Jan 30, 2023 Version 9

RNA-Stable Isotope Probing V.9

DOI
https://dx.doi.org/10.17504/protocols.io.kxygxm23kl8j/v9

Roey Angel[1], Eva Petrova[1], Ana Lara[1]

[1]Soil and Water Research Infrastructure

Anaerobic and Molecular Microbiology Lab, Biology Centre CAS
Tech. support email: **eva.petrova@bc.cas.cz**

Roey Angel
Soil and Water Research Infrastructure

DOI: https://dx.doi.org/10.17504/protocols.io.kxygxm23kl8j/v9

Protocol Citation: Roey Angel, Eva Petrova, Ana Lara 2023. RNA-Stable Isotope Probing. **protocols.io** https://dx.doi.org/10.17504/protocols.io.kxygxm23kl8j/v9Version created by **Roey Angel**

Manuscript citation:
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.

Protocol status: Working
We use this protocol and it's working

Created: January 30, 2023

Last Modified: January 30, 2023

Protocol Integer ID: 76053

Keywords: Stable isotope probing, SIP, RNA, Ultracentrifugation, Density gradient, protocol total nucleic acids extraction from soil, protocol total nucleic acids extraction, purification of rna, extracting rna, stable isotope probing, separation of the rna, stable isotope probing experiment, labelled rna, nitrogen in the rna, stable isotope, rna from unlabelled rna, rna, unlabelled rna, crude na extract protocol, total rna, purification, environmental sample, extraction, labelled substrate, nitrogen, downstream quantification, sip experiment,

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.

Citation

Whiteley AS, Thomson B, Lueders T, Manefield M (2006). RNA stable-isotope probing. Nature protocols.

10.1038/nprot.2007.115 LINK

Citation

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 LINK

Citation

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 LINK

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**).

Citation

Angel R (2018)
. 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 LINK

Citation

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 LINK

Citation

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 LINK

Citation

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 LINK

Citation

Angel R, Panhölzl C, Gabriel R, Herbold C, Wanek W, Richter A, Eichorst SA, Woebken D (2017) . Application of stable-isotope labelling techniques for the detection of active diazotrophs. Environmental microbiology.

https://doi.org/10.1111/1462-2920.13954 LINK

Materials

STEP MATERIALS

- Trizma® hydrochloride / Tris-HCl **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T5941
- Potassium chloride (KCl) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P9333
- Ethylenediaminetetraacetic acid disodium salt dihydrate BioUltra 98.5-101.5% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**E1644-100G
- Cesium Trifluoroacetate (CsTFA) illustra™ **Thermo Fisher Scientific Catalog #**45-000-147
- Hi-Di Formamide **Thermo Fisher Scientific Catalog #** 4311320
- GlycoBlue™ coprecipitant **Thermo Fisher Scientific Catalog #** AM9515
- 3M Na-Acetate pH 5.5 **Thermo Fisher Scientific Catalog #** AM9740
- THE RNA Storage Solution **Thermo Fisher Scientific Catalog #**AM7000
- Random hexamers **Thermo Scientific Catalog #**N8080127
- 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
- SuperScript™ IV First-Strand Synthesis System **Thermo Fisher Scientific Catalog #**18091050
- RNaseOUT™ Recombinant Ribonuclease Inhibitor **Thermo Fisher Scientific Catalog #**10777019

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

Protocol materials

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

Ethylenediaminetetraacetic acid disodium salt dihydrate BioUltra 98.5-101.5% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**E1644-100G

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

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

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

Trizma® hydrochloride / Tris-HCl **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T5941

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

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

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

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

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

Random hexamers **Thermo Scientific Catalog #**N8080127

Potassium chloride (KCl) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P9333

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

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

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

Cesium Trifluoroacetate Solution **FUJIFILM Wako Pure Chemical Corporation Catalog #**031-13681

Safety warnings

Safety information

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 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 start

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:

