A comparison of storage methods for gut microbiome studies in teleosts insights from rainbow trout (Oncorhynchus mykiss)

Hildonen, Mathis; Kodama, Miyako; Puetz, Lara C.; Gilbert, M. Thomas P.; Limborg, Morten T.

Published in:
Journal of Microbiological Methods

DOI:
10.1016/j.mimet.2019.03.010

Publication date:
2019

Document version
Peer reviewed version

A comparison of storage methods for gut microbiome studies in teleosts: Insights from rainbow trout (Oncorhynchus mykiss)

Mathis Hildonen, Miyako Kodama, Lara Puetz, M. Thomas P. Gilbert, Morten T. Limborg

PII: S0167-7012(19)30122-8
DOI: https://doi.org/10.1016/j.mimet.2019.03.010
Reference: MIMET 5599

To appear in: *Journal of Microbiological Methods*

Received date: 13 February 2019
Accepted date: 12 March 2019

Please cite this article as: M. Hildonen, M. Kodama, L. Puetz, et al., A comparison of storage methods for gut microbiome studies in teleosts: Insights from rainbow trout (Oncorhynchus mykiss), Journal of Microbiological Methods, https://doi.org/10.1016/j.mimet.2019.03.010

A comparison of storage methods for gut microbiome studies in teleosts: insights from rainbow trout (*Oncorhynchus mykiss)*

Mathis Hildonen[1], Miyako Kodama[1], Lara Puetz[1], M. Thomas P. Gilbert[1], Morten T. Limborg[1]

1. National History Museum of Denmark, University of Copenhagen, DK-1350 Copenhagen, Denmark

Corresponding author:

Morten T. Limborg

Morten.limborg@gmail.com

National History Museum of Denmark, University of Copenhagen, Øster Voldgade 5-7, DK-1350 Copenhagen, Denmark

Abstract

Immediate freezing is perhaps the most preferred method used for preserving gut microbial samples, but research on sample preservation has been principally based around samples from mammalian species, and little is known about the advantages or disadvantages relating to different storage methods for fish guts. Fish gut samples may pose additional challenges due to the different chemical and enzymatic profile, as well as the higher water content, which might affect the yield and purity of DNA recovered. To explore this, we took gut content and mucosal scrape samples from 10 rainbow trout (*Oncorhynchus mykiss*), and tested whether different preservation methods have any effect on the ability to construct high quality genomic libraries for shotgun and 16S rRNA gene sequencing. Four different storage methods were compared for the gut content samples (immediate freezing on dry ice, 96% ethanol, RNAlater and DNA/RNA shield), while two different methods were compared for mucosal scrape samples (96% ethanol and RNAlater). The samples were thereafter stored at -80°C. Our findings concluded that 96% ethanol outperforms the other storage methods when considering DNA quantity, quality, cost and labor. Ethanol works consistently well for both gut content and mucosal scrape samples, and enables construction of DNA sequencing libraries of sufficient quantity and with a fragment length distribution suitable for shotgun sequencing. Two main conclusions from our study are i) sample storage optimisation is an important part of establishing a microbiome research program in a new species or sample type system, and ii) 96% ethanol is the preferred method for storing rainbow trout gut content and mucosal scrape samples.

Keywords: Aquaculture, Preservation methods, metagenomics, rainbow trout, PCR inhibition

Introduction

The quality and quantity of DNA extracted from biological tissues lies at the heart of the success of most modern genome and microbiome studies. The focus on DNA has changed the prerequisites for sample preservation methods, as the methods used in microbiome research have to a large degree moved from traditional culture-based methods [1], to sequencing of environmental samples for the study of microbiome composition [2].

Metagenomic research applies molecular techniques for analyzing microbial communities [3], which requires maintenance of not only the integrity of the microbial DNA itself [4], but also the community composition, during storage and transportation [5]. Traditionally, immediate freezing at -80°C was the preferred method for preserving fecal microbial communities, as immediate deep freezing allows for later cultivation of a bacterial sample [6]. However, freezing the samples for culture-dependent studies requires a continuous cold chain from sampling site to the lab. In contrast, preservation in stabilizing media have shown to better conserve the microbial community profile when the sample is exposed to repeated freeze and thaw cycles [5]. When conducting targeted gene and metagenomic studies using techniques such as bacterial 16S rRNA amplicon or shotgun sequencing, the ability to culture a sample no longer remains a prerequisite, so preservation media that kill the bacteria are now viable options. The choice of preservation method can help optimize cost and resource efficiency, and may be considered with regards to logistical and legal considerations, as well as sample type, when optimizing protocols for a specific project.

