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## Construction of high-quality rice ribosome footprint library

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### ABSTRACT

A protocol for construction of high-quality rice ribosome footprint library is provided. Rice ribosome/RNA complexes are isolated with a modified low ionic polysome extraction buffer. After nuclease digestion, rice ribosome footprints are extracted using SDS method followed by column purification. High-quality rice ribosome footprint library is constructed via key steps including rRNA depletion, end repair, 3' adapter ligation, reverse transcription, circularization, PCR enrichment and several rounds of purification.

### GUIDELINES

- 1) Isolation of rice polysomes
- 2) Profile analysis for rice polysomes
- 3) Isolation of rice ribosome footprints
- 4) rRNA depletion in rice ribosome footprints
- 5) End repair for rice ribosome footprints
- 6) 3' adapter ligation for repaired rice ribosome footprints
- 7) Reverse transcription
- 8) Purification of rice ribosome footprint library
- 9) Circularization and PCR enrichment for rice ribosome footprint library
- 10) Purification of enriched rice ribosome footprint library
- 11) Notes

### MATERIALS TEXT

Tris;  
HCl;  
KCl;  
MgCl<sub>2</sub>;  
Polyoxyethylene (10) tridecyl ether;  
Deoxycholic acid;  
DTT;  
Cycloheximide;  
DNase I;  
Sucrose;  
SUPERase-in RNase inhibitor;  
Ethanol;  
Urea;  
40% (W/V) Acrylamide/Bis;  
Boron;  
EDTA;  
Ammonium persulphate (APS);  
TEMED;  
6 × Blue/Orange loading dye;  
SYBR Gold nucleic acid gel stain;

Glycogen;  
 Isopropanol;  
 NaCl;  
 NaAc;  
 1.5 mL, 2 mL, and 15 mL DNase/RNase-free tube;  
 200 µL DNase/RNase-free PCR tube;  
 Polypropylene centrifuge tube, 13 × 51 mm;  
 Illustra MicroSpin S-400 HR column;  
 Illumina TruSeq Mammalian Ribo Profile Kit;  
 Ribo-Zero rRNA Removal Kit for Plant Leaf;  
 Zymo RNA clean and concentrator kit R1015;  
 Zymo RNA clean and concentrator kit R1017;  
 0.45 µm COSTAR Spin-X filter;  
 Agencourt AMPure XP beads.

### Isolation of rice polysomes

- 1 Rice tissue is ground to powder with mortar and pestle in liquid nitrogen. About 1 g of the powder is resuspended in 5 mL of ice-cold polysome extraction buffer (PEB) [100 mM Tris-HCl (pH 8.0), 40 mM KCl, 20 mM MgCl<sub>2</sub>, 2% (V/V) polyoxyethylene (10) tridecyl ether (SIGMA), 0.2% (W/V) deoxycholic acid (SIGMA), 1 mM DTT, 100 µg/mL cycloheximide (SIGMA), and 10 U/mL DNase I (Epicentre)]; the suspension is centrifuged at 5,000 g for 10 min at 4 °C followed by another 10 min centrifugation in a new 15 mL DNase/RNase-free tube at 20,000 g at 4 °C in Avanti J-E high-speed centrifuge (Beckman). RNA concentration and A260 units of the supernatant are measured with Nanodrop 2000 spectrophotometers (Thermo) and then used for polysome profile analysis and isolation of ribosome footprints.

### Profile analysis for rice polysomes

- 2 To perform profile analysis, 1,000 A260 units of the isolated polysomes from the step 1 are loaded on a 15-60% (W/V) sucrose gradient that is prepared in a [polypropylene centrifuge tube \(13 × 51 mm\)](#), Beckman) using a peristaltic pump (BT101S, Lead Fluid). The [sucrose](#) gradient with polysome sample is then centrifuged in a SW-55 rotor (Beckman) at 4 °C at 170,000 g for 1.5 h. Fractionation, absorbance assay at 254 nm and data acquisition of the resulted sample are performed using a gradient fractionator system (BRANDEL) with a UA-6 absorbance detector (Teledyne ISCO).

