# Appendix A

## 1. Formaldehyde agarose gel electrophoresis

To prepare the 1.2% gel for RNA analysis, 0.36 g of agarose was mixed with 30 ml of 1x Formaldehyde gel (FA) buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, final pH 7.0). The gel mixture was heated until the agarose dissolved and, then, cooled to 65-70ºC in a water bath. After cooling, 0.54 ml of 37% (12.3 M) formaldehyde was added and well mixed by swirling in a fume hood. The gel electrophoresis chamber was set up. The agarose solution was poured onto the gel tray in a fume hood to a thickness of 3-5 mm and let stand to gel for at least 30 min. For sample preparation, about 5 µg of each RNA sample was well mixed with 5x RNA loading buffer (bromophenol blue solution, EDTA pH 8.0, 37% (12.3 M) formaldehyde, 100% glycerol, formamide, 10x FA gel buffer). Each sample was incubated for 5 min at 65ºC and immediately chilled on ice to denature RNA. The denatured RNA sample was applied to the gel. The electrophoresis was performed at 60 volts for 3 h in FA gel running buffer (10x FA gel buffer, 37% (12.3 M) formaldehyde). Next the gel was stained with GelStar® stain solution (10 µl of stain stock in 50 ml of 1x FA gel buffer) for 30 min by gentle agitation. The RNA pattern was visualized under ultraviolet (UV) light.

## 2. Agarose gel electrophoresis

In this study, gel electrophoresis was used for determining the size of DNA of interest. For the gel, 1.8% (w/v) of agarose gel in 1x TAE buffer (40 mM Tris-

borate, 1 mM EDTA) was melted and poured on a plastic tray, a comb was placed in the gel. After the agarose gel completely set (30-45 min at the room temperature), the comb was carefully removed and the gel was installed on the platform in the electrophoresis tank containing 1x TAE buffer. The DNA samples were mixed with 30% (v/v) gel-loading buffer (25% (v/v) glycerol, 60 mM EDTA, 0.25% (w/v) Bromophenol Blue) and slowly loaded into the slots of the submerged gel using an automatic micropipette. The electrophoresis was conducted at a constant 120 V for 1 h. Next, the gel was stained with 2.5 µg/ml of ethidium bromide (EtBr) solution for 5 min and destained with water for 15 min. After that the DNA patterns were observed under UV light box (Gel Doc model 1000, BIO-RAD, USA).

**3. pGEM-TEasy ligation**

The purified PCR products were ligated with pGEM-TEasy Vector (Promega) according to the following condition. The ligation mixture containing 5 µl of 2x Rapid ligation buffer, T4 DNA ligase, 0.5 µl of pGEM-TEasy Vector (25 ng), 0.5 µl of T4 DNA ligase (3 units/µl) and 4 µl of purified PCR product was incubated at 4°C, overnight.

**4. Transformation into *E. coli* by $CaCl_2$ method (Cohen et al., 1972)**

**4.1 Preparation of *E. coli* competent cells**

An isolated single colony of *E. coli* was inoculated into 3 ml of LB broth, incubated at 37°C overnight by shaking. The overnight culture was inoculated in 25 ml of fresh medium (1:100 dilution) and incubated at 37°C until $OD_{600}$ reached 0.3-0.5. The cell pellets were harvested by centrifugation at 4,500 rpm for 6 min at 4°C

and washed with 20 ml of ice-cold 0.1 M $MgCl_2$. The cell suspension was centrifuged at 4,500 rpm for 6 min at 4°C. The pellets were resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$, then incubated on ice for at least 15 min to establish competency. The cell suspension was centrifuged, then, resuspended in 1.7 ml of ice-cold 0.1 M $CaCl_2$. A volume of 0.3 ml of glycerol was added to the cell suspension to give 15% (w/v) of final concentration. The cell suspension was aliquoted in a volume of 200 µl per tube and kept at –80°C.