While several studies have explored the effect of sample preservation and storage methods on gut microbiome samples, these have mainly focused on mammalian fecal samples, and on how the preservation method affects the microbiome composition [5, 7-10]. In contrast, the challenge of

extracting DNA of sufficient quantity for metagenomics from fish gut samples has received less attention, despite a plethora of studies on fish gut microbiota [11]. While some preservatives work well on fecal and/or mammal samples, they might not work sufficiently on other types of samples such as gut content from fish or other non-mammalian species. The reason for this may be a difference in density, water content and enzymatic and chemical properties [12], which may inhibit DNA extraction and PCR [13]. Previous studies on extracting DNA of sufficient quality and quantity for metagenomic studies have mainly focused on protocols for extraction and purification [14-16] but not on the effect of different preservation methods.

In this study, we compared the efficacy of four methods commonly used to preserve microbial DNA, on samples taken as gut content, and mucosal scrape of gut epithelia, from rainbow trout (*Oncorhynchus mykiss*), a species accounting for a large share of the global aquaculture market [17]. Our emphasis was on the ability to extract microbial DNA of sufficient quality to perform 16S rRNA gene and shotgun sequencing, while keeping costs and labor intensity down when working on large sample sizes, something of increasing necessity to applied research on livestock and aquaculture-related experiments.

Materials and methods

Gut content and mucosal gut scrapes were taken from farmed rainbow trout (*Oncorhynchus mykiss*) and preserved using four common storage methods. The DNA was isolated and measured for total DNA concentration, prior to being assessed for bacterial DNA content and PCR inhibition using quantitative real-time PCR (qPCR). Shotgun libraries were built from the fragmented DNA extracts, and quality assessed for total DNA concentration and fragment length distribution.

Rainbow trout microbial samples

Gut content and mucosal gut scrapes from farmed rainbow trout (*Oncorhynchus mykiss)* were obtained from a commercial farm located in saltwater near Bisserup, Zealand (DK) in December 2017 and originally hatced at Vork Dambrug (Denmark). Gut content and mucosal scrapes were collected from 10 individuals. All 10 fish were randomly sampled from the same genetic cohort with an age of 3 years and insignificant size variation. The fish were too unripe to be reliably sexed individually, but normally the fish farm contains a female to male sex ratio of 60:40 at this life stage; regardless, since the main objective of this study is to solely evaluate the efficiency of different storage methods for preserving the quantity and quality of DNA, and not the microbial community composition, we do not expect any differences in sex or size to affect our conclusions.

Four subsamples of gut content were sampled from each fish. First, the entire mid and distal gut sections were carefully dissected out of the fish and cleaned with saline water. Then we opened the gut with a sterile scalpel and carefully removed between 0.3 – 0.6 ml gut content from the mid gut and immediately stored it in one of the four different preservation methods; i) immediate freezing on dry ice in a Styrofoam container, and thereafter kept on -80°C, while the other three were immersed in ii) 96% ethanol, iii) RNAlater and iv) DNA/RNA Shield (Zymo Research, Irvine, CA), and then kept at -80°C.

Lastly, we also sampled gut epithelial scrape samples for the comparison of two different storage methods. We washed the interior gut epithelial tissue with saltwater and carefully obtained two subsamples of mucosal scrapes with 4-5 scalpel scrapes over a surface area of 4-6 cm2. The scraped tissue samples were then immersed in two different preservatives; i) 96% ethanol and ii) RNAlater, and after 24 hours kept at -80°C until extraction.

Extraction of bacterial DNA from gut content and gut scrape samples

DNA was extracted from ~0.1 g of sample using the MagAttract Powersoil DNA Kit (Qiagen, Venlo, NL), with a modified protocol using bead tubes from the Powersoil DNA Isolation Kit (Qiagen, Venlo, NL) and a KingFisher Duo robot (Thermo Fisher Scientific, Waltham, MA).

