Experimental Set Up and Data Analysis Considerations for DNA- and RNA-SIP Experiments in the Omics Era

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Abstract

Careful and thoughtful experimental design is crucial to the success of any SIP experiment. This chapter discusses the essential aspects of designing a SIP experiment, focusing primarily on DNA- and RNA-SIP. The design aspects discussed here begin with considerations for carrying out the incubation, such as, the effect of choosing different stable isotopes and target biomolecules, how enriched should a labelled substrate be, what concentration to use and how long the incubation should take. Then tips and pitfalls in the technical execution of SIP are listed, including how much nucleic acids should be loaded, how many fractions to collect and what centrifuge rotor to use. Lastly, a brief overview of the current methods for analysing SIP data is presented, focusing on high-throughput amplicon sequencing, together with a discussion on how the choice of analysis method might affect the experimental design.

Running head: Experimental set-up and data analysis

Keywords: DNA-SIP, RNA-SIP, amplicon sequencing, omics, network analysis

1. Introduction

The success of any lab experiment hinges on a thoughtful design of the experimental system, careful execution of protocols and statistically-sound data analysis. While SIP protocols have matured and become standardised over the past 20 years since their introduction, what surrounds the gradient generation and fractionation, i.e., the experimental design and data analysis, have been somewhat neglected. Other chapters in this book provide detailed protocols on how to perform SIP in the lab and how to analyse the data using specific methods. This chapter, on the other hand, discusses general considerations in conceptualising a SIP experiment, designing the experimental set-up and choosing the right analysis method. The focus here is on DNA- and RNA-SIP experiments since these are the most flexible and most widely-used forms of SIP. **Table** 1 summarises the main points to consider during each of the various steps in designing a SIP experiment.

2. Choice of stable isotope

Every SIP experiment is based on incubating the sample in the presence of a heavy isotope labelled substrate. In theory, every element that is present in the target biomolecule – DNA, RNA, phospholipid-derived fatty acids, or proteins – can be labelled and therefore be used in a SIP experiment. The only exception is, of course, phosphorus for which the common form – ^{31}P – is the only stable isotope that exists. In practice, however, SIP experiments almost exclusively use ^{13}C as the isotope of choice, with a tiny minority using ^{18}O and ^{15}N. The choice of substrate and stable isotope as labelling compounds in a SIP experiment is of course directly related to

the metabolic process or microbial guild of interest. Naturally, in SIP target microbes can only be isotopically labelled through assimilatory processes. This is somewhat unfortunate because many of the microbially-mediated biogeochemical processes of interest are energy-yielding dissimilatory processes, involving only electron transfer between two compounds and leave no trace in the biomass. In such cases, the microbial guild of interest can only be labelled indirectly through an assimilatory process that is powered by the dissimilatory process of interest (e.g., using ^{18}O-H_2O or ^{13}C-CO_2 as general substrates for all active organisms and for autotrophs, respectively).

Beyond the question of which biological process or microbial target group to study, the different stable isotopes used for SIP differ in their ability to label nucleic acids and therefore lead to buoyant density (BD) changes. **Table** 2 lists and compares the number of additional neutrons gained per nucleotide in a DNA or RNA molecule by replacing all the atomic positions of a particular element with its heavier stable isotope. The table shows that theoretically the highest mass increase from labelling is achieved by using ^{18}O, with added 12 or 14 neutrons on average for a hypothetical DNA or RNA molecule, respectively. This is, of course, thanks to the fact that labelling with ^{18}O adds two neutrons per atom compared to only one for either ^{13}C, ^{15}N or D, therefore leading to higher overall mass increase despite the lower number of atoms in the molecule. In contrast, N is, unfortunately, the rarest in nucleic acids compared to C, O or H and labelling with ^{15}N can lead to a maximum of 3.75 added neutrons per base, on average, or 2.5 times less in mass increase compared to labelling with ^{13}C. This was confirmed experimentally already over 40 years ago when it was shown that fully ^{15}N-labelled

