Stable Isotope Probing Techniques and Methodological Considerations using ^{15}N

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Abstract

Nitrogen fixation and assimilation processes are vital to the functioning of any ecosystem. Nevertheless, studying these processes using ^{15}N-based stable isotope probing was so far limited because of technical challenges related to the relative rarity of nitrogen in nucleic acids and proteins compared to carbon, and because of its absence in lipids. However, the recent adoption of high-throughput sequencing and statistical modelling methods to SIP studies increased the sensitivity of the method and enabled overcoming some of the challenges. This chapter describes in detail how to perform DNA- and RNA-SIP using ^{15}N.

Running head: ^{15}N-SIP methods

Keywords: nitrogen, ^{15}N, DNA-SIP, RNA-SIP, amplicon sequencing, BNF, diazotrophs

1 1 Introduction

1.1 1.1 Background on ^{15}N-SIP

Nitrogen is the 3rd most abundant element in living cells by weight and is essential for synthesising proteins and nucleic acids (Fenchel et al., 2012). Although the atmosphere is composed of nearly 80% N_2, nitrogen is biologically unavailable in this form and organisms must therefore either acquire fixed nitrogen forms from the environment or produce them themselves (Madigan et al., 2017). Some microorganisms, known as diazotrophs, can reduce dinitrogen gas into ammonia for their own needs in a process termed biological nitrogen fixation (BNF). BNF is one of the most energy costly processes in nature, requiring 16–24 moles of ATP for each mole ammonia produced (Fisher and Newton, 2002). It is therefore not surprising that BNF is tightly regulated on both transcriptional- and post-translational levels in the

cell (Fischer, 1994; Dixon and Kahn, 2004). This makes studying the ecology of diazotrophs in the natural environment challenging since detecting genes, or even transcripts cannot provide a guarantee for the activity of nitrogen fixation. Studying diazotrophy using stable isotopes probing is, therefore, advantageous because it can provide a strong link between activity and genetic identity.

Despite the cardinal importance of nitrogen to life and the centrality of nitrogen in many ecosystems, only a handful of studies involving stable isotope probing using nitrogen (^{15}N-SIP) have been published to date. There are several good reasons why ^{15}N-SIP is not as nearly as popular as ^{13}C-SIP or even ^{18}O-SIP. Probably the most important one is the fact that while N-transforming processes always drew considerable attention from microbiologists, many of them are dissimilatory, used solely for gaining energy and not for building biomass. Nitrogen assimilation is limited to BNF, or otherwise assimilation of fixed nitrogen forms from the environment such as ammonia, nitrate or amino acids (or peptides). However, assimilation of fixed nitrogen forms is widespread amongst most organisms and therefore provides relatively little differentiating power for targeting specific microbial taxa or guilds in natural communities. A second and obvious reason is that of the three types of biomarkers used for SIP, namely nucleic acids, proteins and lipids, only the first two contain nitrogen and can be used as targets for ^{15}N-SIP, while lipids are excluded. An additional important reason for the relative lack of popularity of ^{15}N-SIP is the difficulty in getting the cells to assimilate enough of the isotopic label. First, because of the high energetic costs, diazotrophs will typically only fix so much atmospheric nitrogen as to fulfil their basic requirements, so a high level of ^{15}N assimilation is difficult to achieve. Secondly, and more important, is the fact that nitrogen atoms are much less abundant than carbon in proteins and nucleic acids, thus inevitably leading to a lower maximum mass addition upon labelling. As a result, while ^{13}C-labelling yields a density gain of ca. 0.036 and 0.035 g ml^{-1}, ^{15}N-labelling yields only a density shift of 0.016 and 0.015 g ml^{-1} in fully labelled DNA and RNA, respectively (Lueders et al., 2004; Birnie and Rickwood, 1978; Angel et al., 2018). Lower density shifts of labelled DNA and RNA mean a greater overlap between labelled and unlabelled templates, which creates a significant challenge for analysing ^{15}N-SIP data. In RNA-SIP, a greater overlap makes it more difficult to detect the enrichment of sequences above the background level. The problem is even more critical in DNA-SIP because DNA also migrates as a function G+C content and could cause unlabelled high-G+C sequences to become enriched in the heavy fractions of the gradient without being labelled.