### 4.2 Transformation into *E. coli* competent cells

A volume of 100 µl of *E. coli* competent cells was gently mixed with 0.1-1.0 µg of plasmid DNA. The mixture was left on ice for 30 min to give higher transformation frequency. The cell mixture was incubated at 42°C for 90 sec and placed on ice for an additional 5 min. The transformed cells were mixed with 500 µl of LB broth (1 liter; 10 g of NaCl, 10 g of tryptone, 5 g of yeast extract and deionized water, pH 7.0) and incubated at 37°C for 1 h with constant shaking. Finally, 200 µl of transformed culture was spread on LB selective plate containing 100 µg/ml of ampicillin and incubated at 37°C for 16 h.

## 5. Plasmid extraction and purification from *E. coli*

### 5.1 Small-scale preparation of plasmid DNA from *E. coli*

A single bacterial colony was inoculated into 3.0 ml of LB medium containing 100 µg/ml ampicillin and incubated overnight at 37°C with vigorous shaking. The cell culture was collected using centrifugation at 14,000 rpm for 30 sec

at room temperature in microcentrifuge tube. A supernatant was discarded, the bacterial pellet was resuspended in 350 μl of STET buffer (8% (w/v) glucose, 5% (v/v) Tritron X-100, 50 mM EDTA and 50 mM Tris-HCl, pH 8.0) and 25 μl of a 10 mg/ml lysozyme solution was added. The mixture was mixed and left at room temperature for 2 min, placed in the boiling water bath for exactly 40 sec, and then incubated on ice for 3 min. The bacterial lysate was centrifuged at 14,000 rpm for 10 min at room temperature, then, the pellet of bacterial debris was removed from the microcentrifuge tube with a steriled toothpick. An equal volume of isopropanol was added to this tube and the mixture was incubated at -80°C for 10 min. After centrifugation at 14,000 rpm for 10 min at 4°C, the supernatant was discarded. Finally, the tubes were placed in an inverted position on a paper towel to allow all fluid to drain off. Plasmid DNA was resuspended in 30 μl of 10 mM Tris-HCl, pH 8.0 containing DNase free pancreatic RNase A (2 mg/ml) and stored at –20°C.

### 5.2 Purification of Plasmid DNA

The plasmid DNA was purified by QIAprep spin Miniprep Kit (QIAGEN). The manufacture's instruction was followed. A single bacterial colony was inoculated into 3.0 ml of LB medium containing 100 μg/ml of ampicillin and incubated overnight at 37°C with vigorous shaking. The cell culture was collected using centrifugation at 14,000 rpm for 1 min at room temperature in a microcentrifuge tube. The supernatant was discarded and, then, the bacterial pellet was resuspended in 250 μl of buffer P1. Next, 350 μl of buffer P2 and 350 μl of buffer N3 were added to bacterial suspension, and mixed by inverting 4-6 times. The cell mixture was centrifuged at 14,000 rpm for

10 min. The supernatants were applied to the QIAprep column by decanting or pipetting. The flow though was discarded by centrifugation at 14,000 rpm for 1 min. The column was washed by adding 750 µl of buffer PE and centrifuged at 14,000 for 1 min. Additionally, the flow through was removed by centrifuged at 14,000 rpm for 1 min to get rid of residual wash buffer. The QIAprep column was placed in a clean microcentrifuge tube and, then, plasmid DNA was eluted by adding 30 µl of buffer EB, left at room temperature for 1 min and centrifuged at 14,000 rpm for 1 min.

**6. Automated DNA sequencing**

The ABI PRISM™ BigDye Terminator Cycle Sequencing Kit was used to prepare the DNA samples for sequence analysis. The principle of the protocol is based on fluorescent-labeled terminator cycle sequencing. The PCR reaction was performed in a reaction mixture (20 µl) containing a terminator ready reaction mix [A-dye terminator labeled with dichloro (R6G), C-dye terminator labeled with dichloro (ROX), G-dye terminator labeled with dichloro (R110), T-dye terminator labeled with dichloro (TAMR10), deoxynucleoside triphosphates (dATP, dCTP, dITP, dUTP), $MgCl_2$, Tris-HCl pH 9.0, and Amplitaq DNA polymerase, 200-500 ng of plasmid DNA

**7. Protein concentration determination**

The concentration of protein was determined by Bradford's method. Protein standard BSA was diluted in distilled water to concentrations of 3.125, 6.25, 12.5, 25, 50, 100 µg/ml. A volume of 50 µl of either protein standard or protein samples was

mixed with 100 µl Bradford's reagent (Bio-Rad, dilution in ratio water:reagent = 1:8.) in 96 well plate. The absorbance at 620 nm was determined from protein standard or samples using SpectraMAX® M2 (Molecular devices, USA). The concentration of protein sample was calculated by referring to the concentration of protein standard.