### Isolation of rice ribosome footprints

- 3 For isolation of ribosome footprints, the RNA concentration of rice polysome extract is first adjusted to 400 ng/µL, and then 200 µL aliquot is digested with the nuclease (20 U per 40 µg of RNA) provided by the [TruSeq Mammalian Ribo Profile Kit](#) (Illumina) for 1.4 h in a dry bath (Thermo) that is set at 25 °C with shaking speed of 600 rpm. This digestion is stopped by adding 15 µL of SUPERase-in (Thermo) to the reaction mixture. The ribosome footprints are isolated immediately by loading the entire digested lysate on equilibrated [Illustra MicroSpin S-400 HR columns \(GE Healthcare\)](#) with 3 mL of PEB, followed by spinning at room temperature (RT) with the speed of 600 rpm for 2 min. After adding 20 µL of 10% (W/V) SDS into the filtrate, RNAs are extracted using [Zymo RNA clean and concentrator kits R1017 and R1015](#) (Zymo Research), respectively, according to the manufacturer's manuals with some modifications.

### Purification and concentration of rice ribosome footprints by R1017 kit

- 3.1 Add 2 volume of RNA Binding Buffer into the isolated ribosome footprints and mix well. Thereafter, one volume of 100% (V/V) ethanol is added into the mixture and mix well. The resulted solution is transferred to a column that is provided in the R1017 kit and centrifuged with 12,000 g at RT for 30 s. The filtrate is transferred to the same column and the centrifugation is repeated once more. Discard the filtrate and an on-column DNase I treatment is performed for the ribosome footprints that are bound in the column: the column is first washed by adding 400 µL of RNA Wash Buffer followed by a 30 s centrifugation with 12,000 g at RT; then 80 µL of DNase I treatment solution (5 µL DNase I + 75 µL DNase I Digestion Buffer) is loaded into the washed column and incubated at RT for 15 min to make sure that the DNAs are completely removed in the ribosome footprint sample. After the 15 min incubation, the column is washed by 400 µL of RNA Pre Buffer with 12,000 g centrifugation at RT for 30 s, followed by 700 µL and 400 µL of RNA Wash Buffer with 12,000 g centrifugation at RT for 30 s and 2 min, respectively. Finally, the ribosome footprints bound by the column are eluted by the column with 50 µL RNase-free H<sub>2</sub>O and two rounds of 16,000 g centrifugation at RT for 1 min (safety stop point and the obtained ribosome footprint solution can be stored at -80 °C ~ -65 °C).

### Purification and concentration of rice ribosome footprints by R1015 kit

- 3.2 Add 2 volume of RNA Binding Buffer into the obtained ribosome footprints from the step of 3.1 and mix well. Thereafter, one volume of 100% (V/V) ethanol is added into the mixture and mix well. The resulted solution is transferred to a column that is provided in the R1015 kit and centrifuged with 12,000 g at RT for 30 s. The filtrate is transferred to the same column and the centrifugation is repeated once more. Discard the filtrate and the column is washed by 400 µL of RNA Pre Buffer with 12,000 g centrifugation at RT for 30 s, followed by 700 µL

and 400  $\mu\text{L}$  of RNA Wash Buffer with 12,000 g centrifugation at RT for 30 s and 2 min, respectively. Finally, the ribosome footprints bound by the column are eluted by 2 min incubation of the column with 26  $\mu\text{L}$  RNase-free  $\text{H}_2\text{O}$  and two rounds of 16,000 g centrifugation at RT for 1 min (Safety stop point and the obtained ribosome footprint solution can be stored at  $-80^\circ\text{C} \sim -65^\circ\text{C}$ ).