One successful way to overcome this was published in 2007 and used a two-step centrifugation protocol and the DNA-intercalating agent bis-benzimide (Buckley et al., 2007a). Briefly, the method works as follows: a first density gradient is prepared and centrifuged following a standard DNA-SIP protocol. Then, the heavy fractions corresponding to a density of ca. 1.725–1.735 mg ml^{-1} are collected and pooled

together. These fractions presumably contain labelled DNA of relatively low G+C content with unlabelled DNA of high G+C content. The DNA in these fractions is then used for a second centrifugation step in a CsCl density gradient containing bis-benzimide. During the second centrifugation step, bis-benzimide significantly decreases the BD of low G+C content DNA thus resolving it from unlabelled high G+C DNA (**Fig. 1C)**. However, more recent works employing ^{15}N-DNA-SIP tended to avoid this two-step protocol and instead rely on the ability of high-throughput sequencing coupled with statistical modelling to detect labelled taxa and avoid false positives via the use of parallel no-label controls (Pepe-Ranney et al., 2015) (see **Fig. 1 B** and **Chapters 9** and **11**). The first published attempt at ^{15}N-RNA SIP is attributed to Addison and colleagues in 2010 (Addison et al., 2010), although the authors finally concluded that ^{15}N-labelled RNA could not be definitely resolved from unlabelled RNA. However, it should be noted that the protocol used in that work deviated somewhat from the standard RNA-SIP protocol in several aspects, including using much higher amounts of RNA, higher centrifugation speed but lower temperature and shorter centrifugation time. Finally, a successful demonstration of a ^{15}N-RNA-SIP protocol was published in 2018 and using a standard RNA-SIP protocol in combination with amplicon sequencing and statistical modelling (Angel et al., 2018).

1.2 1.2 Experimental considerations

As in any SIP experiment, incubating the sample in the presence of the ^{15}N-labelled substrate should be prolonged enough to ensure that the DNA or RNA are sufficiently labelled above the detection limit. In contrast, long incubation times will almost inevitably result in the labelling of non-diazotrophic microbes through cross-feeding. The issue of cross-feeding is of general concern in SIP experiments and has been mostly discussed for ^{13}C-based SIP experiments (e.g., McDonald et al. 2005; DeRito et al. 2005), but diazotrophs have also been shown to release substantial amounts of fixed nitrogen through cross-feeding or leaching (Belnap, 2001; Adam et al., 2015). Diazotrophy is a slow and costly process, and, incubation times are accordingly relatively long compared to incubations with a ^{13}C-labelled substrate. Consequently, ^{15}N-SIP incubations targeting diazotrophs would require incubating the samples for several days or even weeks, depending on the specific level of activity of the system (Angel et al., 2018; Buckley et al., 2007b; Pepe-Ranney et al., 2015). However, for targeting the assimilation of biologically available N-forms such as ammonium, nitrate or amino-acids, incubation times should be reduced to several hours to few days, since the process is much more rapid and requires only little energy from the cells (Alonso-Pernas et al., 2017; Bell et al., 2011). Because of the greater overlap between labelled and unlabelled sequences in ^{15}N-SIP compared to ^{13}C-SIP gradients the chance of detecting false negatives and false

positives increases. This can be remediated to some degree by increasing the number of replicates in the experiment.

1.3 1.3 Data analysis

In essence, data analysis for ^{15}N-SIP experiments is not different from what is used in other DNA- and RNA-SIP experiments. ^{15}N-SIP experiments were analysed successfully using both traditional comparison of clone libraries (Buckley et al., 2007b) and statistical modelling of high-throughput amplicon data (HR-SIP)(Pepe-Ranney et al., 2015; Angel et al., 2018) and qSIP (Morrissey et al., 2018). The much greater sensitivity achieved through high-throughput sequencing and statistical modelling is particularly advantageous for analysing ^{15}N-SIP experiments, because the target guild is typically small and only partially labelled, and because the separation between labelled and unlabelled nucleic acids is low. Figure 1 illustrates the results of a ^{15}N-SIP experiment using only two phylotypes that differ in their G+C content and where only the low-G+C-content organism can assimilate ^{15}N. Using standard DNA-SIP procedure the labelled and unlabeled phylotypes cannot be visually differentiated, because of G+C-content based density shift (**Fig.** 1 **A and B**). However, using a second centrifugation step in the presence of bis-benzimide helps to resolve the two phylotypes (**Fig.** 1 **C**). However, the two phylotypes can also be resolved using a standard one-step centrifugation if the results are statistically modelled using qSIP or HR-SIP (**Fig** 1 **A and B**). On the other hand, in RNA-SIP because G+C content has relatively little effect on buoyant density in the presence of formamide, the small mass addition from ^{15}N labelling is visible. Nevertheless, using statistical modelling to detect labelled phylotypes is nevertheless advantageous or even necessary in most real-life cases because of the increased sensitivity.