**8. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

The method of SDS-PAGE was performed as described by Laemmli, 1970. The gel solution was prepared as shown in Table 7. Electrophoresis was carried out in the descending direction on the Tris-glycine buffer (25 mM Tris-HCl, pH 6.8, 192 mM glycine and 0.1% (w/v) SDS) using a constant 80 V for 30 min and 200 V for 45 min or until the tracking dye reached the edge of the gel.

**9. Restriction endonuclease digestion**

The reaction contained 2 µg of DNA, 1 µl of restriction enzyme (1-10 units), 1x reaction buffer and sterile distilled water to give a total volume of 20 µl. The restriction enzymes used in this study including their restriction sequences and optimal temperatures are shown in Table 8. After digestion was completed, the digested products were analyzed by agarose gel electrophoresis.

**Table 7. Preparation of SDS-Polyacrylamide gel**

| Solution | Stacking gel (4%) | Resoluting gel (12%) |
|---|---|---|
| Water | 1.45 ml | 2.13 ml |
| Acrylamide (40%) (acrylamide : N,N'-methylenebisacrylamide, 29:1) | 0.25 ml | 1.5 ml |
| 1 M Tris-HCl (pH 6.8) | 0.25 ml | - |
| 1.5 M Tris-HCl (pH 8.8) | - | 1.265 ml |
| 10%SDS | 20 µl | 50 µl |
| 10%APS | 20 µl | 50 µl |
| TEMED | 2 µl | 2 µl |

SDS = Sodium Dodecyl Sulfate

APS = Ammonium Persulfate

TEMED = N,N,N,'N'- tetramethylenediamine

**Table 8. The restriction endonuclease enzymes, recognition sequences, and optimal temperatures.**

| Restriction enzyme | Recognition sequence | Optimal temperature (ºC) |
|---|---|---|
| ↓ ↑ *Bam*HI | G GATT C<br>C CTAG G | 37 |
| ↓ ↑ *Eco*RI | G AATT C<br>C TTAA G | 37 |
| ↓ ↑ *Sal*I | G TCGA C<br>C AGCT G | 37 |
| ↓ ↑ *Xho*I | C TCGA G<br>G AGCT C | 37 |

**10. Agarose gel electrophoresis**

In this study, gel electrophoresis was used for determining the size of DNA of interest. For the gel, 1.0% (w/v) of agarose gel in 1x TBE buffer (89 mM Tris base, 89 mM Boric acid, 2.5 mM EDTA, pH 8.0) was melted, 2.5 μg/ml of ethidium bromide (EtBr) solution was added, and poured on a plastic tray; a comb was placed in the gel. After the agarose gel was completely set (1 h at the room temperature), the comb was carefully removed and installed on the platform in the electrophoresis tank containing 1xTBE buffer. The DNA samples were mixed with 30% (v/v) gel-loading buffer (25% (v/v) glycerol, 60 mM EDTA, 0.25% (w/v) Bromophenol Blue) and slowly loaded into the slots of the submerged gel using an automatic micropipette. The electrophoresis was carried out at a constant 150 V for 1 h. After that, the DNA patterns were observed under UV light box (Gel Doc model 2000, BIO-RAD, USA).

**11. Gel purification**

The DNA fragment and vector were purified by QIAquick Gel Extraction Kit (QIAGEN). The manufacture's instruction was followed. The expected DNA fragment or vector was excised from the agarose gel and transferred to a microcentrifuge tube. Three volumes of gel solubilization and binding buffer (QG) were added to 1 volume of the gel (100 mg of gel ~ 100 μl), then the mixture was incubated at 50 °C for 10 min or until the gel slice had completely dissolved. After that, 1 gel volume of isopropanol was added to the sample and the mixture was applied to the spin cartridge placed into 2 ml wash tube, then centrifuged at maximum speed for 1 min. The flow-through solution was discarded. The cartridge was washed

by adding 0.5 ml of buffer QG and centrifuged at maximum speed for 1 min. The flow-through was discarded and the cartridge was washed by adding 0.75 ml of buffer PE, then, centrifuged at maximum speed for 1 min. The flow-through was discarded and the cartridge was centrifuged for an addition 1 min at maximum speed to remove residual wash buffer. After that, 30 µl of distilled water was added to this cartridge placed on a new microcentrifuge tube, left standing at room temperature for 1 min, and centrifuged for 1 min at maximum speed to elute DNA. Finally, the eluted DNA was determined by running in agarose gel electrophoresis.

