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ORIGINAL ARTICLE

Optimizing the Use of Solid-Phase Reversible Immobilization Beads for High-Throughput Full-Length 16S rDNA Sequencing Library Construction

Yinmei Li[1,2], Ziqiang He[1], Mimi Kong[2] and Dong Jin[1,*]

Abstract

Objective: Solid-phase reversible immobilization (SPRI) beads are widely used for high-throughput sequencing library construction to purify and recover nucleic acids. This research was aimed at investigating the effects of SPRI bead ratio, incubation time, and elution time on nucleic acid recovery during full-length 16S rDNA high-throughput sequencing library construction.

Methods: The effects of different SPRI bead ratios, incubation times, and elution times were compared for three different initial sample amounts. An L9(3^3) orthogonal experiment was designed to determine the optimal combination of these factors.

Results: The incubation time of three factors including SPRI beads ratio, incubation time, and elution time had a statistically significant effect on the recovery rate for the initial sample amount of 1500 ng and 3000 ng. The orthogonal experiment results indicated that incubation time had the greatest impact among the three factors.

Conclusion: Incubation time significantly influences recovery rate in full-length 16S rDNA high-throughput sequencing library construction. The use of 0.8× SPRI beads, 15 minutes of incubation, and 10 minutes of elution resulted in the highest recovery rate. SPRI beads offer a viable method for recovering full-length 16S rDNA amplicons.

Keywords: SPRI beads, full-length 16S rDNA high-throughput sequencing, library construction, recovery rate, nucleic acid purification and recovery

***Corresponding author:**
E-mail: jindong@icdc.cn (DJ)

[1]State Key Laboratory of Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Changping District, Beijing 102206, China
[2]Department of Epidemiology, School of Public Health, Shanxi Medical University, Taiyuan, Shanxi, 030001, China

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INTRODUCTION

Solid-phase reversible immobilization (SPRI) bead technology is a method for nucleic acid purification and recovery based on the reversible immobilization of solid-phase carriers. This technology uses the adsorption of DNA onto carboxyl-modified polymeric magnetic beads under specific polyethylene glycol and salt ion concentrations [1-3]. The process allows for selective yet reversible binding of DNA, thus enabling DNA selection, elution, recovery, and purification by using low-ionic-strength buffers or water [1,3].

Compared with other methods of nucleic acid purification and recovery, such as resin membrane and gel extraction, SPRI beads offer advantages that make them highly suitable for next-generation sequencing library

construction. These advantages include convenience, time efficiency, high recovery rate, and the ability to easily scale up to high-throughput and automated operations [1,4,5]. By addition of the beads to the library in varying targeted ratios and application of a magnetic field, beads of the desired size range can be separated from the rest of the library [1,4,6,7].

High-throughput sequencing of the 16S rRNA gene (16S rDNA) is extensively used to study microbial communities in the digestive and reproductive tracts of humans, farm animals, poultry, and wild animals [8–11]. The advent of third-generation sequencing technology, particularly the Pacific Biosciences (PacBio) sequencing platform, has made high-throughput sequencing of full-length 16S rDNA a crucial tool for investigating animal populations and identifying potential pathogens [8–10]. Nucleic acid purification and recovery are crucial steps in the preparation of high-throughput sequencing libraries. With the PacBio sequencing platform, the standard procedure for constructing a full-length 16S rDNA sequencing library involves mixing PCR products of full-length 16S rDNA from different samples, SPRI bead purification of the 16S rDNA PCR products, damage repair, end repair, tail addition, adapter ligation, SPRI bead purification (twice), and addition of sequencing primer and enzyme [12]. Several factors influence the efficiency of recovery during SPRI bead purification and recovery, including the bead ratio, incubation time, and elution time [3]. Additionally, proper resuspension of the beads before pipetting, use of fresh 80% ethanol, and adequate drying of the beads before elution are important operational steps affecting the recovery rate [3]. To construct a PacBio library for full-length 16S rDNA sequencing, DNA purification and recovery using SPRI beads must be performed at least three times. However, commercially available PacBio magnetic beads can be expensive. Furthermore, the recommended 0.6× PacBio SPRI beads used in library construction may not ensure a high recovery rate for all initial sample concentrations. Additionally, SPRI bead purification and recovery alone can take as long as 40 minutes in library construction. Gel DNA extraction is commonly used to recover 16S rDNA amplification products, owing to its low cost. However, gel extraction has substantial drawbacks, including the use of potentially harmful reagents, the potential for sample cross-contamination, the relative complexity and time-consuming nature of the experimental process, imprecise size selection of the recovered fragments and potential contamination with of low molecular weight compounds during gel cutting [13]. This study was aimed at assessing the influence of three factors—SPRI bead ratio, incubation time, and elution time—on the efficiency of sample purification in high-throughput full-length 16S rDNA sequencing library construction. The investigation involved varying the initial sample amount to determine an optimal and cost-effective process for purifying and recovering samples for 16S rDNA sequencing library construction with the PacBio platform.