1.4 1.4 Procedures

Methodological details for conducting both DNA-SIP and RNA-SIP are given below. In general, the steps for performing SIP are independent of the isotope used, so the protocols below can be used for processing samples from any DNA- or RNA-SIP experiment. For DNA-SIP, a protocol including a secondary centrifugation step in the presence of bis-benzimide is also detailed as an optional deviation from the standard DNA-SIP protocol. Although this method is considered outdated by now, it is provided here for completeness. Since DNA- and RNA-SIP protocols share many similarities with each other, much of the protocol is given for both methods together, and deviations for each specific method are highlighted. All protocols assume that an environmental sample has been incubated in the presence

of a ^{15}N-labelled substrate and that total DNA or RNA have been extracted from the sample following incubation (*see* **Notes 1** and **2**). Methods for extracting DNA or RNA from environmental samples are well established and go beyond the scope of this chapter. Many commercial kits are available for this purpose, depending on the type of sample, as well as also general-purpose lab protocols (e.g., Angel and Angel 2012).

2 2 Materials

2.1 2.1 Gradient preparation

1. An ultracentrifuge, capable of achieving 177,000 × g and equipped with a vertical or a fixed-angle rotor for tube volumes of 2–8 ml (typically 5–6 ml; e.g., VTi 90 from Beckman Coulter)
2. Compatible polyallomer ultracentrifugation tubes and caps (one for each sample, e.g., Optiseal 4.9 ml)
3. Refractometer (typically, Reichert's AR200 digital refractometer)
4. DNA samples in TE or water (0.5–5 µg; **for DNA SIP**)
5. CsCl solution (prepare a 7.163M CsCl solution by dissolving 603 g CsCl in 500 ml of filter-sterilised molecular-grade water; confirm that the density is ca. 1.89 g ml^{-1}; store at RT; **for DNA SIP**)
6. RNA samples in TE or water (300–500 ng; **for RNA SIP**)
7. CsTFA solution (ca. 2 g ml^{-1}; store at 4 °C; **for RNA-SIP**)
8. Hi-Di Formamide (Thermo), or any other deionised formamide (**for RNA-SIP**)
9. Gradient Buffer (GB): prepare a 0.1 M Tris-HCl (pH 8.0), 0.1 M KCl and 1 mM EDTA in RNase-free water, filter-sterilise (0.1 µm) into a clean glassware and autoclave
10. Gradient Buffer (GB): prepare a 0.1 M Tris-HCl (pH 8.0), 0.1 M KCl and 1 mM EDTA in RNase-free water, filter-sterilise (0.1 µm) into a clean glassware and autoclave
11. One 50-ml tube per gradient
12. RNase-free water (for calibrating the refractometer)
13. Bis-benzimide (Hoechst 33258, 10 mg ml^{-1} solution; for **DNA-SIP using bis-benzimide**)

2.2 2.2 Gradient fractionation

1. Refractometer
2. 1.5-ml non-stick tubes
3. Test tube utility clamp mounted on a stand
4. 20-ml syringe
5. A flexible tube (approx. 30 cm; for instance an elastic HPLC tube) attached to the syringe on one end with a Luer-Lock connection fitting, and with an additional Luer-Lock connection fitting for a disposable needle on the other end
6. RNase-free water for displacing the gradient solution (enough to displace the entire volume of an ultracentrifugation tube times the number of gradients)
7. Variable-speed, automatic syringe pump
8. Disposable needles: 23G and 26G

