RNA-Stable Isotope Probing V.7

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SoWa RI Anaerobic and Molecular Microbiology (public)
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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.

Whiteley AS, Thomson B, Lueders T, Manefield M (2007). RNA stable-isotope probing. Nature protocols.
http://10.1038/nprot.2007.115

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

Dumont MG, Pommerenke B, Casper P, Conrad R (2011). DNA-, rRNA- and mRNA-based stable isotope probing of aerobic methanotrophs in lake sediment.. Environmental microbiology.
https://doi.org/10.1111/j.1462-2920.2010.02415.x

DOI

dx.doi.org/10.17504/protocols.io.bx3dpqi6

PROTOCOL CITATION

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MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Angel, R., and Conrad, R. (2013). Elucidating the microbial resuscitation cascade in biological soil crusts following a simulated rain event. Environ Microbiol 15, 2799–2815. doi:10.1111/1462-2920.12140.

WHAT'S NEW

Added an option for preparing CsTFA solution from powder

KEYWORDS

Stable isotope probing, SIP, RNA, Ultracentrifugation, Density gradient

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53061

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).

Angel R (2019). Experimental Setup and Data Analysis Considerations for DNA- and RNA-SIP Experiments in the Omics Era. Methods in molecular biology (Clifton, N.J.). https://doi.org/10.1007/978-1-4939-9721-3_1

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Sieradzki ET, Koch BJ, Greenlon A, Sachdeva R, Malmstrom RR, Mau RL, Blazewicz SJ, Firestone MK, Hofmockel KS, Schwartz E, Hungate BA, Pett-Ridge J (2020). Measurement Error and Resolution in Quantitative Stable Isotope Probing: Implications for Experimental Design. mSystems.
https://doi.org/pii:e00151-20.10.1128/mSystems.00151-20

Youngblut ND, Barnett SE, Buckley DH (2018). HTSSIP: An R package for analysis of high throughput sequencing data from nucleic acid stable isotope probing (SIP) experiments. PloS one.
https://doi.org/10.1371/journal.pone.0189616

Hungate BA, Mau RL, Schwartz E, Caporaso JG, Dijkstra P, van Gestel N, Koch BJ, Liu CM, McHugh TA, Marks JC, Morrissey EM, Price LB (2015). Quantitative microbial ecology through stable isotope probing. Applied and environmental microbiology.
https://doi.org/10.1128/AEM.02280-15

Angel R, Panhölzl C, Gabriel R, Herbold C, Wanek W, Richter A, Eichorst SA, Woebken D (2018). Application of stable-isotope labelling techniques for the detection of active diazotrophs. Environmental microbiology.
https://doi.org/10.1111/1462-2920.13954

MATERIALS TEXT

STEP MATERIALS

Trizma® hydrochloride / Tris-HCl **Merck Millipore Sigma Catalog #T5941** Step 1.1

Potassium chloride (KCl) **Sigma Aldrich Catalog #P9333** Step 1.1

Ethylenediaminetetraacetic acid disodium salt dihydrate BioUltra 98.5-101.5% **Sigma Aldrich Catalog #E1644-100G** Step 1.1

Cesium Trifluoroacetate (CsTFA) illustra™ **Thermo Fisher Scientific Catalog #45-000-147** Step 3

Hi-Di Formamide **Thermo Fisher Scientific Catalog # 4311320** Step 5

GlycoBlue™ coprecipitant **Thermo Fisher Scientific Catalog # AM9515** Step 25

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3M Na-Acetate pH 5.5 **Thermo Fisher**