A total of 60 µl of lysis solution was added to the sample and incubated for 10 minutes at 65°C in a heat block. Samples were bead-beaten in a Tissuelyser II (Qiagen, Venlo, NL) for 10 minutes at 30Hz prior to two cycles of freeze thawing (incubated at -80°C until completely frozen and subsequently thawed at 65°C), as modified from [14]. Four µl of proteinase K was added to the sample and incubated on a rotator for one hour at 56°C then cooled to room temperature. The samples were centrifuged at 10,000 x g for 2 minutes, and the supernatant was transferred to a 2 ml collection tube with 450 µl IRT solution, vortexed for 5 seconds and incubated for 10 minutes at 4°C. Samples were subsequently centrifuged at 13,000 x g for 2 minutes, after which the entire volume of supernatant was transferred to a clean 2 ml collection tube, which was then centrifuged again at 13,000 x g for 2 minutes.

The supernatant was transferred to a clean Kingfisher Deep Well 96 plate as summarized in Table S1, and the KingFisher MO BIO PowerMag Soil robotic program Version 2 was run to extract DNA. Extracted DNA was quantified using the Qubit dsDNA Broad Range Assay kit, transferred to 1.5 ml lo-bind Eppendorf tubes and stored at -20°C.

Assessment of bacterial DNA and inhibition level

To assess DNA extraction efficiency, the relative amounts of bacterial DNA and detection of inhibitors was estimated through qPCR targeting the V3-V4 region of the bacterial16S rRNA gene

[18]. Undiluted and 1:20 dilutions were used, and extraction blanks and PCR negatives were included in every run. When comparing the amount of bacterial DNA among the preservation methods, the 1:20 dilution was used, to minimize the impact of inhibition on the Ct values. Inhibition was assessed by looking at the difference in Ct value between the undiluted and diluted sample. In an uninhibited and optimally amplifying sample, a ΔCt value of one cycle should account for a double amount of PCR substrate, and a 1:20 dilution should result in a ΔCt of ~4.3. In contrast, if inhibition is present, the ΔCt between dilutions should decrease.

Genomic DNA was qPCR amplified to target the V3-V4 variable region of the bacterial 16S rRNA gene (~465 bp) using the primer pair Bact-341F (5'-CCTAYGGG RBGCASCAG-3') and Bakt-806R (5'-GGACTACNNGGGTATCTAAT-3') [18] with Illumina Nextera overhang adapters (Illumina Inc., San Diego, CA, USA). The qPCR mixture amplifying community bacterial DNA used 1X AccuPrime SuperMix II (Invitrogen, Eugene, OR, USA), 0.5 µM (each) forward and reverse primers, 2 µl DNA (following dilution series), 1 µl SYBR green and DNase/RNase-free water to a final volume of 20 µl. The qPCR profile consisted of an initial denaturation step at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 52°C for 15 s, and 68°C for 40 s and a final step to estimate the dissociation curve from 55°C-95°C. All qPCR reactions were run on the Stratagene Mx3005p (Agilent Technologies, Santa Clara, CA).

Sequencing libraries

We subsequently tested the ability to build shotgun metagenomic sequencing libraries from the DNA extractions. For each preservation method, we built metagenomic sequencing libraries on three biological samples that exhibited varying results regarding total DNA and qPCR Ct value.

This was performed to observe how low, medium and high discrepancy in DNA concentration among the preservation methods would affect the quality of sequencing libraries.

A M220 Focused Ultrasonicator (Covaris, Woburn, MA) was used to shear the DNA to about 300bp fragments. Each sample was then processed with the BEST [19] library building protocol. The protocol includes four steps: blunt end-repair with T4 DNA polymerase, ligating with T4 DNA ligase, fill-in with Bst 2.0 Warmstart polymerase, a SPRI magnetic bead purification, and elution in EB buffer. The total DNA content of the libraries were measured on the Qubit Fluorometer HS Assay. Diluted libraries were run on a Tapestation 4200 High Sensitivity Assay (Agilent Technologies, Santa Clara, CA) to assess the fragment length distribution and concentration.

