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RNA-Stable Isotope Probing

Roey Angel¹, Eva Petrova¹, Ana Lara¹¹Soil and Water Research Infrastructure

1 Works for me dx.doi.org/10.17504/protocols.io.zrqf55w

SoWa RI Anaerobic and Molecular Microbiology (public)
Tech. support email: eva.petrova@bc.cas.czEva Petrova
Soil and Water Research Infrastructure

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>

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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.

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KEYWORDS

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

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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



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, Wobken D (2018). Application of stable-isotope labelling techniques for the detection of active diazotrophs. *Environmental microbiology*. <https://doi.org/10.1111/1462-2920.13954>

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Hi-Di Formamide	4311320	Thermo Fisher Scientific
Random hexamers	N8080127	Thermo Scientific
RNase AWAY™ Surface Decontaminant	7002PK	Thermo Fisher Scientific
Cesium Trifluoroacetate (CsTFA) illustra™	45-000-147	Thermo Fisher Scientific

NAME	CATALOG #	VENDOR
3M Na-Acetate pH 5.5	AM9740	Thermo Fisher Scientific
GlycoBlue™ coprecipitant	AM9515	Thermo Fisher Scientific
THE RNA Storage Solution	AM7000	Thermo Fisher Scientific
Bovine Serum Albumin (BSA)	B14	Thermo Fisher Scientific
dNTP Mix (10 mM each)	R0191	Thermo Fisher Scientific
USB Dithiothreitol (DTT) 0.1M Solution	707265ML	Thermo Fisher Scientific
SuperScript™ IV First-Strand Synthesis System	18091050	Thermo Fisher Scientific
RNaseOUT™ Recombinant Ribonuclease Inhibitor	10777019	Thermo Fisher Scientific
Ethylenediaminetetraacetic acid disodium salt dihydrate BioUltra 98.5-101.5%	E1644-100G	Sigma Aldrich
Trizma® hydrochloride / Tris-HCl	T5941	Merck Millipore Sigma
Potassium chloride (KCl)	P9333	Sigma Aldrich

MATERIALS TEXT

Apparatus


- 1) For gradient preparation
 - Working bench in a climatized 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 climatized 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

EQUIPMENT

NAME	CATALOG #	VENDOR
Disposable needles Sterican® long bevel facet, 25 mm, 0.45 mm, Brown	c718.1	Carl Roth
DNA LoBind Tubes	0030108051	Eppendorf
AR200 Automatic Digital Refractometer	13950000	
Thermo Scientific TUBE PA ULTRACRIMP 6ML PK/50	03945	Thermo Fisher Scientific
Thermo Scientific TOOL ULTRACRIMP EA	03920	Thermo Fisher Scientific
Masterflex Fitting, Polypropylene, Straight, Male Luer Lock to Hose Barb Adapter, 1/16" ID; 25/PK	30800-16	Cole-Parmer
Disposable needles Sterican® long bevel facet, 30 mm, 0.60 mm, Blue	X129.1	Carl Roth
NE-300 Just Infusion™ Syringe Pump	NE-300	World Precision Instruments

NAME	CATALOG #	VENDOR
Masterflex L/S® Precision Pump Tubing, Peroxide-Cured Silicone, L/S 14; 25 ft	96400-14	Cole-Parmer
Masterflex Fitting, Polycarbonate, Straight, Female Luer to Low-Profile Semi-Rigid Barb Hose Adapter, 1/16" ID; 25/PK	45501-16	Cole Parmer

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 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
by Thermo Fisher Scientific
Catalog #: [7002PK](#)

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) pH8.0

 **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

by Merck Millipore Sigma

Catalog #: T5941



Potassium chloride (KCl)

by Sigma Aldrich

Catalog #: P9333

CAS Number: 7447-40-7



Ethylenediaminetetraacetic acid disodium salt dihydrate BioUltra 98.5- 101.5%

by Sigma Aldrich

Catalog #: 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**

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

📄 9.696 mL CsFTA

📄 2.166 mL Gradient Buffer

🌡 Room temperature



Adjust the volumes if using different-sized ultracentrifugation tubes.



Cesium Trifluoroacetate (CsTFA)

illustra™

by Thermo Fisher Scientific

Catalog #: 45-000-147

CAS Number: 9048-46-8



Thermo Scientific TUBE PA ULTRACRIMP

6ML PK/50

Ultracentrifugation tubes

Thermo Fisher Scientific 03945 [↗](#)

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

5 

Add 3.56% vol HiDi (422 μ l if the volume was not corrected).

📄 422 μ l HiDi



Hi-Di Formamide

by Thermo Fisher Scientific

Catalog #: 4311320

- 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 µl 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 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 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.

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 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.



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 [↗](#)



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 volume to **1 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.