#### rRNA depletion in rice ribosome footprints

- 4 rRNA depletion and recovery of rice ribosome footprints between 28 and 30 nt are performed with the [Ribo-Zero rRNA Removal Kit](#) for Plant Leaf (MRZPL1224, Illumina) and PAGE purification, respectively, according to the TruSeq Ribo Profile Kit manual. The detail for rRNA depletion and purification is as follow:

##### Preparation of rRNA depletion magnetic beads

- 4.1 Transfer 225  $\mu\text{L}$  of rRNA depletion magnetic beads to a 1.5 mL DNase/RNase-free tube and place the tube on a magnetic stand until the liquid in the tube becomes clear. Discard the supernatant, add 225  $\mu\text{L}$  RNase-free  $\text{H}_2\text{O}$  into the tube to resuspend the rRNA depletion magnetic beads by vortex. Then the tube is placed on the magnetic stand until the mixture becomes clear and discard the supernatant. The rRNA depletion magnetic beads are washed again by 225  $\mu\text{L}$  of RNase-free  $\text{H}_2\text{O}$  and the resulted beads are finally resuspended in 65  $\mu\text{L}$  of Magnetic Bead Resuspension Solution.

##### Hybridization of probes with rRNA in rice ribosome footprints

- 4.2 Prepare hybridization mixture in a 200  $\mu\text{L}$  DNase/RNase-free PCR tube based on Table 1, pipette the solution well and place the tube in a PCR machine (Bio-Rad) that is set as  $68^\circ\text{C}$  for 10 min. Thereafter the tube is centrifuged shortly at low speed and incubated at RT for 5 min.

Component	Weight/Volume
Ribosome footprint RNA	5 $\mu\text{g}$
Volume of ribosome footprint RNA	26 $\mu\text{L}$
Ribo-Zero Removal Solution	10 $\mu\text{L}$
Ribo-Zero Reaction Buffer	4 $\mu\text{L}$
Total	40 $\mu\text{L}$

Table 1 Hybridization mixture

##### rRNA depletion

- 4.3 Transfer the solution obtained from the step of 4.2 to the tube that contains 65  $\mu\text{L}$  of rRNA depletion magnetic beads from the step of 4.1, pipette the mixture well and incubate it at RT for 10 min. Place the tube on the magnetic stand until the liquid becomes clear and then transfer the supernatant to a new 1.5 mL DNase/RNase-free tube. Keep the rRNA-depleted ribosome footprints in ice bath (safety stop point and the rRNA-depleted ribosome footprints can be stored at  $-25^\circ\text{C} \sim -15^\circ\text{C}$  overnight or at  $-80^\circ\text{C} \sim -65^\circ\text{C}$  for one month).

##### Purification of rice ribosome footprints by R1015 kit

- 4.4 The rRNA-depleted ribosome footprints are purified and recovered with Zymo RNA clean and concentrator kit R1015 according to the manufacturer's manuals with some modifications: make the volume of rRNA-depleted ribosome footprint solution to 100  $\mu\text{L}$  by adding RNase-free  $\text{H}_2\text{O}$ ; then add 200  $\mu\text{L}$  of RNA Binding Buffer and 450  $\mu\text{L}$  of 100% (V/V) ethanol to the 100  $\mu\text{L}$  rRNA-depleted footprint solution and mix well; transfer the resulted mixture to a column that is provided in the R1015 kit and centrifuge with 12,000 g at RT for 30 s. The obtained filtrate is transferred to the same column and the centrifugation is repeated once more. Discard the filtrate and the column is washed by 400  $\mu\text{L}$  of RNA Pre Buffer with 12,000 g centrifugation at RT for 30 s, followed by 700  $\mu\text{L}$  and 400  $\mu\text{L}$  of RNA Wash Buffer with 12,000 g centrifugation at RT for 30 s and 2 min, respectively. Finally, the ribosome footprints bound in the column are eluted by 2 min incubation of the column with 11  $\mu\text{L}$  RNase-free  $\text{H}_2\text{O}$  and two rounds of 16,000 g centrifugation at RT for 1 min.