**12. DNA precipitation**

The DNA was precipitated by using seeDNA$^{TM}$ Co-precipitant kit (Amersham Biosciences). The manufacture's instruction was followed. The ligation mixture was precipitated by adding 2 µl of seeDNA, 0.1 volumes of 3M sodium acetate pH 5.2, and mixed. Two volumes of ethanol were added to the mixture, briefly vortexed, and incubated at room temperature for 2 min. The sample was centrifuged at 14,000 rpm for 5 min and the supernatant was discarded. A pink pellet was observed at the bottom of the tube. The pellet was rinsed with 500 µl of 70% (v/v) ethanol and centrifuged at 14,000 rpm for 5 min. The residual ethanol was removed by air drying. The pink pellet was resuspended in 10 µl of sterile distilled water.

**13. Transformation into *E. coli* XL1-blue by electroporation method**

The electroporation cuvette and microcentrifuge tube were chilled on ice. The electroporator was setted at 1,700 V. Forty microlitters of eletroporation-competent cells (Stratagene) and 2 μl of ligation mixture (from step 12) were added to the pre-chilled tube and gently mixed. The cell-DNA mixture was transfered to electroporation cuvette. The cuvette was placed into electroporator and pulsed. Next, the cuvette was removed from the electroporator and immediately added SOB (tryptone, yeast extract, NaCl, deionized water, $MgCl_2$, $MgSO_4$) medium to the cell. The cell was transferred to a sterile 15 ml tube and incubated at 37°C for 1 h by shaking at 250 rpm. After that, 10, 100, 990 μl of transformation mixture was plated on LB agar plate containing 100 μg/ml of ampicillin. Finally, the plates were incubated at 37°C for 16-18 h.

**14. Large-scale preparation of plasmid DNA from *E. coli***

The plasmid DNA was purified by QIAGEN plasmid Mexi Kit (QIAGEN). The manufacture's instruction was followed. To prepare starter, a positive clone was inoculated into 5 ml of LB medium containing 100 μg/ml of ampicillin and incubated for 8 h at 37°C with vigorous shaking. The bacterial culture was inoculated into 1 liter of LB medium containing 100 μg/ml of ampicillin and incubated for 12-16 h at 37°C with vigorous shaking. The cell culture was harvested using centrifugation at 6,000 rpm for 20 min at 4°C. The supernatant was discarded and the bacterial pellet was resuspended in 10 ml of buffer P1. Next, 10 ml of buffer P2 was added to the suspension, gently mixed by inverting 4-6 times and, then, incubated at room

temperature for 5 min. A volume of 10 ml of buffer P3 was added to the cell mixture, immediately mixed by inverting for 4-6 times, and incubated on ice for 20 min. The cell mixture was centrifuged at 12,500 rpm for 1 h at 4°C. The QIAGEN column was equilibrated by adding 10 ml of buffer QBT and allowed to empty by gravity flow. After centrifugation, the supernatant was applied to the QIAGEN column and allowed to empty by gravity flow. The column was washed twice by adding 30 ml of buffer QC. The QIAGEN column was placed in clean centrifuge tube and, then, plasmid DNA was eluted by adding 15 ml of buffer QF. The elute DNA was precipitated by adding isopropanol, mixed, and centrifuged at 12,500 rpm for 30 min at 4°C. The supernatant was discarded and the DNA pellet was washed with 5 ml of 70% ethanol, and centrifuged at 12,500 rpm for 30 min at 4°C. The DNA pellet was dried for 10 min and redissolved in 500 μl of TE buffer (10 mM Tris-HCl, pH 7.0, 1 mM EDTA, pH 7.0).

**15. Automated DNA sequencing**

The DNA was sequenced by using PE BIOSYSTEMS sequencers (Lark Technologies).