DNA in CsCl has a BD gain of ca. 0.016 g ml^{-1} compared to a BD gain of ca. 0.036 g ml^{-1} with ^{13}C [1]. Similarly, RNA fully labelled with ^{15}N showed a BD gain of 0.015 g ml^{-1} [2] compared to 0.035 for ^{13}C [3]. The lower maximum mass addition to DNA and RNA through ^{15}N-labelling means a smaller shift of labelled nucleic acids away from unlabelled nucleic acids in an isopycnic gradient compared to ^{13}C-labelling. Still, this more modest shift in BD is nevertheless sufficient to detect labelling in DNA originating from a single organism, as was shown already in the classical work of Meselson and Stahl [4]. However, for DNA-based SIP this creates a major challenge since double-stranded DNA migrates in a BD gradient not only as a function of its mass but also as a function of its hydration state. The latter is ultimately determined by the G+C content of the DNA and causes an undesired migration of unlabelled high-GC DNA towards the denser regions of the gradient [5]. Already in the first attempts to develop ^{15}N-SIP, it was noticed that due to the relatively small migration of ^{15}N-labelled DNA, unlabelled DNA with high-G+C content could overlap with even fully-labelled DNA of lower G+C content, and obscure the ability to differentiate labelled from unlabelled taxa [6, 7]. This is further intensified by the fact that A-T base pairs contain only seven nitrogen atoms compared to eight in a G-C base-pair, resulting in a lower, albeit minor labelling of the A-T base pair [8].

Surprisingly, while ^{18}O labelling should theoretically increase the mass of DNA by 23% and of RNA by 47% compared to labelling with ^{13}C, in practice the observed shifts in BD in ^{18}O-SIP gradients are not much different than in ^{13}C-SIP gradients (0.04 g ml-1)[9, 10], indicating that not all positions can be replaced with a heavy isotope.

Deuterium has been used in SIP experiments coupled with either Raman microspectroscopy [11] or metabolomics [12], but because of the toxicity of deuterated water (heavy water) at high concentrations, it is probably not suitable for DNA or RNA-SIP.

Considering these, it is easy to understand why carbon is the most widely used isotope in SIP. Carbon is abundant enough in biomolecules to allow for easy labelling. In many cases, carbon-based substrates are used for both assimilatory and dissimilatory processes in the cell, so biomass labelling is easily achieved using any of a selection of different substrates. In contrast, many N-transforming processes are dissimilatory, while at the same time many N-assimilation processes are common between different functional groups of microorganisms and therefore provide relatively little differentiating power. Similarly, oxygen is also found abundantly in various terminal electron acceptors used for respiration, which are therefore unsuitable for SIP, or alternatively in water, which is assimilated into the biomass by all known organisms.

3. Setting up an experiment

SIP experiments are usually relatively complex, laborious and time-consuming, and can, therefore, fail because of various reasons and at different stages. Thus, the experimental 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, through possible pitfalls and down to the desired method of data analysis. Before deciding on a SIP experiment, it is impor-

tant to gain some preliminary knowledge of the system in question and the microbial guild to be targeted. For SIP to be successful, sufficient substrate needs to be processed and assimilated by the microbes during the incubation period. Therefore, one of the first and most important preliminary tests to perform is to measure the rate and dynamics of the process in question to estimate the length of the incubation period that is needed. Although the relationship between substrate consumption and level of labelling depends on the assimilation efficiency and the size of the active microbial guild and is therefore difficult to establish, some insights and ballpark estimates can nevertheless be made. Also, it is advisable to measure the enrichment level of the total DNA or RNA extracted from the sample to assess if detection of labelled microbes will be feasible [2, 13, 14]. Again, while it is impossible to draw a general direct relation between the level of enrichment of nucleic acids and the outcome of the SIP, because this will depend on whether or not the label is concentrated within a small group of highly labelled microbes or shared amongst many members, but a qualitative relationship can nevertheless easily be drawn for specific environments and microbial guilds.