2.3 2.3 DNA-SIP fraction precipitation

1. GlycoBlue Coprecipitant (15 mg ml^{-1}) or molecular-grade glycogen (see **Note 5**)
2. PEG 6000 solution (prepare a 30% PEG and 1.6 M NaCl solution by dissolving 150 g of polyethylene glycol 6000 and 46.8 g NaCl in molecular-grade water to a final volume of 500 ml and autoclave. Final solution is 30% PEG 6000 and 1.6 M NaCl)
3. Ethanol (prepare a 70% solution using molecular grade ethanol and molecular-grade water)

2.4 2.4 RNA-SIP fraction precipitation

1. GlycoBlue Coprecipitant (15 mg ml^{-1}) or RNA-grade glycogen (see **Note 5**)
2. Ethanol (100%; molecular grade)
3. Sodium acetate solution (3 M; pH 5.2, RNase free)
4. Ethanol (70%; molecular grade in RNase-free water)
5. *Optional:* RNA Storage Solution (Ambion)

3 3 Methods

3.1 3.1 Gradient preparation and centrifugation

1. Prepare all solutions in advance.
2. Equilibrate the CsCl (**for DNA-SIP**) or CsTFA (**for RNA-SIP**) solution to room temperature for about 60 min (if stored at 4 °C).
3. Calibrate the refractometer using pure water.
4. Prepare the gradient mixture depending on the type of SIP (see below):

3.1.1 3.1.1 Preparation of DNA-SIP gradient mixture

1. For each gradient, mix GB, DNA sample and CsCl solution to reach the desired density (typically 1.725 g ml^{-1}) in a separate 50-ml tube. The volume of CsCl solution needed to achieve a specific density is given according to equation 1.

$$V_{CsCl} = V_{mix} \cdot \frac{(\rho_{mix} - \rho_{(GB+DNA)})}{(\rho_{CsCl} - \rho_{(GB+DNA)})} \quad (1)$$

Where: V_{mix} is the volume of the entire gradient (typically the volume of the ultracentrifugation tube), ρ_{mix} is the desired final density of the gradient mixture, ρ_{CsCl} is the density of the CsCl solution, and $\rho_{(GB+DNA)}$ is the density of the gradient buffer and DNA mixture. The rest of the volume should be filled with the mixture of GB and DNA. The density of GB is around 1.01 g ml^{-1}, while that of DNA solution is very close to 1 g ml^{-1}. Although the exact density of the GB + DNA solution can be calculated, depending on the volume of the DNA sample, the effect of the latter on the overall density is negligible, and it is safe to assume that the density remains unchanged. Hence equation 1 can be re-written as follows:

$$V_{CsCl} = V_{mix} \cdot \frac{(\rho_{mix} - 1.01)}{(\rho_{CsCl} - 1.01)} \quad (2)$$

Assuming a tube volume of 4.9 ml, a CsCl solution with $\rho = 1.89$ and a desired density of 1.725 g ml^{-1} then V_{CsCl} should be 3980 µl and the rest (920 µl) should be a mixture of GB and DNA. It is recommended to prepare a volume larger by about 2–3% than needed to account for volume differences between tubes and for pipetting errors.

2. Validate the final density using a refractometer and adjust accordingly if the reading differs from nD-TC = 1.4031 +/- 0.0002.

3. Balance each tube pair according to the instructions of the ultracentrifuge's manufacturer.

4. Centrifuge at 177,000 × g_{av} (49,500 RPM for the VTi 90 rotor) at 20 °C for >36 h at maximum acceleration and minimum deceleration (no brake).

3.1.2 3.1.2 Preparation of gradient mixture for two-step DNA-SIP using bis-benzimide

1. Collect and pool fractions corresponding to densities between 1.725–1.735 mg ml^{-1} (see **section** 3.2) and discard the rest.

2. Recover the DNA from these pooled fractions through precipitation. Resuspend in about 20–30 µl of TE (see **section** 3.3.1).

3. For the secondary centrifugation gradient, prepare a fresh CsCl gradient by following the steps above (see **section** 3.1.1), but replace 8 µl of the GB with 8 µl of bis-benzimide (10 mg ml^{-1}).

4. Load the recovered DNA from the first centrifugation step.

5. Proceed with centrifugation, fractionation and DNA recovery as usual for DNA-SIP (see **sections** 3.1.1 and 3.3.1).

6. The fractions corresponding to densities between ca. 1.690 and 1.710 mg ml-1 should now contain the labelled DNA while fractions corresponding to densities between 1.710 and 1.713 mg ml^{-1} should contain unlabelled high-G+C DNA.

