

## Protocol for Low Stringency Prolamine Zipcode RBP Capture Experiment

### Preparation of developing rice seed extract

All steps were performed at 4°C or on ice unless indicated. Developing rice seeds (*Oryza sativa* cv. Kitaake) were collected 12-14 days after flowering (DAF), frozen in liquid N<sub>2</sub>, and stored at -80 °C until use. Approximately 20 grams of seed were frozen in liquid N<sub>2</sub> and ground in a coffee grinder. The resulting powder was ground using a mortar and pestle in 25 ml grinding buffer (20 mM Tris-Cl, pH 7.5, 0.4 M sucrose, 1% Triton, 1 mM DTT, 10 μM leupeptin, 1 μM pepstatin, 1 mM PMSF). The extract was filtered through 2 layers of Miracloth and overlaid atop a 20 ml 60% sucrose cushion in 20 mM Tris-Cl, pH 7.5. The sample was centrifuged 2 min at 1000 rpm in a SW28 rotor to pellet starch. The upper layer and interface was removed and overlaid onto a new sucrose cushion and centrifuged at 15000 rpm for 15 min in a SW28 rotor to pellet the protein bodies. The upper layer and interface was removed and centrifuged for 45 min at 50000 rpm in a SW55 rotor. The resulting supernatant was dialyzed overnight against 20 mM HEPES, pH 7.5, with two changes of buffer. Protein concentration was measured using 5X Protein Assay Reagent (Cytoskeleton).

### RBP capture assay

Routinely, 75 μL of 10 mg/mL Streptavidin coated magnetic beads (Roche) were washed twice with 1 mL of oligo binding buffer (OBB; 10 mM Tris-HCl, pH 7.5, 150 mM NaOH). 5' biotinylated RNA oligos from the prolamine 5' CDS zipcode (GAGUUUGAUGUUUUAGGUCAAAGUUAUU AGGCAAUA) were purchased from Integrated DNA Technologies (Coralville, IA). 150 pmol of RNA in 150 μL of OBB were added per mg beads and incubated on ice for 30 min. Unbound RNA was then removed by washing twice with 1 mL of oligo wash buffer (OWB; 10 mM Tris-HCl, pH 7.5, 1 M NaCl). Before the protein extract was added, the beads were washed twice with Protein Binding Buffer (PBB, 20 mM Hepes-KOH, pH 7.5, 50 mM KCl). 1 ml of protein extract (3-5 mg/ml) was added to the beads as well as 50 mM KCl, 1 mM DTT, 10 μM leupeptin, 1 μM pepstatin, 1 mM PMSF, and 10 U/ml RNase inhibitor. Samples were rotated overnight at 4°C. After removal of unbound proteins, the beads were washed 5 times with 1 ml PBB. Bound proteins were first eluted with 30 μl 0.3 M MgCl<sub>2</sub> PBB, then 30 μl 1 M MgCl<sub>2</sub> PBB. The beads were reused for a second capture experiment after washing twice with 1 ml 1 M MgCl<sub>2</sub> PBB, then twice with 1 ml PBB. Fresh protein extract was added as above and samples were allowed to rotate 4-6 hours. The beads were washed and eluted as above. An RBP capture experiment typically consisted of 12 separate tubes of RNA-bound beads and protein extract done in replicate. Multiple experiments were performed until a suitable amount of material from the 0.3 M MgCl<sub>2</sub> elution (~35 μg protein) was obtained for SDS-PAGE and subsequent analysis by mass spectrometry.

Proteins from the 0.3 M MgCl<sub>2</sub> elution were precipitated with TCA (4 parts sample to 1 part 100% TCA) on ice for 60 min. After centrifugation at 15000 rpm for 15 min at 4°C in a microcentrifuge, the protein pellet was washed three times with 300 μl ice cold acetone. The pellet was resuspended in 6 M urea SDS PAGE sample buffer and incubated for 10 min at 30°C, then vortexed at room temperature for 20 min. Proteins were separated on a 12% acrylamide SDS PAGE gel and stained with SYPRO Ruby. The entire lane of proteins was cut into ~3 mm bands and frozen at -80°C.

