Filter, Heat, Spin: A Simple and Inexpensive Method for DNA Preparation from Freshwater for use in High-Throughput Molecular Source Tracking

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Abstract

Molecular source tracking (MST) can improve community health by enabling the identification of the source species of fecal bacteria contamination in waterways. However, widespread

adoption of this method at a large scale is hindered by the cost of commercial extraction kits and the technical expertise required to use them. We developed a simpler, highly efficient, scalable, accessible, and semi-quantitative method to extract DNA from environmental water samples via heat lysis. After filtering water samples onto a polycarbonate membrane, the membrane is suspended in AE buffer, heated, and then centrifuged. The liquid supernatant is then used directly in quantitative PCR (qPCR) analysis. Our filter-heat-spin (FHS) extraction method was then compared to a commercial DNA extraction kit (Qiagen DNeasy). The FHS extraction yielded 5.1 times more DNA (on average) than the traditional extraction kit. Additionally, it maintained identical long-term stability as compared to the kit extraction over a five-week period. Further, the cost per FHS extraction is roughly $0.05 per sample and requires 15 minutes to complete, while the typical kit extraction is roughly $4.48 per sample and requires 50 minutes to complete. The proposed extraction method allows for efficient and inexpensive water sample processing, which in turn, reduces barriers for the implementation of MST techniques.

Introduction

Aging infrastructure can pose risks including exposure to potentially pathogenic microbes, harmful chemicals, and heavy metals (Sörme & Lagerkvist, 2002; Griffin et al., 2003; Venkatramanan et al., 2015; Olds et al., 2018). There is a correlation between these risks and Fecal Indicator Bacteria (FIB) levels making this statistic a good index for the health of an aquatic system (Mallin et al., 2007). This data can inform management decisions as well as infrastructure planning (Mallin et al., 2007; Soller et al., 2010; Schoen, Soller & Ashbolt, 2011). Molecular Source Tracking (MST) is an even more powerful technique as it allows for the identification of the FIB source species (e.g. dog, human) in creeks, lakes, and other bodies of

water (Sidhu et al., 2013). The biological source and quantity of FIB can point to the underlying pollution source (e.g. runoff, leaching pipes) which, in turn, allows watershed managers to better develop possible solutions to protect public health and environmental quality.

There are a multitude of studies that utilize the MST of FIB to detect nonpoint sources of pollution for the purposes of infrastructure planning and public health management. (Ervin et al., 2014; Riedel et al., 2015 Paruch et. al 2022). A popular assay used for the detection and quantification of human-associated FIB in freshwater samples is based on amplifying the HF183 16S ribosomal DNA cluster which is specific to human-associated FIB (Bernhard & Field, 2000; Haugland et al., 2010; Green et al., 2014; U.S. EPA, 2019). While the knowledge gained from molecular assays is highly useful, they typically require extensive sample preparation that includes the extraction and purification of DNA from water samples using molecular biology kits (such as the Qiagen DNeasy Blood and Tissue Kit) (U.S. EPA, 2019). These kits utilize various buffer solutions to resuspend bacteria, lyse cells, bind DNA to a matrix, wash away any contaminating proteins and/or biomolecules, and finally, elute the highly pure DNA off the matrix for further analysis. However, these commercial molecular biology kits are expensive, require trained technicians, and are time intensive. In fact, sample preparation via traditional extraction methods for a qPCR assay accounts for approximately half the total cost of MST (Riedel et al., 2014).

A simpler, less expensive, and faster method of sample preparation for MST analysis would eliminate the need for a commercial DNA extraction kit, thus reducing costs and preparation time. Instead of relying on traditionally used lysis buffers, the proposed method uses heat to lyse

the microbes and inactivate proteins to isolate DNA from water samples for the HF183 TaqMan qPCR assay protocol. Heat lysis has been shown to be an effective method of bacterial DNA extraction from blood culture fluid; for this application, it is also more sensitive, economical, and higher throughput than traditional methods (Kulski & Pryce, 1996). Water samples are filtered, heated, and spun (FHS) with the resulting supernatant being used for qPCR analysis. Identical water samples were processed with both the commercial kit and the FHS extractions. Across 15 unique samples, the FHS extraction gave similar concentrations of DNA and similar copy numbers compared to the commercial kit. FHS has a lower concentration of DNA, but a higher yield because of the higher final extraction volume. The advantages of FHS include lower cost, higher efficiency, and less time. Labs will be able to dramatically increase throughput, thus, allowing for more widespread adoption of MST. For example, high throughput sampling via FHS can be advantageous when tracing FIBs (Myers, 1992; Kim et al., 2013, Paruch et. al 2022) or disease outbreaks such as COVID-19 and polio in wastewater (Deshpande, Shetty & Siddiqui, 2003; Patel et al., 2020; Udugama et al., 2020; Carducci et al., 2020; Larsen & Wigginton, 2020).