# Appendix B

## 1. Chemical stock solution and buffer

### 1 M Tris-HCl

Dissolve 121.1 g of Tris base in 800 ml of distilled water. Adjust the pH to the desired value by adding concentrated HCL. Adjust the volume of the solution to 1000 ml with distilled water and sterilize by autoclaving.

### 1 M $MgCl_2.6H_2O$

| | |
|---|---|
| $MgCl_2.6H_2O$ | 203.3 g |

Dissolve in 800 ml of distilled water and adjust the volume to 1000 ml with distilled water. Sterilize by autoclaving and store at room temperature.

### 1 M $MgSO_4$

| | |
|---|---|
| $MgSO_4$ | 12 g |

Dissolve in a final volume of 100 ml distilled water. Sterilize by autoclaving and store at room temperature.

### 10 N NaOH

| | |
|---|---|
| NaOH | 400 g |

Prepare this solution with extreme care in plastic beakers. Dissolve in 800 ml of distilled water and adjust the volume to 1000 ml with distilled water. Store the solution in a plastic container at room temperature.

**5 M NaCl**

| | |
|---|---|
| NaCl | 292 g |

Dissolve in 800 ml of distilled water and adjust the volume to 1000 ml with distilled water. Sterilize by autoclaving and store the solution at room temperature.

**0.1 M $MgCl_2$**

| | |
|---|---|
| $MgCl_2.6H_2O$ | 20.33 g |

Dissolve in 1000 ml with distilled water and then sterilize by autoclaving.

**0.1 M $CaCl_2$**

| | |
|---|---|
| $CaCl_2.2H_2O$ | 14.7 g |

Dissolve in 1000 ml with distilled water and then sterilize by autoclaving.

**0.5 M EDTA (pH 8.0):**

| | |
|---|---|
| EDTA | 186.1 g |
| Distilled water | 800 ml |

Adjust the pH to 8.0 with NaOH and bring up to 1000ml.

**20% SDS (w/v):**

| | |
|---|---|
| SDS | 200 g |

Dissolve in 900 ml of distilled water. Heat to 68ºC and stir with a magnetic stirrer to assist dissolution. Adjust the volume to 1000 ml with distilled water and store at room temperature. Sterilization is not necessary.

**Ammonium Persulfate (10% w/v)**

| | |
|---|---|
| Ammonium persulfate | 1 g |
| Distilled water | 10 ml |

Dissolve 1 g ammonium persulfate in 10 ml of distilled water and store at 4º C.

**Ethidium bromide (10 mg/ml)**

| | |
|---|---|
| Ethidium bromide | 1 g |
| Distilled water | 100 ml |

Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer the solution to a dark bottle and store at room temperature.

**Phosphate-buffered Saline (PBS):**

| | |
|---|---|
| NaCl | 8 g |
| KCl | 0.2 g |
| $Na_2HPO_4$ | 1.44 g |
| $KH_2PO_4$ | 0.24 g |

Dissolve the ingredients in 800 ml of distilled water. Adjust the pH to 7.4 with HCl. Add distilled water to 1000 ml. Sterilize the buffer by autoclaving and store at room temperature.

**STET buffer**

| | |
|---|---|
| Glucose | 80 g |
| Triton X-100 | 50 ml |
| $Na_2EDTA$ | 18.61 g |
| Tris-base | 12.1 g |

Adjust pH to 8.0 with HCl and bring up to 1000 ml. Sterilize by autoclaving.

**Lysis buffer (RIPA):**

150 mM NaCl

1% (v/v) NP-40

0.25% (w/v) Sodium deoxycholate

1 mM EDTA

50 mM Tris, pH 7.4

Adjust the volume to 100 ml with distilled water.

**RNase A (10 mg/ml)**

| | |
|---|---|
| RNase A | 10 g |

Dissolve and bring up to 1 ml with sterile distilled water. Boil in water for 5 min and store at -20ºC.