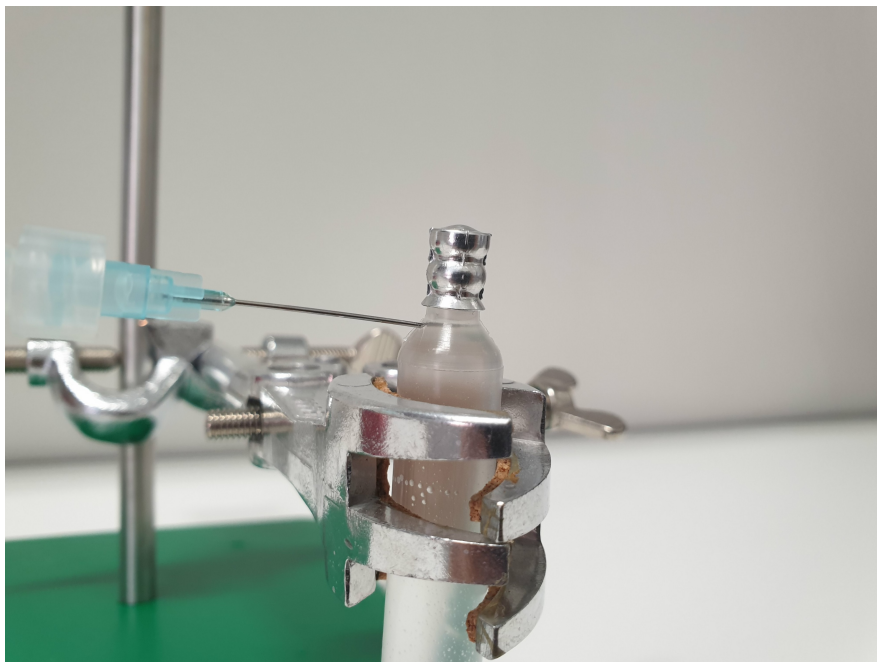


Using a different syringe (other than 30 ml) will require adjusting the inner diameter setting on the pump

- 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 the precision pump tube.



Be careful not to pierce through the other end of the tube!

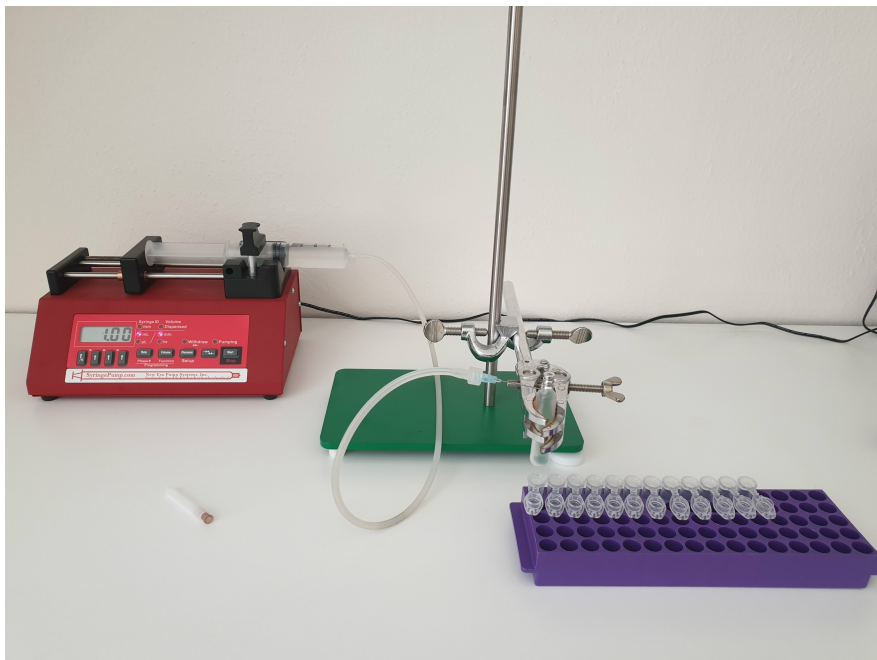
- 20 Take a new, sterile **26G** needle, carefully puncture a hole at the bottom of the ultracentrifugation tube and remove the needle. The tube should not leak at this stage.



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:



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.

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 be in the range of $1.77\text{--}1.80\text{ g ml}^{-1}$, 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.



To each tube add **2 µl** of GlycoBlue, **0.1 volumes** Na-Acetate (**3 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

by Thermo Fisher Scientific

Catalog #: **AM9515**



3M Na-Acetate pH 5.5

by 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



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

by 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






by 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

by Thermo Fisher Scientific
Catalog #: 18091050



RNaseOUT™ Recombinant Ribonuclease Inhibitor

by Thermo Fisher Scientific
Catalog #: 10777019



Bovine Serum Albumin (BSA)

by Thermo Fisher Scientific
Catalog #: B14












dNTP Mix (10 mM each)

by Thermo Fisher Scientific
Catalog #: R0191



USB Dithiothreitol (DTT) 0.1M Solution

by Thermo Fisher Scientific
Catalog #: 707265ML
CAS Number: 3483-12-3

- 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



16S Bacteria 338F-516P-805R BSA

by Eva Petrova,

Soil and Water Research Infrastructure

PREVIEW

RUN

38.1

Reagent	Final conc.	1 tube (20µl)	plate (20µl x 100)
PCR H ₂ O		4.6	460
iQ TM Supermix	1x	10	1000
MgCl ₂ (25mM)	4.0 mM	0.8*	80*
BSA (20µg/µl)	0.2 µg/µl	0.2	20
338F (10µM)	0.5 µM	1.0	100
805R (10µM)	0.5 µM	1.0	100
516P (10µM) [†]	0.2 µM	0.4	40
Template		2	2 x 100

* Buffer contains MgCl₂ at final conc. of 3.0 mM

38.2

1. 95°C – 5'
2. **x 40** {
 - a. 95°C – 30"
 - b. 22°C – 60" take a 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](#))



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