Note

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 MilliporeSigma (Sigma-Aldrich) Catalog #**T5941

Potassium chloride (KCl) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P9333

Ethylenediaminetetraacetic acid disodium salt dihydrate BioUltra 98.5-101.5% **Merck MilliporeSigma (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

1.3 If preparing CsTFA solution from powder:

1. Pour 17.2 ml gradient buffer directly into a 50 g CsTFA vial.
2. Mix by hand at Room temperature and then allow for the residues to fully dissolve during several hours or overnight.
3. If particle impurities are visible, filter the solution into a fresh bottle using a 0.1 or 0.2 µm filter.
4. Store at 4 °C .

Note

1. CsTFA will not dissolve well in pure water, and the pH will be too low
2. CsTFA powder is highly hygroscopic. It is, therefore, recommended to dissolve the entire content of the bottle immediately.
3. It is recommended to dissolve the powder first, using only around 16.5 mL of gradient buffer and adjust it afterwards.
4. Confirm that the density is 2 g ml^{-1} using an analytical scale and adjust the solution if necessary.

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**

Equipment

AR200 Automatic Digital Refractometer	NAME
Digital Refractometer	TYPE
Reichert	BRAND
13950000	SKU
https://www.reichertai.com/products/handheld-digital/ar200-automatic-digital-refractometer/	LINK

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

Note

Adjust the volumes if using different-sized ultracentrifugation tubes.

Cesium Trifluoroacetate Solution **FUJIFILM Wako Pure Chemical Corporation Catalog #**031-13681

Equipment

Thermo Scientific TUBE PA ULTRACRIMP 6ML PK/50	NAME
Ultracentrifugation tubes	TYPE
Thermo Fisher Scientific	BRAND
03945	SKU
https://www.fishersci.com/shop/products/tube-pa-ultracrimp-6ml-pk-50/03945	LINK

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 [M] 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.**

Note

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 ca. 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)

[M] 150 ng/µl RNA (75-600 ng)

Note

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).

Equipment

Thermo Scientific TOOL ULTRACRIMP EA	NAME
Tube crimper	TYPE
Thermo Fisher Scientific	BRAND
03920	SKU
https://www.fishersci.com/shop/products/tool-ultracrimp-ea/03920	LINK

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.

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.

Note

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).

Equipment

DNA LoBind Tubes	NAME
Microcentrifuge tubes	TYPE
Eppendorf	BRAND
0030108051	SKU
https://online-shop.eppendorf.com/OC-en/Laboratory-Consumables-44512/Tubes-44515/DNA-LoBind-Tubes-PF-56252.html	LINK

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.

Equipment

NE-300 Just Infusion™ Syringe Pump	NAME
Automatic syringe pump	TYPE
New Era Pump Systems, Inc.	BRAND
NE-300	SKU
https://www.syringepump.com/NE-300.php	LINK

Equipment

Masterflex L/S® Precision Pump Tubing, Peroxide-Cured Silicone, L/S 14; 25 ft	NAME
Silicone tube	TYPE
Masterflex	BRAND
96400-14	SKU
https://www.coleparmer.com/i/masterflex-l-s-precision-pump-tubing-peroxide-cured-silicone-l-s-14-25-ft/9640014	LINK

Equipment

Masterflex Fitting, Polycarbonate, Straight, Female Luer to Low-Profile Semi-Rigid Barb Hose Adapter, 1/16" ID; 25/PK	NAME
Luer fitting	TYPE
Masterflex	BRAND
45501-16	SKU
https://www.coleparmer.com/i/masterflex-fitting-polycarbonate-straight-female-luer-to-low-profile-semi-rigid-barb-hose-adapter-1-8-id-25-pk/4550116	LINK

Equipment

Masterflex Fitting, Polypropylene, Straight, Male Luer Lock to Hose Barb Adapter, 1/16" ID; 25/PK	NAME
Luer fitting	TYPE
Masterflex	BRAND
30800-16	SKU
coleparmer.com/i/masterflex-fitting-polypropylene-straight-male-luer-lock-to-hose-barb-adapter-1-16-id-25-pk/3080016	LINK