**2. Preparation of K199**

| | |
|---|---|
| M199 | 50 ml |
| Magnesium sulfate hepta-hdrous | 33 mg |
| Sodium hydrogen phosphate | 0.5 mg |

| | |
|---|---|
| Sodium chloride | 110 mg |
| Calcium chloride dihydrous | 9 mg |
| HEPES | 23.8 mg |
| L-glutamine | 0.10 mg |

Adjust the pH to 7.6 and adjust the volume of the solution to 100 ml with distilled water. Sterilize the solution by passing it through a 0.22 μM Milipore filter, and store at 4ºC.

**3. Solutions for electrophoresis**

**50X TAE, Electrophoresis buffer:**

| | |
|---|---|
| Tris-base | 242 g |
| Glacial acetic acid | 57.1 ml |
| 0.5 M EDTA, pH 8.0 | 100 ml |

Dissolve the ingredients in distilled water and bring up to volume 1000 ml. Working solution in the gel and the buffer is 1X.

**5X TBE, Electrophoresis buffer:**

| | |
|---|---|
| Tris-base | 54 g |
| Boric acid | 27.5 g |
| 0.5 M EDTA, pH 8.0 | 20 ml |

Dissolve the ingredients in distilled water and bring up to volume 1000 ml. Working solution in the gel and the buffer is 1X.

**10X Formaldehyde gel buffer (pH 7.0), Electrophoresis buffer:**

| | |
|---|---|
| MOPS | 41.9 g |
| Sodium acetate | 6.8 g |
| 0.5 M EDTA | 20 ml |

Dissolve the ingredients in sterile DEPC-treated water. Adjust the pH to 7.0 with NaOH. Adjust the volume of the solution to 1000 ml with DEPC-treated water. Sterilize the solution by passing it through a 0.45 μM Milipore filter, and store at room temperature protected from light.

**1X Fomaldehyde gel running buffer**

| | |
|---|---|
| 10x FA gel buffer | 100 ml |
| 37% (12.3 M) formaldehyde | 20 ml |

Adjust the volume of the solution to1000 ml with DEPC-treated water RNase-free water and store at room temperature.

**Tris-glycine buffer, Electrophoresis buffer:**

| | |
|---|---|
| Tris base | 15.1 g |
| Glycine | 94 g |
| 20% SDS | 25 ml |

Dissolve the ingredients in 800 ml of distilled water. Adjust the volume to 1000 ml with distilled water to.

**Gel-loading buffer:**

25% (v/v) glycerol

60 mM EDTA

0.25% (w/v) Bromophenol Blue

**5X Formaldehyde Gel-loading buffer:**

| | |
|---|---|
| Bromohenol blue solution | 16 µl |
| 500 mM EDTA, pH 8.0 | 80 µl |
| 37% (12.3 M) formaldehyde | 720 µl |
| 100% glycerol | 2 ml |
| Formamide | 3.084 ml |
| 10X Formaldehyde Gel loading buffer | 4 ml |

Adjust the volume of the solution to 10 ml with DEPC-treated water and store at -20ºC.

**4X SDS Gel loading buffer:**

200 mM Tris-HCl, pH 6.8

8% (w/v) SDS (electrophoresis grade)

0.4% (w/v) bromophenol blue

40% (v/v) glycerol

8% (v/v) 2-ß-mercaptoethanol

400 mM DTT

Adjust the volume of the solution to 50 ml with distilled water and store at -80ºC.

## 4. Media and antibiotics for Bacterial culture

**Ampicillin (100 mg/ml)**

| | |
|---|---|
| Ampicillin | 100 mg |

Dissolve in 1 ml of sterile distilled water. Store at -20ºC.

**Tetracyclin (10 mg/ml)**

| | |
|---|---|
| Tetracyclin | 10 mg |

Dissolve in 1 ml of absolute ethanol. Store at -20ºC.

**Zeocin (500 mg/ml)**

| | |
|---|---|
| Zeocin | 500 mg |

Dissolve in 1 ml of sterile distilled water. Store at 4ºC or -20ºC.

**LB (Luria-Bertani) broth** (supplement with 100 μg/ml ampicillin)

| | |
|---|---|
| 1% (w/v) trytone or peptone | 10.0 g |
| 0.5% (w/v) yeast extract | 5.0 g |
| NaCl | 5.0 g |

Adjust the volume of the solution to 1000 ml with distilled water and sterilize by autoclaving for 20 min at 15 psi. Add 1 ml of ampicillin (100 mg/ml) into warm medium (50ºC).

