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Note

Development of a SYBR safe™ technique for the sensitive detection of DNA in cesium chloride density gradients for stable isotope probing assays

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Abstract

SYBR safe™, a fluorescent nucleic acid stain, was evaluated as a replacement for ethidium bromide (EtBr) in cesium chloride (CsCl) density gradients for DNA stable isotope probing (DNA-SIP) assays. The separation of ^{12}C- and ^{13}C-labelled DNA using SYBR safe™ gave similar results to those obtained using EtBr with pure cultures and environmental samples exposed to a ^{13}C-labelled substrate, while the detection limit of DNA was enhanced by the use of SYBR safe™ by at least 5 times. The results demonstrated that SYBR safe™ is a safe, sensitive and effective alternative to the use of ethidium bromide in CsCl density gradients for DNA-SIP assays.

Keywords: Stable isotope probing; Cesium chloride density gradient; SYBR safe™; Ethidium bromide

Stable isotope probing (SIP) of DNA is a technique that has become an important tool in microbial ecology (Radajewski et al., 2000). In this technique, compounds labelled with stable isotopes like ^{13}C or ^{15}N are provided as substrates for the growth of microorganisms in environmental samples. These microorganisms incorporate the stable isotopes into their cellular compounds, including phospholipid fatty acids (PLFA), RNA and DNA, which can be used as biomarkers to identify the bacterial population implicated in the metabolism of the substrate of interest. ^{13}C-labelled DNA is the biomarker that has been the most extensively used in SIP studies. The ^{13}C-labelled DNA can be separated from non-labelled ^{12}C-DNA by cesium chloride (CsCl) density gradient ultracentrifugation and screened through various phylogenetic and functional analyses. Using DNA stable isotope probing (DNA-SIP), the active microbial populations implicated in the degradation of various compounds have been identified (Friedrich, 2006).

One of the critical steps of the DNA-SIP technique is the CsCl density gradient ultracentrifugation. During this step, the ^{13}C-DNA is separated from the ^{12}C-DNA based on their respective buoyant densities. High concentrations of ethidium bromide (EtBr) are commonly added in the CsCl gradients to allow for the visualization of the DNA under UV light following the ultracentrifugation. This technique has numerous disadvantages. EtBr, because of its tendency to intercalate between DNA bases, is known as a carcinogenic and mutagenic agent (Singer et al., 1999). Moreover, several studies have shown that UV exposure can damage DNA through photochemical reactions (Cariello et al., 1988; Emanuele et al., 2005) and subsequently compromise its use in downstream molecular biology analyses (Hartman, 1991; Gründemann and Schomig, 1996). Finally, EtBr wastes require safe disposal involving considerable environmental and monetary costs.

In the present study, we tested the use of SYBR safe™ (Invitrogen, Carlsbad, CA) as a replacement for EtBr in the CsCl density gradient ultracentrifugation step of SIP assays. SYBR safe™ is a fluorescent nucleic acid stain that has a lower mutagenic potential than EtBr and is classified as non-hazardous (http://probes.invitrogen.com/media/publications/494.pdf). Moreover, it was developed to be visualized optimally using a blue light transilluminator that does not damage DNA. The results obtained in this study also demonstrated that the use of

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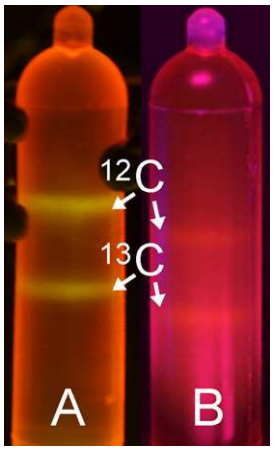

Fig. 1. Comparison of the CsCl density gradient ultracentrifugation of 5 μg of ^{12}C- and ^{13}C-DNA from *M. trichosporium* with SYBR safe™ (A) or EtBr (B).

SYBR safe™ improves the detection limit of DNA in CsCl density gradients.