Equipment

Disposable needles Sterican® long bevel facet, 30 mm, 0.60 mm, Blue	NAME
Disposable needles	TYPE
Sterican	BRAND
X129.1	SKU
https://www.carlroth.com/com/en/starter-kit-dna-synthesis-for-approx-100x-30mers/disposable-needles-sterican-long-bevel-facet/p/x129.1	LINK

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. Volume should be set to "off" and diameter to "22 mm". 30s

Note

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

Note

Using a different syringe (other than 20 ml) will require adjusting the inner diameter setting on the pump

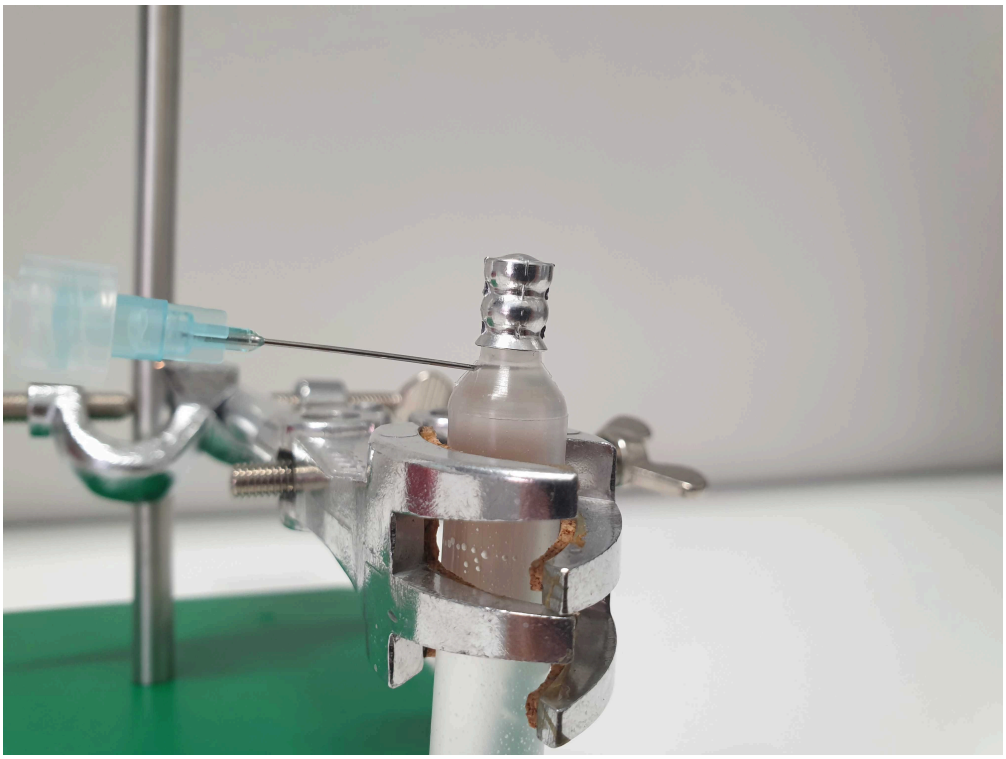
17 Switch the pump on to test the system and also to get rid of air trapped inside the needle and any air bubbles in the tube. Switch the pump back off.

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.

Note

Make sure the tube stays upright during handling.

