Agricultural Research & Technology:
Open Access Journal

Research Article
Volume 1 Issue 2 - January 2016

Agri Res & Tech: Open Access J


# Critical Level of $^{13}C$ Enrichment for the Successful Isolation of $^{13}C$ Labeled DNA

**Teng-xiang Lian[1,2], Guang-hua Wang[1], Zhen-hua Yu[1], Xiao-bing Liu[1] and Jian Jin[1]***

[1]*Key Laboratory of Mollisols Agroecology, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, China*

[2]*University of Chinese Academy of Sciences, China*

**Submission:** December 11, 2015; **Published:** January 22, 2016

***Corresponding author:** Jian Jin, Key Laboratory of Mollisols Agroecology, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Harbin 150081, China, Tel: +86 451 8669 1286; Fax: +86 451 8660 3736; Email: jinjian29@hotmail.com

**Abstract**

In the stable-isotope probing (SIP) technology, the $^{13}C$-labelled substrate is normally used to incorporate $^{13}C$ into nucleic acids. A proper abundance of $^{13}C$ in substrate is critical to the success of SIP, because the $^{13}C$ level not only determines whether $^{13}C$ in nucleic acids is sufficient to be detective, but also affects the enrichment bias in the labeled microbes. However, such information is very rare. In this study, a serial of $^{13}C$-labelled glucose from 0 to 50 atom% $^{13}C$ was used to incubate with Escherichia coli and then performed DNA-SIP. Our results showed that the detective level of $^{13}C$-DNA could be reduced to 2 atom% $^{13}C$ of glucose (1.30 atom% $^{13}C$ in DNA extract), while the ideal level was 10 atom% $^{13}C$ glucose (2.25 atom% $^{13}C$ in DNA). The critical level of $^{13}C$ for the separation of $^{13}C$-DNA provides a new reference of DNA-SIP in order to trace active microbial populations utilizing specific C substrates in environments.

**Keywords:** Stable isotope probing; $^{13}C$-DNA SIP; $\delta^{13}C$; Glucose; Microbes

**Abbreviations:** SIP: Stable-Isotope Probing; DNA: Deoxyribonucleic Acid; RNA: Ribonucleic Acid; PLFA: Phospholipid-Derived Fatty Acid; *E.coli: Escherichia coli;* CsTFA: Cesium Trifluoroacetate; GB: Gradient Buffer; OD600: Optical Density at 600nm

## Introduction

Stable-isotope probing (SIP) of nucleic acids has become a focal method in microbial ecology since it can identify microorganisms being involved in the metabolism of specific substrates [1-7]. The $^{13}C$-labelled substrate has been typically used in this technique to incorporate $^{13}C$ into deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and phospholipid-derived fatty acid (PLFA) [5,7]. The isolation of the $^{13}C$-labeled DNA or RNA can provide the solid information of nutrient cycling in relation to phylogeny and function of uncultivated microorganisms in the natural environment [8].

Two requirements have to be satisfied in a successful isolation of the $^{13}C$-labeled DNA ($^{13}C$-DNA). The first one is that the labeled DNA should be distinguished above a background of abundant unlabeled molecules, which depends on the minimum substrate concentration and duration of the incubation. The other is the avoidance of too much substrate that may lead to cross-feeding of the substrate and enrichment bias [5,9]. Therefore, identifying the proper concentration of substrate is the key for an acceptable SIP experiment that can reflect the nature of microorganisms incorporating the substrate turnover.

However, various concentrations of $^{13}C$ labeled substrate were used in the SIP studies. For example, Radajewski et al. [8] stated that approximately 15–20 atom% $^{13}C$ was necessary for separating $^{13}C$-DNA from $^{12}C$-DNA and analyzing the link between identity and function of microorganisms, while around 30 atom% $^{13}C$ substrate was recommended for DNA-SIP in other studies [10,11]. Fan et al. [10] reported that the maize residue with 31 atom% $^{13}C$ was necessary to effectively identify the active microorganisms which decomposed the residues. In a study of commercial strains isolation, 32 atom% $^{13}C$ of biphenyl was used to successfully probe polychlorinated biphenyls (PCB)-degrading populations in PCB-contaminated river sediment [11]. Although the protocol of the DNA-SIP technology *per se* has been well documented [4,12], the critical level of the $^{13}C$ enrichment in substrate for differentiating $^{13}C$-labeled DNA has not been quantitatively tested. Narrowing such knowledge gap will be helpful to precisely assessing the metabolic "functional" genes on the specific substrates. In the present study, we incubated the *Escherichia coli* (*E.coli*) with a serial of $^{13}C$ enrichments in glucose to identify the critical level of $^{13}C$ enrichment in the substrate for the effective separation of $^{13}C$-DNA. It is predicted that the minimum enrichment of $^{13}C$ labeled substrate for the

distinguishable detection of $^{13}$C-DNA would be in a range of 10–30 atom% $^{13}$C substrate.