##### PAGE purification of rice ribosome footprints

- 4.5 Prepare 15 mL of 1.00 mm 15% (W/V) urea-PAGE [6.3 g [urea](#) (SIGMA)] 5.625 mL [40% \(W/V\) Acrylamide/Bis](#) solution (Ambion), 0.75 mL  $10\times$  TBE buffer, 12  $\mu\text{L}$  10% (W/V) APS (SIGMA) solution and 9  $\mu\text{L}$  [TEMED](#) (SIGMA)]. Add 1  $\mu\text{L}$  of [6  \$\times\$  Blue/Orange loading dye](#) (Promega) to 5  $\mu\text{L}$  of control RNAs (28 nt control RNA: NNGUACACGGAGUCGACCCGCAACGCNN; and 30 nt control RNA: NNGUACACGGAGUCAAGACCCGCAACGCNN; 'N' represents A, or U, or C, or G.) that are provided in the TruSeq Mammalian Ribo Profile Kit (Illumina), and 2  $\mu\text{L}$  of 6  $\times$  Blue/Orange loading dye to the purified ribosome footprint solution (approximately 10  $\mu\text{L}$ ) from the step of 4.4, respectively. Mix the control RNA and footprint samples well and keep them in ice bath immediately after being treated in the dry bath at  $95^\circ\text{C}$  for 5 min. The cooled control and ribosome footprint samples are then separated in the 15% urea-PAGE by electrophoresis. The urea-PAGE is stained by pre-cooled [SYBR Gold](#) (Thermo) nucleic acid staining solution at  $4^\circ\text{C}$  for 10 min when the bromophenol blue reaches the bottom of gel. Gel slices that contain control RNAs and 28 ~ 30 nt ribosome footprints are recovered under a dark-field transilluminator (TGreen) and transferred to 2 mL DNase/RNase-free tubes. The gel slices are well ground and resuspended by adding 2  $\mu\text{L}$  of 10% (W/V) SDS, 40  $\mu\text{L}$  of 5 M acetic ammonium and 400  $\mu\text{L}$  of RNase-free  $\text{H}_2\text{O}$ . Place the 2 mL tubes on a Tube Revolver (MIULAB) and elute the control RNAs and ribosome footprints from the gel slices by continuously rotation with the speed of 30 rpm at  $4^\circ\text{C}$  overnight. Thereafter,

transfer the liquids from the 2 mL DNase/RNase-free tubes to [0.45 µm COSTAR Spin-X filters](#) (Corning) and centrifuge them with 12,000 g at RT for 10 min. Add 2 µL of [glycogen](#) (Invitrogen) and 700 µL [isopropanol](#) into the filtrates, mix well and then keep the filtrates at -25 °C ~ -15 °C overnight. After centrifugation with 16,000 g at 4 °C for 30 min ~ 1 h, the pellets of control RNA and ribosome footprint samples are washed with 80% (V/V) ethanol, air-dried and finally resuspended in 8 µL and 20 µL of RNase-free H<sub>2</sub>O, respectively.

### End repair for rice ribosome footprints

- 5 Prepare reaction mixture for end repair in a 200 µL DNase/RNase-free PCR tube based on Table 2. Pipette the mixture well and place the tube in a PCR machine (Bio-Rad) that is set as 37 °C for 1 h. The end-repaired ribosome footprints are purified and recovered with Zymo RNA clean and concentrator kit R1015: make the volume of end-repaired ribosome footprint solution to 100 µL by adding RNase-free H<sub>2</sub>O; then add 200 µL of RNA Binding Buffer and 450 µL of 100% (V/V) ethanol to the 100 µL rRNA-depleted footprint solution and mix well; transfer the resulted liquid to a column that is provided in the R1015 kit and centrifuge with 12,000 g at RT for 30 s. The obtained filtrate is transferred to the same column and the centrifugation is repeated once more. Discard the filtrate and the column is washed by 400 µL of RNA Pre Buffer with 12,000 g centrifugation at RT for 30 s, followed by 700 µL and 400 µL of RNA Wash Buffer with 12,000 g centrifugation at RT for 30 s and 2 min, respectively. Finally, the ribosome footprints bound by the column are eluted via 2 min incubation of the column with 11 µL RNase-free H<sub>2</sub>O and two rounds of 16,000 g centrifugation at RT for 1 min. Place the recovered ribosome footprint sample in ice bath for subsequent 3' adaptor ligation.