Assessing DNA content for index PCR

To assess the amount of cycles needed for index PCR, the libraries were diluted to 1:20 and run on the Stratagene Mx3005p qPCR. The qPCR mixture used 1X PCR Buffer, 0.2 µM (each) forward and index primers (BGI), 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.2 µl BSA (20mg/ml), 0.8 µl SYBR green, 0.1 U/µl Taq Gold (Thermo Fisher Scientific, Waltham, MA), 1 µl DNA (following dilution) and DNase/RNase-free water to a final volume of 20 µl. The qPCR profile consisted of an initial denaturation step at 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 60°C for 60 s, and 72°C for 60 s and analyzed using the Stratagene Mx3005p.

Library indexing and quality control

After the fragment length distribution and DNA concentration was measured, the libraries from the samples preserved in ethanol were selected for further downstream steps.

Using the results from the library qPCR, an optimal cycle number for index PCR was calculated for each sample individually. The index PCR mixture used 1X PCR Buffer, 0.2 µM (each) forward and index primers (BGI), 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.1 U/µl Taq Gold, 20 µl DNA and DNase/RNase-free water to a final volume of 100 µl. Three PCR negatives were included. The PCR profile consisted of an initial denaturation step at 95°C for 12 min, followed by 3-16 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C for 40 s, and a final step of 72°C for 5 min.

Fifty µl of product was then purified using SPRI magnetic beads and eluted in 30µl EB buffer. Total DNA content of the purified product was then measured using the Qubit fluorometer HS Assay. The purified product was run on the Agilent 4200 Tapestation High Sensitivity Assay, to assess fragment length distribution and concentration.

Statistical analysis and graphics

We tested whether DNA concentration and Ct value were different between samples. First, the Kruskal-Wallis test was performed to jointly compare the four gut content preservation methods. When the Kruskal-Wallis test was significant we followed up with the Wilcoxon Signed-Rank Test to reveal which pairs of samples (two-tailed) were significant from each other. Statistical analyses were conducted in Microsoft Excel, using the add-in Real Statistics (http://www.real-statistics.com/), and figures were made with R (https://www.r-project.org/).

Results

One of the sampled rainbow trout had an empty gut, and was not included, leaving a total of nine different specimens from which gut content samples were taken.

Total DNA concentration

Gut content preserved in ethanol had the highest mean DNA content compared to all other storage methods (13.55 ng/µL), however, it also had the highest amount of within group variation as the concentrations for two out of nine sample DNA extractions being too low to quantify on the Qubit HS Assay (Fig. 1). Both the extracts from the frozen samples and those preserved in DNA/RNA Shield had very low DNA content (0.28 ng/µL and 0.45 ng/µL respectively; Fig. 1), with several too low to measure on the Qubit. DNA from samples preserved in RNAlater were the only ones with consistently readable results, although the average total DNA was lower than from those preserved in ethanol (2.36 ng/µL). The extractions of samples stored in RNAlater and ethanol had significantly higher total DNA content compared to samples stored frozen and in DNA/RNA Shield ($P<0.05$; n=9), while there was no significant difference between RNAlater and ethanol ($P=0.203$; n=9), or between frozen and DNA/RNA shield ($P=0.078$; n=9).

The extractions from mucosal scrape samples stored in ethanol had a significantly higher ($P<0.05$; n=10) total DNA concentration (125.23 ng/µL) than from the samples stored in RNAlater (75.57 ng/µL; Fig. 2). Two mucosal scrape samples preserved in ethanol were too high to measure with the Qubit HS Assay, and were included in the calculation with an estimate of the lowest possible result outside of the Qubit HS Assay range (i.e. 120 ng/µL). As the ethanol preserved samples showed the highest DNA concentrations, the potentially higher concentration of the samples (i.e. >120 ng/µL)

would only further reinforce our conclusion that ethanol is the best performing storage buffer when it comes to preserving high concentrations of microbial DNA.

