Short Communication

Do it yourself! – Experiences with self-synthesized CsTFA for RNA-SIP analyses

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Abstract

Cesiumtrifluoroacetate (CsTFA) is a key chemical for RNA-based stable isotope analyses to link the structure and function of microbial communities. We report a protocol to easily synthesize CsTFA from Cesiumcarbonate ($Cs_2CO_{3)}$ and Trifluoroacetate (TFA) and show that self-synthesized CsTFA behaves similar to commercial CsTFA in the separation of isotopically labelled and unlabelled *E. coli* RNA.

Text

RNA-based Stable Isotope Probing (RNA-SIP) is an elegant technique to link the structure (community composition) and function (physiological properties) of complex microbial communities (Ghori et al., 2019; Lueders et al., 2016). To do so, microbial communities are incubated with an isotopically labelled (mostly ^{13}C) substrate under conditions that are as close as possible to the *in-situ* environmental conditions. During the incubation, some microorganisms assimilate the provided substrate and integrate the label into their RNA. After incubation, RNA is extracted from the incubations and separated into labelled and unlabelled fractions by buoyant density-gradient ultracentrifugation. Comparative sequencing of labelled vs. unlabelled RNA and subsequent bioinformatics analyses finally reveal the taxonomic identity of the organisms that have largely assimilated the provided substrate. Introduced into molecular microbial ecology almost 20 years ago (Manefield et al., 2002a; Manefield et al., 2002b), RNA-SIP provided new and interesting insight into the functionality of microbial communities, e.g. in the fields of environmental microbiology and bioremediation (Vogt et al., 2016) or animal - microbiome interactions (Berry and Loy, 2018). We successfully used this technique to identify prebiotics-assimilating bacteria from intestinal tracts (Egert et al., 2018).

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Cesium-Trifluoracetate (CsTFA) is a chaotropic salt which is used in RNA-SIP analyses to create the buoyant density gradients for the separation of labelled and unlabeled RNA during ultracentrifugation. To prepare the gradient forming solution, all RNA-SIP protocols known to us recommend the use of a CsTFA stock solution with a concentration of ~ 6 mol/L and a density of ~ 2 g/mL, which, however, is currently not commercially available. Unfortunately, the solubility of powdery CsTFA in water is limited to 2 mol/L (Alfa Aesar by Thermo Fisher Scientific, 2021). To overcome this shortage of CsTFA, we aimed at synthesizing a sufficiently concentrated solution using Cs_2CO_3 and TFA (Trifluoroacetate) as reactants. Synthesis turned out to be straightforward using the following approach.

Glassware to be used was sealed with aluminum foil at the openings and RNA-sterilized by heating in an oven overnight at 250 °C. For the synthesis, 97.9 g (0.3 mol) of Cs_2CO_3 (Cesium carbonate 99.9% trace metal basis, Sigma-Aldrich, Taufkirchen, Germany) were weighed directly into a 250 mL two-neck round bottom flask equipped with a magnetic stirrer, a sterile septum and a condenser (Fig. 1). Subsequently, 10 mL of sterile, nuclease-free, autoclaved, DEPC-treated water (Carl Roth, Karlsruhe, Germany) were added resulting in a thick, yet still stirrable suspension to which 46.2 mL (0.6 mol) of TFA (Trifluoroacetic acid ReagentPlus, 99%, Sigma-Aldrich) were added in small portions via a sterile syringe over 40 minutes. The resulting clear, but still CO_2 producing solution was stirred for additional 60 minutes. Afterwards, the pH was adjusted to approximately pH 7 by adding small amounts of Cs_2CO_3 or TFA, respectively, and controlled by means of pH paper. Finally, the solution was adjusted to 100 mL in a graduated flask and density was controlled by weighing to be ~ 2 g/mL (1.96 - 2.03 g/mL).