3.1.3 3.1.3 Preparation of gradient mixture for RNA-SIP

1. For each gradient, mix GB, RNA sample (300–500 ng), and CsTFA stock solution in a separate 50 ml tube according to equation 2 for a final density of 1.825 g ml^{-1}, but using only 97% of the final volume to leave room for the formamide (below). Assuming 4.9 ml final volume, mix 3900 µl CsTFA with 850 µl GB and adjust if the refractive index differs from nD-TC = 1.3702 +/- 0.0002. Again, it is advisable to prepare a slightly larger volume than needed.

2. Add 3.59% vol. formamide (170 µl if mixed as above). Adjust if the refractive index differs from nD-TC = 1.3725 ± 0.0002.

3. Balance each tube pair.

4. Centrifuge at 130,000 × g_{av} (42,400 rpm for the VTi 90 rotor) at 20 °C >65 h at maximum acceleration and minimum deceleration (no brake).

3.2 3.2 Gradient fractionation

1. Stop the ultracentrifuge.
2. Fill a 20-ml syringe with RNase-free water; remove any air bubbles.
3. Attach the flexible tube to the syringe and mount it on the pump.
4. Set the syringe pump to the desired speed. To collect 20 fractions, set the speed to 0.75 ml min^{-1} and collect in 20-second steps (make sure the correct syringe volume is also set).
5. Connect a new 23G needle to the tube and test the flow. Wait until water starts to come out of the needle.
6. Prepare 20, 1.5 ml non-stick tubes per gradient in a rack (assuming 20 fractions will be collected).
7. Carefully remove the rotor from the centrifuge and release the screws or the lid. Ensuring that mechanical disturbance is minimal is crucial at this point.
8. Mount 1 ultracentrifugation tube on the utility clamp about 1 cm above the opening of the first collection tube.
9. Carefully puncture the ultracentrifugation tube horizontally with the needle connected to the flexible tube, just below the bottom of the neck in the ultracentrifugation tube (the top level of the liquid volume).
10. Using a new 26G needle, carefully puncture a hole at the bottom of the ultracentrifugation tube and remove the needle. The ultracentrifugation tube should not leak at this stage.
11. Place the rack under the ultracentrifugation tube so that the first collection tube is positioned right below the hole at the bottom of the tube.
12. Start the pump and then start the stopwatch immediately after the first drop falls out of the ultracentrifugation tube.
13. After 20 seconds, shift the rack so that the solution starts dropping to the second collection tube. Continue in a similar fashion until all tubes are filled.
14. Discard the used ultracentrifugation tube and continue with the next gradient.

15. After finishing fractionating all ultracentrifugation tubes, measure the density of every fraction using the refractometer starting from the last fraction (the lightest). The density of the fractions should increase at a linear rate.

3.3 3.3 Recovery of nucleic acids

3.3.1 3.3.1 DNA recovery

1. To each 1.5 ml tube containing a gradient fraction, add 2 µl of GlycoBlue Coprecipitant (or 30 µg molecular-grade glycogen) (see **Notes 4** and **5**) and approximately 2 volumes of the PEG solution. Mix by inversion.
2. Incubate the tubes for 2 h at RT.
3. Centrifuge at $>$ 13,000 $\times$ g for 30 minutes at 4 °C.
4. Decant the supernatant, add 1 ml of 70% ethanol.
5. Centrifuge for $>$ 13,000 $\times$ g for 10 minutes at 4 °C.
6. Decant the supernatant and leave the tubes open to dry at room temperature for ca. 15 min (preferably under an open flame or in a biological hood) to evaporate the remaining ethanol.
7. Resuspend in 30 µl TE buffer or sterile water. Store at 4 °C up to several days or frozen at -20 °C or -80 °C indefinitely.
8. Proceed with PCR amplification and sample preparation for sequencing using any standard protocol.