3.1. Which bio-molecule to target

SIP was first designed to identify labelled microbes through the incorporation of a stable isotope into their DNA [15]. While this is still the most commonly used 'flavour' of SIP, other types of SIP quickly followed, since in essence nearly every stable bio-molecule in the cell can be used as a target for SIP. Targeting DNA is advantageous because DNA is the gold-standard for taxonomic classification of organisms and for hypothesising about potential functions. It is also popular because DNA amplification and sequencing

technologies are affordable and wide spread in most molecular and microbiological labs. A protocol for targeting RNA instead of DNA in a SIP experiment [13] then quickly followed. RNA-SIP offers the same taxonomic resolution power as DNA-SIP but because RNA synthesis is uncoupled to cell replication it offers higher sensitivity, though at the cost of a somewhat more laborious and sensitive lab work. A further advantage of RNA-SIP is that unlike DNA, RNA does not migrate based on its G+C content in a density gradient, so the potential for detecting false-positives is theoretically lower (see **Sections** 2**,** 3.6 **and** 4.3 and in **Chapter 9** of this book). Targeting PLFA [16] is another popular way for running SIP that even predates the use of DNA-SIP for detecting active microbes in the environment. Because of the use of an isotope-ratio mass spectrometer (IRMS), which is capable of a much finer mass separation compared to density gradient, PLFA-SIP offers significantly higher sensitivity over DNA or RNA SIP, which can be important when studying organisms with very low specific activity such as deep subsurface microorganisms [17] or bacteria that oxidise atmospheric methane [18]. However, in addition to excluding the use of ^{15}N-labelled substrates, PLFA inherently offers a much limited capacity for taxonomic affiliation of microbes compared to DNA or RNA and can only differentiate between groups at broad level [19]. Targeting proteins and metabolites is also an option (e.g. Baran et al. 12, Jehmlich et al. 20), thus providing a direct and unquestionable proof of processing a labelled substrate. However these methods are very laborious, low throughput and require significant in-house experience in sample processing, and analysis of the output data. Lastly, identification of isotopically labelled microbes at the single-cell

levels is also gaining interest lately using tools such as NanoSIMS [21] and SIP-Raman [22] microspectroscopy, however their application is still limited because they are costly, low-throughput and relay on equipment that is found in only a handful of labs around the world.

3.2. Duration of incubation

As mentioned, incubation length will depend on the one hand on the rate in which the process in question is proceeding and its specific assimilation efficiency. Incubation in the presence of the labelled substrate should allow enough time for the nucleic acids to become sufficiently labelled to be detected above the background. For very fast processes such as water uptake, incubation time can be as short as a few hours [23, 10], while for very slow processes, such as nitrogen fixation, incubation can be as long as several days to weeks [2, 24, 25]. Incubation time should also vary if targeting DNA or RNA. Labelling of RNA can be detected earlier because it does not require cell replication and because its synthesis is not semi-conservative as DNA replication (although this does not preclude a significant dilution of newly synthesised RNA with light isotope as a result of recycling of building blocks within the cell). In general, it is assumed that DNA or RNA molecules should be labelled to at least 30 atomic % to differentiate them from unlabelled molecules in a BD gradient [26, 27]. On the other hand, long incubation times bear the risk of labelling community members that do not perform the metabolic activity in question but were labelled through cross-feeding. Because microbes are interlinked through a network of trophic interactions, any labelled element will eventually be spread amongst many members of the community, regardless of how specific the process in question is. Cross-feeding in isotope-labelling

experiments has been acknowledged from the start and has been shown for nitrogen as well as carbon (e.g., [28, 29]). Although typically considered to be an unwanted side effect in SIP experiments, cross-feeding has also been taken advantage of many times to study substrate flow patterns microbial interactions on a temporal scale [30, 31]. Since cross-feeding in a microbial community cannot simply be put to a halt, the typical way of dealing with this issue is to sample at several time points, limit the incubation time to the minimum necessary for labelling and combine complementary lines of evidence when concluding that a specific taxon indeed performs the metabolism in question.