Materials & Methods

Sample Collection:

Water samples were obtained from 15 separate collections at two different sites along Waller Creek within the University of Texas at Austin main campus via previously described methods (U.S. EPA, 2019). Samples were collected from two locations within the University of Texas at Austin Campus, one denoted SB and the other CS. Collection dates were: SB 1-3 July 23, 2019;

SB 4-6 and CS 10-12 January 27, 2022; and SB 7-9 and CS 13-15 June 2, 2022. Waller Creek is known to have a high amount of fecal bacteria, particularly human associated fecal bacteria (Jackson & Herrington, 2012). Before collecting the samples, 500 mL HDPE plastic amber bottles were acid washed with 10% hydrochloric acid. At the site, the bottles were rinsed with creek water 3 to 5 times before collecting approximately 500 mL of creek water in each bottle. The bottles were then stored for no longer than two hours on ice in an insulated container to prevent exposure to sunlight and high temperatures.

Filtration:

Filter towers were placed on a filter manifold using Nalgene analytical filter funnels equipped with 0.22 micron pore size polycarbonate filter membranes. Following the filtration of 200 mL samples, filters were folded and placed in 1.5 mL microcentrifuge tubes. The filters extracted with the DNeasy kit had acid washed glass beads added while the filters extracted by the FHS method were resuspended in Qiagen AE Buffer.

Commercial Extraction Kit:

The Qiagen DNeasy blood and tissue kit (Qiagen 69504) is one of the currently accepted standard methods for extracting DNA from freshwater samples. We followed standard manufacturer protocols for performing DNA extractions with the recommended U.S. EPA modifications. In brief, the microcentrifuge tube containing the filter and glass beads was resuspended in 500 µL of ATL buffer and 40 µL of proteinase K. The sample was then run in a bead beater for 2 minutes before being centrifuged at 12,000 x g for 2 minutes. Next, the liquid contained within the microcentrifuge tube was transferred into a new 2 mL microcentrifuge tube

before being centrifuged at 12,000 x g for 1 minute. Then 225 µL of liquid was transferred into another new microcentrifuge tube and the standard protocol for the Qiagen DNeasy Blood and Tissue Kit was followed. The eluted solution was collected in a microcentrifuge tube and stored in a -20˚C freezer. This method yields approximately 100 µL of starting DNA template for qPCR.

FHS only:

As shown in **Figure 1**, the FHS method begins by resuspending the filter in 500 µL AE buffer (10 mM Tris-Cl and 0.5 mM EDTA, pH 9.0). After vortexing the microcentrifuge tube for 10 seconds, it was then heated for 10 minutes at 95°C in a heat block. This solution was then vortexed again for 10 seconds and centrifuged at 12,000 x g for 2 minutes. Following centrifugation, the liquid was decanted into a separate microcentrifuge tube. This method yields approximately 450 µL of starting DNA template for qPCR which was distributed among aliquots and frozen at -20°C.

Figure 1: Schematic of the FHS method used for DNA extraction from freshwater samples. Created with BioRender.com.

qPCR:

The qPCR reaction was run on a BioRad 96-Well CFX Connect Real-Time PCR Detection System using the standard protocol for the New England Biolabs LUNA 2X probe-based qPCR mastermix, with 40 cycles. The cycles were as follows: 95˚C for 10 minutes for activation followed by 40 cycles of 95˚C for 15 seconds and 60˚C for 60 seconds (followed by a plate

read). Reactions were run in a total volume of 20 µL with 10 µL of LUNA master mix, 1.66 µL forward primer, 1.66 µL reverse primer, 1.32 µL probe, 0.36 µL of molecular biology grade water, and 5 µL of either sample, gBlock dilutions (as the positive control), or molecular biology grade water (to serve as no template controls). Each sample was run as 4 technical replicates with the average of the 4 replicates used in the data analysis, with outliers removed accordingly. The sequences for the forward and reverse primers along with the probe are in **Table 1**. The HF183 gBlock standard (**Table 2**) standard curve ranged between 10^2 and 10^5 copies per µL. These concentrations were used to make a master standard curve because prior literature has shown that the range of HF183 in water samples is typically within that range (Riedel et al., 2015). Each qPCR plate also included an internal HF183 standard to normalize runs across different plates.

Table 1. Primers and probe sequences for HF183 (Haugland et al., 2010).

Name	**Sequence (5' to 3')**
HF183 Forward Primer	ATC ATG AGT TCA CAT GTC CG
HF183 Reverse Primer	CGT AGG AGT TTG GAC CGT GT
HF183 Probe	[6FAM]CTGAGAGGAAGGTCCCCCACATTGGA[BHQ-1]

Table 2. Positive control sequence for HF183.

HF183 Positive Control	**Sequence (5' to 3')**
ATCATGAGTTCACATGTCCGCATGATTAAAGGTATTTTCCGGTAGACGATGGGGATGCGTTCCATTAGC TCGAGATAGTAGGCGGGGTAACGGCCCACCTAGTCAACGATGGATAGGGGTTCTGAGAGGAAGGTCC CCCACATTGGAACTGAGACACGGTCCAAACTCCTACG	

Time Course Study:

Six samples were collected at two sites along Waller Creek and extracted using both the commercial kit and FHS methods outlined. Following extraction, the samples were aliquoted and stored at -20°C. The qPCR assay outlined above was performed at one week intervals over the course of five weeks.