Scientific Catalog # AM9740 Step 25

THE RNA Storage Solution **Thermo Fisher**

Scientific Catalog #AM7000 Step 32

Random hexamers **Thermo**

Scientific Catalog #N8080127 Step 33

Bovine Serum Albumin (BSA) **Thermo Fisher**

Scientific Catalog #B14 Step 35

dNTP Mix (10 mM each) **Thermo Fisher**

Scientific Catalog #R0191 Step 35

USB Dithiothreitol (DTT) 0.1M Solution **Thermo Fisher**

Scientific Catalog #707265ML Step 35

SuperScript™ IV First-Strand Synthesis System **Thermo Fisher**

Scientific Catalog #18091050 Step 35

RNaseOUT™ Recombinant Ribonuclease Inhibitor **Thermo Fisher**

Scientific Catalog #10777019 Step 35

Apparatus

1) For gradient preparation
- Working bench in a climatised room at 20 °C
- Icebox
- 50 ml tube (for up to 8 gradients)
- Ultracentrifuge (capable of achieving 177,000 g) and a vertical rotor (e.g. Sorvall WX Ultra 100 Ultracentrifuge, TV-1665 rotor). A fixed-angle
- Ultracentrifugation tubes (e.g. Ultracrimp, PA centrifugation tubes 6 ml)
- Ultracentrifugation tube caps
- Refractometer
- Purified RNA samples (DNA-free) with a concentration >20 ng μl^{-1}
- Micropipettes and tips
- Lab-scale

2) For fractionation
- Working bench in a climatised room at 20°C
- Refractometer
- Low-binding tubes (one per fraction; 1.5 ml)
- Test tube utility clamp mounted on a stand
- Automatic syringe pump (e.g. NewEra's NE-300 Syringe Pump)
- 20 ml syringe
- Precision pump peroxide-cured silicone tube (or similar), 1/16", about 0.5-1 m long
- Luer fittings (1/16"), male and female, to fit the tube on a syringe on one end and a disposable needle on the other end
- Disposable syringe needles: 23G and 26G
- Stopwatch
- Micropipettes and tips

SAFETY WARNINGS

CsTFA 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.

HiDi-formamide may damage fertility or the unborn child if swallowed. Suspected of causing cancer if swallowed. May cause damage to organs through prolonged or repeated exposure.
Do not breathe fumes or spray. Wear protective gloves/protective clothing/eye protection/face

Citation: Roey Angel, Eva Petrova, Ana Lara (09/08/2021). RNA-Stable Isotope Probing. https://dx.doi.org/10.17504/protocols.io.bx3dpqi6

protection.

- **Storage and waste**
- Store below eye level to prevent injuries in case of a spill.
- Dispose of CsTFA and HiDi-formamide in a sealed container as hazardous waste.

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.

RNase AWAY™ Surface Decontaminant **Thermo Fisher Scientific Catalog #7002PK**

Solutions for SIP 1h

1 Prepare the following solutions:

All glassware and plasticware must be clean and free of RNA and RNAse. Glassware can be baked at **180 °C** for **04:00:00**

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 **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**

Store at **Room temperature**

1.2 Molecular-grade ethanol solution ([M] **75 % (v/v)**):

75 ml Absolute ethanol

25 ml RNase-free water

Store at **-20 °C**

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1.3

If preparing CsTFA solution from powder, mix **17.2 ml gradient buffer** with **50 g CsTFA** mix at **Room temperature** until fully dissolved. If particle impurities are visible filter using a 0.1 or 0.2 µm filter.

Cesium trifluoroacetate **Thermo Fisher Scientific Catalog #44633.18**

Gradient preparation 1h

2 Calibrate the refractometer with RNAse-free water at **20 °C** .

30 µl RNAse-free water

Following calibration, the device should read **1.3330 ± 0.0002 nD-TC**

AR200 Automatic Digital Refractometer
Digital Refractometer
Reichert 13950000

3

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

9.696 ml CsFTA

2.166 ml Gradient Buffer

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

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4 Mix by inverting several times, pipette **30 µl** and measure the density in a refractometer. Make sure the density is: **1.3702 ± 0.0002 nD-TC.** Otherwise, add either CsTFA or GB to correct.

5

Add **3.56 % (v/v)** HiDi (**422 µl** if the volume was not corrected).

Hi-Di Formamide **Thermo Fisher Scientific Catalog # 4311320**

6 Measure the density. Make sure the density is: **1.3725 ± 0.0002 nD-TC.**

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

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.

8 Add the RNA sample. For downstream PCR purposes, ca. 200-350 ng is more than enough. Preferably, use a highly concentrated RNA solution to avoid diluting the gradient.

4 µl RNA (1-8 µl)

150 ng/µl RNA (75-600 ng)

The amount of RNA should not exceed 100 ng per 1 ml of gradient mixture.

9 Weigh each tube together with the caps and make sure every opposite pair of tubes weighs no more than 0.1 g apart 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 type of tubes).

