ABSTRACT

The following protocol describes how to perform an RNA-Stable Isotope Probing experiment. The scope of this protocol only covers the parts involving separating labelled RNA from unlabelled RNA using ultracentrifugation in a caesium trifluoroacetate density gradient and downstream quantification to evaluate whether the labelling and separation of the RNA were successful. Total RNA should be extracted from an environmental sample or an enrichment culture that was incubated with an isotopically-labelled substrate. Labelling can be of the carbon, oxygen or nitrogen in the RNA (or any combination of the 3). For environmental samples, we recommend extracting RNA using our protocol Total Nucleic Acids Extraction from Soil and purifying it using the Purification of RNA from Crude NA Extract protocol. This protocol is based on the following papers: Whiteley et al. (2007); Dumont et al. (2011); Angel and Conrad (2013). For a comprehensive discussion on how to design a SIP experiment and how to analyse the resulting data, we recommend referring to the recent book on the subject: Stable Isotope Probing: Methods and Protocols, especially chapters: 1-3 and 9-18.


Angel R, Conrad R (2013). Elucidating the microbial resuscitation cascade in biological soil crusts following a simulated rain event.. Environmental microbiology. https://doi.org/10.1111/1462-2920.12140

GUIDELINES

- **Design of SIP experiments.** SIP experiments are usually relatively complex, laborious, and time-consuming, and can, therefore, fail because of various reasons and at different stages. Therefore, the design of a SIP experiment should be carefully considered in advance and cover all aspects and phases, including preliminary knowledge of the environment and the targeted process, the nature and duration of the incubation, how many and what types of controls to include, how many fractions to collect and how deep to sequence. These considerations extend beyond the scope of this protocol. Comprehensive discussions and tips on how to best design a SIP experiment can be found at Angel (2019) and Sieradzki et al. (2020).

- **RNA handling.** Since RNA is very sensitive to both chemical and enzymatic degradation, some precautionary measures should be taken. The RNA molecules are protected from degradation while in the CsTFA gradient but are sensitive to degradation during the precipitation and washing steps and downstream applications. For more info see Total Nucleic Acids Extraction from Soil.

- **Reducing the volume required for the refractometer.** The typical handheld-refractometer such as the Reichert AR200 has a large lens size requiring 50-100 µl of liquid to cover its surface adequately. To minimise the volume of wasted sample, it is possible to cover the lens with a piece of strong dark adhesive tape, to which a hole was made using a perforator.

- **Timing.** The timings for each step listed SIP protocol assume that only two gradients are being processed simultaneously. We recommend processing more than 4-8 gradients at a time, but not more.

- **Data analysis.** Several statistical frameworks have been developed in recent years to analyse SIP datasets such as qSIP (Hungate et al., 2015), HR-SIP (Youngblut et al., 2018) and HR-RNA-SIP (Angel et al., 2018).


MATERIALS TEXT

STEP MATERIALS

- **Trizma® hydrochloride / Tris-HCl** Merck Millipore
  Sigma Catalog # T5941
- **Potassium chloride (KCl)** Sigma
  Aldrich Catalog # P9333
- **Ethylenediaminetetraacetic acid disodium salt dihydrate BioUltra 98.5-101.5%** Sigma
  Aldrich Catalog # E1644-100G
- **Cesium Trifluoroacetate (CsTFA) illustra™** Thermo Fisher
  Scientific Catalog # 45-000-147
- **Hi-Di Formamide** Thermo Fisher
  Scientific Catalog # 4311320

Citation: Roey Angel, Eva Petrova, Ana Lara (09/01/2020). RNA-Stable Isotope Probing. https://dx.doi.org/10.17504/protocols.io.bjaakiae

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SAFETY WARNINGS

CaTFA is considered hazardous by the OSHA Hazard Communication Standard (29 CFR 1910.1200). Causes respiratory tract, eye and skin irritation. May be harmful if swallowed. Do not ingest. Avoid breathing vapour or mist. Use only with adequate ventilation. Avoid contact with eyes, skin and clothing. Keep container tightly closed. Wash thoroughly after handling.