MATERIALS AND METHODS

Construction of simulated 16S rDNA amplicon samples

The simulated samples for construction of the full-length 16S rDNA PacBio library were obtained through gel electrophoresis to identify and extract the 16S rDNA amplicons from wild animal fecal samples, by using the universal primer set 27F-1492R (5′-agagtttgatcmtggctcag-3′ and 5′-ggytaccttgttacgactt-3′) with the barcode supplied with the PacBio sequencing platform [14,15]. A QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) was used to extract the amplicons from the gel. The quantity of extracted DNA was measured with a Qubit 4.0 fluorometer (Thermo-Fisher, California, USA) to ensure accurate sample quantification. For construction of simulated samples, three different amounts of sample amplicons were mixed; the amounts used were 500 ng (representing the minimum initial sample concentration), 1500 ng (representing a common initial sample concentration), and 3000 ng (representing a high sample concentration). Each sample had a volume of 100 μL. These simulated samples were created to mimic the range of initial sample concentrations that may be encountered during library construction, thus enabling investigation of factors affecting the efficiency of sample purification and recovery in full-length 16S rDNA sequencing library construction with the PacBio platform.

Purification and recovery of DNA with SPRI beads

Purification and recovery of DNA with SPRI beads were performed according to the PacBio operation manual (https://www.pacb.com/support/documentation/). In brief, the volume of AMPure PB beads (PacBio, San Diego, USA) was adjusted according to the sample volume. The tube containing the sample was pipetted and centrifuged 15 times to mix the beads, then incubated at room temperature. Subsequently, the tube was centrifuged to collect the beads, and a magnetic bead rack (Thermo-Fisher, California, USA) was used to separate and collect the beads. The cleared supernatant was carefully removed with a pipette, and the beads were washed twice with freshly prepared 80% ethanol. After removal of residual ethanol, the purified DNA was eluted with the Elution Buffer (PacBio, San Diego, USA). The concentration of purified DNA was measured with a Qubit 4.0 fluorometer (Thermo-Fisher, California, USA), and its integrity was evaluated with 2% agarose gel electrophoresis.

Effects of SPRI bead ratio, incubation time, and elution time on DNA purification and recovery

SPRI bead ratios of 0.5×, 0.6×, and 0.8× were used, according to the PacBio manual and the characteristics of the full-length 16S rDNA library. Purification and recovery experiments were conducted with three initial sample

amounts: 500 ng, 1500 ng, and 3000 ng. The incubation time was set to 10 minutes, and the elution time was set to 2 minutes. Each experiment was repeated three times with the same bead ratio.

To assess the influence of incubation time on the purification and recovery process, we tested various incubation times (Table 1). The experiments used an SPRI bead ratio of 0.6×, an elution time of 2 minutes, and three different initial sample amounts (500 ng, 1500 ng, and 3000 ng). Each experiment was repeated three times.

Three different elution times were tested: 2 minutes, 5 minutes, and 10 minutes. These times were determined on the basis of the protocol for constructing the full-length 16S rDNA library. To account for the effects of incubation time on the purification and recovery rate, we used different incubation times for each initial sample amount: 10 minutes for 500 ng, 15 minutes for 1500 ng, and 25 minutes for 3000 ng. A magnetic bead ratio of 0.6× was used, and each experiment was repeated three times.