## Materials and Methods

### Incubation of the *E.coli* with $^{13}$C-glucose

Six levels of $^{13}$C enrichment in glucose were designed in this study. They were 0, 2, 5, 10, 20 and 50 atom % $^{13}$C in glucose (Sigma-Aldrich) as substrate. Each level comprised 3 replications. Using M9 medium with 4% of glucose [13,14], 15 µl of *E. coli* solution (optical density at the 600 nm, OD600 = 0.8) was added to the medium and incubated at 37°C for 96 hrs on an end-over-end shaker. The OD600 values of the medium were determined at the beginning and the end of incubation with a spectrometer.

### Extraction of *E.coli* DNA

DNA of *E.coli* was extracted from each treatment according to Casas and Rohwer [15]. DNA samples were visualized by electrophoresis in a 1% agarose gel. The concentration of DNA was measured using a NanoDrop 1000 (Thermo Scientific, Wilmington, DE) and the $\delta^{13}$C of the total *E.coli* DNA was determined by the Mat 253 isotope ratio mass spectrometer (Thermo Fisher, Germany). $^{13}$C enrichments were expressed relative to Pee Dee Belemnite standard as either $\delta^{13}$C or atom fraction $^{13}$C excess.

### DNA-SIP

Cesium trifluoroacetate (CsTFA, Amersham Pharmacia Biotech) density gradient centrifugation was performed to separate the $^{13}$C-labled DNA from total *E.coli* DNA [16]. Control gradients were run with the DNA from the unlabeled *E.coli* for each course to calibrate the centrifugation system. Approximately 7000 ng of each DNA sample was loaded into a centrifuge solution with a starting buoyant density of 1.60 g ml$^{-1}$. This centrifugation medium consisted of 3.2 ml of a 1.99 g ml$^{-1}$ the CsTFA solution and 1.8 ml of gradient buffer (GB). The CsTFA solution with DNA was transferred to 4.9 ml Ultracrimp tubes (Beckman, USA) using 5 ml syringes. The tubes were centrifuged in a vertical rotor (Vti 65.2, Beckman) at 179,000 g (43,500 rpm) for 40 hrs at 20°C. The gradients were fractionated into 14 fractions by being displaced with water at a flow rate of 11.3 µl/s using a syringe pump (New Era Pump Systems, Inc. New York, USA). An AR200 digital refractometer (Reichert Inc., Depew, New York, USA) in the nD-TC mode was used to measure the buoyant density of gradient fractions. DNA in each fraction was precipitated with isopropanol (885 µl) and 1/10 volume (30 µl) of 3 M sodium acetate (pH 5.2). The DNA pellets were then washed and redissolved in $ddH_2O$. The redissolved DNA was amplified using a universal primer pair for the 16S *rDNA* gene, i.e. 357f (5'- CCT ACG GGA GGC AGC AG -3') and 517r (5'- ATT ACC GCG GCT GCT GG -3') [17]. The cycling profile was 95°C for 10 min and 28 cycles of 95°C for 15 s, 60°C for 10 s and 72°C for 20 s, and then 72°C for 10 min. The PCR products from the 1$^{st}$ to 10$^{th}$ fractions were run on a 1% agarose gel.

The F (fractional abundance) was calculated as followed [18]:

$$F = \frac{^{13}C}{^{12}C + ^{13}C} = \frac{\left(\frac{\delta^{13}C}{1000} + 1\right) \times R_{PDB}}{\left(\frac{\delta^{13}C}{1000} + 1\right) \times R_{PDB} + 1}$$

Where $R_{PDB}$ = 0.0112372 (the absolute isotope ratio of the PDB $^{13}$C standard) [19].