**LB (Luria-Bertani) broth** (supplement with 10 μg/ml tetracyclin)

| | |
|---|---|
| 1% (w/v) trytone or peptone | 10.0 g |
| 0.5% (w/v) yeast extract | 5.0 g |
| NaCl | 5.0 g |

Adjust the volume of the solution to 1000 ml with distilled water and sterilize by autoclaving for 20 min at 15 psi. Add 1 ml of tetracyclin (10 mg/ml) into warm medium (50ºC)

**LB agar** (supplement with 100 μg/ml ampicillin)

| | |
|---|---|
| 1% (w/v) trytone or peptone | 10.0 g |
| 0.5% (w/v) yeast extract | 5.0 g |
| NaCl | 5.0 g |
| 1.8% agar | 18.0 g |

Adjust the volume of the solution to 1000 ml with distilled water and sterilize by autoclaving for 20 min at 15 psi. Add 1 ml of ampicillin (100 mg/ml) into warm medium (50ºC). The medium was poured into glass or plastic plate.

**LB agar** (supplement with 10 μg/ml tetracyclin)

| | |
|---|---|
| 1% (w/v) trytone or peptone | 10.0 g |
| 0.5% (w/v) yeast extract | 5.0 g |
| NaCl | 5.0 g |
| 1.8% agar | 18.0 g |

Adjust the volume of the solution to 1000 ml with distilled water and sterilize by autoclaving for 20 min at 15 psi. Add 1 ml of tetracyclin (10 mg/ml) into warm medium (50ºC). The medium was poured into glass or plastic plate.

**SOB medium**

| | |
|---|---|
| Tryptone | 20 g |
| Yeast extract | 5 g |
| NaCl | 0.5 g |

Adjust the volume of the solution to 1000 ml with distilled water and sterilize by autoclaving for 20 min at 15 psi. Just before use, added 10 ml of a sterile solution of 1M $MgCl_2$ and 10 ml of a sterile solution of 1M $MgSO_4$.

**5. Reagent for apoptotic assay:**

**250 mM Campothecin:** Dissolve in DMSO and store at -20ºC.

**100 mM Cisplatin:** Dissolve in DMSO and store at -20ºC.

**50 mM Cycloheximide:** Dissolve in ethanol and store at -20ºC.

**10 mM Doxorubicin:** Dissolve in distilled water and store at -20ºC.

**200 mM Etoposide:** Dissolve in DMSO and store at -20ºC.

**500 mM 5-Fluorouracil:** Dissolve in DMSO and store at -20ºC.

**200 mM Hydrogenperoxide:** Dissolve in distilled water and store at -20ºC.

**10 mM Hydroxyura:** Dissolve in distilled water and store at -20ºC.

**1 mM Staurosporine:** Dissolve in DMSO and store at -20ºC.

**10 mM Thapsigargin:** Dissolve in DMSO and store at -20ºC.

**1 µM TNF-α:** Dissolve in 1XPBS and store at -20ºC.

**6. Solution for Western blot analysis**

**Electroblotting buffer:**

| | |
|---|---|
| Glycine | 7.9 g |
| Tris-base | 5.8 g |
| Methanol | 200 ml |

Dissolve the ingredients in distilled water and bring up to volume 1000 ml with distilled water.

**5X TTBS buffer:**

| | |
|---|---|
| Tween® 20 | 5 ml |
| NaCl | 45 g |
| Tris-HCl | 31.75 g |
| Tris-base | 5.8 g |

Dissolve the ingredients in distilled water and bring up to volume 1000 ml with distilled water.

**Blocking buffer:**

| | |
|---|---|
| Low fat dry milk | 5 g |
| 1X TTBS | 100 ml |

**Washing buffer:**

| | |
|---|---|
| Low fat dry milk | 10 g |
| 1X TTBS | 1000 ml |

**Hybridization buffer:**

| | |
|---|---|
| Low fat dry milk | 1 g |
| 1X TTBS | 100 ml |

**7. Solution for immunofluorescent stain**

**Wash buffer**

0.1% (v/v) Tween®20 in PBS

**Fix solution: 4% Paraformaldehyde**

| | |
|---|---|
| Paraformaldehyde | 4 g |

Dissolve in 50 ml distilled water and then add 1 ml of 1 M NaOH solution. Stir the mixture gently on a heating block (~65ºC) until the paraformaldehyde is dissolved. Next add 10 ml of 10X PBS and allow the mixture to cool to room temperature. Adjust the pH to 7.4 using 1 M HCl and then adjust the final volume to 100 ml with distilled water. Filter the solution through a 0.45 µM membrane filter to remove any particulate matter, and store at -20ºC.