Thermo Scientific TOOL ULTRACRIMP EA
Tube crimper
Thermo Fisher Scientific 03920

11 Place the tubes in the rotor, screw only the caps for the positions that contain tubes using a torque wrench up to about 120 in.-lb.

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Ultracentrifugation 2d 17h

12

Centrifuge

130000 x 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 a longer time period 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.

Fractionation 1h

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

DNA LoBind Tubes
Microcentrifuge tubes
Eppendorf **0030108051**

14 Fill a 20 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.

NE-300 Just Infusion™ Syringe Pump
Automatic syringe pump
New Era Pump Systems, Inc. **NE-300**

Masterflex L/S® Precision Pump Tubing, Peroxide-Cured Silicone, L/S 14; 25 ft
Silicone tube
Masterflex **96400-14**

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Masterflex Fitting, Polycarbonate, Straight, Female Luer to Low-Profile Semi-Rigid Barb Hose Adapter, 1/16" ID; 25/PK
Luer fitting

Masterflex **45501-16**

Masterflex Fitting, Polypropylene, Straight, Male Luer Lock to Hose Barb Adapter, 1/16" ID; 25/PK
Luer fitting

Masterflex **30800-16**

Disposable needles Sterican® long bevel facet, 30 mm, 0.60 mm, Blue
Disposable needles

Sterican **X129.1**

16 Set the Rate to **1 ml min^{-1}** and collect fractions in **00:00:30** steps. If using a 6 ml tube, this will yield 12 fractions. 30s
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 20 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.

Make sure the tube stays upright during handling.

19

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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!
If the other end of the tube was accidentally pierced, a small amount of petroleum gel can be used to seal the hole.

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.

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:

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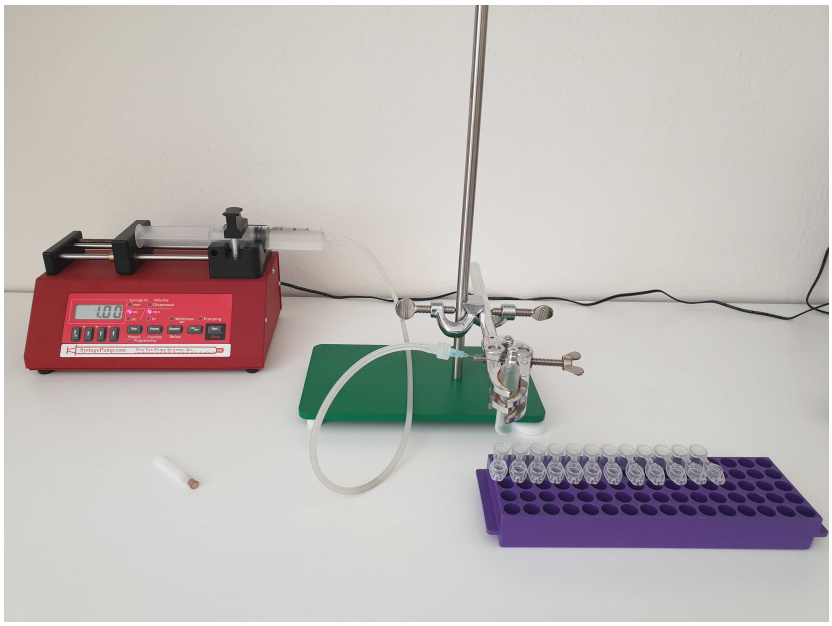

The SIP fraction collection set-up ready to start

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

23 After **00:00:30** (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. 30s

24

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

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):

$$\rho = 31.495n - 41.439$$

And can be easily determined in the lab by weighing a known volume of several fractions and establishing a calibration curve.

The gradient should range between 1.75 and 1.84 g ml^{-1}, assuming a vertical rotor was used (a fixed-angle rotor will yield a steeper gradient, meaning a wider range of densities).