Statistical Analysis:

All qPCR reactions were run in 4 technical replicates per sample. Quadruplicate Cq values were averaged, if only three out of four replicates amplified then an average Cq was calculated with the fourth replicate assigned a Cq value of 40. Cq averages within the range of quantification of the master standard curve spanning 4 orders of magnitude were converted to starting quantity (copies per µL). Non-detects were assigned to samples if three or more replicates did not amplify within 40 cycles or if the calculated concentration was below the limit of detection of the qPCR assay. GraphPad Prism Version 10.2.3 was used to generate figures and perform statistical analyses.

Results

The total DNA yield (total copy number) for each extraction method at two sampling sites is shown in **Figure 2.** These values were calculated from the average starting quantities of each qPCR technical replicate (four technical replicates per sampling site) and the total volume yielded for the respective extraction methods. The FHS extraction method produced 450 µL and the commercial kit produced 100 µL of template. Based on these calculations, the FHS method

yields 5.1 times more DNA than the commercial kit. However, as seen in **Figure 3,** the commercial extraction yielded a more concentrated sample than the FHS method. Furthermore, both methods gave reliable results with very low variability within each extraction method. It should be noted that the variance in **Figures 2** and **3** between trials can be attributed to the samples being collected on different days (see methods). While all of the samples were collected at base flow (>7 days post-rain), FIB levels vary due to season and climatological conditions.

Figure 2: Total DNA yield (total copy number) across both methods from 15 independent samples with error bars representing the standard deviation of the mean. The numbered abbreviations each represent the location and sample number of the 15 different collections along Waller Creek within The University of Texas at Austin main campus. These collections took place at two different sites (SB and CS) across three different days. In most samples, the FHS method had a higher total DNA yield than the commercial kit. On average, the FHS method was found to yield 5.1 times more total DNA than the commercial kit.

Figure 3: DNA copies per µL by method with error bars representing the standard deviation of the mean. The numbered abbreviations each represent the location and sample number of the 15 different collections along Waller Creek within The University of Texas at Austin main campus. These collections took place at two different sites (SB and CS) across three different days. In most of the cases, the commercial extraction has a higher concentration of DNA per µL than the FHS method.

The next step of the investigation aimed to determine whether the presence of such impurities in the FHS extracted samples would lead to increased DNA degradation over time. Over a period of 6 weeks, we examined the relative stability of the extraction methods. DNA extracted from each method was aliquoted and stored at -20°C. One vial was thawed each week and compared to the calculated starting quantity as detected by qPCR targeting the HF183 gene to the initial calculated starting quantity of qPCR done at the time of DNA extraction. As seen in **Figure 4,** there was no significant difference in DNA degradation between the methods.

Figure 4: DNA stability across extraction methods over the course of five weeks. Each data point represents six simultaneous water collections. Data at each time point was normalized to the starting quantity (**SQ**) at time zero to examine deviation from the original starting quantity. Within each collection there are four technical replicates. Error bars represent the standard error of the mean.

As a proof of concept, the FHS method was applied to samples collected at 8 different sites along Waller Creek in Austin, TX. **Figure 5** shows the copies of HF183 DNA and FIB colony forming units per 100 mL at different sites along the length of the creek. The trend in HF183 copies does not directly correlate with *E. coli* levels, however, this demonstrates that HF183 is detectable in water samples containing FIB. The lack of a direct trend here suggests that other species sources (such as canine or avian) are responsible for such contamination.

Figure 5: Representative data collected using the FHS extraction and MST with HF183 to detect human-associated FIB from Waller Creek in Austin, TX. The samples were collected along the creek on July 22, 2021 and processed the same day. The *E. coli* was quantified using 3M Petrifilm plates and ranged from 300 to 2033 cfu/100mL. HF183 amplified at all sites ranging from 2809 to 14403 copies/100mL.

Discussion

The FHS DNA extraction protocol was developed to test the viability of a high-throughput method for quickly extracting DNA from freshwater samples. Across 15 independent samples from two different sampling sites and three different dates, the FHS extraction method yields viable DNA that is equally stable when compared to the commercial kit extraction method. The commercial kit yields a volume of 100 µL, while the FHS method yields a volume of 450 µL. While the concentration of the commercial extraction method is higher, the total HF183 copies produced with the FHS method is greater (**Figures 2 and 3**). The variability between samples is due to the different locations and different dates that the samples were collected.

The lack of purification steps in the FHS extraction compared to the commercial kit extraction might lead to the presence of nucleases and PCR inhibitors in samples that could potentially alter their stabilities or impair quantification. However, as seen in **Figure 4,** the DNA extracted by both methods had similar stabilities over a period of five weeks. Weekly qPCR runs were performed across a five-week period and the copies of DNA per µL at each week were found to follow a similar trend. The results of this experiment suggest that the high temperature (95˚C) in

the FHS extraction was enough to inhibit or denature enzymes that could lead to DNA degradation. However, it should be noted that the presence of inhibitors in the template solution could potentially disrupt DNA detection, and diluting the template may be necessary to overcome such issues (Eichmiller, Miller & Sorensen, 2016; Lance & Guan, 2019; Kumar, Eble & Gaither, 2020).