Using SAS (SAS Institute, 1994), data were analyzed statistically. The difference of $^{13}$C enrichment in the extract DNA between treatments was assessed with protected ANOVA tests at $P < 0.05$ level [20].

Since ultracentrifuge creates gradient fractions, the stability of buyout densities between treatments greatly affects the quality of the $^{13}$C labeled DNA fractionation [12]. This stability was determined by calculating the coefficient of variability (*CV*) of buyout densities across treatments. The smaller the *CV*, the more consistent the fractionation of $^{13}$C-DNA was between treatments.

## Results

### *E.coli* growth supplied with $^{13}$C substrate and DNA extraction

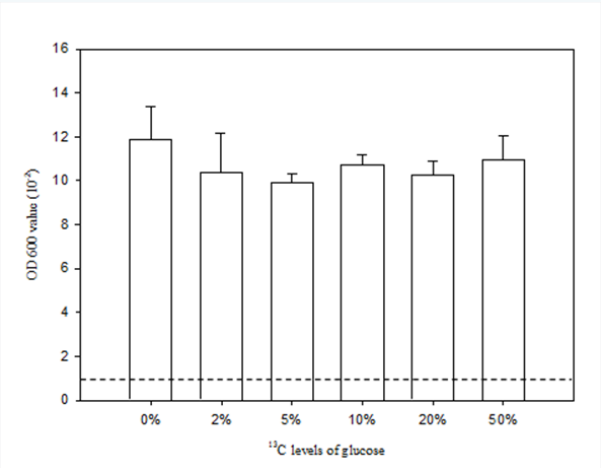


**Figure 1:** Optical densities at the 600 nm (OD600) for *Escherichia coli* that were grown in the M9 medium [1,18] for 96 hrs. The dash line indicates the initial OD600 value at the beginning of incubation.

After 96 hrs of incubation, there was no significant difference in optical density (OD600) of M9 medium between treatments (Figure 1). Relatively pure DNA was extracted from the *E.coli* and the concentrations of DNA were in a range from 441 to 760 ng/µl (Figure 2). The $\delta^{13}$C of DNA increased significantly with the


How to cite this article: Teng-xiang L, Guang-hua W, Zhen-hua Y, Xiao-bing L, Jian J. Critical Level of $^{13}$C Enrichment for the Successful Isolation of $^{13}$C Labeled DNA. Agri Res & Tech: Open Access J. 2016; 1(2): 555556.

increase of $^{13}C$ enrichment in glucose. The F values had the same trend (Table 1).

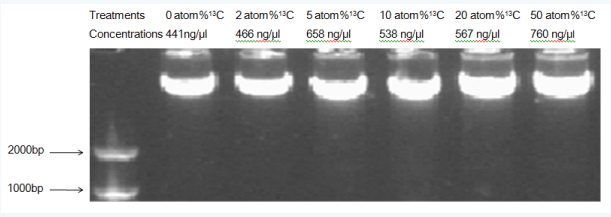


**Figure 2:** Agarose gel electrophoresis of total DNA extracted from the *Escherichia coli* incubated in the M9 medium for 96 hrs. *E.coli* was supplied with different levels of $^{13}C$ enrichment in glucose, i.e. 0, 2, 5, 10, 20 and 50 atom% $^{13}C$.

**Table 1:** The $\delta^{13}C$ and F values of the DNA extracted from *Escherichia coli* mediums. *E.coli* was supplied with different levels of $^{13}C$ abundance in glucose (0, 2, 5, 10, 20 and 50 atom% $^{13}C$)

| Treatments (atom% $^{13}C$) | $\delta^{13}C$ (‰ PDB) | F (%) |
|---|---|---|
| 0 | -27.01f | 1.08f |
| 2 | 169.57e | 1.30e |
| 5 | 522.81d | 1.68d |
| 10 | 1044.76c | 2.25c |
| 20 | 3043.08b | 4.35b |
| 50 | 10716.09a | 11.63a |

F indicates fractional abundance, i.e. $^{13}C/(^{12}C + ^{13}C)$. Different letters represent significance of difference at $P < 0.05$ level.