# Appendix C

## 1. U2OS

**Table 9. Properties of U2OS cell**

| Category | Biological of Cultured Cell |
|---|---|
| Organism | Homo sapiens (Human) |
| Source | Organ: bone<br>Disease: osteosarcoma |
| Cellular Products | osteosacoma derived growth factor (ODGF) (Heldin et al., 1986) |
| Growth properties | adherent |
| Morphology | epithelial |
| Receptors | insulin-like growth factor I (IGF-I); insulin-like growth factor II (IGF II) (Raile et al., 1994) |
| Antigen expression | Blood Type A; Rh+; HLA A2, Aw30, B12, Bw35, B40 (+/-) |
| Cytogenetic analysis | Ponten and Saksela (1967) reported that cell line U2OS is chromosomally highly altered, with chromosome counts in the hypertriploid range. These authors did not find the hypodiploid cell population.<br>Instead, most of the population has slightly higher counts than first described. |

**Table 9. Continued**

| Category | Biological of Cultured Cell |
|---|---|
| | Very few normal chromosomes are present, but a high number of stable marker chromosomes are identified. Different chromosomal rearrangement involving the same chromosomes (N1, N7, N9, and N11 particularly), are seen. Twenty-two markers are found including: t (9qter →9q21::1p36 →1P::?), 7p+, iso(17q), t(15q;?), 4q+, del(3)(q21), 5q(aberrant) and others. |
| Comments | Ponten and Saksela (1967) derived U2OS line (originally 2T) in 1964 from a moderately differentiated sarcoma of the tibia of a 15 year old girl. Viruses were not detected during co-cultivation with WI-38 cells or in CF tests against SV40, RSV or adenoviruses. Mycoplasma contamination was detected and eliminated in 1972. |
| Propagation | ATCC complete growth medium: McCoy's 5a medium with 1.5 mM L-glutamine, 90%; fetal bovine serum, 10% Temperature: 37ºC |
| Subculture | Remove medium, and rinse with 0.25% trypsin, 0.03% EDTA solution. Remove the solution and an additional 1 to 2 ml of trypsin-EDTA solution. Allow the flask to |

**Table 9. Continued**

| Category | Biological of Cultured Cell |
|---|---|
| | sit at room temperature (or at 37ºC) until the cells detach.<br>Add fresh culture medium, aspirate and dispense into new culture flasks.<br>Subcultivation ratio: A subcultivation ratio of 1:3 to 1:6 is recommended.<br>Medium renewal: 2 to 3 times per week |
| Preservation | Culture medium, 95%; DMSO, 5% |

## 2. Amino acid

**Table 10. Abbreviations and molecular weight for Amino acids**

| Amino acid | Three-letter abbreviation | One-letter symbol | Molecular weight (Da) |
|---|---|---|---|
| Alanine | Ala | A | 89 |
| Arginine | Arg | R | 174 |
| Asparagine | Asn | N | 132 |
| Aspartic acid | Asp | D | 133 |
| Asparagine or Aspartic acid | Asx | B | - |
| Cysteine | Cys | C | 121 |
| Glutamine | Gln | Q | 146 |
| Glutamic acid | Glu | E | 147 |
| Glutamine or Glutamic acid | Glx | Z | - |
| Glycine | Gly | G | 75 |
| Histidine | His | H | 155 |
| Isoleucine | Lle | I | 131 |
| Leucine | Leu | L | 131 |
| Lysine | Lys | K | 146 |
| Methionine | Met | M | 149 |
| Phenylalanine | Phe | F | 165 |
| Proline | Pro | P | 115 |
| Serine | Ser | S | 105 |
| Threonine | Thr | T | 119 |
| Tryptophan | Try | W | 204 |
| Tyrosine | Tyr | Y | 181 |
| Valine | Val | V | 117 |