Orthogonal experiment for determining the optimal combination of SPRI bead ratio, incubation time, and elution time

To determine the best combination of SPRI bead ratio, incubation time, and elution time, we used an L9(3^3) orthogonal table (Table 2) [16]. This experiment was aimed at determining the optimal weights of the three factors for achieving the highest recovery efficiency. The initial sample amount used in the experiment was 1500 ng, and the volume was 100 μL. Each combination in the orthogonal table was repeated three times to ensure the robustness of the results. The recovery results were validated by gel electrophoresis.

TABLE 1 | Incubation times of different initial sample amounts.

Level (k)	Incubation time		
	500 ng	1500 ng	3000 ng
1	5	5	10
2	10	10	15
3	15	15	20
4	20	20	25
5	-	-	30

TABLE 2 | Factor levels in the orthogonal experiment.

Level (k)	Factor		
	Bead ratio (A)	Incubation time (B)	Elution time (C)
1	0.5	5	2
2	0.6	10	5
3	0.8	15	10

Statistical analysis

To determine the significant differences among variables, we performed the rank sum test and range analysis in SPSS statistics software version 23. The threshold for statistical significance was $P<0.05$. The rank sum test was used to analyze differences in nucleic acid recovery rates across different SPRI bead ratios, incubation times, and elution times, considering the various initial sample amounts. Additionally, range analysis was conducted to evaluate the recovery rate outcomes for different combination conditions presented in the orthogonal table [15]. The average recovery rates (k1, k2, and k3) were calculated for the three combinations of SPRI bead ratios. A higher value of k indicated a higher recovery rate for that ratio, and was used to identify optimal SPRI bead ratios. Similar calculations were performed to determine the optimal incubation time and elution time. Ultimately, the optimal combination of experimental parameters was determined by selecting the optimal values for all three factors. The range value (R) was computed to illustrate the extent of variation in the recovery rate when each factor was altered. A higher R value indicated greater influence of the respective factor on the experimental results. The R values for the three factors (SPRI bead ratio, incubation time, and elution time) were calculated individually.

Ethical statement

The study was reviewed and approved by the ethics committee of the National Institute for Communicable Diseases Control and Prevention, China CDC, according to Chinese ethic laws and regulations, under number ICDC-2016004.

RESULTS

Effects of purification and recovery parameters on SPRI bead recovery efficiency with different initial sample amounts

SPRI bead ratio

The average recovery rates were evaluated with different initial sample amounts with various SPRI bead ratios. The results were analyzed and depicted with box plots (Fig 1). To assess the statistical significance of the differences in recovery rates, we performed a Kruskal–Wallis H rank sum test. For the initial sample amount of 500 ng, the recovery rates showed no significant differences among SPRI bead ratios ($H=0.622$, $P>0.05$). Similarly, for initial sample amounts of 1500 ng and 3000 ng, the recovery rates showed no significant variation among magnetic bead ($P>0.05$). The respective Kruskal-Wallis H values were calculated as $H=5.956$ for the 1500 ng sample amount and $H=2.463$ for the 3000 ng sample amount.

Incubation time

Different incubation times were tested according to the protocol for constructing the full-length 16S rDNA library to assess the effects on recovery rates. Initially, a