The FHS method is faster to perform which allows it to be higher throughput. Vastly decreased sample processing time makes MST a more accessible method for monitoring FIB source species. The commercial kit also requires six different pipetting steps while the FHS extraction method only requires two. Fewer steps means that the FHS method has a lower chance of introducing variability between samples which, in turn, allows for personnel with less training to be able to successfully extract DNA with reliable yields. Tracking FIB contamination in watersheds necessitates a large number of data points across both time and space. The time and resources needed to use a standard DNA extraction kit make it difficult to achieve the high level of throughput that is necessary for such large-scale projects (**Table 3**). The FHS method solves many of these problems, and as seen in **Figure 5**, performing MST across a watershed becomes possible. Our group has been studying urban creek FIB levels in Austin, Texas, and to date have processed over 200 samples using the FHS method.

Table 3: Comparison of common DNA extraction methods used for MST from water samples. Data for extraction kits gathered from the manufacturer's standard protocol.

Method	Cost per extraction	Approximate Time	Final Volume	qPCR Yield Range (copy number/µL)

	(U.S.D.)	(minutes)	(µL)	
FHS Method	$0.05	15	450	1-11
Commercial Kit	$4.48	50	100	1-37

Figure 5: Representative data collected using the FHS extraction and MST with HF183 to detect human-associated FIB from Waller Creek in Austin, TX. The samples were collected along the creek on July 22, 2021 and processed the same day. The *E. coli* was quantified using 3M Petrifilm plates and ranged from 300 to 2033 cfu/100mL. HF183 amplified at all sites ranging from 2809 to 14403 copies/100mL.

The ability for high-throughput processing of water samples for qPCR analysis can be useful for a range of studies. Pinpointing the sources of FIB contamination requires the processing of many samples at the same time (8-12 sample sites in duplicate). Similar examples are investigations of how rainfall affects FIB levels across multiple regions (Myers, 1992; Kim et al., 2013). High-throughput sampling can also be advantageous when tracing disease outbreaks such as COVID-19 and polio in wastewater (Deshpande, Shetty & Siddiqui, 2003; Patel et al., 2020; Udugama et al., 2020; Carducci et al., 2020; Larsen & Wigginton, 2020). Our lab has also used the FHS extraction with TaqMan DogBac (data not shown), used for identifying canine associated fecal indicator bacteria (Kildare et al., 2007; Kapoor, Lu & Wendell, 2012). Dog feces are another significant source of fecal pollution in urban freshwater systems. Running DogBac assays alongside HF183 allows for comparison of FIB sources. Using the FHS extraction, we have been able to detect dog associated FIB in freshwater systems. This method could also be

applied to detect bovine and pig specific fecal bacteria markers, expanding its applicability from urban environments to rural nonpoint molecular source tracking (Kildare et al., 2007; Mieszkin et al., 2009).

Conclusion

The FHS method offers several advantages that allow for the rapid, scalable, and semi-quantitative extraction of DNA for use in qPCR. The speed and decreased costs of the FHS method make it practical to answer experimental questions that were previously difficult and costly to answer. We have demonstrated the usefulness of this method in our own research group mapping the species source of FIB contamination across the city of Austin, TX. While the City of Austin monitors the FIB levels in Austin's creeks to identify areas of high contamination, they have not identified the species source of the FIB contamination (City of Austin, 2021). Solutions to these problem areas require a fuller understanding of the sources of contamination that we can now provide using the FHS methodology to process samples from locations around the city.

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Table 1. Primers and probe sequences for HF183 (Haugland et al., 2010).

Name	Sequence (5' to 3')
HF183 Forward Primer	ATC ATG AGT TCA CAT GTC CG
HF183 Reverse Primer	CGT AGG AGT TTG GAC CGT GT
HF183 Probe	[6FAM]CTGAGAGGAAGGTCCCCCACATTGGA[BHQ-1]

Table 2. Positive control sequence for HF183.

HF183 Positive Control	Sequence (5' to 3')
ATCATGAGTTCACATGTCCGCATGATTAAAGGTATTTTCCGGTAGACGATGGGGATGCGTTCCATTAGC TCGAGATAGTAGGCGGGGTAACGGCCCACCTAGTCAACGATGGATAGGGGTTCTGAGAGGAAGGTCC CCCACATTGGAACTGAGACACGGTCCAAACTCCTACG	

Results:

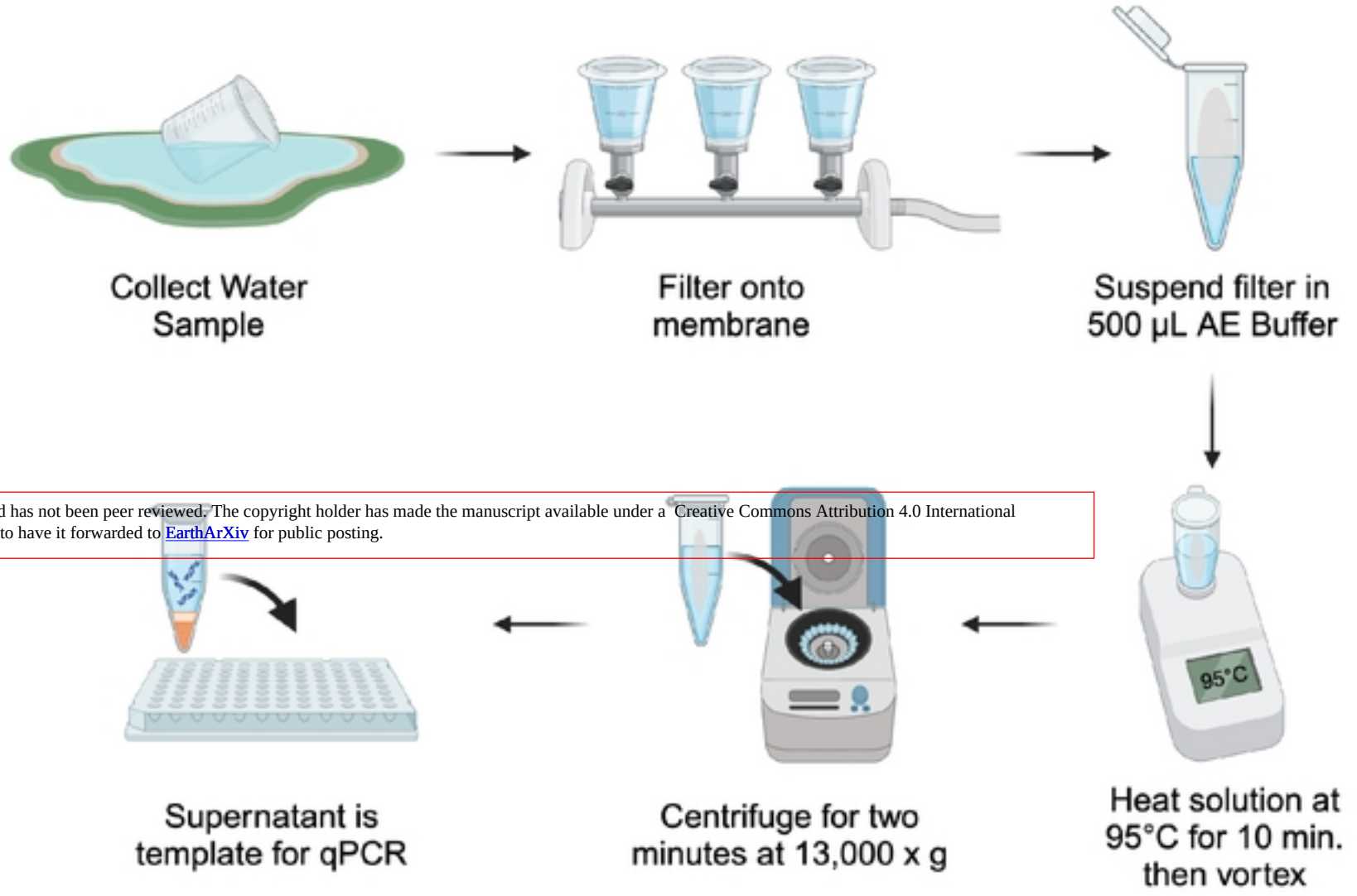

Figure 1: Schematic of the FHS method used for DNA extraction from freshwater samples. Created with BioRender.com.