The first step of this study was to verify that a similar separation of the ^{12}C- and ^{13}C-DNA could be obtained using SYBR safe™ instead of EtBr in the CsCl density gradients. ^{12}C- and ^{13}C-DNA was obtained through the cultivation of *Methylosinus trichosporium* on ^{12}C- or ^{13}C-methane. DNA was extracted following the protocol described by Pospiech and Neumann (1995) and was quantified using the PicoGreen® dsDNA quantitation assay (Invitrogen, Carlsbad, CA). Five micrograms of each ^{12}C- and ^{13}C-DNA from *M. trichosporium* was loaded into the ultracentrifugation tubes and duplicates were performed for each DNA stain. For the ultracentrifugation with EtBr, the tubes were prepared as described by Radajewski et al. (2004). For the ultracentrifugation with SYBR safe™, we determined that the tubes had to be prepared according to the following protocol. The volume of the DNA extract was adjusted to 700μL with TE (10:1) buffer. One gram of CsCl was dissolved in this solution and 25μL of 10 000× SYBR safe™ were added. The resulting mixture was loaded into a 13 × 51mm polyallomer Quick-Seal centrifuge tube (Beckman, Fullerton, CA, USA). The tubes were filled with a 1g mL^{-1} CsCl solution and were heat-sealed. The final density of the solution was 1.72g mL^{-1}, as determined by refractometry (Reichert Abbe Mark II Refractometer, Reichert, Depew, NY, USA). Tubes were ultracentrifuged (L8-70M ultracentrifuge, Beckman, Fullerton, CA, USA) at 265 000×*g* for 16h in a Vti80 rotor (Beckman, Fullerton, CA, USA). Tubes containing SYBR safe™ were visualized using the Safe Imager™ blue light transilluminator (Invitrogen, Carlsbad, CA) while tubes containing EtBr were visualized using a long-wave UV hand light (Model UVL 56, UVP, Upland, CA, USA).

Typical results for the ultracentrifugation of 5μg of each ^{12}C- and ^{13}C-DNA from *M. trichosporium* with SYBR™ safe or EtBr are presented in Fig. 1. Results were consistent between replicates (data not shown). Ultracentrifugation with SYBR safe™ resulted in the efficient separation of the two DNA bands (Fig. 1A). Band separation was similar to what was observed when the ^{12}C- and ^{13}C-DNA were ultracentrifuged with EtBr (Fig. 1B).

The detection limit of DNA in CsCl gradients prepared with SYBR safe™ as a dye was determined. Five ultracentrifugation tubes were set up, ultracentrifuged and visualized as described previously for SYBR safe™, each one containing 2000ng of ^{12}C-DNA and either 0, 100, 200, 500, 1000 or 2000ng of ^{13}C-DNA from *M. trichosporium* (Fig. 2). Using this approach, we found that a band containing 100ng of DNA was faint but detectable in the CsCl density gradient, while a 200ng band was easily visualized. The ultracentrifugation in CsCl density gradients of 100ng and 200ng of ^{12}C-DNA from *M. trichosporium* was repeated to confirm the value of the detection limit (data not shown).

The potential of using SYBR safe™ for the ultracentrifugation in CsCl density gradients of DNA from environmental samples exposed to a ^{13}C-labelled substrate was also evaluated. Two microcosms containing 20g of an Arctic surface soil sample collected at Eureka (Ellesmere Island, Nunavut) were incubated at 4°C with 20mL of NMS medium until 5 or 40mL of $^{13}CH_4$ were consumed. DNA was extracted from 10g of soil from each of the two microcosms following the protocol described by Fortin et al. (2004), but without performing the PVPP purification step. Ultracentrifugation tubes containing the

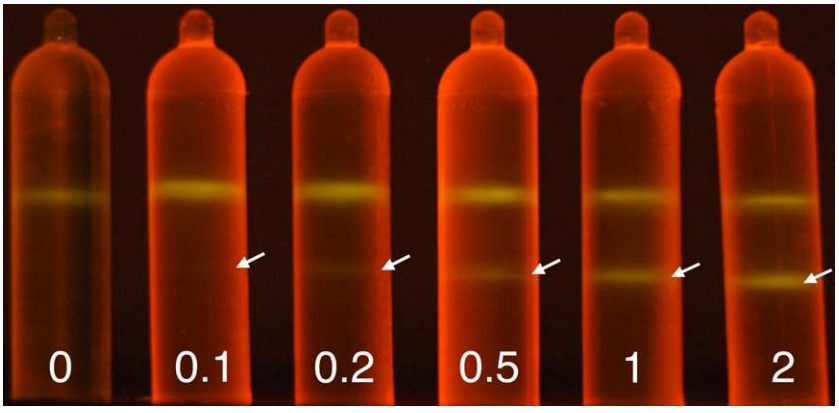

Fig. 2. CsCl density gradient ultracentrifugation with SYBR safe™ of 0, 0.1, 0.2, 0.5, 1.0 and 2.0 μg of ^{13}C-labelled DNA with a constant amount of 2.0 μg of ^{12}C-DNA, both from *M. trichosporium*. Arrows indicate the position of the ^{13}C-DNA band.