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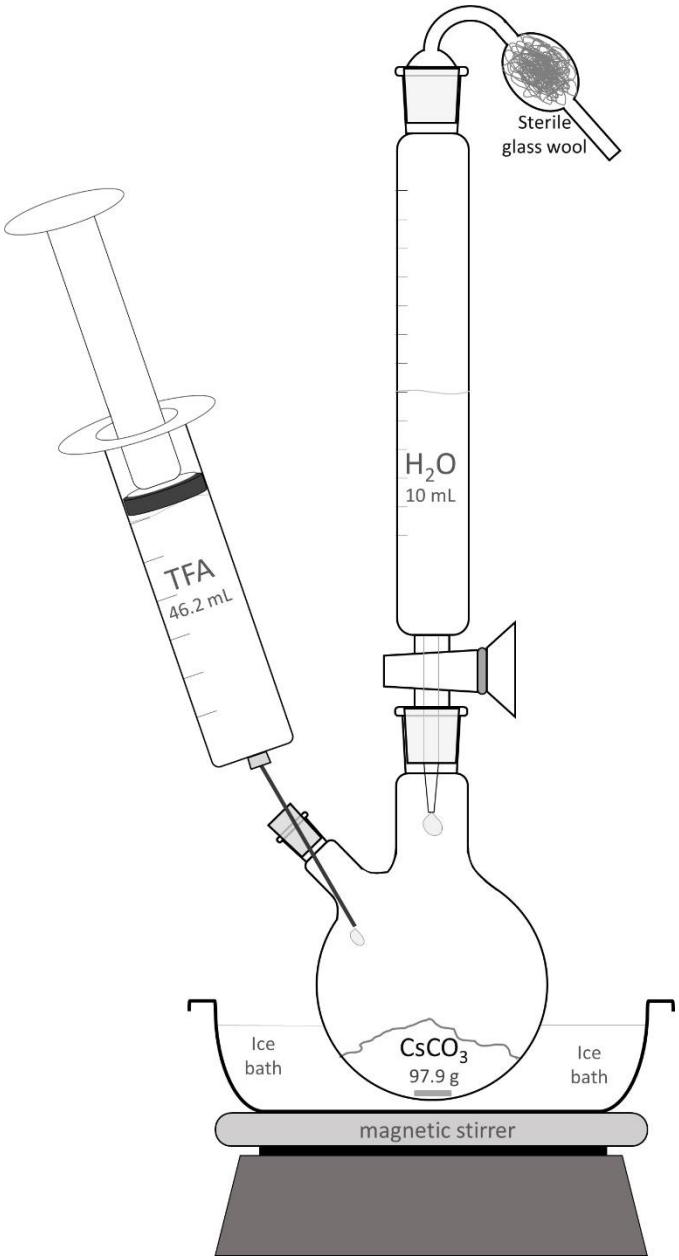

Figure 1: Schematic layout for the synthesis of CsTFA with Cs_2CO_3 and TFA. All components in direct contact with the chemicals were rendered RNA-sterile. Indicated masses and volumes are calculated to produce CsTFA with approximately 2 g/mL after adjusting to a final volume of 100 mL with water.

The suitability of the self-synthesized CsTFA (s-CTFA) solution for RNA-SIP analyses was tested using a 1:1 mixture of unlabeled and fully ^{13}C-labeled *E. coli* RNA as described previously (see below). In brief, labeled *E.coli* RNA was extracted from *E. coli* cells grown in

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minimal medium with ^{13}C-glucose as sole carbon source, quantified spectrometrically, and subsequently mixed with *E. coli* RNA extracted from cells grown with ^{12}C-glucose. We previously used the same approach to compare different rotor types (Herrmann et al., 2017) as well as gradient solutions with and without formamide (Weis et al., 2020) for their performance in RNA-SIP analyses. Using a well-established and previously published RNA-SIP centrifugation and analysis protocol (Weis et al., 2019), self-synthesized and commercial CsTFA (c-CsTFA, GE Healthcare, Freiburg, Germany) showed very similar separation results (Fig. 2). In both cases, the labelled and unlabelled RNA, quantified by means of a low range Ribogreen assay (Quant-it RiboGreen RNA Assay Kit, Thermo Fisher Scientific, Schwerte, Germany) from the harvested gradient factions, were nicely separated into two distinct peaks, located at densities that were very similar to previously published densities for labeled (1.8-1.82 g/mL) and unlabeled (1.775-1.795 g/mL) RNA (Weis et al., 2020).