**Table 2:** Buyout densities from the 1st to 14th fraction of DNA-SIP in the treatments of $^{13}C$-labeled glucose (0, 2, 5, 10, 20 and 50 atom% $^{13}C$).

| Fractions | $^{13}C$ abundance in glucose (atom% $^{13}C$) | | | | | | CV |
|---|---|---|---|---|---|---|---|
| | 0 | 2 | 5 | 10 | 20 | 50 | (%) |
| 1 | 1.6187 | 1.6169 | 1.6187 | 1.6187 | 1.6169 | 1.6187 | 0.0574 |
| 2 | 1.6151 | 1.6151 | 1.6151 | 1.6151 | 1.6151 | 1.6151 | 0.0000 |
| 3 | 1.6115 | 1.6115 | 1.6115 | 1.6115 | 1.6115 | 1.6115 | 0.0000 |
| 4 | 1.6080 | 1.6080 | 1.6080 | 1.6080 | 1.6080 | 1.6080 | 0.0000 |
| 5 | 1.6044 | 1.6044 | 1.6044 | 1.6027 | 1.6044 | 1.6044 | 0.0433 |
| 6 | 1.6010 | 1.6010 | 1.6010 | 1.5992 | 1.6010 | 1.6010 | 0.0459 |
| 7 | 1.5975 | 1.5958 | 1.5975 | 1.5958 | 1.5958 | 1.5975 | 0.0583 |
| 8 | 1.5941 | 1.5924 | 1.5924 | 1.5907 | 1.5924 | 1.5941 | 0.0803 |
| 9 | 1.5907 | 1.5890 | 1.5890 | 1.5873 | 1.5890 | 1.5907 | 0.0805 |
| 10 | 1.5873 | 1.5856 | 1.5856 | 1.5840 | 1.5856 | 1.5873 | 0.0788 |
| 11 | 1.5840 | 1.5823 | 1.5823 | 1.5807 | 1.5823 | 1.5840 | 0.0789 |
| 12 | 1.5823 | 1.5807 | 1.5807 | 1.5790 | 1.5790 | 1.5807 | 0.0790 |
| 13 | 1.5710 | 1.5742 | 1.5171 | 1.5742 | 1.5742 | 1.5758 | 1.4851 |
| 14 | 1.4707 | 1.4611 | 1.4738 | 1.4738 | 1.4611 | 1.4823 | 0.5589 |

The buyout density (Table 2) ranged from 1.582 g $ml^{-1}$ to 1.622 g $ml^{-1}$. The density in each fraction was consistent across treatments with small *CV*. This suggested that the performance of ultracentrifugation was consistent and successful between courses.

## PCR products of the fractions

For the unlabeled control, the DNA band was visible from the 8th to 10th fraction (Figure 3). However, appearance of DNA band started in the 7th fraction under the 2 atom% $^{13}C$ treatment. The distinguishable DNA band moved towards smaller fractions with the increase of $^{13}C$ enrichment. At 50 atom% $^{13}C$, DNA band was clearly observed in the 2nd–10th fraction.

## Discussion

The insignificant OD600 values between treatments (Figure 1) suggested that the growth of *E.coli* was not affected by the addition of glucose with different $^{13}C$ enrichments. Furthermore, the relatively high concentration of DNA and the clear bands of total DNA indicated that the extracted DNA (Figure 2) was


**How to cite this article:** Teng-xiang L, Guang-hua W, Zhen-hua Y, Xiao-bing L, Jian J. Critical Level of $^{13}C$ Enrichment for the Successful Isolation of $^{13}C$ Labeled DNA. Agri Res & Tech: Open Access J. 2016; 1(2): 555556.

appropriate for the DNA-SIP process.

In this study, the DNA band in the 7th fraction appeared for the 2 atom% $^{13}C$-glucose treatment, while non DNA band in the same fraction for the unlabeled control, revealing that the detectable level of $^{13}C$ substrate for $^{13}C$-DNA-SIP under the pure culture condition can be reduced to 2 atom% $^{13}C$ in glucose. However, the better separation of $^{13}C$-DNA was achieved in the ≥10 atom% $^{13}C$ treatment since the DNA band was visible in the 5th, 6th and 7th fraction under that treatment.