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**BEFORE STARTING**

1. Prepare all buffers and solutions in advance (see Step 1).
2. Wipe all surfaces and apparatus with an RNase eliminating solution (e.g. RNAse Away).
3. Equilibrate CSTFA to room temperature (about 30-60 min).
4. Prepare one 50 ml tube (for up to 8 gradients; depending on the size of the centrifugation tubes) and one ultracentrifugation tube for each gradient.

---

### Solutions for SIP **1h**

1. **Prepare the following solutions**
   Use clean and preferably baked glassware (make sure all non-glass components can withstand the high temperatures).

1.1 **Gradient buffer (0.1 M Tris-HCl, 0.1 M KCl, 1 mM EDTA) [pH 8.0]**
   - 15.76 g Tris-HCl
   - 7.455 g KCl
   - 0.37224 g EDTA

   Dissolve the salts in RNase-free water and fill up to 1000 ml. Filter sterilise (0.1-0.2 μm). Autoclave.

   - [Trizma® hydrochloride / Tris-HCl](https://www.sigmaaldrich.com/catalog/product/T5941)
   - [Potassium chloride (KCl)](https://www.sigmaaldrich.com/catalog/product/P9333)
   - [Ethylene diaminetetraacetic acid disodium salt dihydrate BioUltra 98.5-101.5%](https://www.sigmaaldrich.com/catalog/product/E1644-100G)

   Store at **Room temperature**

1.2 **Molecular-grade ethanol solution (75%)**
   - 75 mL Absolute ethanol
   - 25 mL RNase-free water

   Store at **-20 °C**

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2 Calibrate the refractometer with RNAse-free water at \( \pm 20 \, ^\circ\text{C} \).

\[ 30 \, \mu\text{l} \, \text{RNAse-free water} \]

Following calibration, the device should read \( 1.3330 \pm 0.0002 \, \text{nD-TC} \).

AR200 Automatic Digital Refractometer

Digital Refractometer

Reichert 13950000

3

For every two gradients and if using Ultracrimp, PA centrifugation tubes (6 ml), mix the following in a 50 ml tube:

\[ 9.696 \, \text{mL CsFTA} \]
\[ 2.166 \, \text{mL Gradient Buffer} \]
\[ \text{Room temperature} \]

Adjust the volumes if using different-sized ultracentrifugation tubes.

Cesium Trifluoroacetate (CsTFA) illustra™ Thermo Fisher Scientific Catalog #45-000-147

Thermo Scientific TUBE PA ULTRACRIMP 6ML PK/50 Ultracentrifugation tubes

Thermo Fisher Scientific 03945

4 Mix by inverting several times, pipette 30 \( \mu\text{l} \), and measure the density in a refractometer. Make sure the density is: \( 1.3702 \pm 0.0002 \, \text{nD-TC} \). Otherwise, add either CsTFA or GB to correct.

5 Add 3.56% vol HiDi (422 \( \mu\text{l} \) if the volume was not corrected).

422 \( \mu\text{l} \) HiDi

Hi-Di Formamide Thermo Fisher Scientific Catalog # 4311320

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6 Measure the density. Make sure the density is: $1.3725 \pm 0.0002$ nD-TC.

Due to potential variance between batches, it is recommended to add a slightly lower volume of HiDi at first to avoid exceeding the recommended value.

7 Transfer approx. 5.8 ml of the mixture to each centrifugation tube using a micropipette. Make sure the volume reaches only the bottom of the neck.

5.8 mL gradient mix solution

8 Add the RNA. For downstream PCR purposes, ca. 200-350 ng are more than enough. Preferably, use a highly concentrated RNA solution to avoid diluting the gradient (ca 2-8 μl). The amount of RNA should not exceed 100 ng per 1 ml of gradient mixture.