Component	Volume
Ribosome footprints	20 µL
TruSeq Ribo PNK Buffer	7.5 µL
TruSeq Ribo PNK	3 µL
RNase-free H <sub>2</sub> O	44.5 µL
Total	75 µL

Table 2 Reaction mixture for end repair

### 3' adapter ligation for repaired rice ribosome footprints

- 6 Add 1 µL of TruSeq Ribo Profile 3' Adapter (AGATCGGAAGAGCACACGTCT) into the control RNA sample from the step of 4.5 and the end-repaired ribosome footprint sample from the step 5, respectively, and then place these samples in the PCR machine that is set as 65 °C for 2 min followed by 4 °C for ever. Thereafter, 3.5 µL of TruSeq Ribo Profile Ligation Buffer, 1 µL of 100 mM DTT and 1.5 µL of TruSeq Ribo Profile Ligase are added into both the control RNAs and ribosome footprint sample, mixed well and then placed in the PCR machine that is set as 23 °C for 2 h. Finally, 2 µL of TruSeq Ribo Profile AR Enzyme is added into both the control and ribosome footprint samples, mixed well and kept at 30 °C for 2 h in the PCR machine. The resulted samples are placed in ice bath.

### Reverse transcription

- 7 Add 4.5 µL of TruSeq Ribo Profile RT Reaction Mix, 1.5 µL of 100 mM DTT, 1 µL of EpiScript RT and 6 µL of RNase-free H<sub>2</sub>O into both the control RNA and ribosome footprint samples from the step 6, mix well and then keep them at 50 °C for 30 min in the PCR machine. Thereafter, 1 µL of TruSeq Ribo Profile Exonuclease is added into the control and ribosome footprint samples, mixed well and kept at 37 °C for 30 min, at 80 °C for 15 min and at 4 °C for ever in the PCR machine (safety stop point and the obtained control and ribosome footprint libraries can be kept at -20 °C or lower for long-term storage). Thereafter, add 1 µL of TruSeq Ribo Profile RNase Mix into the control and ribosome footprint libraries, mix well and place these samples in the PCR machine that is set as 55 °C for 5 min to remove the remaining RNAs in the libraries.

### Purification of rice ribosome footprint library

8

#### Purification of library by R1015 kit

- 8.1 Make the volume of control and ribosome footprint libraries to 50 µL by adding RNase-free H<sub>2</sub>O; then add 100 µL of RNA Binding Buffer and 150 µL of 100% (V/V) ethanol to the 50 µL control and ribosome footprint libraries. Mix the libraries well, transfer the resulted liquids to columns that are provided in the R1015 kit and centrifuge with 12,000 g at RT for 30 s. The obtained filtrates are transferred to the same columns again and the centrifugation is performed once more. Discard the filtrates and the columns are washed by 400 µL of RNA Pre Buffer with 12,000 g centrifugation at RT for 30 s, followed by 700 µL and 400 µL of RNA Wash Buffer with 12,000 g centrifugation at RT for 30 s and 2 min, respectively. Finally, the control and ribosome footprint libraries bound by the columns are eluted via 2 min incubation of the columns with 11 µL RNase-free H<sub>2</sub>O and two rounds of 16,000 g centrifugation at RT for 1 min. Place the recovered libraries in ice bath.