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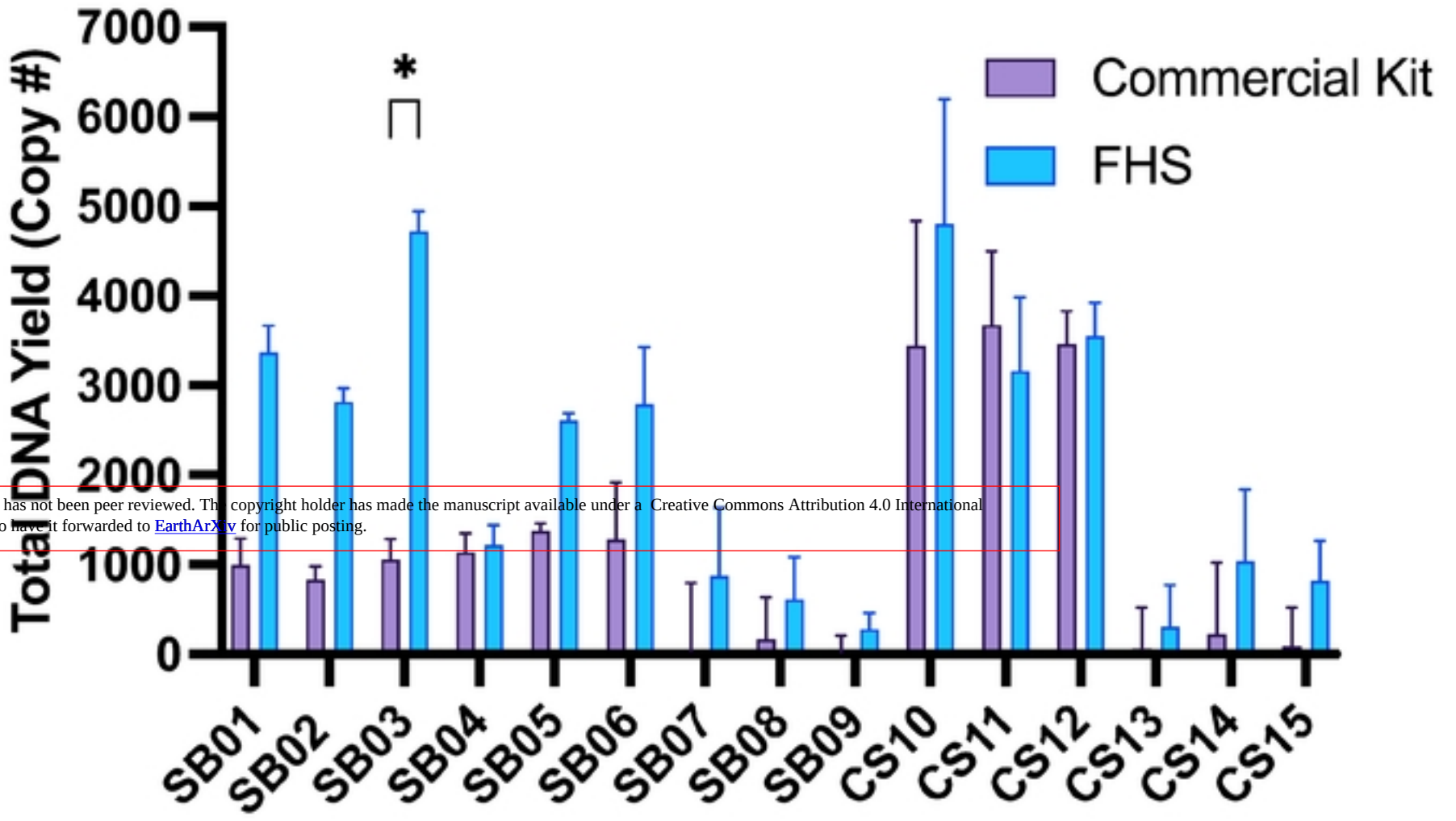

Figure 2: Total DNA yield (total copy number) across both methods from 15 independent samples with error bars representing the standard deviation of the mean. The numbered abbreviations each represent the location and sample number of the 15 different collections along Waller Creek within The University of Texas at Austin main campus. These collections took place at two different sites (SB and CS) across three different days. In most samples, the FHS method had a higher total DNA yield than the commercial kit. On average, the FHS method was found to yield 5.1 times more total DNA than the commercial kit.