### Mass spectrometry analysis

In-gel trypsin digestion was done according to a protocol obtained from the UCLA Pasarow Mass Spectrometry Laboratory. In brief, excised protein bands were destained, cut into ~1 mm cubes, and dehydrated for 30 min in acetonitrile. After drying in a SpeedVac, gel pieces were incubated first in 10 mM DTT, 100 mM  $\text{NH}_4\text{HCO}_3$  for 60 min at 56°C, followed by 55 mM iodoacetamide, 100 mM  $\text{NH}_4\text{HCO}_3$  for 45 min at room temperature in the dark. After a brief wash in 100 mM  $\text{NH}_4\text{HCO}_3$ , gel pieces were dehydrated in acetonitrile and then reswelled with 100 mM  $\text{NH}_4\text{HCO}_3$  for 30 min. Following a second dehydration in acetonitrile, the gel pieces were dried in a SpeedVac and reswelled with 50 mM  $\text{NH}_4\text{HCO}_3$ , 5 mM  $\text{CaCl}_2$ , 12.5 ng/ $\mu\text{L}$  Trypsin Gold (Promega) for 45 min on ice. Excess solution was removed and 20  $\mu\text{L}$  of 50 mM  $\text{NH}_4\text{HCO}_3$ , 5 mM  $\text{CaCl}_2$  was added before incubation overnight at 37°C. Peptides were extracted after a brief wash in 20 mM  $\text{NH}_4\text{HCO}_3$  by incubating three times in 5% formic acid, 50% acetonitrile for 20 min. Following a final incubation in acetonitrile, post-digestion washes and extractions were pooled and dried.

Samples were analyzed at the UCLA Pasarow Mass Spectrometry Laboratory by nano-liquid chromatography with data-dependent tandem mass spectrometry (nLC-MSMS) using a hybrid linear ion trap Fourier Transform Ion Cyclotron Resonance mass spectrometer (LTQ FT Ultra, Thermo Fisher Scientific, San Jose, California). After dissolution in 10  $\mu\text{L}$  0.1% formic acid, 1% acetonitrile (v/v) samples were injected onto a trapping column (3 cm, 100  $\mu\text{M}$ , C18, Micro-Tech) previously equilibrated in 100% A (A, 0.1% formic acid, 1% acetonitrile in water; B, 0.1% formic acid in acetonitrile) at a flow rate of 2  $\mu\text{L}/\text{min}$ . Following 10 min washing, the trapping column was eluted through a pre-equilibrated analytical column (15 cm, 75  $\mu\text{M}$ , C18, Micro-Tech) at a flow rate of 300 nL/min using a compound linear gradient (3 min at 95% A; 85% A, 15% B at 8 min; 65% A, 35% B at 18 min; 25% A, 75% B at 30 min and 90% A, 10% B at 50 min). Column eluent was directed to an uncoated pulled silica nanospray tip (Picotip FS360-20-10-N-5-C12, New Objective) at 2.4 kV for ionization without nebulizer gas. The mass spectrometer was operated in data-dependent mode with a precursor survey scan (350-2000 m/z) at 100,000 resolution, followed by data-dependent MSMS in the ion trap for the top six most intense precursor ions while employing optimized dynamic exclusion settings in recruiting those ions. Individual sequencing experiments were matched to protein sequences obtained from the NCBI *Oryza* database using Sequest software (ThermoFinnigan, San Jose) using trypsin as the default enzyme, a fixed modification of carbamidomethylation of cysteines, and a variable modification of methionine oxidation. Only hits with at least two fully or partially tryptic peptides were considered. MSMS spectra of doubly charged ions with cross correlation scores (Xcorr) greater than 2.5 and of triply charged ions greater than 3.7, as well as a normalized difference in correlation score of greater than 0.1, were considered a significant hit.