Density Gradient Fractionation of RNA from RNA-SIP Experiments

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Abstract

This protocol is following RNA-SIP experiments and RNA extraction to fractionate the RNA according to density. The protocol is based on Leuders et al. 2010.

Citation: Julie Huber Density Gradient Fractionation of RNA from RNA-SIP Experiments. **protocols.io** dx.doi.org/10.17504/protocols.io.iunceve
Published: 06 Jul 2017

Protocol

Step 1.
Label 15ml tubes with sample names. Do not run more then 6-8 samples. Always include at least one No RNA control.

Step 2.
Add 5.1ml of CsTFA. Using a 5ml syringe and 0.2um filter, add 5ml of CsTFA to each tube. Then, using a 1ml syringe and 0.2um filter add the 0.1ml.

Step 3.
Add 185ul formamide to each tube. Formamide should be stored in small aliquots in -20C freezer to decrease degradation.

Step 4.
Add 1ml minus volume of 750ng of RNA of Gradient Salt Solution. E.g. If you are going to add 20ul of RNA, then add 980ul of Gradient Salt Solution. Recipe for solution can be found in Neufeld et al. 2010, JoVe article.

Step 5.
Add 750ng of RNA. Can add 500-750ng, but no lower.

Step 6.

Mix gently by inverting. DO NOT vortex.

Step 7.
Set refractometer to nd-TC setting. Calibrate using MilliQ water.

Step 8.

Using 75ul from each tube, measure Refractive Index (RI) on refractometer. Each tube should measure 1.3729±0.0002. If RI is too high or too low, adjust using CsTFA (if too low) or Gradient Salt solution (too high). Add 50-100ul to adjust. Then mix and re-measure. DO NOT proceed until each tube is within range. Also measure pure CsTFA each time to check if there are changes in density.

Step 9.

Use 5ml serological pipette to fill each 5ml Beckman centrifuge tube. Fill to bottom of neck. Do not overfill! Place black caps on top. Note, Beckman tubes should be rinsed with 70% ethanol and dried ahead of time. Determine mass of each filled tube on scale. It is easiest if you place the tube in a small beaker before measuring. Make sure each tube has a comparable mass.

Step 10.
Pair up tubes with similar masses and load into rotor. MAKE SURE IT IS BALANCED. Place gold caps on top of tubes, then thread in the caps. Use ratchet to tighten.

Step 11.

Place rotor in ultracentrifuge. Run @37,000 rpm, 20C, for >60 hours. I run for 64 hours. Make sure program has NO BRAKE for deceleration option.

Step 12.

Ahead of time, do the following:

-Make 70% ethanol, place in freezer

-Aliquot 100% isopropanol into 50ml tube, place in freezer

-Make 50ml of colored MilliQ water with food dye or Resazurin (I use 500ul)

-Autoclave tubing for low-flow pump

-Set-up ring stand with test-tube clamp

-Sterilize and label 1.5 tubes for each sample. Label 1-12. Can use label maker.

-Place tubes in racks. I do 24 tubes per rack (2 samples)

Step 13.
Take rotor out of ultracentrifuge, unload tubes and place in black rack.

Step 14.
Set-up pump and ring-stand. Make sure pump is set to 0.42ml/min.

Step 15.
Prime tubing with colored MilliQ water.

Step 16.
Screw needle on end of tubing, prime again.

Step 17.
Place 5ml tube in clamp and ready 1.5ml tubes. Make sure tube 1 is the FIRST tube filled, this is the heaviest.

Step 18.
Puncture the bottom of 5ml tube with needle.

Step 19.
Place tube rack underneath 5ml tube. Make sure first tube has cap open.

Step 20.
Puncture top of 5ml tube with the primed needle attached to tubing.

Step 21.
Set timer for 12 minutes. Simultaneously, press start on pump and timer.10. Shift rack every minute to begin collection into new 1.5ml tube. There should be about 420ul per tube.

Step 22.
Measure and record the RI for each fraction. The middle fractions, tubes 6-7 should be 1.3729. If this is not the case, may need to re-run.

Step 23.
Repeat fractionation for each sample.

Step 24.

Add 1 volume of ice-cold 100% Isopropanol to each tube. Mix gently by inverting. Incubate in freezer for >30min. Can do overnight, although we have not tried this.

Step 25.

Spin tubes for 25min in microcentrifuge in COLD ROOM to precipitate RNA.

Step 26.

Pipet off liquid. Try not to touch bottom, disturb the pellet.

Step 27.

Add 150ul of 70% ethanol.