(Analysis of mRNA(cDNA))

〇RNase free Water

0.1%DEPC water:

DDW 1000ml

DEPC 1ml

1. Weigh 1ℓ of MilliQ using a graduated cylinder.

2. Remove 1ml of MilliQ using auto pipette.

3. Pour 1ml of DEPC in laminar hood and close the lid with parafilm and mix it.

4. Incubate it for 24 hours in laminar hood. Vaporize DEPC if something was attached with DEPC in laminar hood.

5.Loose the lid, and wrap the lid with aluminum foil.

6.Autoclave it for 20 min at 121℃.

7.Store it at RT.

8.Wash an inside and a basket of autoclave with RO water and scrub brush.

9.Exchange the water of autoclave.

Diethylpyrocarbonate; DEPC：volatile, aromaticity



RNA extraction from adhesive cell:

Materials：

cells

TRLZOL

chloroform

isopropyl alcohol

75％Ethanol

RNA-free water

Autoclaved tip, eppendrof tube

Phenol chloroform mixture：avoid light. Be stored at 4℃.

Phenol /0.1mMTris-HCl(pH8.0)(lower layer) 22cc

chloroform　　　　　　　　　　 　　 22cc

Upper layer of phenol/0.1mMTris-HCL(pH8.0)　1cc

Because these solution are dripping, be careful of it.

Centrifuge it at 3000rpm, for 10min.

Aqua layer and organic layer become transparent.

75％ethanol：

99.5%Ethanol　 37.5cc

RNA-free water　　　　　　　　12.5cc

1. Wipe the desk with 70% Ethanol
2. Aspirate the medium of the cells in 10cmdish(55cm2), Add 8ml of TRIZOL Reagent 8ml and passing the cell lysate several times through a pipette to lyse the cells.

※The amount of TRIZOL Reagent added is based on the area of the culture dish (1 ml per 10 cm2 and not on the number of cells present. An insufficient amount of TRIZOL Reagent may result in contamination of the isolated RNA with DNA.

1. Incubate the homo for 5min at RT.
2. Transfer it into 15ml tube, divide it into 8 1.5ml eppendrof tube each 1ml. Store it at －80℃.
3. Add 0.2ml of chloroform per aml of TRIZOL, and vortex it for 15 sec.
4. Incubate it for 3 min at RT.
5. Centrifuge it at 3500rpm(12000×g), 4℃ for 30min.
6. Following centrifugation, the mixture　separates into a lower red, phenol-chloroform phase(DNA, protein), an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase.
7. Transfer the aqueous phase to a fresh tube.



1. Add 0.2ml of phenol-choloroform.
2. Centrifuge it for 30min at 3500rpm, 4℃.
3. Transfer the aqueous phase to a fresh tube.
4. Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol and 16.7μg/μl glycogen. Use 0.5 mL of isopropyl alcohol and 1μl of 16.7μg/μl glycoge of per 1 mL of TRIZOL Reagent used for the intial homogenization.
5. Place them at −20℃, for more than 10 min.
6. Centrifuge it at 3500rpm, 4℃ for 30 min.
7. Remove the supernatant. Wash the RNA pellet once with 1ml of 75% ethanol/RNaseFree-water.
8. Mix the sample by vortexing.
9. Centrifuge at 3500rpm(no more than 7,500 x g) for 30 min at 4°C.
10. Remove the supernatant, air-dry for 5 minutes. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility.
11. Add 10μl of RNA-free water.
12. Store it at −80℃.

Estimated the amount of RNA:

Added 19μl of DEPC-water to 1μl RNA solution

Measure absorbance.

Prepared mixture by adding following component.

1μl of RNA sample.

5μl of Fromamide

3μl of 37% Fromaldehyde

0.5μl of 20×MOPS Buffer.

Incubated the loading sample for 15 min at 65℃.

Added 1μl of 1mg/ml etidium bromide.

Gel electrophoresis.

First-Strand cDNA synthesis:

totalRNA:1pg〜5μg

polyA＋RNA:1pg〜500ng

Example:

RNA sample(adjust it to 3μg) S(1192.0ng/μl):2.5μl＋DEPC water8.5μl

 　 　　　　　 H(969.2ng/μl) :3.1μl＋DEPC water7.9μl

10μM Oligo(dT) 　1μl

10mM dNTP Mix 　1μl

Incubate for 5 min at 65℃ and place them on ice for 1 min.

Mix with following components.

4μl of 5× First-Strand Buffer

1μl of 0.1M DTT

1μl of 40 U/μl RNaseOUT

1μl of 200 U/μl SuperScript Ⅲ Reverse Transcriptase

Incubate for 1hour at 55℃.

Inactivate reaction by incubating for 10min at 70℃.

Store at -20℃.

