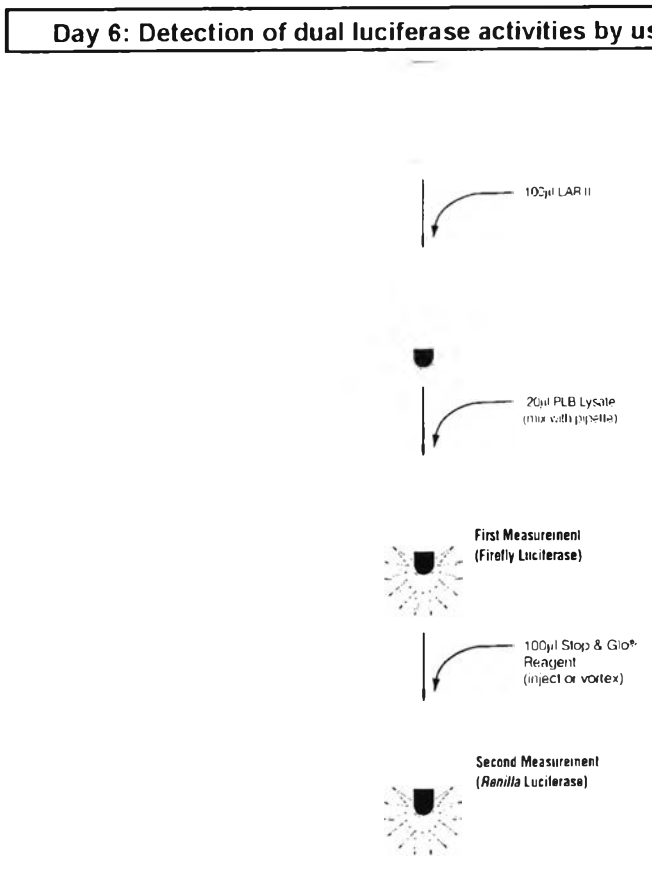
a. Calculate the ratio of firely to renilla luciferase activities

b. Relative luciferase activity was calculated and shown as fold induction relative to the luciferase activity of the control vector pcDNA$^{TM}$ 3.1/myc-His B. All experiments were performed in triplicate and repeated two times. The results were reported as fold induction ± SD. Statistical analyses were performed using ANOVA.

## Overall diagram for transient co-transfection studies

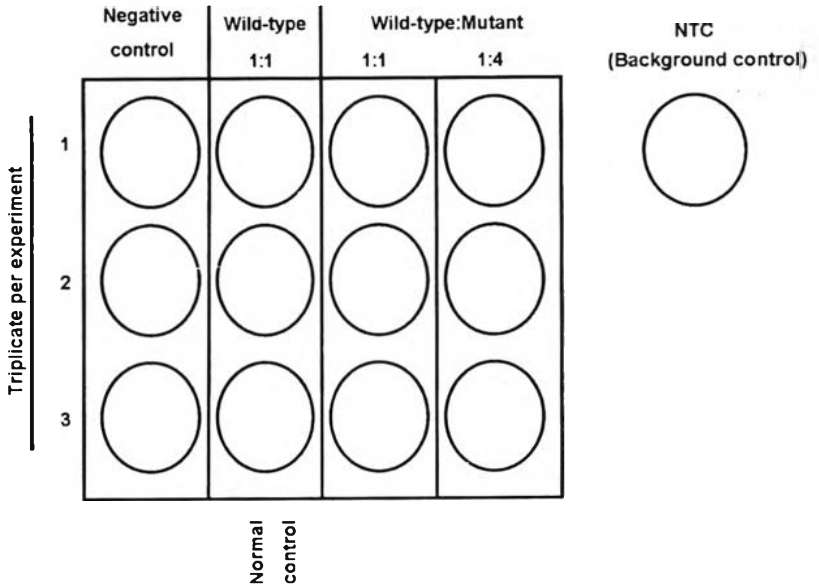



Day 3- 5: Cells were treated with 10-7 M T3 and Harvest cell
Negative control
Wild-type
Mutant TRß1s
NTC
(Background control)
No T3
10-7M T3
Triplicate per experiment
1
2
3
control
Incubation for 48 hours, cells were lysed and extracts for
assayed Firefly and Renilla-TK luciferase activities


Day 6: Detection of dual luciferase activities by using luminometer
100µl LAR II
20µl PLB Lysate
First Measurement
(Firefly Luciferase)
100µl Stop & Glo®
Reagent
(inject or vortex)
Second Measurement
(Renilla Luciferase)