3.3. Substrate enrichment level and concentration

Substrates used in SIP experiments are in almost all cases "fully" labelled, i.e., all positions are enriched with the labelled isotope to the highest level possible (>97 atomic %). This, of course, stems from the need to achieve high levels of labelling in nucleic acids to detect labelled microbes. However, labelling of carbon only at specific positions could also be employed, for example, to study microbial guilds that would attack the substrate at a specific position of interest, while excluding others. The substrate concentration can also affect the rate and strength of labelling, however, presenting a sample with unrealistically high-concentrations can lead to undesired consequences such as drastic community changes or a rapid enrichment of a fast-growing sub-population with low substrate affinity. Therefore it is best to remain within the range (typically on the higher end) of substrate concentrations that are expected to be found in the environment.

3.4. Amount of nucleic acids to load

Typical DNA-SIP gradients are prepared with 0.5–5 μg of DNA, but there does not seem to be a hard limit for the amount of DNA that can be loaded on a gradient. For PCR purposes this amount should be more than enough to target the rRNA or any other functional gene. For metagenomic or metatranscriptomic sequencing of the fractions larger amounts of the template will be needed. This can be achieved either by pooling together several fractions from several different gradients or by multiple displacement amplification (e.g., Chen et al. 32). In RNA-SIP gradients, overloading with RNA will cause aggregation that will prevent efficient separation. The typical recommended amount is around 500 ng for a 5.5 ml gradient [33]. However, this issue was never been studied systematically.

3.5. Number of fractions to collect, and sequencing depth

Regardless of which method is used for analysing the data, success in a SIP experiment is determined by the ability to detect microbial phylotypes that are present in the denser fractions of a labelled gradient and are either absent or have lower abundance in the lighter fractions of the same gradient, or in the denser fraction of a control gradient. The detection limit in SIP experiments is itself not a fixed value but will depend on the sequencing depth, the number of fractions being collected from each gradient, and on which method is being used to analyse the data (see **Section** 4). Using state of the art sequencing technologies it is now easy to obtain thousands of sequences per fraction. However, this, of course, comes at a cost, which might not be necessary. It is therefore advisable, if possible, to first obtain an estimate of the size of the microbial guild in question in relation to the

total microbial population, using for example qPCR with primers targeting a functional gene or fluorescent microscopy. The smaller the size of the target community, the harder it will be to detect its labelling above the detection limit. Naturally, this will almost inevitably be an overestimation since only a part of the population will be active during the experiment and will eventually incorporate the substrate, but this will at least give a minimum threshold for the sequencing depth needed. The number of fractions collected can also affect the detection limit. While a higher number of fractions will most likely increase the sensitivity, it also entails higher sample processing efforts and costs. In addition, more fractions also mean less template per fraction and thus also an increased difficulty to amplify the target and a higher chance of contamination with foreign nucleic acids from the environment. Typically 12–20 fractions are collected, of which about 10–16 end up being analysed because the lightest and heaviest fractions contain little to no nucleic acids.

3.6. Unlabelled controls

As in any lab experiment, appropriate label controls should be set up in parallel to minimise the detection of false-positives. Many of the older published works included only one or two controls, usually at the last time point or at the highest amendment level. Recently, however, particularly with the growing use of high-throughput sequencing and statistical models to detect labelled OTUs the need to include more no-label controls in the experiment to correctly detect labelled phylotypes has been growing, but on the other hand also became easier to achieve. The exact number and type of no-label controls will depend on the exact statistical method used to analyse the data, but also on the type of SIP being performed since DNA-SIP is more

prone to detecting false positives than RNA-SIP because of the effect of the G+C-content on DNA BD (see **Section** 4). Ideally, every labelled sample will have its parallel no-label control. However, this is very laborious and costly, and might not be needed. Since RNA-SIP does not suffer from the bias caused by G+C-based migration as in DNA-SIP, it is possible to compare fractions within a gradient, rather than between gradients, and thus reduce the number of controls (see **Section** 4). Similarly, methods that are only interested in identifying labelling of a phylotype (e.g., differential abundance) but not necessarily quantifying it (e.g., qSIP) remain robust even when some controls are omitted (see **Section** 4 and **Chapter 11**).