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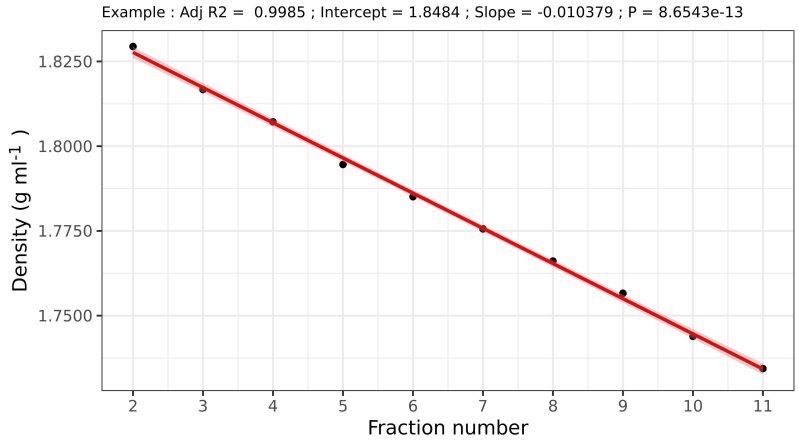

Typically the first and last fractions are discarded because they contain little to no nucleic acids.

RNA precipitation 2h

25

To each tube add **2 µl** of GlycoBlue, **10 % (v/v)** Na-Acetate (**3 Molarity (M)**), and **250 % (v/v)** 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.

26

Incubate at **-80 °C** for **00:30:00** .

27

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

28

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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:

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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)**
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

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

38.1

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Name	Type	Sequence	Target region[1]
BAC338F	Forward	ACT CCT ACG GGA GGC AG	338-354
BAC516P[2]	Probe	TGC CAG CAG CCG 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

38.2

Reagent	Final concentration	1 tube (20 µl)	plate (20 µl x 100)
PCR H_2O		4.6	460
iQ™ Supermix	1x	10	1000
$MgCl_2$ (25 mM)	4.0 mM	0.8[1]	80
BSA (20 µg µl^{-1})	0.2 µg µl^{-1}	0.2	20
338F (10 µM)	0.5 µM	1.0	100
805R (10 µM)	0.5 µM	1.0	100
516P (10 µM)	0.2 µM	0.4	40
Template		2	2 x 100

1 Buffer contains $MgCl_2$ at final conc. of 3.0 mM

38.3

1. **95 °C** for **00:05:00**
2. x 40 {
   2.1 **95 °C** for **00:00:30**
   2.2 **62 °C** for **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}

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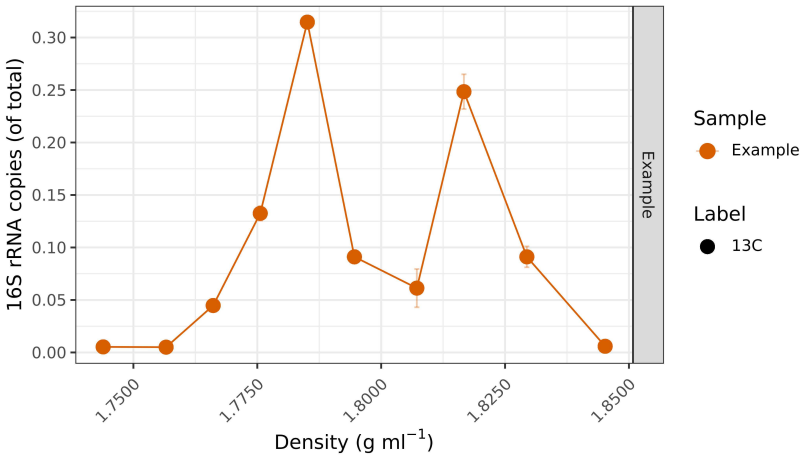

An example of successful labelling with ^{13}C, seen via the presence of a peak in the copy numbers around 1.82 g ml$_{-1}$

Plot_SIP_example.RMD | Frac_density_example.csv | qPCR_SIP_example.csv

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)

Youngblut ND, Barnett SE, Buckley DH (2018). HTSSIP: An R package for analysis of high throughput sequencing data from nucleic acid stable isotope probing (SIP) experiments. PloS one. https://doi.org/10.1371/journal.pone.0189616

Angel R (2019). Experimental Setup and Data Analysis Considerations for DNA- and RNA-SIP Experiments in the Omics Era. Methods in molecular biology (Clifton, N.J.). https://doi.org/10.1007/978-1-4939-9721-3_1

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