#### Purification of library by 10% urea-PAGE

- 8.2 Prepare 15 mL of 1.00 mM 10% (W/V) urea-PAGE [6.3 g urea, 3.75 mL 40% (W/V) Acrylamide/Bis solution, 0.75 mL 10 × TBE buffer, 12 μL 10% (W/V) APS solution and 9 μL TEMED]. Add 2 μL of 6 × Blue/Orange loading dye to both the control and ribosome footprint libraries (approximately 10 μL) from the step of 8.1. Mix the libraries well and then keep them in ice bath immediately after being treated in the dry bath at 95 °C for 5 min. The cooled control and ribosome footprint libraries are then separated in the 10% urea-PAGE by electrophoresis. The urea-PAGE is stained by pre-cooled SYBR Gold nucleic acid staining solution at 4 °C for 10 min when the bromophenol blue migrates out of gel. Gel slices that contain 70 ~ 90 nt control and ribosome footprint libraries are recovered under the dark-field transilluminator and transferred to 2 mL DNase/RNase-free tubes. The gel slices are well ground and resuspended by adding 2 μL of 10% (W/V) SDS, 40 μL of 5 M acetic ammonium and 400 μL of RNase-free H<sub>2</sub>O. Place the 2 mL tubes on the Tube Revolver and elute the control and ribosome footprint libraries from the gel slices by continuously rotation with the speed of 30 rpm at 4 °C overnight. Thereafter, transfer the mixture from the 2 mL tubes to 0.45 μm COSTAR Spin-X filters and centrifuge with 12,000 g at RT for 10 min. Add 2 μL of glycogen and 700 μL of isopropanol into the filtrates, mix well and then keep the filtrates at -25 °C ~ -15 °C overnight. After 16,000 g centrifugation at 4 °C for 30 min ~ 1 h, the pellets of control and ribosome footprint libraries are washed with 80% (V/V) ethanol, air-dried and finally resuspended in 10 μL nuclease-free H<sub>2</sub>O, respectively (safety stop point and all library samples can be stored at -20 °C or lower for a long term).

### Circularization and PCR enrichment for rice ribosome footprint library

- 9 Prepare circularization reaction mixture based on Table 3 and pipette it well. The mixture is kept in a PCR machine at 60 °C for 2 h and then ice-cooled. Prepare PCR enrichment mixture according to Table 4, pipette the mixture well and then start the enrichment reaction based on the following PCR procedure: 98 °C for 30 s; 94 °C for 15 s, 55 °C for 5 s, 65 °C for 10 s, 9-13 cycles; 4 °C for ever.

Component	Volume
cDNA	10 μL
TruSeq Ribo Profile CL Reaction Mix	4 μL
ATP	2 μL
MnCl <sub>2</sub>	2 μL
CircLigase	2 μL
Total	20 μL

Table 3 Reaction mixture for circularization

Component	Volume
Circularized cDNA	X μL
TruSeq Ribo Profile Forward PCR Primer*	2 μL
TruSeq Ribo Profile Index PCR Primers**	2 μL
2 × Phusion MasterMix	25 μL
Nuclease-free H <sub>2</sub> O	Y μL
Total	50 μL

Table 4 PCR reaction mixture for library enrichment

Note: \* the sequence of Forward Primer (AATGATACGGCGACCAACCGAGATCTACACGTTTCAGAGTTCTACAGTCCGACG)

\*\* the sequences of Index Primers (CAAGCAGAAGACGGCATACGAGAT**ATCACG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT) or (CAAGCAGAAGACGGCATACGAGAT**CGATGT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT) or (CAAGCAGAAGACGGCATACGAGAT**TTAGCG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT) or (CAAGCAGAAGACGGCATACGAGAT**TGACCA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT) or (CAAGCAGAAGACGGCATACGAGAT**ACAGTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT) or (CAAGCAGAAGACGGCATACGAGAT**GCCAAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT) or (CAAGCAGAAGACGGCATACGAGAT**CAGATC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT) or (CAAGCAGAAGACGGCATACGAGAT**ACTTGA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT) or (CAAGCAGAAGACGGCATACGAGAT**GATCAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT) or (CAAGCAGAAGACGGCATACGAGAT**TAGCTT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT) or (CAAGCAGAAGACGGCATACGAGAT**TGCTAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT) or (CAAGCAGAAGACGGCATACGAGAT**CTTGTA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT)