3.7. Type of rotor

Traditionally a vertical rotor was preferred over a fixed-angle one for SIP experiments because it provides a shallower gradient and therefore a higher degree of separation between densities. Recent modelling work suggests, however, that this comes at the cost of a higher diffusion of nucleic acids throughout the gradient (and thus leading to a higher background) [34]. Both rotor types were successfully used for ^{15}N-SIP, but to date, no experimental comparison was published.

4. Data analysis

4.1. Analysis of barcoded amplicon data for SIP

Arguably, the most significant advancement in the field of DNA- and RNA-SIP in recent years came from the introduction of high-throughput sequencing techniques and their adoption to the study of microbial communities using barcoded amplicon sequencing [35, 36, 37]. The ability to

sequence dozens of samples simultaneously to a very high depth meant that it was now possible to identify rare taxa that were labelled but also taxa that are only partially labelled. Before the adoption of high-throughput sequencing (HT-sequencing), successful labelling of DNA or RNA was done visually, either by detecting a second band of nucleic acids under UV light following ethidium bromide staining or fractionating the gradient into multiple fractions, amplifying the nucleic acids using PCR or qPCR and evaluating the intensity of the bands or copy numbers. The use of fingerprinting techniques such as DGGE and TRFLP enabled not only a more sensitive comparison between fractions but also a direct, albeit qualitative, insight into how many phylotypes were labelled. However, it still suffered from low resolution and a high degree of noise that are inherent to these methods. Moreover, the unequivocal identification of the labelled microbes was still low-throughput, laborious and costly since it required the construction of clone libraries followed by Sanger sequencing. Barcoded amplicon sequencing allows for robust, semi quantitative comparison of different fractions along a density gradient, as well as an identification of the identity of which microbes became labelled and which did not. Moreover, the ability to obtain thousands of sequences per sample meant that even labelling of minor members of the community could be detected — something that could not be achieved with standard molecular fingerprinting techniques or Sanger sequencing. The adoption of HT-sequencing technologies also called for new analytical methods that could take advantage of this increase in sensitivity through statistical modelling and enable robust detection of either minor or partially labelled members of the active guild [38, 39]. However, alongside with added sensitivity barcoded

amplicon sequencing also presents some challenges for comparing samples because it is difficult to control the number of sequences per sample, also known as the library depth. The problem is not unique to analysing SIP experiments and poses a major analytical challenge in the field of microbiome studies and comparative transcriptomics (RNA-Seq). In essence, most statistical methods used for comparison assume that across different samples, templates with identical relative abundance should have equal chances of being sequenced and thus any observed differences are an indication that the true abundance of the given sequence differs between the samples. In ecology, the issue is known as "sampling effort". Traditionally, the most common way to alleviate the problem of unequal sequencing depths was to randomly sub-sample sequences from each sample down to the smallest sample size so that all samples become equal (a process sometimes called "rarefaction"). This practice, however, came under scrutiny in recent years and sparked some heated polemic papers on how to best handle microbiome data [40]. While the severity of the bias caused by random sub-sampling is debated, it is generally accepted that this is a sub-optimal way to deal with the problem. Another common approach is to convert all abundances to relative abundances and compare the different sequences on a fraction (or percentage) basis. This, however, leads to other problems since it maintains the correlation between sequencing depth and the number of unique sequences (or OTUs) while at the same time drastically reducing the number of degrees of freedom by coercing the sum of abundance in each sample to 100% [41]. More recent methods try to "eat the cake and leave it whole" by attempting to equalise the variance between samples through a scaling factor while not

discarding any data (covered in [42]). Whichever method is chosen, it is important to remember that no statistical trick can solve the inherent problems that stem from large differences in library sizes and these should be handled at the level of sample preparation or sequencing and not data analysis.