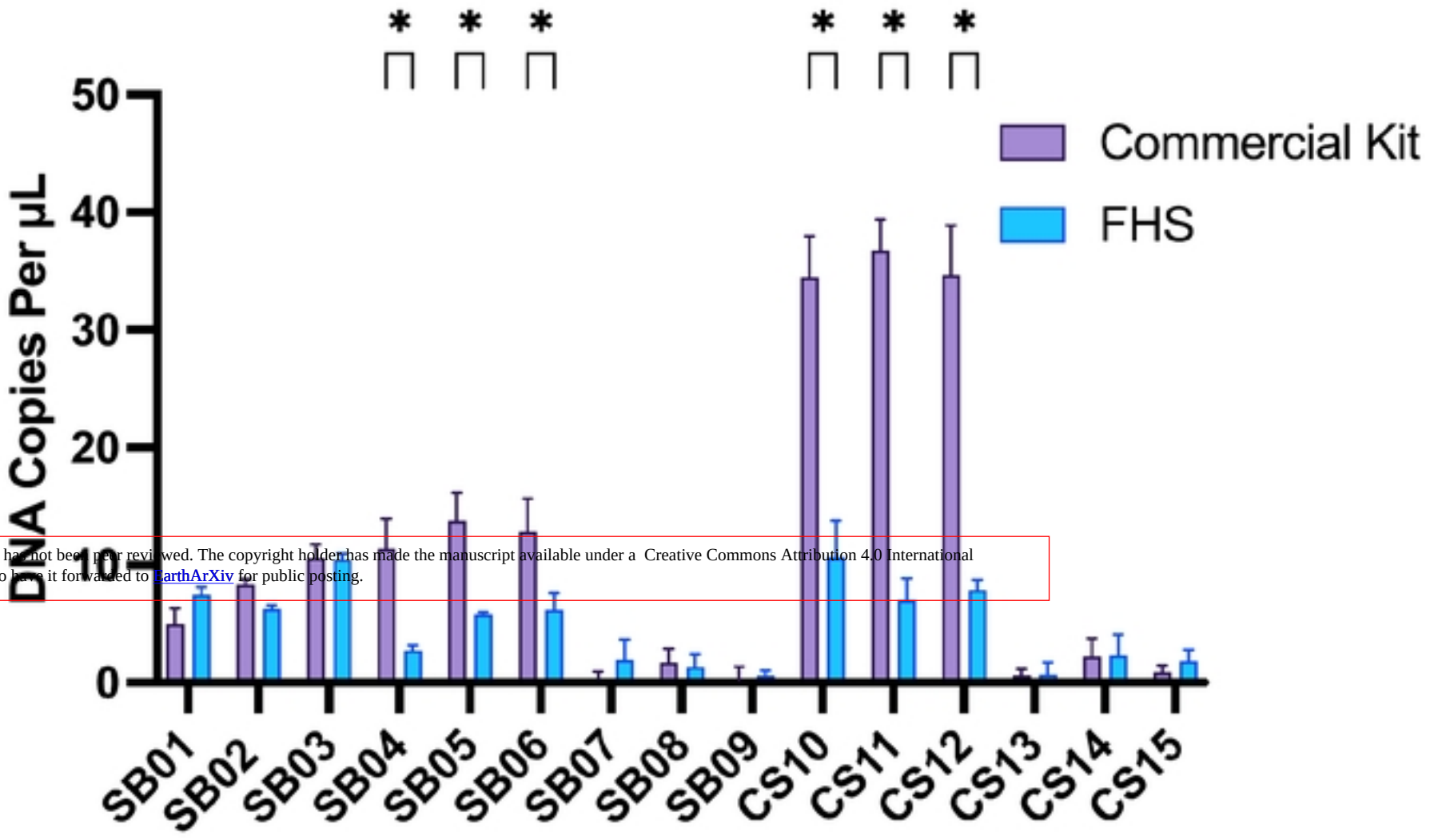

Figure 3: DNA copies per µL by method with error bars representing the standard deviation of the mean. The numbered abbreviations each represent the location and sample number of the 15 different collections along Waller Creek within The University of Texas at Austin main campus. These collections took place at two different sites (SB and CS) across three different days. In most of the cases, the commercial extraction has a higher concentration of DNA per µL than the FHS method.