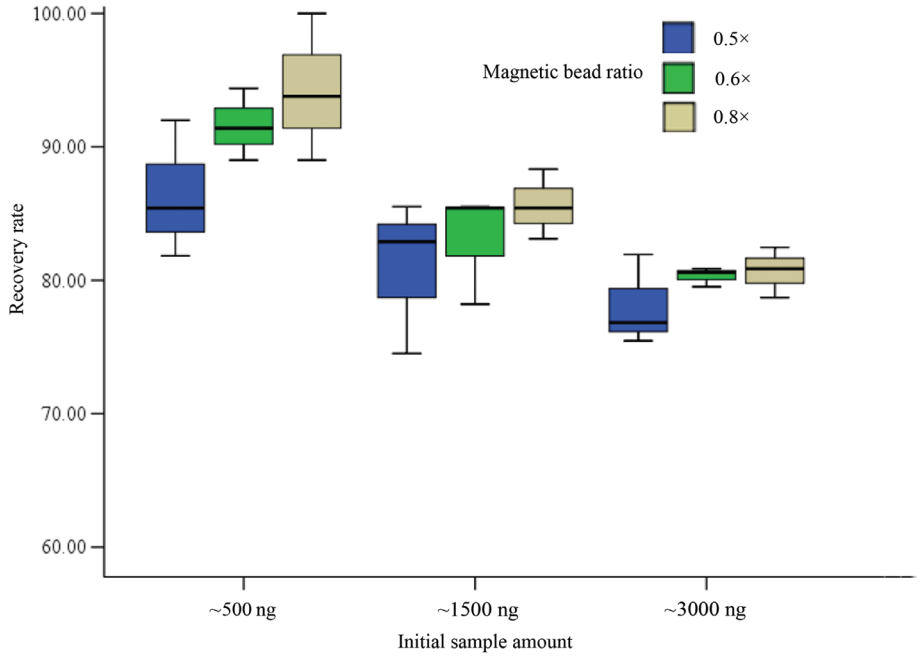

FIGURE 1 | Box plot of recovery rates with different SPRI bead ratios. The x-axis represents the initial sample amount, the y-axis represents the recovery rate, and the boxes depict the recovery rate of DNA with different SPRI bead ratios, with other conditions held constant.

high sample amount of 3000 ng was used, requiring an initial incubation time of 10 minutes. For the remaining samples with lower initial sample amounts, an incubation time of 5 minutes was used. To evaluate the effect of incubation time on recovery rates, we designed a gradient with 5-minute intervals. The average recovery rates were plotted as box plots (Fig 2), and statistical analysis was performed with the Kruskal–Wallis H rank sum test. For the initial sample amount of 500 ng, the statistical analysis yielded an H value of 0.630, indicating no significant difference in recovery rates across various incubation times ($P > 0.05$). However, for the initial sample amount

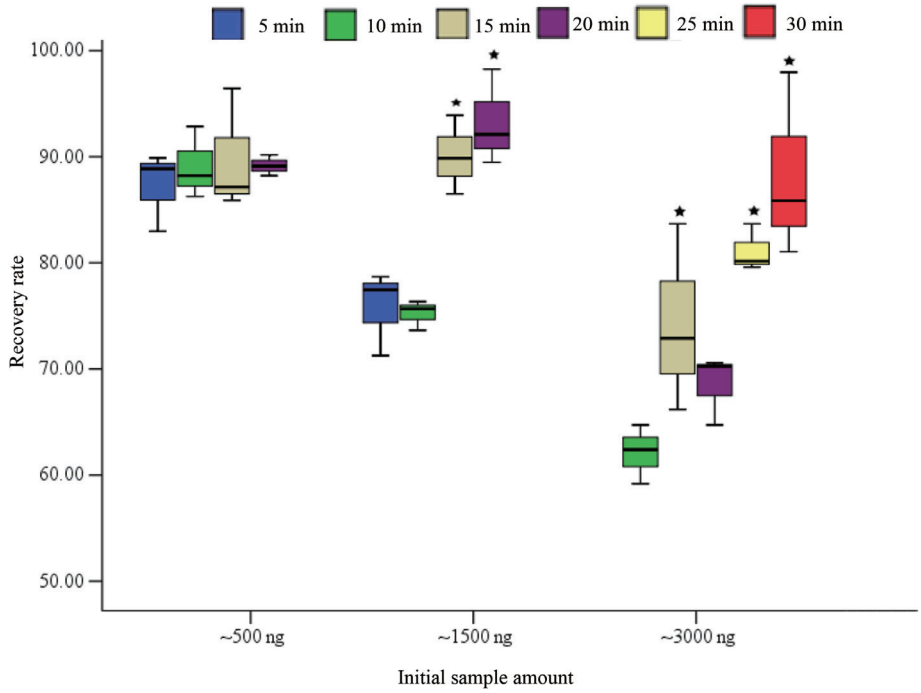

FIGURE 2 | Box plots of recovery rates with different incubation times. The x-axis represents the initial sample amount, the y-axis represents the corresponding recovery rates, and the box plots show the recovery rates of DNA with different incubation times, with other conditions held constant. *Denotes a statistically significant difference.

of 1500 ng, the statistical analysis revealed an H value of 8.530, demonstrating a significant difference in recovery rates with varying incubation times ($P < 0.05$). Similarly, for the initial sample amount of 3000 ng, the statistical analysis yielded an H value of 11.324, indicating a significant difference in recovery rates at different incubation times ($P < 0.05$).