4.2. Differential abundance analysis and quantitative analysis

The most common methods for comparing fractions in SIP experiments were developed for analysing RNA-Seq datasets. The parallels are apparent; typical RNA-Seq experiments are designed as a case-control study and the analytical challenge is to identify which sequences are differentially expressed (either up-regulated or down-regulated) compared to the control, while overcoming the natural variance and differences in library sizes. Similarly, in SIP experiments one would like to identify which sequences are "differentially abundant" in the fractions where labelled nucleic acids are expected to be present compared to those where unlabelled nucleic acids are present. An important difference to RNA-Seq experiments is, however, that only enriched sequences in the 'heavy' fractions are of interest, while depleted sequences should only occur when labelling is strong enough to displace unlabelled sequences from the 'light' fractions to a noticeable degree. Nearly all existing data analysis methods should apply to both DNA- and RNA-SIP, albeit with some differences. This book offers two recent and very robust ways to analyse SIP datasets: quantitative SIP (qSIP; **Chapter 11**) and High-Resolution SIP (HR-SIP; **Chapter 9**). Both yield similar results, but they nevertheless differ in some details (discussed in [43]). While High-Resolution SIP, like all other differential abundance methods, aims only at detecting labelled phylotypes, qSIP also attempts to quantify the level of enrichment

per phylotype, but requires additional quantitative data from qPCR and also a matching unlabelled control sample for every labelled sample, to reliably detect growth.

4.3. Data analysis for RNA-SIP experiments

Since both HR-SIP and qSIP are carefully detailed in this book, repeating the steps here would be redundant. However, because the methods were published for DNA-SIP, some differences to RNA-SIP should be noted. In principle, both methods rely on a comparison of the gradient fractions from labelled samples to those from unlabelled control samples (between-gradient comparison). Moreover, both assume and make use of the fact that while DNA and RNA will concentrate around their theoretical BD, they diffuse throughout the gradient in a Gaussian shape so that amplifiable amounts of nucleic acids are present in every fraction in the gradient [2, 34]. However, because the course of development of a microbial community is controlled by stochastic processes in addition to deterministic ones, parallel incubations from the same parent community often lead to different communities after a while, even if conditions are kept as similar as possible. Consequently, it was demonstrated that these stochastic variations reduce the detection accuracy and it was recommended that the Bray-Curtis dissimilarity between communities of labelled and unlabelled samples that are being compared should ideally be >0.2 [34]. Between-gradient comparisons are crucial for DNA-SIP because as mentioned above, the DNA of different taxa will migrate in the gradient also based on their G+C content. Moreover, the migration based on G+C content is not constant per phylotype. Instead, it will vary based on the size of the DNA fragment surrounding the gene of target,

which varies stochastically in most DNA extraction methods [7]. In RNA-SIP however, the buoyant density of RNA is less affected by G+C content, and one can assume that in a gradient from an unlabelled sample the relative abundance of each taxon should remain relatively constant throughout the different fractions. In contrast, in a gradient from a labelled sample, some taxa will be more abundant in the heavy fractions compared to the lighter ones, while the relative abundance of unlabelled taxa will remain constant throughout the gradient or decline in the heavy fractions if the labelled taxa make up a significant proportion of the entire community. In any case, since in RNA-SIP differential migration of taxa is only expected as a response of labelling, detection of labelled taxa can also be done in a within-gradient fashion by comparing the relative abundances of taxa in the heavy fractions (i.e., ca. 1.72–1.76 g ml^{-1} for DNA-SIP or 1.80–1.84 g ml^{-1} for RNA-SIP) with those in the light fractions (i.e., ca. 1.68–1.72 g ml^{-1} for DNA-SIP or 1.77–1.80 g ml^{-1} for RNA-SIP). However, some label-free controls should nevertheless be set up (e.g., paralleling the beginning and end time points or the highest and lowest treatment extremes) and analysed because they can help to fine-tune the statistical cutoff parameters so that false positives can be avoided [2].