4 pl RNA (1-8)

150 μg/μl RNA (75-600)

9 Weigh each tube together with the caps and make sure every opposite pair of tubes is no more than 0.1 g different from each other. Otherwise, adjust the weight by adding gradient mix solution.

10 Close the caps (using an appropriate crimper or by hand, depending on the manufacturer).

Thermo Scientific TOOL ULTRACRIMP EA
Tube crimper
Thermo Fisher Scientific 03920

11 Place the tubes in the rotor, screw only the cap with tubes in them using the torque wrench up to about 120 in.-lb.

Ultracentrifugation 2d 17h

12 Centrifuge

$130000 \times g$, 20°C, 65:00:00, (37,900 rpm for the TV-1665 rotor)

Maximum acceleration and deceleration.

Because the density gradient will stabilise over time, centrifuging for longer time periods will make no difference but can be used for timing reasons. However, after the centrifugation has stopped the gradient will slowly diffuse back to its original state. Therefore, the gradients are best fractionated immediately.

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13. Prepare a rack filled with 2.0 ml low-binding collection tubes (one per fraction).

<table>
<thead>
<tr>
<th>DNA LoBind Tubes</th>
<th>Microcentrifuge tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eppendorf</td>
<td>0030108051</td>
</tr>
</tbody>
</table>

14. Fill a 30 ml syringe with RNase-free water. Remove any air bubbles.

15. Attach a female Luer fitting to one end of a precision pump tube (about 0.5 m long) and a male Luer fitting to the other end. Attach the syringe to the precision pump tube on the female Luer fitting side. Attach a sterile 23G needle to the other end of the tube on the male Luer fitting side. Lightly press the syringe piston to get water into the tube and mount the syringe on an automatic syringe pump.

<table>
<thead>
<tr>
<th>NE-300 Just Infusion™ Syringe Pump</th>
<th>Automatic syringe pump</th>
</tr>
</thead>
<tbody>
<tr>
<td>New Era Pump Systems, Inc. NE-300</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Masterflex L/S® Precision Pump Tubing, Peroxide-Cured Silicone, L/S 14; 25 ft Silicone tube</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Masterflex 96400-14</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Masterflex Fitting, Polycarbonate, Straight, Female Luer to Low-Profile Semi-Rigid Barb Hose Adapter, 1/16&quot; ID; 25/PK Luer fitting</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Masterflex 45501-16</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Masterflex Fitting, Polypropylene, Straight, Male Luer Lock to Hose Barb Adapter, 1/16&quot; ID; 25/PK Luer fitting</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Masterflex 30800-16</td>
<td></td>
</tr>
</tbody>
</table>
16 Set the volume to $1 \text{ ml min}^{-1}$ and collect fractions in 30 s steps. If using a 6 ml tube, this will yield 12 fractions. Volume should be set to "off" and diameter to "22 mm".

For collecting more or fewer fractions, adjust the speed or collection rate.

Using a different syringe (other than 30 ml) will require adjusting the inner diameter setting on the pump.

17 Switch the pump on to test the system and also to get rid of air trapped inside the needle and any air bubbles in the tube. Switch the pump back off.

18 Stop the ultracentrifuge. Remove the rotor and open the screw-caps. Take the first tube out of the rotor and carefully mount it on a stand with a clamp holder just above the collection tubes.

19 Pierce the ultracentrifugation tube, just below the neck, using the needle attached to to the precision pump tube.
Be careful not to pierce through the other end of the tube!

20 Take a new, sterile **26G** needle, carefully puncture a hole at the bottom of the ultracentrifugation tube and remove the needle. The tube should not leak at this stage.