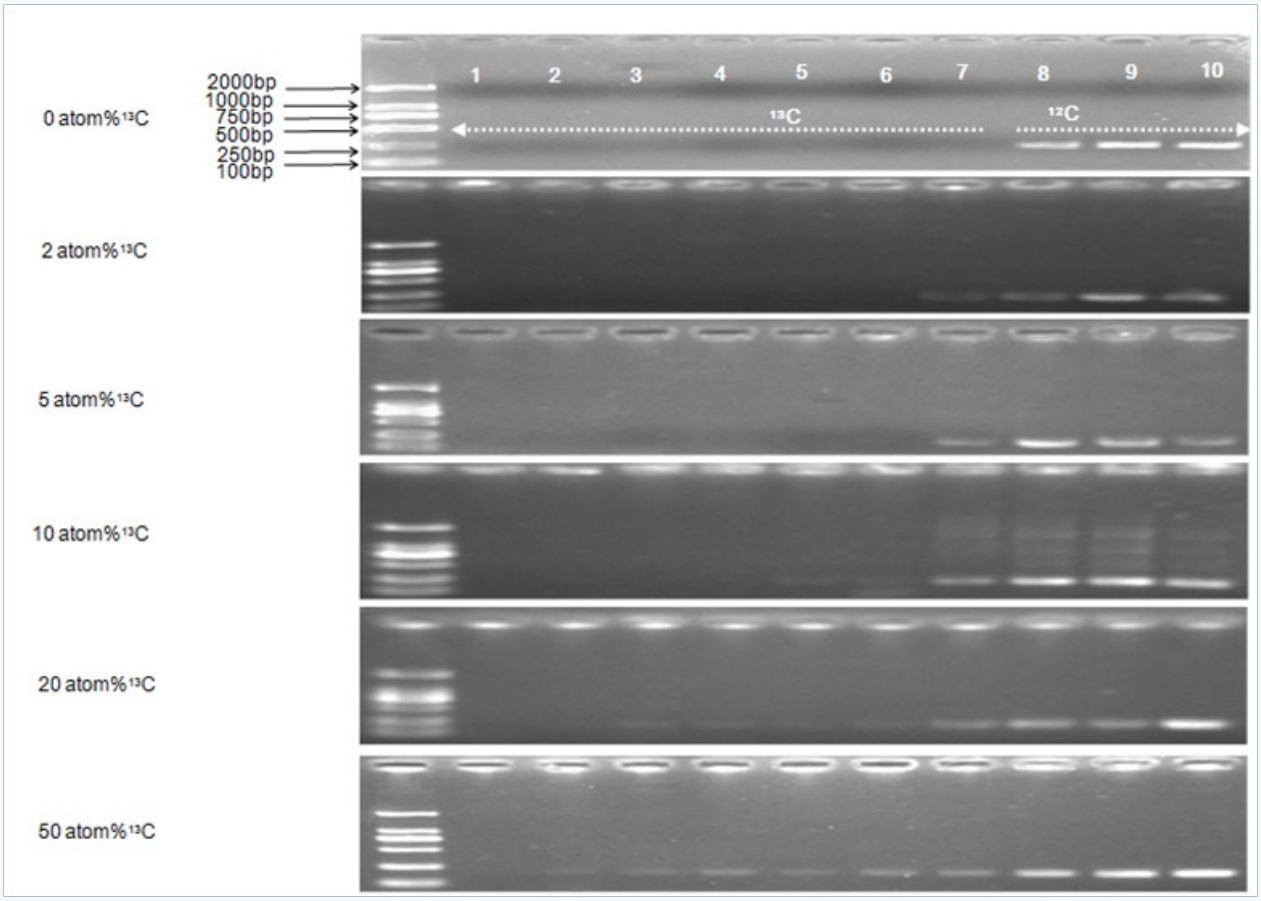


**Figure 3:** Aliquots of gradient fractions of DNA-SIP were run on 1% agarose gels and a 1-kb ladder is included as a marker. DNA samples were extracted from the M9 mediums of Escherichia coli supplied with 0, 2, 5, 10, 20 and 50 atom% $^{13}C$ in glucose.