Elution time

The recovery rate results, represented by box plots (Fig 3), demonstrated an increased recovery rate with longer elution times. To evaluate the statistical significance of this trend, we performed a Kruskal–Wallis H rank sum test on the recovery rate results for different elution times while maintaining the same initial sample amount. For an initial

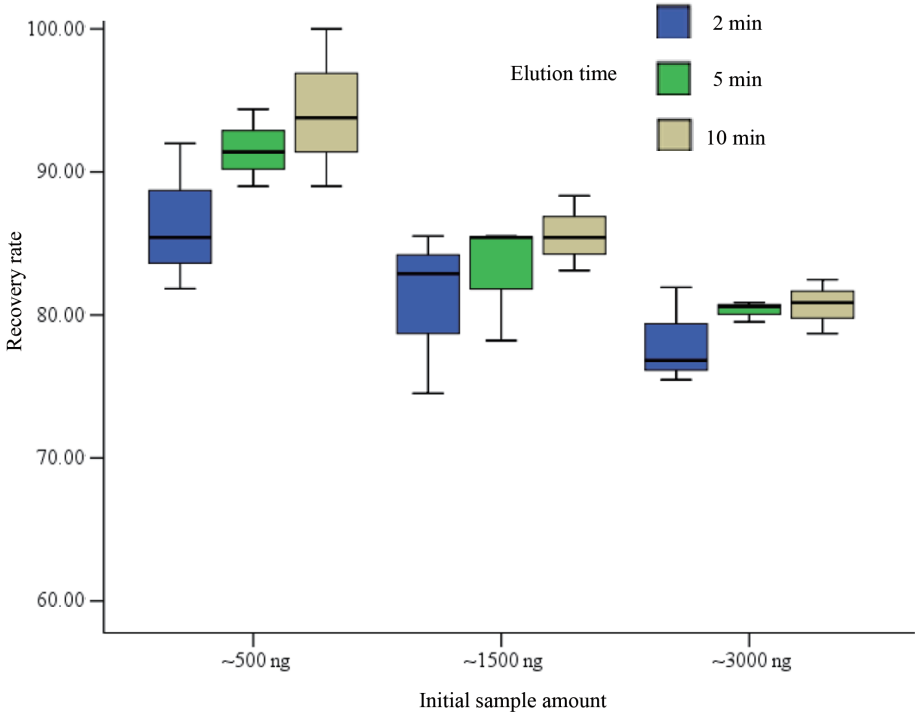

FIGURE 3 | Box plots of recovery rates with different elution times. The x-axis represents the initial sample amount, the y-axis represents the corresponding recovery rates, and the box plots illustrate the recovery rates of DNA with different elution times, with other conditions held constant.

TABLE 3 | Results of the orthogonal experiment.

Number	Bead ratio (A)	Incubation time (B)	Elution time (C)	Average DNA recovery rate (%)
1	1	1	1	77.11
2	1	2	2	83.78
3	1	3	3	82.45
4	2	1	2	77.57
5	2	2	3	84.60
6	2	3	1	84.30
7	3	1	3	84.00
8	3	2	1	85.10
9	3	3	2	88.97
k_1	81.11	79.56	82.17	
k_2	82.15	84.49	83.43	
k_3	86.01	85.23	83.68	
R	4.90	5.67	1.51	

sample amount of 500 ng, the statistical analysis yielded an H value of 2.622, thus suggesting no significant difference in recovery rates across elution times ($P > 0.05$). Similarly, for the initial sample amount of 1500 ng, the statistical analysis revealed an H value of 1.107, thereby indicating no significant difference in recovery rates across elution times ($P > 0.05$). For an initial sample amount of 3000 ng, the statistical analysis yielded an H value of 1.277, a value also suggesting no significant difference in recovery rates across elution times ($P > 0.05$).