19 Pierce the ultracentrifugation tube, just below the neck, using the needle attached to to the precision pump tube.



Note

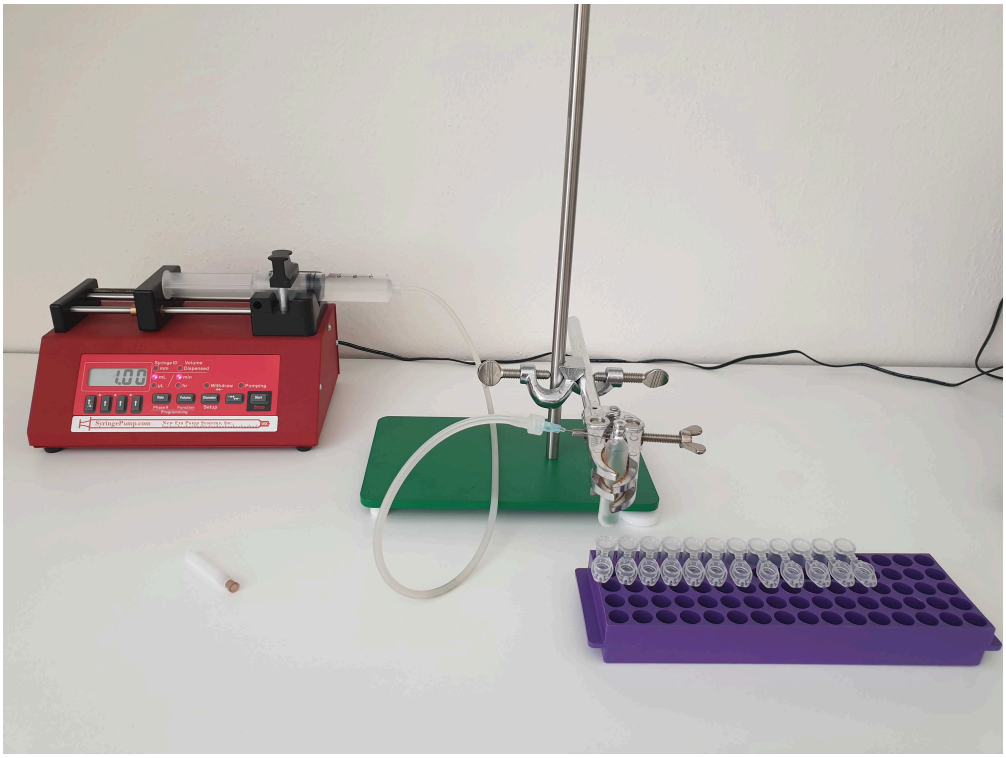
Be careful not to pierce through the other end of the tube!
If the other end of the tube was accidentally pierced, a small amount of petroleum gel can be used to seal the hole.

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.

Equipment

Disposable needles Sterican® long bevel facet, 25 mm, 0.45 mm, Brown	NAME
Disposable needles	TYPE
Sterican	BRAND
c718.1	SKU
https://www.carlroth.com/com/en/starter-kit-dna-synthesis-for-approx-100x-30mers/disposable-needles-sterican-long-bevel-facet/p/c718.1	LINK

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:



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

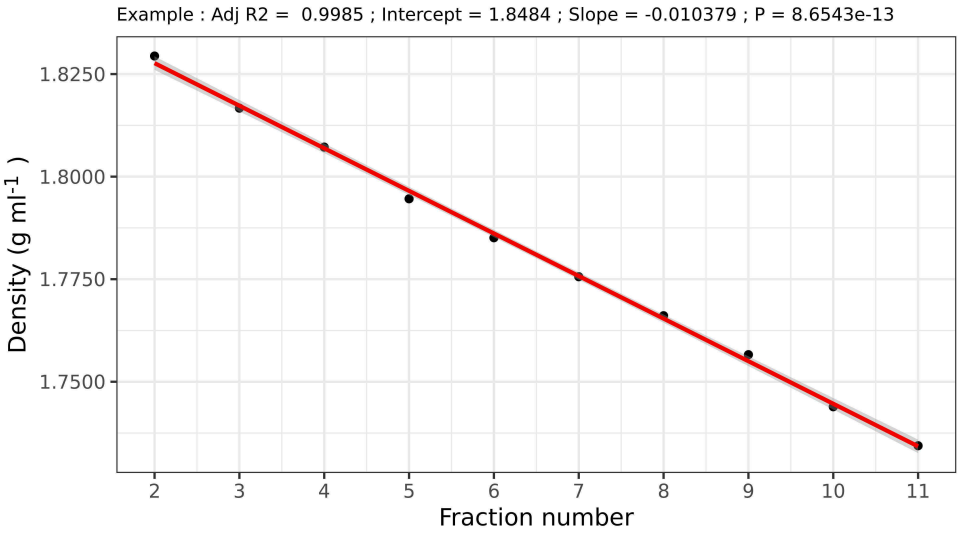
The density of the fractions should increase at a linear rate as you progress from the lighter to the heavier fraction.