The detectable level of $^{13}C$ substrate for $^{13}C$-DNA-SIP in this study is lower than that in a study by Radajewski et al. [8], reporting that 20 atom% $^{13}C$ in substrate would be feasible to incorporate $^{13}C$ into DNA, and resolve $^{13}C$-DNA from $^{12}C$-DNA. The difference is probably because the carbon source of $^{13}CH_3OH$ used in that study could only label the methylotrophic bacterium i.e. *Methylobacterium extorquens* and this bacterium was not dominant in the soil microbial communities, while the $^{13}C$-labeled glucose was the sole C source in the pure incubation solution in this study. Thus, the threshold of $^{13}C$ enrichment in glucose for the separation of $^{13}C$-DNA in this study was not as high as other studies. In addition, compared with $^{15}N$ substrate for $^{15}N$-DNA-SIP, the minimum requirement of $^{13}C$ enrichment in substrate was much lower even under the similar pure culture condition [1]. The minimum level of a $^{15}N$-labelled substrate, i.e. $NH_4NO_3$ has been quantified as low as 40 atom% $^{15}N$ for the clear separation of $^{15}N$-DNA of *Pseudomonas putida* [1]. The difference is attributed to the fact that the C concentration of DNA is theoretically in a range of 41.2 to 46.1%, while the N concentration of DNA varies only between 13.9 and 15.8% [1]. This is also reflected in the shifts in buoyant density in the CsCl gradients. For $^{13}C$-labeled DNA this shift is approximate 0.036 g/ml, while it is only 0.013–0.016 g/ml for $^{15}N$-labeled DNA [21,22].

Nevertheless, the $^{13}C$ enrichment in the extracted DNA for the minimum requirement of DNA-SIP is probably more practicable than that in substrates when this technology are used in the environmental experiments, especially in the soil experiments, because a number of uncertain factors such as carbon-conversion efficiency and growth rate of the target organisms [5] may lower the convert efficiency of $^{13}C$ from substrate to DNA. A number of


**How to cite this article:** Teng-xiang L, Guang-hua W, Zhen-hua Y, Xiao-bing L, Jian J. Critical Level of $^{13}C$ Enrichment for the Successful Isolation of $^{13}C$ Labeled DNA. Agri Res & Tech: Open Access J. 2016; 1(2): 555556.

studies have also stated that the requirement for supplemental nutrient addition for C assimilation of microbes and the amount of carbon incorporated into nucleic acid mostly depend on the targeted microorganisms and the characteristics of the samples being analyzed [4,12]. On the point of these views, the $^{13}C$ enrichment in the extracted DNA is likely to be more accurate for predicting the successful SIP. In this study, the detectable level of $^{13}C$ enrichment for DNA-SIP in the extracted DNA was 1.3 atom% $^{13}C$ (Table 1).

It is worth to note that $^{13}C$-DNA bands were successively distinguishable from light to heavy fractions with the increase of $^{13}C$ enrichment in substrate (Figure 3). This indicates that $^{13}C$ gradually and proportionally incorporates into the C frame of the DNA molecular. However, in some DNA-SIP studies [5,23], the $^{13}C$ DNA bands were observed in the heavy fractions (the 4$^{th}$ and 5$^{th}$ fractions) rather than the fractions in between such as the 6$^{th}$ and 7$^{th}$ fractions. The difference may be attributed to the $^{13}C$ enrichment of substrate, incubation time and microbial specificity in utilizing $^{13}C$ substrates.

## Conclusion

In this study, we confirmed that the level of $^{13}C$ enrichment in glucose for detectable $^{13}C$-labeled DNA could be reduced to 2 atom% $^{13}C$ (1.30 atom% $^{13}C$ in DNA extract). The critical level of $^{13}C$ for the isolation of $^{13}C$-DNA provides a new reference of DNA-SIP in order to trace active microbial communities utilizing specific C substrates in environments.

## Acknowledgement

The project was funded by the Key Project of Chinese Academy of Sciences (KZZD-EW-TZ-16-01), the Hundred Talents Program, Heilongjiang Provincial Funds for Distinguished Young Scientists (JC201413) and the National Natural Science Foundation of China (41271261).