Orthogonal experimental results

Table 2 summarizes the levels of the factors in the orthogonal experiment, and the corresponding experimental results are presented in Table 3. The validated recovery results were visualized through gel electrophoresis, as shown in the Supplemental figure. On the basis of analysis of the results and consideration of the range (R) values for the factors, the sequence of factor significance within the scope of this orthogonal table was as follows: factor B > factor A > factor C. Incubation time had the greatest effect on the recovery rate, and was followed by the magnetic bead ratio and elution time. On the basis of the combinations in the orthogonal table, the optimal combination was A3, B3, C3, corresponding to a magnetic bead ratio of 0.8×, an incubation time of 15 minutes, and an elution time of 10 minutes. With this combination, the average DNA recovery rate was 89.27%—a value higher than those achieved with the other combinations listed in the orthogonal table.

DISCUSSION

The 16S rRNA gene, which is present in all prokaryotes, contains valuable phylogenetic information and has several advantageous characteristics, such as easy amplification and alignment, as well as a comprehensive alignment database [8,9]. Consequently, it has become the most commonly used molecular marker for bacterial identification and colony studies [14,15]. Recent advancements in third-generation sequencing technology, notably the PacBio platform, now allow for accurate acquisition of near-full-length 16S rRNA sequences, thus enabling the detection and classification of bacterial species in microbiome analysis [8]. SPRI bead purification plays a critical role in constructing full-length 16S rDNA sequencing libraries. This step not only eliminates small fragments such as short primers, adapters, and enzymes, but also ensures a high recovery rate while preserving sample integrity [7,13]. However, following the recommended SPRI bead ratios and operational procedures may not always ensure optimal recovery rates. Additionally, multiple purification steps can be time-consuming, thus posing challenges when urgent outbreak-associated requirements arise.

To address these concerns, we sought to examine the effects of SPRI bead ratio, incubation time, and elution time on the purification and recovery rate of SPRI beads in construction of 16S rDNA sequencing libraries. The experimental results indicated that, regardless of the initial sample amount (500 ng, 1500 ng, or 3000 ng), the DNA recovery rates were similar with magnetic bead ratios of 0.5×, 0.6×, and 0.8×. Additionally, we observed no significant differences in recovery rate among elution times of 2 min, 5 min, and 10 min. Statistical analysis further revealed that, for the 500 ng initial sample amount, the recovery rates did not significantly differ among incubation times of 5 min, 10 min, 15 min, and 20 min. However, for the 1500 ng and 3000 ng initial sample amounts, we observed a statistically significant difference in recovery rate with varying incubation times. On the basis of these findings, we concluded that extending the incubation time can improve the recovery rate. Importantly, in this experiment, only the incubation time showed a statistically significant effect on the purification and recovery efficiency of full-length 16S rDNA with magnetic beads. Although an increasing trend in recovery rate was observed with longer elution times, the difference was not statistically significant. These results demonstrated that different initial sample amounts yield different purification and recovery outcomes with the same purification steps. Moreover, the recovery efficiency was lower when protocol parameters for purifying larger sample amounts were used.

To achieve a higher recovery rate, increasing the incubation time is recommended. According to the analysis of incubation times required to achieve 80% recovery for different initial sample amounts, 500 ng required 5 minutes, 1500 ng required 15 minutes, and 3000 ng required >25 minutes of incubation (Fig 2). Therefore, larger initial sample amounts require longer incubation times to achieve better recovery results.

The results of the orthogonal experiment indicated that, among the investigated factors, incubation time had the greatest effect on the recovery rate at the levels specified in the orthogonal table, and was followed by the magnetic bead ratio and elution time. For a standard initial sample amount of 1500 ng, a high recovery rate was achieved with a 0.8× magnetic bead ratio, incubation for 15 minutes, and elution for 10 minutes.

In conclusion, this study investigated the purification and recovery conditions of SPRI beads for constructing 16S rDNA libraries. The results suggested that a magnetic bead ratio of 0.8×, incubation for 15 minutes, and elution for 10 minutes achieves a high recovery rate. We recommend increasing the incubation time when the sample amount increases, to increase nucleic acid recovery and facilitate efficient construction of full-length 16S rDNA libraries.

Importantly, this experiment used an orthogonal method to explore the optimal parameters for purification and recovery. Therefore, the recovery effects under parameters outside these specific combinations cannot be compared or generalized. Further studies may be needed to evaluate the effects of other factors, and optimize the purification and recovery process for different sample amounts and library construction protocols.

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CONFLICTS OF INTEREST

None to declare.

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