## Dominant negative effect

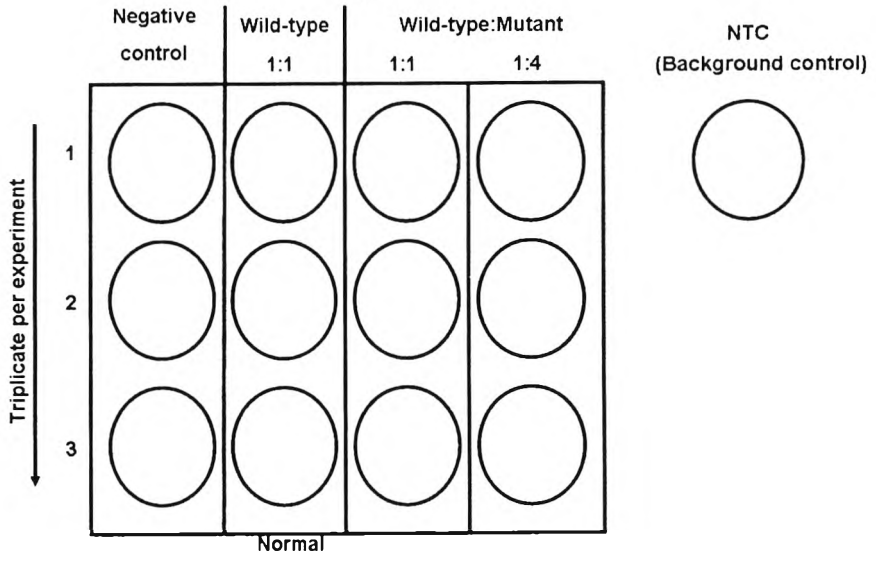
### Day 1: Seeding cells for 24 hours before transfection




### Day 2: Transfection

Add 1.2ml transfection solution (1ml DMEM+200 µl DNA- LF™ 2000 Reagent complexes )

Incubate the cells at 37°C in a $CO_2$ incubator for 5 hours




Remove the transfection solution and replace it with 1 ml of DMEM+10% charcoal-stripped FBS+1%PenStrep, and incubated for 24 h in $CO_2$ incubator

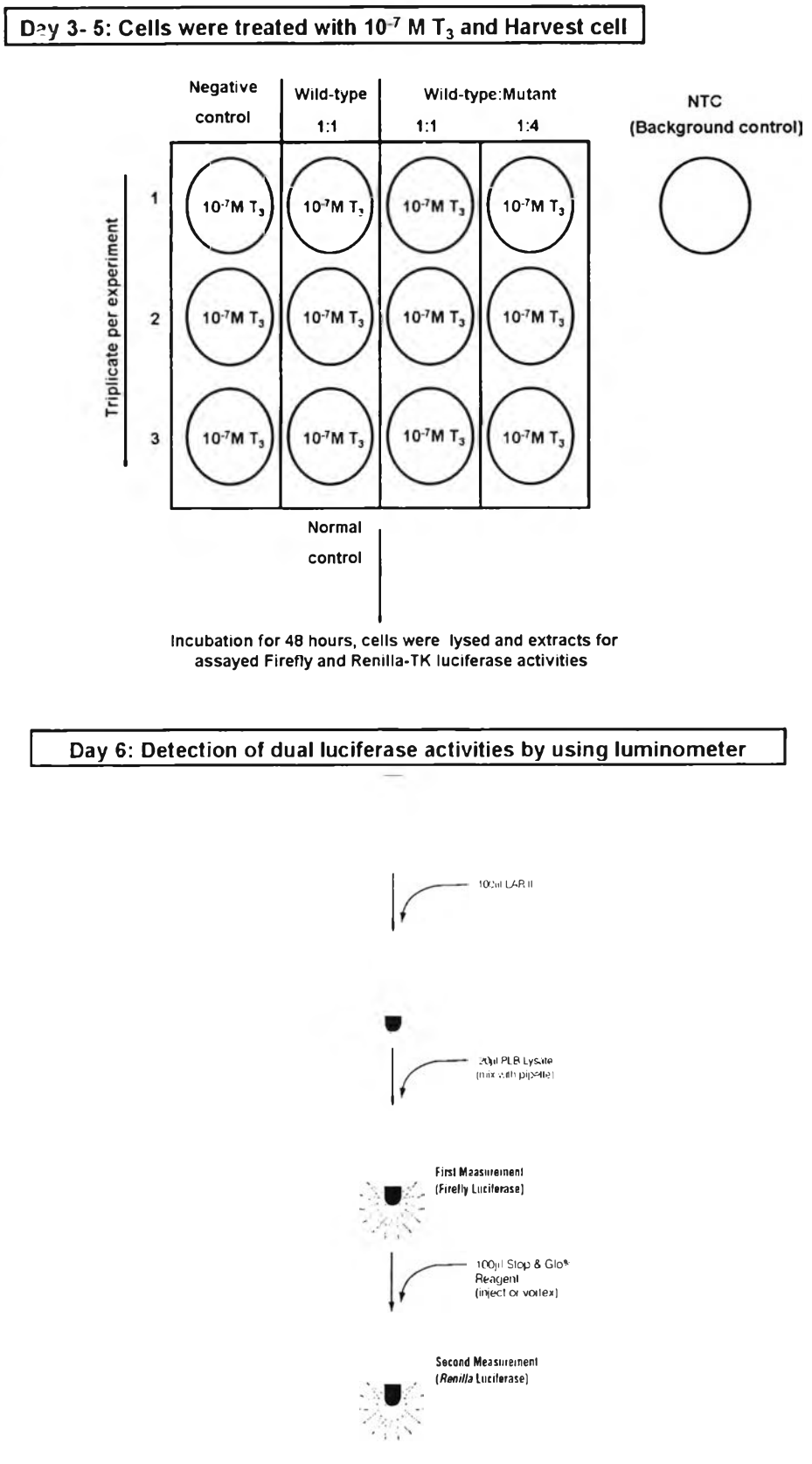



**Figure 17** Schematic diagram for measuring the transcriptional activity and dominant negative effect in COS-7 cells using luciferase reporter assays.