### Purification of enriched rice ribosome footprint library

#### Purification of enriched rice ribosome footprint library by Agencourt AMPure XP beads

- 10.1 Transfer the enriched control and ribosome footprint libraries from the step 9 to 90  $\mu\text{L}$  of Agencourt AMPure XP beads (Beckman) in 1.5 mL DNase/RNase-free tubes, pipette the mixture well and place the tubes on the magnetic stand until the liquid becomes clear. Discarding the supernatant, resuspend the beads in 200  $\mu\text{L}$  of 70% (V/V) ethanol by pipetting and then keep the tubes at RT for 5 min. Place the tubes on the magnetic stand until the mixture becomes clear. Discard supernatant and repeat the washing. The double-washed beads are air-dried and resuspended with 16  $\mu\text{L}$  nuclease-free  $\text{H}_2\text{O}$ . Place the tubes on the magnetic stand until the liquid becomes clear. Transfer the supernatant into new 1.5 mL DNase/RNase-free tubes and place the tubes in ice bath.

#### Purification of enriched rice ribosome footprint library by 8% native PAGE

- 10.2 Prepare 15 mL of 1.00 mm 8% (W/V) native PAGE [3 mL 40% (W/V) Acrylamide/Bis solution, 0.75 mL  $10\times$  TBE buffer, 12  $\mu\text{L}$  10% (W/V) APS solution and 9  $\mu\text{L}$  TEMED]. Add 3  $\mu\text{L}$  of  $6\times$  Blue/Orange loading dye into both the enriched control and ribosome footprint libraries (approximately 15  $\mu\text{L}$ ) from the step of 10.1. Mix the enriched libraries well and then separate them in the 8% native PAGE by electrophoresis. The native PAGE is stained by pre-cooled SYBR Gold (Thermo) nucleic acid staining solution at 4  $^{\circ}\text{C}$  for 10 min when the bromophenol blue reaches the bottom of gel. Gel slices that contain 140 ~ 160 nt enriched ribosome footprint library are recovered under the dark-field transilluminator and transferred to a 2 mL DNase/RNase-free tube. The gel slices are well ground and resuspended by adding 400  $\mu\text{L}$  of 0.4 N NaCl solution. Place the 2 mL tube on the Tube Revolver and elute the enriched ribosome footprint library from the gel slices by continuously rotation with the speed of 30 rpm at 4  $^{\circ}\text{C}$  overnight. Thereafter, transfer the mixture from the 2 mL tube to a 0.45  $\mu\text{m}$  COSTAR Spin-X filter and centrifuge with 12,000 g at RT for 10 min. Add 2  $\mu\text{L}$  of glycogen, 40  $\mu\text{L}$  of 3 M NaAc (pH5.2) and 1 mL of 100% (V/V) ethanol into the filtrate, mix well and then keep the filtrate at -80  $^{\circ}\text{C}$  ~ -65  $^{\circ}\text{C}$  overnight. After 16,000 g centrifugation at 4  $^{\circ}\text{C}$  for 30 min ~ 1 h, the pellet of enriched ribosome footprint library is washed with 80% ethanol, air-dried and finally resuspended in nuclease-free  $\text{H}_2\text{O}$  for subsequent sequencing analysis.

#### Notes

- 11
- 1) All solutions, reagents, tips and tubes etc. that are used in this protocol must be DNAase/RNAase-free.
  - 2) Polysome extraction buffer should be used immediately after being prepared and the concentration of each component should be strictly quantified, particularly for deoxycholic acid, of which high concentration could result in precipitation of ions in the buffer and failure of ribosome footprint isolation.
  - 3) For storage, the polysome extracts are better to be kept at -80 ~ -65  $^{\circ}\text{C}$  immediately after being frozen with liquid nitrogen.
  - 4) The fresh polysome extracts are better to be used for profile analysis.
  - 5) The amount of nuclease and digestion time could be influenced by tissue types and thus the optimization should be done sometimes.
  - 6) Do not use UV light to visualize the SYBR Gold-stained PAGE gel.
  - 7) Positive control should be included when the PAGE purification is done for rice ribosome footprints or library construction to facilitate the recovery of the samples with expected size.
  - 8) For the enrichment of library by PCR, the amount of template and cycle number should be optimized and excessive template or PCR cycles have to be avoided.



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