4.4. Network analysis using SIP data

Network analysis – the prediction of microbial associations from presence-absence or abundance data is gaining popularity in ecological studies in general and microbiome studies in particular [41]. This type of analysis has also been used in concert with SIP to detect, for example, positive and negative correlation between phylotypes of ammonia-oxidising archaea, nitrite

oxidising bacteria and methanotrophs [44], clusters of anaerobic and aerobic bacteria in rewetted biological soil crusts [10], or to identify community members that interact with methane-oxidizing bacteria [45]. However, in contrast to a standard network analysis on microbiome data, the interpretation of the results from a SIP experiment might not be so straightforward. First, most probably only the "heavy" fractions from the labelled gradients should be analysed because changes in the "light" fractions are either already reflected in the "heavy" fractions (i.e., phylotypes becoming labelled and hence depleted in the "light" fractions), or not directly related to substrate incorporation (e.g., growth and death of phylotypes in the general community). Secondly, while the interpretation of positive correlations in the heavy fractions are relatively easy to interpret (i.e., two phylotypes acquire label under similar conditions), it is not entirely clear what negative interactions mean if anything at all. Thirdly, it is important to bear in mind that network analysis does not reveal the mode of the interaction between two interacting phylotypes and a positive correlating could mean that both use the same substrate, that there is cross feeding occurring (and thus the interaction is positive-positive or at least positive-neutral), or that one phylotype is praying on another (positive-negative interaction). Lastly, it should be noted that many replicates are required for a network to be stable (at least 25) and that communities should be reasonably similar in all samples [46]. For SIP studies this probably translates into an analysis of at least 25 "heavy" gradient fractions, coming from both labelled and no-label control incubations. However, when analysing data from DNA-SIP experiments care should be taken when analysing multiple fractions from the same gradient since this

could simply be a result of similar G+C contents.

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Tables

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Table 1: What should be considered during each of the various steps in the design of a SIP experiment.

Experimental design step	**What to consider**
Which stable isotope to use?	Choice of stable isotope primarily depends on the substrate being used, but different stable isotopes differ in their ability to label nucleic acids and lipids
Prior to incubation	Measure or estimate the turnover rate of the substrate that will be used for labelling
Target molecule	Will dramatically affect what type of data will be produced and what can be learned from it
Incubation duration	Short incubation times might lead to insufficient labelling of the target molecule but long incubation times increase the risk of cross-feeding
Substrate enrichment level and concentration	Substrate should almost always be fully labelled, concentration should be within a realistic range for the sample
Amount of nucleic acids to load	Varies for DNA- and RNA-SIP. Will also depend on the downstream application
Number of fractions to collect and sequencing depth	More fractions means higher sensitivity but also higher contamination potential and sequencing costs
Unlabelled controls	Should always be included but the exact number will depend on the requirements of the data analysis method
Type of rotor	Traditionally vertical but fixed angle has been recently suggested to be advantageous
Data analysis	Consider how many gradients, fractions and types of samples (e.g. controls, time series, various concentration levels etc.) will be needed for the chosen data analysis pipeline

Table 2: Number of additional neutrons per nucleotide in a DNA or RNA molecule given full labelling (all respective atoms are replaced by a heavier stable isotope).

	Carbon-13	**Oxygen-18**	**Nitrogen-15**	**Deuterium**
Adenine	10	10/12	5	9
Guanine	10	12/14	5	10
Cytosine	9	12/14	3	9
Thymine/Uracil	10/9	14/16	2	8
Mean	9.75/9.5	12/14	3.75	9

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