3.3.2 3.3.2 RNA recovery

1. To each 1.5-ml tube containing a fraction, add 2 µl of GlycoBlue Coprecipitant (or 30 µg RNA-grade glycogen), 2.5 volumes of 100% ethanol and 0.1 volumes of sodium acetate (assuming 250-µl fractions were collected and 40 µl of each was used for density measurement, add 21 µl of Na-acetate and 625 µl of 100% ethanol). Mix by inversion.
2. Incubate the tubes for 30 min at -80 °C.
3. Centrifuge at $>$ 13,000 $\times$ g for 30 min at 4 °C.
4. Decant the supernatant, add 1 ml of ice-cold 75% ethanol, invert the tube several times.
5. Centrifuge at $>$ 13,000 $\times$ g for 15 min at 4 °C.

6. Remove as much as possible from the supernatant first using a 1-ml tip, spin down the remaining drops in the tube, and remove the rest of the liquid with a 100-µl tip. Be careful not to disrupt the pellet.

7. Leave tubes open to dry at room temperature for ca. 15 min (preferably under an open flame or in a biological hood) to evaporate the remaining ethanol.

8. Resuspend the pellets in 10-µl RNase-free water or RNA Storage Solution. Proceed immediately to synthesising cDNA or store at -20 °C to -80 °C.

9. Synthesise cDNA using any commercial reverse transcription kit (see **Note 6**).

10. Proceed with PCR amplification and sample preparation for sequencing using any standard protocol.

4 4. Notes

1. **Substrate enrichment level.** Typical SIP experiments involve using high substrate concentrations to achieve maximum labelling. Since $^{15}N_2$ is also non-toxic, there is no limitation in supplying the incubation vials with atmospheric or even super-atmospheric concentrations of $^{15}N_2$ gas (e.g., in anoxic incubations). However, this might not be necessary since even in very active systems only a small fraction of the dinitrogen gas eventually gets fixed. To save on costs, some of the gas can be replaced with another inert gas such as helium or argon. We have incubated several types of soil under an atmosphere of 40:40:20 ($^{15}N_2$, He, O_2) and noticed no difference in labelling compared to incubating the samples under 80:20 ($^{15}N_2$, O_2; data not shown), although this should probably be best confirmed for every type of sample.

2. **Substrate contamination issues.** Bottles of $^{15}N_2$ are nearly always sold at a purity of around 99% (and >97% isotopic enrichment). However, the single remaining percent of foreign substance can turn out to be detrimental, because it was found out that a significant fraction of it is in the form of ^{15}N-labelled ammonia and nitrate (Dabundo et al., 2014). Ideally, every batch of labelled gas should be tested for potential contamination either by direct measurement of ammonia and nitrate, or, for example, indirectly by incubating a culture of a non-diazotrophic microorganism in the presence of the gas as a sole nitrogen source and then testing if the label has accumulated in the biomass.

3. **Amount of template to use in a gradient.** For DNA SIP, template amounts of 0.5–5 µg are typically used in the literature. There does not seem to be an upper limit to how much DNA can

be loaded, but adding a large amount of an aqueous DNA solution will eventually significantly reduce the average density of the gradient. The final amount of DNA that should be loaded on a gradient will depend on the size of the target guild compared to the total population and the downstream applications (i.e. PCR based analysis vs shotgun genomics). In contrast, much lower template amounts are used for RNA SIP because it has been reported that RNA will precipitate in a CsTFA density gradient in concentrations above approx. 80 ng of RNA per ml of gradient solution (Lueders et al., 2003). Luckily for targeting rRNA this is rarely an issue because rRNA accounts for over 80% of the total rRNA in a bacterial cell (with SSU rRNA alone accounting for ca. 27%), however for a transcriptomic analysis enrichment of mRNA might be needed (Dumont et al., 2013).

4. **Type of glycogen.** Standard molecular-grade (or RNA-grade) glycogen may also be used, but the pellet will most likely not be visible and can be accidentally lost in the washing process. Using dyed glycogen such as GlycoBlue helps to prevent loss of precipitated nucleic acids.

5. **Purity of glycogen.** Non-molecular-grade glycogen may contain residual nucleic acids, and molecular-grade glycogen which is not RNA-grade may contain residual RNA. The presence of foreign DNA or RNA can significantly obscure the results of the SIP experiment and it therefore highly recommended to use an appropriate glycogen, and to verify by PCR that the glycogen is free of contaminating nucleic acids.