![](image)

Disposable needles Sterican® long bevel facet, 25 mm, 0.45 mm, Brown
Disposable needles Sterican c718.1

21 Open all the collection tubes in the rack and make sure the first tube is positioned just below the bottom hole of the ultracentrifugation tube.
Your set-up should look like this:

![](image)

The SIP fraction collection set-up ready to start

22 Start the pump, as soon as the first drop falls off the ultracentrifugation tube start the stopwatch

23 After **30 s** (or your chosen time interval), shift the rack so that the drops will fall into the second collection tube. Continue in a similar fashion until all tubes have been filled. Close the tubes to avoid contamination and label them.

24 Measure the density of each fraction using the refractometer. Start from the last (the lightest) fraction.

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30 µl of each fraction

The density of the fractions should increase at a linear rate as you progress from the lighter to the heavier fraction. The conversion between refractive index (n) to density (ρ) is (empirically):

\[ ρ = 31.495n - 41.439 \]

And can be easily determined in the lab by weighing a known volume of several fractions and establishing a calibration curve. The gradient should be in the range of 1.77-1.80 g ml\(^{-1}\), assuming a vertical rotor was used (a fixed-angle rotor will yield a steeper gradient, meaning a wider range of densities).

Typically the first and last fractions are discarded because they contain little to no nucleic acids.

RNA precipitation

To each tube add 2 µl of GlycoBlue, 0.1 volumes Na-Acetate (\(3 M\) Molarity (M)), and 2.5 volumes of absolute ethanol. Assuming 500 µl fractions were collected and 30 µl were spent for determining the density, add 47 µl Na-acetate and 1175 µl ethanol (absolute).

GlycoBlue™ coprecipitant Thermo Fisher
Scientific Catalog # AM9515

3M Na-Acetate pH 5.5 Thermo Fisher
Scientific Catalog # AM9740

GlycoBlue is particularly advantageous here because otherwise, the pellet is completely invisible.

Incubate at -80 °C for 00:30:00.

Centrifuge at 14000 rpm, 4°C, 00:30:00.

Decant the supernatant, wash once with 1 mL 75% ethanol, ice-cold, invert the tube several times.

The pellet should be stable at this point and not detach from the tube's wall.
29  
Centrifuge at **14000 rpm, 4°C, 00:10:00**.

30  
Remove as much as possible from the supernatant first using a 1 ml tip, spin down the remaining drops in the tube, and remove them with a 100 µl tip.

The pellet is unstable at this point. Be careful not to pipette the pellet with the liquid!

31  
Leave the tubes open at room temperature for around 5 min (preferably under a flame or in a laminar-flow hood) in order to evaporate the remaining ethanol. Alternatively, the pellets can be dried under a filtered stream of air. **00:05:00 maximum time for drying**

The pellets might not be completely dry at this point, but the remaining liquid should be pure water.

32  
Resuspend the pellets in **10 µl** RNase-free water or the RNA Storage solution.

**THE RNA Storage Solution** Thermo Fisher  
Scientific Catalog #AM7000

cDNA synthesis **2h**

33  
For each fraction, prepare the following mixture in a PCR tube:

1. **10 µl** template RNA
2. **3 µl** random hexamers (**50 Micromolar** (µM)) diluted 20x in RNase-free water:
   **2.5 Micromolar** (µM)

**Random hexamers** Thermo  
Scientific Catalog #N8080127

34  
Incubate the mixture at **65 °C** for **00:05:00** in a thermocycler and chill at **4 °C** for at least **00:01:00**.