## References

1. Cadisch G, Espana M, Causey R, Richter M, Shaw E, et al. (2005) Technical considerations for the use of $^{15}N$-DNA stable-isotope probing for functional microbial activity in soils. Rapid Commun Mass Spectrom 19(11): 1424-1428.
2. Ginige MP, Hugenholtz P, Daims H, Wagner M, Keller J, et al. (2004) Use of stable-isotope probing, full-cycle rRNA analysis, and fluorescence in situ hybridization-microautoradiography to study a methanol-fed denitrifying microbial community. Appl Environ Microbiol 70(1): 588-596.
3. Manefield M, Whiteley AS, Griffiths RI, Bailey MJ (2002) RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. Appl Environ Microbiol 68(11): 5367-5373.
4. Neufeld JD, Dumont MG, Vohra J, Murrell JC (2007) Methodological considerations for the use of stable isotope probing in microbial ecology. Microb Ecol 53(3): 435-442.
5. Neufeld JD, Vohra J, Dumont MG, Lueders T, Manefield M, et al. (2007) DNA stable-isotope probing. Nat Protoc 2(4): 860-866.
6. Radajewski S, Webster G, Reay DS, Morris SA, Ineson P, et al. (2002) Identification of active methylotroph populations in an acidic forest soil by stable-isotope probing. Microbiology 148(Pt 8): 2331-2342.
7. Uhlík O, Jecná K, Leigh MB, Macková M, Macek T (2009) DNA-based stable isotope probing: a link between community structure and function. Sci Total Environ 407(12): 3611-3619.
8. Radajewski S, Ineson P, Parekh NR, Murrell JC (2000) Stable-isotope probing as a tool in microbial ecology. Nature 403(6770): 646-649.
9. Morris SA, Radajewski S, Willison TW, Murrell JC (2002) Identification of the functionally active methanotroph population in a peat soil microcosm by stable-isotope probing. Appl Environ Microbiol 68(3): 1446-1453.
10. Fan F, Yin C, Tang Y, Li Z, Song A, et al. (2014) Probing potential microbial coupling of carbon and nitrogen cycling during decomposition of maize residue by $^{13}C$-DNA-SIP. Soil Biol Biochem 70: 12-21.
11. Sul WJ, Park J, Quensen JF, Rodrigues JL, Seliger L, et al. (2009) DNA-stable isotope probing integrated with metagenomics for retrieval of biphenyl dioxygenase genes from polychlorinated biphenyl-contaminated river sediment. Appl Environ Microbiol 75(17): 5501-5506.
12. Dunford EA, Neufeld JD (2010) DNA stable-isotope probing (DNA-SIP). J Vis Exp (42): pii: 2027, doi: 10.3791/2027.
13. Barman DN, Haque MA, Islam SMA, Yun HD, Kim MK (2014) Cloning and expression of ophB gene encoding organophosphorus hydrolase from endophytic Pseudomonas sp. BF1-3 degrades organophosphorus pesticide chlorpyrifos. Ecotoxicol Environ Saf 108: 135-141.
14. Sancar A, Hack AM, Rupp WD (1979) Simple method for identification of plasmid-coded proteins. J Bacteriol 137(1): 692-693.
15. Casas V, Rohwer F (2007) Phage metagenomics. Methods Enzymol 421: 259-268.
16. Haichar FZ, Achouak W, Christen R, Heulin T, Marol C, et al. (2007) Identification of cellulolytic bacteria in soil by stable isotope probing. Environ Microbiol 9(3): 625-634.
17. Muyzer G, Dewaal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59(3): 695-700.
18. Boutton TW (1991) Stable carbon isotope ratios of natural materials: I. Sample preparation and mass spectrometric analysis. Carbon isotope techniques 155-171.
19. Stewart D, Metherell A (1999) Carbon ($^{13}C$) uptake and allocation in pasture plants following field pulse-labelling. Plant Soil 210(1): 61-73.
20. Steel R, Torrie J (1980) Principles and Procedures of Statistics: A Biometrical Approach. McGrawiHill Co, New York, USA, pp. 633.
21. Buckley DH, Huangyutitham V, Hsu S-F, Nelson TA (2007) Stable isotope probing with $^{15}N$ achieved by disentangling the effects of genome G+ C content and isotope enrichment on DNA density. Appl Environ Microb 73(10): 3189-3195.
22. Cupples AM, Shaffer EA, Chee-Sanford JC, Sims GK (2007) DNA buoyant density shifts during $^{15}N$-DNA stable isotope probing. Microbiol Res 162(4): 328-334.
23. Xia W, Zhang C, Zeng X, Feng Y, Weng J, et al. (2011) Autotrophic growth of nitrifying community in an agricultural soil. ISME J 5(7): 1226-1236.


**How to cite this article:** Teng-xiang L, Guang-hua W, Zhen-hua Y, Xiao-bing L, Jian J. Critical Level of $^{13}C$ Enrichment for the Successful Isolation of $^{13}C$ Labeled DNA. Agri Res & Tech: Open Access J. 2016; 1(2): 555556.