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).



Note

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, [M] 10 % (v/v) Na-Acetate ([M] 3 Molarity (M)), and [M] 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

Note

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

Note

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.

Note

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

Note

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 ([M] 50 micromolar (µM) diluted 20x in RNase-free water: [M] 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)
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.

Note

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

Protocol

NAME

qPCR: Bacterial SSU rRNA 338F-516P-805R

CREATED BY

Roey Angel

Preview

38.1

	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 × 100

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

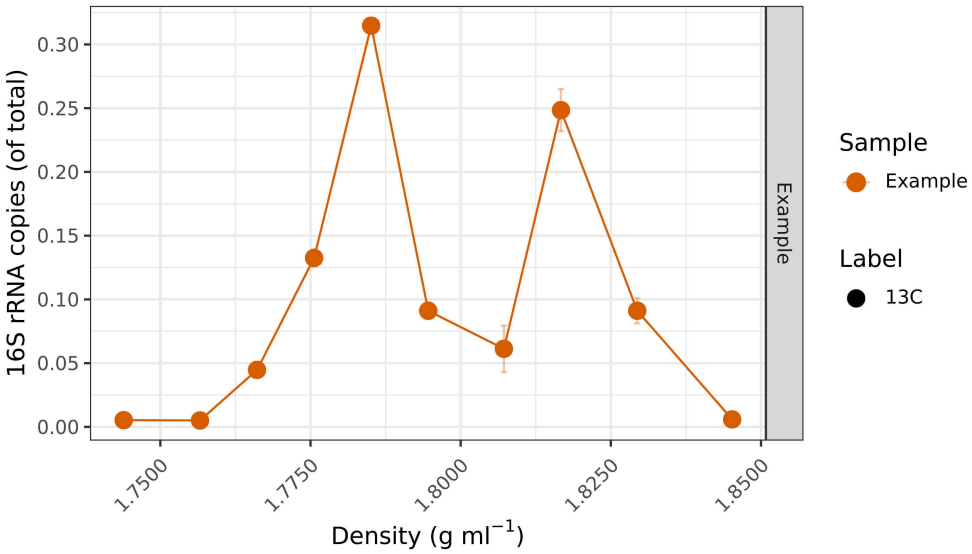
38.3

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.

Expected result

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}



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

Note

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**)

Citation

Youngblut ND, Barnett SE, Buckley DH (Invalid date)
. 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 LINK

Citation

Angel R (Invalid date)
. 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 LINK

Citations

Whiteley AS, Thomson B, Lueders T, Manefield M. RNA stable-isotope probing
10.1038/nprot.2007.115

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

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

Angel R. Experimental Setup and Data Analysis Considerations for DNA- and RNA-SIP Experiments in the Omics Era
https://doi.org/10.1007/978-1-4939-9721-3_1

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. Measurement Error and Resolution in Quantitative Stable Isotope Probing: Implications for Experimental Design
https://doi.org/pii:e00151-20.10.1128/mSystems.00151-20

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

Youngblut ND, Barnett SE, Buckley DH. HTSSIP: An R package for analysis of high throughput sequencing data from nucleic acid stable isotope probing (SIP) experiments
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. Quantitative microbial ecology through stable isotope probing
https://doi.org/10.1128/AEM.02280-15