35  
Prepare the following mixture (times the number of fractions) and add **7 µl** into each tube:

1. **4 µl** 5x Reaction buffer
2. **1 µl** 10 mM dNTP mix
3. **1 µl** 0.1 M DTT (optional)
4. **0.2 µl** RNase OUT (40 U/µl; optional)
5. **0.2 µl** BSA (20 µg/µl)

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6. **0.1 µl** SuperScript IV RT (200 U/µl)
7. **0.5 µl** RNase-free water

   - **SuperScript™ IV First-Strand Synthesis System** Thermo Fisher
     Scientific Catalog #18091050

   - **RNaseOUT™ Recombinant Ribonuclease Inhibitor** Thermo Fisher
     Scientific Catalog #10777019

   - **Bovine Serum Albumin (BSA)** Thermo Fisher
     Scientific Catalog #B14

   - **dNTP Mix (10 mM each)** Thermo Fisher
     Scientific Catalog #R0191

   - **USB Dithiothreitol (DTT) 0.1M Solution** Thermo Fisher
     Scientific Catalog #707265ML

36. Incubate the mixture in a thermocycler for **00:10:00** at **23 °C**, followed by **01:00:00** at **50 °C** and then **00:10:00** at **80 °C**. Chill at **4 °C**.

37. Dilute **1 µl** cDNA in **14 µl** RNase-free water for use as qPCR template. No dilution is required for use as a PCR template.

   This dilution step here is required to not exceed the range of detection of the qPCR assay. Higher or lower dilutions might be required depending on the amount of RNA that was loaded on the gradient and the recovery efficiency.

**Evaluate the level of enrichment** 2h 30m

38. Evaluate the level of isotopic enrichment using a qPCR assay. We recommend:

```
qPCR: Bacterial SSU rRNA 338F-516P-805R
by Roey Angel,
Soil and Water Research Infrastructure
```

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Name | Type | Sequence | Target region
--- | --- | --- | ---
BAC338F | Forward | ACT CCT ACG GGA GGC AG | 338-354
BAC516P | Probe | TGC CAG CAG CGG TAA TA | 516-536
BAC805R | Reverse | GAC TAC CAG GGT ATC TAA TC | 785-805

1. Relative to \( E.\) coli SSU rRNA gene
2. The probe must be dual-labelled either with 5'-6-FAM, 3'-BHQ1 or any other valid combination

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final concentration</th>
<th>1 tube (20 μl)</th>
<th>plate (20 μl x 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR H₂O</td>
<td></td>
<td>4.6</td>
<td>460</td>
</tr>
<tr>
<td>iQ(^\text{TM}) Supermix</td>
<td>1x</td>
<td>10</td>
<td>1000</td>
</tr>
<tr>
<td>MgCl₂ (25 mM)</td>
<td>4.0 mM</td>
<td>0.8(^1)</td>
<td>80</td>
</tr>
<tr>
<td>BSA (20 μg μl(^{-1}))</td>
<td>0.2 μg μl(^{-1})</td>
<td>0.2</td>
<td>20</td>
</tr>
<tr>
<td>338F (10 μM)</td>
<td>0.5 μM</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>805R (10 μM)</td>
<td>0.5 μM</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>516P (10 μM)</td>
<td>0.2 μM</td>
<td>0.4</td>
<td>40</td>
</tr>
<tr>
<td>Template</td>
<td></td>
<td>2</td>
<td>2 x 100</td>
</tr>
</tbody>
</table>

\(^1\) Buffer contains MgCl₂ at final conc. of 3.0 mM

38.3
1. \( \theta\) 95 °C for \( \Theta\) 00:05:00
2. x 40 {
   2.1 \( \theta\) 95 °C for \( \Theta\) 00:00:30
   2.2 \( \theta\) 62 °C for \( \Theta\) 00:00:30 take snapshot
}

39

Plot the cDNA copy numbers against the density of each fraction. It is common to normalise the qPCR results to the highest copy number in the gradient or to the total copy numbers of all the fractions in the gradient.

Expect a peak of unlabelled RNA at around 1.78 g ml\(^{-1}\) and a peak of labelled RNA at around 1.82 g ml\(^{-1}\)

If the amount of labelled RNA is too small it might not be visible through qPCR. However, it might still be detectable through qSIP or HT-SIP analysis (see e.g. Youngblut et al., 2018, Angel, 2019)


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https://doi.org/10.1007/978-1-4939-9721-3_1

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