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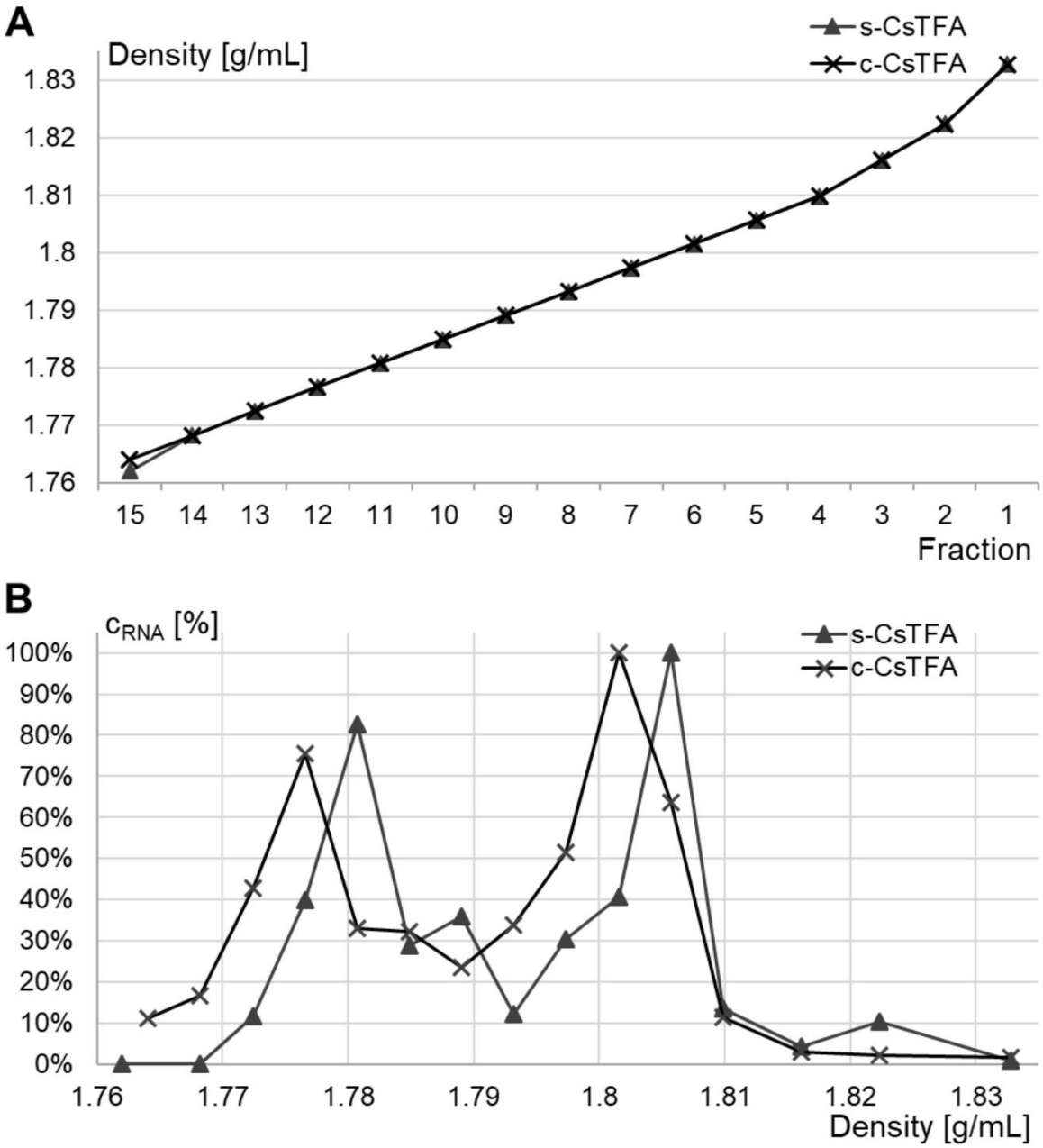

Figure 2: Density gradients and RNA contents. Density gradients (A) produced with self-synthesized (s-CsTFA) and commercial (c-CsTFA) Cesiumtrifluoroacetate both showed a nice linear distribution with densities ranging from 1.7641 g/mL (c-CsTFA) or 1.762 g/mL (sCsTFA) in the 15th fractions to 1.8328 g/mL in both first fractions. RNA was extracted from *Escherichia coli*, incubated with unlabelled glucose and uniformly ^{13}C labelled glucose, respectively, mixed in a 1:1 ratio, and separated by ultracentrifugation using CsTFA density gradients, resulting in a density dependent distribution (B). RNA concentration was quantified

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by a low-range Ribogreen assay and is given as relative RNA content, whereby the highest RNA concentration of each gradient was set as 100%.

Notably, although CsTFA is ascribed an RNAse inactivating activity (Farrell, 2010; Mirkes, 1985), care must be taken to prevent contamination of the s-CsTFA with RNAses. In particular we recommend the use of clean and thoroughly heat-sterilized or RNase free equipment, high purity starting materials, and an RNAse-free handling procedure, avoiding unnecessary contact with potential RNAse sources, such as water, air or skin. Otherwise, dissolved RNA might be rapidly degraded within the gradient solutions. For instance, we observed an almost complete degradation of 850 ng (100 ng/mL) of RNA within 48 h when the CsTFA was prepared without the preventive measures suggested above (data not shown). Since the reaction is exothermic, cooling the flask with ice is advisable (Fig. 1). In addition, we only observed sufficient RNA separation with s-CsTFA solution that had been finally adjusted with TFA or Cs_2CO_3 to an approximate neutral pH of 7.0.

In summary, if care is taken to avoid contamination with RNAses, our results clearly suggest that self-synthesized CsTFA at neutral pH can replace commercially available CsTFA for the use in RNA-SIP analyses.

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