DNA extracted from each microcosm were set up as described above, ultracentrifuged at 177 000×*g* for 40h and visualized with the Safe Imager™ blue light transilluminator.

CsCl density gradient ultracentrifugation of DNA from an environmental sample incubated under ^{13}C-methane using SYBR safe™ resulted in two bands corresponding to the ^{12}C-DNA and ^{13}C-DNA (Fig. 3). After the consumption of 5mL of ^{13}C-methane, the two DNA bands were clearly visible (Fig. 3A), while a longer incubation with up to 40mL of ^{13}C-methane led to a stronger ^{13}C-DNA band and a much fainter ^{12}C-DNA band (Fig. 3B). SYBR safe™ was also used for the ultracentrifugation in CsCl density gradients of DNA from numerous other environmental samples exposed to ^{13}C-methane, with similar results.

The results obtained in this study showed that SYBR safe™ is an effective replacement for EtBr in CsCl density gradients. The separation of the ^{12}C- and ^{13}C-DNA bands, which is crucial for SIP analysis, was achieved using SYBR safe™ as a dye in the CsCl density gradient, both for pure culture DNA and for DNA from an environmental sample previously exposed to a ^{13}C-labelled compound. Our results also demonstrated that the use of SYBR safe™ with the Safe Imager™ blue light transilluminator provided a sensitive detection of DNA in CsCl density gradients. While we were not able to detect less than 2μg of DNA with EtBr (data not shown) and detection limits between 0.5 and 2μg have been reported by others for EtBr (Cadisch et al., 2005; Neufeld et al., 2007), we were able to visualize a band containing amounts as low as 100ng of DNA using SYBR safe™. This increased sensitivity can play an important role in SIP analysis, where the labelling of DNA with ^{13}C can be challenging. The success of a SIP analysis resides not only in the labelling of enough DNA to be able to detect it, but also on short incubation times that limit the extent of cross-feeding. The use of a more sensitive dye provides the opportunity to detect lower amounts of DNA and, therefore, to reduce incubation times.

An interesting aspect of the use of SYBR safe™ in CsCl density gradients is that it is optimally visualized using the Safe Imager™, a blue light transilluminator that does not damage the DNA. The use of a blue light transilluminator can be of critical importance when the DNA retrieved from the gradient is to be used in molecular techniques that require intact, undamaged DNA. For example, the ^{13}C-DNA retrieved from a CsCl density gradient ultracentrifugation tube can be used to create a metagenomic library (Dumont et al., 2006). Such an approach, that has the potential of increasing the amount of information obtained through SIP studies, includes a cloning step that can be negatively affected by previous exposure of the DNA to UV (Hartman, 1991; Gründemann and Schomig, 1996).

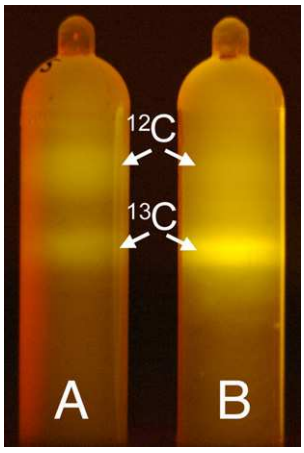

Fig. 3. CsCl density gradient ultracentrifugation with SYBR safe™ of DNA extracted from an environmental sample that had metabolized either 5 mL (A) or 40 mL (B) of ^{13}C-methane.

DNA stable isotope probing is a powerful tool in microbial ecology and it has the potential to provide a great deal of information on microbial activity in environmental samples. However, the application of this technique can be limited by the ability to detect the labelled DNA. Here, we developed and tested a modification of the CsCl density gradient step of the SIP assay that permits a more sensitive detection of DNA. This modification, which consisted of replacing ethidium bromide by SYBR safe™ in the CsCl solution, is simple and, when combined with the use of the Safe Imager™ blue light transilluminator, provides several other advantages including increased safety for the user, much less potential damage to the resulting DNA, and does not generate hazardous waste material. Therefore, we suggest that SYBR safe™ is applicable to DNA-SIP and a significant improvement over the currently employed EtBr-based technique.

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