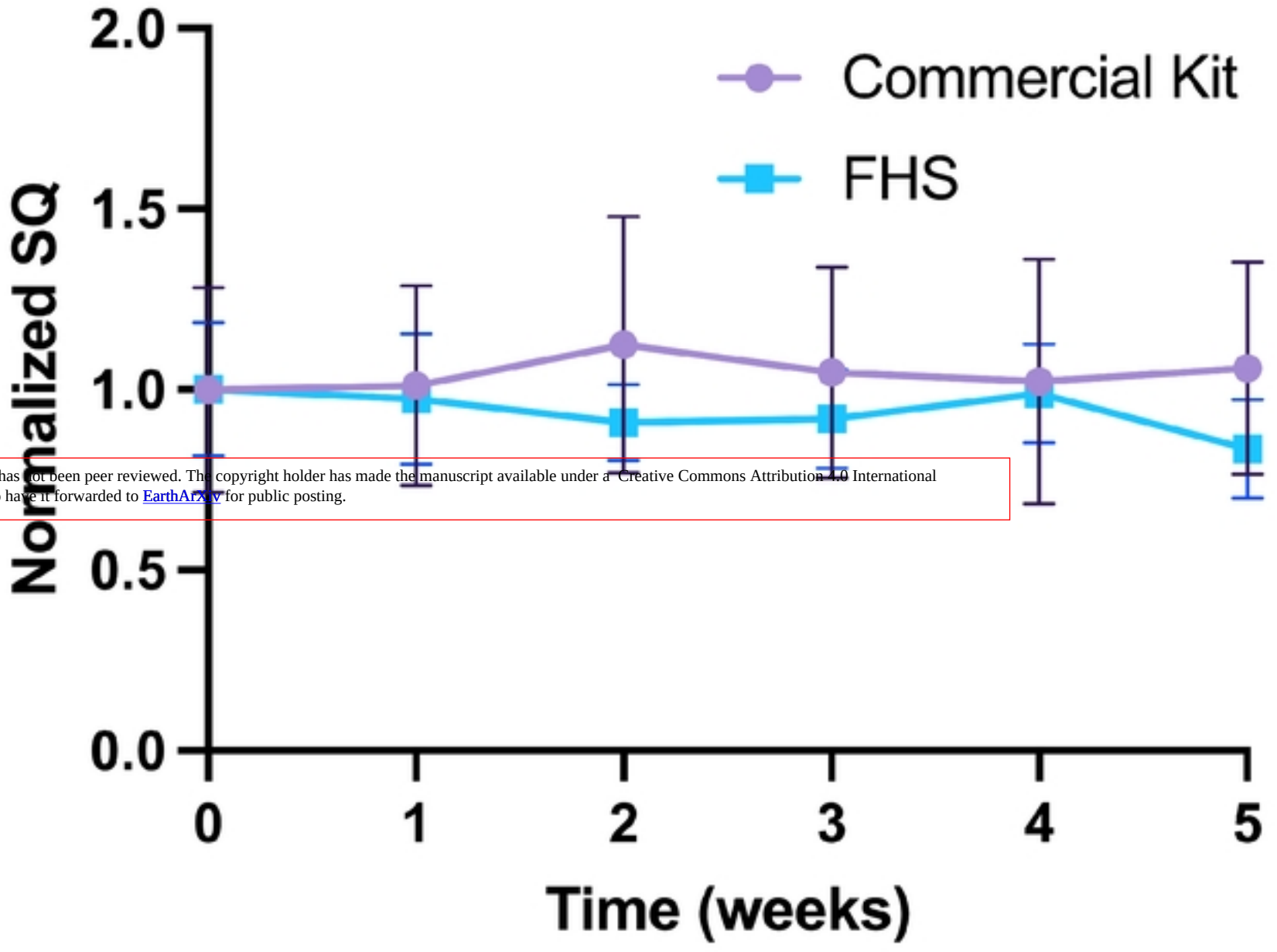

Figure 4: DNA stability across extraction methods over the course of five weeks. Each data point represents six simultaneous water collections. Data at each time point was normalized to the starting quantity (**SQ**) at time zero to examine deviation from the original starting quantity. Within each collection there are four technical replicates. Error bars represent the standard error of the mean.

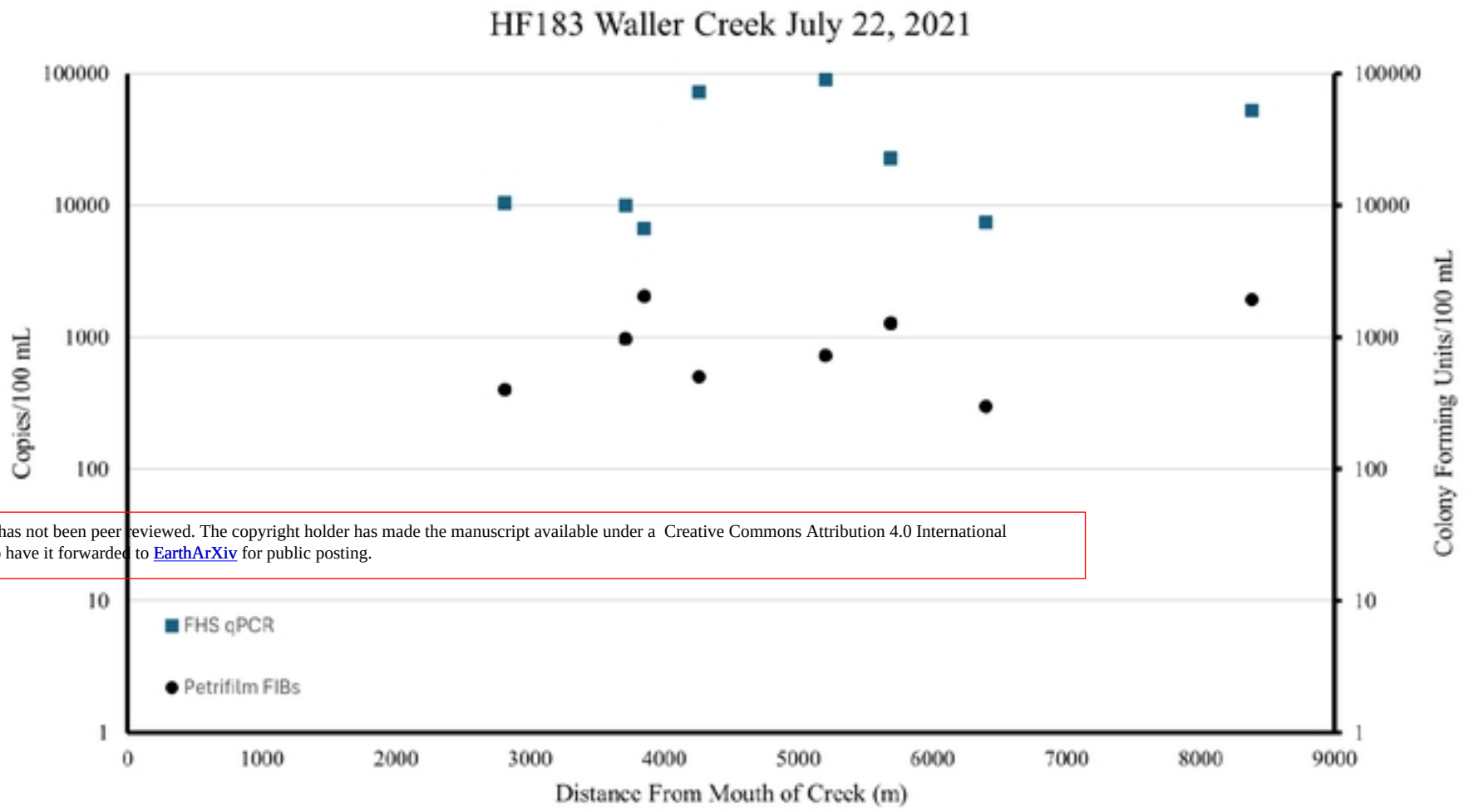

Figure 5: Representative data collected using the FHS extraction and MST with HF183 to detect human-associated FIB from Waller Creek in Austin, TX. The samples were collected along the creek on July 22, 2021 and processed the same day. The *E. coli* was quantified using 3M Petrifilm plates and ranged from 300 to 2033 cfu/100mL. HF183 amplified at all sites ranging from 2809 to 14403 copies/100mL.