6. **cDNA synthesis.** Because the RNA concentration in each fraction is very low (typically 1–300 ng, depending on how the gradient was designed and fractionated) the reverse transcriptase can be safely diluted 10-20 times before use without any noticeable effect on the reaction yield. Store at -20 to -80 °C.

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Figures

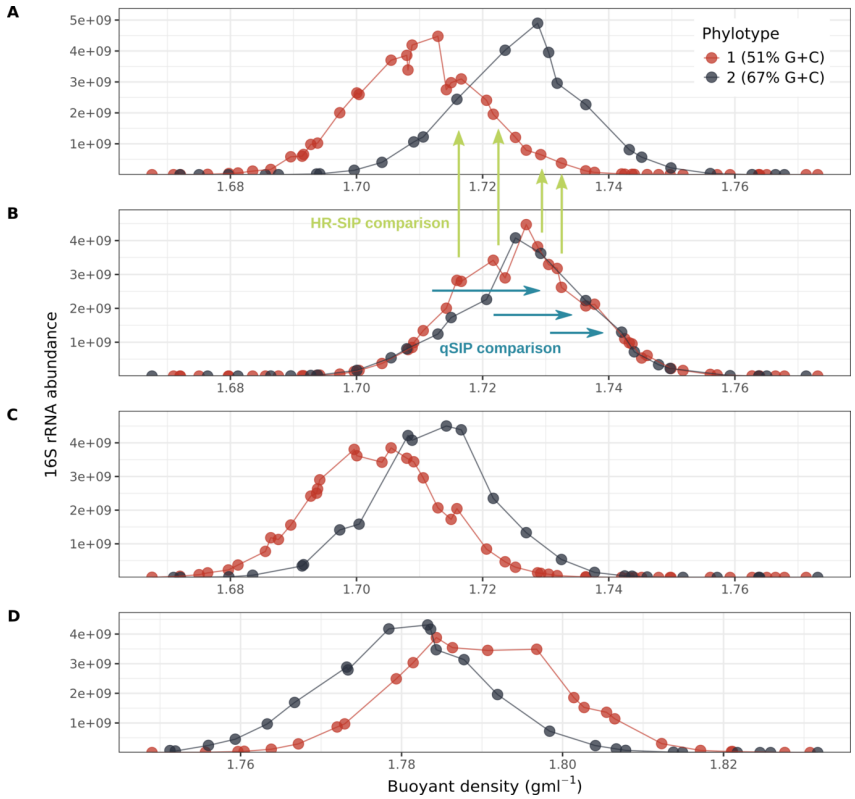

Figure 1: **Figure 1:** Illustration showing the hypothetical results of a ^{15}N-SIP experiment analysed using different methods. A simplified community composed of only two phylotypes: phylotype 1 and phylotype 2 with genomic G+C contents of 51% and 67%, respectively. Each panel shows a simulated distribution of the two phylotypes in different SIP gradients. The x-axis shows the density of the fractions in the gradient while the y-axis shows the abundance of the two phylotypes in each fraction (e.g. obtained using qPCR). **A.** In a ^{14}N-control DNA-SIP, phylotype 2 is centred around the denser parts of the gradient compared to phylotype 1 because of its higher G+C content. **B.** In a ^{15}N-labelled DNA-SIP, only phylotype 1 incorporated the label and as a result migrated towards the denser fractions by about 0.016 g ml-1. However, this minor shift doesn't allow for visual separation from the unlabelled phylotype 2. Green arrows illustrate the binary comparison of the abundance (typically relative abundance) of each phylotype in its heavy fractions of a labelled gradient against its abundance in the heavy fractions of an unlabelled (control) gradient. Significantly higher relative abundance in the heavier fractions of a labelled gradient indicate labelling. Blue arrows illustrate the comparison done using qSIP where the mean shift of the buoyant density of each phylotype is calculated to determine its level of enrichment. **C.** In a secondary gradient using bis-benzimide both phylotypes migrate to the lower-density fractions but the low-G+C phylotype reduced its buoyant density more than the high-G+C phylotype and the two can be visually separated. **D.** In an RNA-SIP gradient, because G+C content has relatively little effect on buoyant density in the presence of formamide, the small mass addition from 15N labelling is visible. However, the statistical modelling using e.g. HR-SIP or qPCR will dramatically increase the detection power of the method (see **Chapter 9** and **11** for further discussion.)

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