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 65-70^{o}C 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 65^{o}C 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 100 V for 30 min. 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).

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^{TM} 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, dTTP, dUTP), $MgCl_2$, Tris-HCl pH 9.0, and Amplitaq DNA polymerase, 200-500 ng of plasmid DNA

7. 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.

Table 9. 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'-tetramethyl-ethylenediamine

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

Restriction enzyme	Recognition sequence	Optimal temperature (^{o}C)
↓ ↑ *Bam*HI	G GATT C C CTAG G	37
↓ ↑ *Eco*RI	G AATT C C TTAA G	37
↓ ↑ *Xho*I	C TCGA G G AGCT C	37

9. 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.

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 68°C 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 4°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.

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 -20°C.

1 M HEPES

HEPES	260.30 g

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

1 M KCl

KCl	74.56 g

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

1 M DTT

DTT	15.43 g

Dissolve in 100 ml with distilled water and then sterilize by filtrattion.

PMSF (10 mg/ml)

PMSF	1 g

Dissolve in 100 ml with distilled water and then sterilize by filtration.

1 M Imidazole

Imidazole	6.80 g

Dissolve in 100 ml with distilled water and then sterilize by filtration.

1M Lithium acetate pH 7.5

Lithium acetate	6.60 g

Dissolve in 80 ml with distilled water and adjust pH to 7.5 then bring the volume up to 100 ml and sterilize by autoclaving.

50% PEG3350

PEG3350	50 ml
Distilled water	50 ml

Mix well and sterilize by autoclaving.

2. Preparation of K199

M199	50 ml
Magnesium sulfate hepta-hydrous	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 4^{o}C.

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.

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 -20°C.

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 - 80°C.

4. Media and antibiotics for bacterial and yeast culture

Ampicillin (100 mg/ml)

Ampicillin	100 mg

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

Kanamycin (50 mg/ml)

Kanamycin	50 mg

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

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 (50°C).

LB (Luria-Bertani) broth (supplement with 50 µg/ml kanamycin)

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 kanamycin (50 mg/ml) into warm medium (50°C)

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 (50°C). The medium was poured into glass or plastic plate.

LB agar (supplement with 50 µg/ml kanamycin)

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 kanamycin (50 mg/ml) into warm medium (50°C). The medium was poured into glass or plastic plate.

YPDA broth

2% Peptone	20 g
1%Yeast extract	10 g

2%Glucose	20 g

Adjust the volume of the solution to 1000 ml with distilled water and sterilize by autoclaving for 20 min at 15 psi. Allow medium to cool to 55°C and then add sterile adenine hemisulfate supplement to final concentration 0.003% per litter of media.

YPDA agar

2% Peptone	20 g
1%Yeast extract	10 g
2%Glucose	20 g
2% Agar	20 g

Adjust the volume of the solution to 1000 ml with distilled water and sterilize by autoclaving for 20 min at 15 psi. Allow medium to cool to 55°C and then add sterile adenine hemisulfate supplement to final concentration 0.003% per litter of media.

Synthetic dropout (SD) broth

Yeast nitrogen base without amino acid	6.7 g
Glucose	20 g
DO supplement (-Trp/-Leu or -Trp/-Leu/-His/-Ade)	0.06 g

Adjust the volume of the solution to 1000 ml with distilled water and sterilize by autoclaving for 20 min at 15 psi.

Synthetic dropout (SD) agar

Yeast nitrogen base without amino acid	6.7 g
Glucose	20 g
DO supplement (-Trp/-Leu or -Trp/-Leu/-His/-Ade)	0.6 g
Agar	20 g

Adjust the volume of the solution to 1000 ml with distilled water and sterilize by autoclaving for 20 min at 15 psi.

1 M 3-AT

3-AT	8.40 g

Dissolve in 100 ml with distilled water and then sterilize by filtration.

5. Solution for X-Gal assay

Z buffer

$Na_2HPO_4.7H_2O$	16.1 g
$NaH_2PO4.H_2O$	5.50 g
KCl	0.75 g
$MgSO_4.7H_2O$	0.24 g

Adjust the pH to 7.0 and adjust the volume of the solution to 1000 ml with distilled water. Sterilize the solution by autoclaving

X-gal (20 mg/ml)

X-gal	20 mg

Dissolve in 1 ml of DMF (N, N-dimethylformamide). Store in the dark at -20°C.

Z buffer/X-gal solution

Z buffer	100 ml
β-mercaptoethanol	0.27 ml
X-gal solution (20 mg/ml)	1.67 ml

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.

Blocking buffer/Hybridization buffer

Low fat dry milk	3 g
1X PBS	100 ml

Washing buffer

1X PBS	1000 ml
Tween20	3 ml

7. Amino acid

Table 11. 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