Preparation of reagents and materials for RNA experiment:

1)0.1%DEPC-DW

1. Add 1ml of DEPC to 999ml of DW.
2. Incubate overnight at RT to complete dissolving.
3. Autoclave

2)20×MOPS Buffer

1. Dissolve following materials in 800 ml of 0.1% DEPC-DW.

83.7g of MOPS(MW209.3)

13.61g of Sodium acetate trihydrate

7.45g of EDTA・2Na dehydrate(MW 372.24)

Put stirrer into 1ℓ beaker. Pour 1ℓ of 0.1% DEPC-DW weighing by measuring cylinder into the beaker　to １ℓ　line 1ℓ, taking care of meniscus. Remove 200ml of DEPC-DW. Weigh and add the reagents. Mix it on a stirrer.

1. Add 15ml of 10N NaOH to adjust pH to 7.0 and drop it with pipette. Check for pH of the buffer using a pH paper.
2. Volume up to 1 liter with 0.1% DEPC-DW using an auto pipetor.
3. Autoclave or filtrate the buffer through 0.2μm filter.
4. Wrap the bottle with Aluminum foil to block out light.



MOPS：

EDTA

Chelate complex salt

Binding to metallic ion by ligand containing multiple coordination position (multidentate ligand)

3)Alkaline Agarose gel

1. Dissolve 0.9g of agarose powder in 77.1ml of DW.
2. Measure the weight.
3. Heat the agarose slurry by microwave oven.
4. After complete dissolving of agarose, refill the water evaporated.
5. Cool the agarose solution in 55℃ water bath for more than 20 min.※ Wrap it with Sran wrap and make a hole on it.
6. Add 5ml of 20×MOPS buffer and 17.9ml of 37% formaldehyde.
7. Pour the solution to chamber, and put it for around 40 min.
8. Store it at 4℃ in 1× MOPS buffer.

4)Preparation Gel Loading Sample

1. Prepare mixture by adding following components.

1μl of RNA sample

5μl of Formamide

3μl of 37% Formaldehyde

0.5μl of 20×MOPS Buffer

1. Incubate the loading sample for 15min at 65℃.
2. Add 1μl of 1mg/ml etidium bromide.(dilute 5mg/ml EtBr using DEPC water at the rate of 1:5.)
3. Gel electrophoresis.

1×MOPS buffer 100V 20min.

You must be able to detect 28S, 18S and 5S of RNA bands.

PCR reaction:

Materials：

siliconized 1.5μl tube

96well plate

TE (for primer dilution ,serial dilution)

Methods：

Serial dilution:

S:145ng/μl

|  |  |  |  |
| --- | --- | --- | --- |
|  | 1/10(15ng/μl) | 1/3(5ng/μl) | 1/3(1.66ng/μl) |
| cDNA | 1(μl) | 3(1/10) | 2(1/30) |
| TE | 9 | 6 | 4 |
| Remains | 7 | 7 | 6 |

※Use the same tip to absorb DNA in an inner face.

H:150ng/μl

|  |  |  |  |
| --- | --- | --- | --- |
|  | 1/12(15ng/μl) | 1/3(5ng/μl) | 1/3(1.66ng/μl) |
| cDNA | 1(μl) | 3(1/10) | 2(1/30) |
| TE | 11 | 6 | 4 |
| Remains | 9 | 7 | 6 |

|  |  |  |
| --- | --- | --- |
|  | ×1 | ×7 |
| 10×ExTaq Buffer | 2 | 14 |
| dNTP | 2 | 14 |
| 10μM primer Fw(final conc. 0.5μM) | 1 | 7 |
| 10μM primer Rv(final conc. 0.5μM) | 1 | 7 |
| Ex Taq | 0.2 | 1.4 |
| MilliQ | 12.8 | 89.6 |

Primers:

|  |
| --- |
| rAmelex202F |
| rAmelex202R |
| rAMBN201F |
| rAMBN201R |
| rNtf4F |
| rNtf4R |
| rKrt14201F |
| rKrt14201R |
| rGAPDH201F |
| rGAPDH201R |

cDNA 1μl(<200ng)

solution 19μl

total 20μl

vortex spin down

Cycle Condition:

Predenature 94℃ 1min

Denature 　 94℃　 30sec

Anealing 58℃　 30sec GAPDH:20cycles others:27cycles

Extension 72℃　 1min 30sec

72℃　5min

4℃　∞

Increase the number of cycles if no bands are seen. Find the linear point before saturation.

Annealing temp.= primer Tm.　−　7℃.

Electrophoresis：

Materials:

2% agarose gel

1×１TAE buffer

ladder

Mixture:

PCR product solution 5μℓ

10×Loading Buffer 1μℓ

Apply 7μl of the mixture and ladder into the well on 2% agarose gel 7μl1.

Electrophorese them in 1×TAE buffer containing EtBr for 15 min.

(EtBr: plus charge, DNA: minus charge)

1×TAE buffer containing EtBr:

50×TAE 60ml

MilliQ 3L

EtBr(5mg/ml) 0.3ml