Protocol for RT-PCR

Isolation of total RNA

1) Aspirate the growth media. Add 1ml of Isogen to each dish. Scrape the cell and transfer Isogen to a new 1.5ml tube. Incubate for 5min at RT.

2) Add 200μl of chloroform → Shake vigorously for 15sec. → Store for 2~3min at RT.

3) Centrifuge at 15000rpm for 15min at 4C.

4) Transfer aqueous phase to a new 1.5ml tube. Add 500μl of isopropanol. Store for 5~10min at RT.

5) Centrifuge at 15000rpm for 10min at 4C. Remove the supernatant.

6) Add 1ml of 70% EtOH. Vortex briefly.

7) Centrifuge at 15000rpm for 5min at 4C. Remove the supernatant.

8) Dry the pellet briefly and resolve the pellet with 30μl of DEPC H_2O.

9) Measure the concentration of RNA using Spectrophotometer. Make 0.25μg/μl solution with DEPC H_2O. (* DEPC : Diethyl polycarbonate)

10) Store at -20C

Reverse transcription and PCR

A) Digestion of DNA

1) RNA (0.25μg/μl in TE) 8μl(=2μg)
10x DNase I Reaction Buffer 1μl
DNase I 1μl
incubate for 15min at RT.

2) Add 1μl of 25mM EDTA and incubate for 10min at 65C to inactivate DNase.

B) Reverse Transcription

Denature

1) Add the following reagents and vortex.

Oligo(dT) primer	1μl
10mM dNTP	1μl

2) Incubate for 5min at 65C. → Put on ice for 1min.

Amnneal

3) Add cDNA synthesis mixture and vortex.

[cDNA synthesis mixture]

5x First Strand Buffer	4μl
0.1M DTT	1μl
RNase OUT	1μl
RT(Superscript III reverse transcriptase	1μl

DNA synthesis **Terminate reaction**

4) 50 C 60 min → 70 C 15 min

5) Add 1μl of RNaseH → 37C 20 min (to **remove RNA**.)

c) RT- PCR

1) Mix 3μl of ssDNA and 12μl of D.W. (5x dilution).

2) Make the PCR reaction mixture in the PCR tube

l

template	1
10x buffer	2.5
MgCl2(25mM)	2
dNTPs	1
Taq polymerase	0.2
Forward Primer (50μM)	0.2 μl
Reverse Primer (50μM)	0.2 μl
D.W.	17.9 μl

Total 25 µl

7) Set the PCR program

Stage 1: resp=1, 94 C 5min

Stage 2: resp=25,

step1 **(Denature)** 94C 30sec,

step2**(Anneal)** 55C 30sec ,

Step3 (**Extension**)72C 30sec

Stage 3 : resp=1, 72 C 5 min

stage 4 : reso=1 , 20 C 1∞

8) Start the reaction

9) Prepare TAE gel

(TAE buffer (40mM Tiris-acetate , 1mM EDTA , pH 8.3) + 1.5% Agarose**)**

Apply samples to the gel

Electrophoresis at 100 V for 15 – 20 min.