Comparison of 16S rDNA concentrations and assessment of PCR inhibition

Bacterial DNA content was estimated in a preliminary qPCR. The DNA from gut content samples stored in RNAlater amplified on average earlier than DNA from the samples stored in ethanol (29.94 vs. 31.18 cycles) (Fig. 3), indicating that they have a higher amount of 16S substrate for PCR, despite the lower amount of total DNA measured. Both the DNA from the frozen samples (33.97 cycles) and the samples preserved in DNA/RNA Shield (34.68 cycles) amplified later than the DNA from the samples preserved in ethanol and RNAlater (Fig. 3). The DNA from the samples preserved in DNA/RNA Shield amplified later than the frozen samples, despite exhibiting a higher total DNA concentration, the difference was however not significant (P=0.16; n=9).

The DNA from gut content samples stored in RNAlater PCR amplified significantly earlier than DNA from all the other preservation methods for gut content samples (P<0.05, n=9). These results were also seen in the mucosal scrape samples (Fig. 4), where those stored in RNAlater amplified significantly earlier than the ones stored in ethanol (30.57 vs. 33.08 cycles, P<0.05, n=10).

We also considered the qPCR results to assess the occurrence of inhibition among the preservation methods. In the gut content samples, moderate levels of inhibition were observed across all the preservation methods, with lower ΔCt values than the expected 4.3 cycles (Fig. 5).

The DNA from mucosal scrape samples stored in RNAlater exhibited the least amount of overall inhibition of all preservation methods (Fig. 5), with a mean ΔCt value of ~4.7, close to the optimum 4.3 cycles. The DNA from mucosal scrapes stored in ethanol showed moderate amounts of inhibition (Fig. 5).

Quality control of pre-indexing shotgun metagenomic sequencing libraries

The total DNA Qubit HS measurement showed low mean amounts of DNA in the frozen (0.17 ng/µL), DNA/RNA Shield (0.15 ng/µL) and RNAlater (0.25 ng/µL) gut content libraries, while the libraries for those preserved in ethanol had a slightly higher DNA content (0.68 ng/µL) (Fig. 6).

The mucosal scrape libraries had higher average DNA concentration than the gut content libraries (3.22 ng/µL vs. 0.31 ng/µL) (Fig. 7), but showed large variation in concentration (ranging between 0.314 ng/µl and 9.23 ng/µl for ethanol, 1.23 ng/µl to 7.78 ng/µl for RNAlater).

Only the libraries built from the samples stored in ethanol had a consistent fragment length distribution and peaks close to the target 300bp and within the targeted size region of 100-700bp for both gut content and mucosal scrape samples (Figs. 8 and 9). The libraries built on the frozen samples and the samples preserved in DNA/RNA Shield performed very poorly, with overall concentrations either very low or too low to measure, and no peaks or visible fragments within the target region were observed except for one of the frozen samples (Fig. 8). The libraries built on samples stored in RNAlater showed a relatively high concentration and fragment sizes within the

targeted length interval for the mucosal scrape samples (Fig. 9), but for the gut content samples, the fragment size was larger than the targeted size range (Fig. 8).

Discussion

Our results indicate that preservation in 96% ethanol outperforms other methods for preserving Rainbow trout gut content and mucosal scrape samples for high throughput sequencing libraries.

DNA concentration and inhibition

For the gut content samples, preservation in ethanol and RNAlater yielded a significantly higher amount of total DNA compared to preservation by immediate freezing and in DNA/RNA shield. This contradicts some earlier reports, where DNA isolated from frozen samples for mammal gut microbiome studies were found to contain higher concentrations of total DNA than from samples stored in 96% ethanol and RNAlater [9, 20, 21]. A possible reason for this is that earlier studies were principally conducted on fecal samples [9, 20, 22], which are more solid and dense than the often more liquid and low density gut content samples. Both of the higher yielding preservation methods, ethanol and the ammonium salts-based RNAlater [23], dehydrate and concentrate the sample through osmosis, and after pelleting by centrifugation, the mass contains more DNA. On the contrary, DNA/RNA shield lyses the cells and dilutes the sample, while the frozen sample is not concentrated through osmosis as cells are maintained intact. Another possible explanation could be that ethanol, RNAlater or the PBS used to remove the RNAlater are changing the chemical properties, such as the pH of the sample, something that has shown an effect on extraction efficiency in earlier studies on eDNA [14]. It should be noted that we coupled the use of DNA/RNA shield from Zymo Research with the MagAttract Powersoil DNA Kit from Qiagen; extracting

samples stored in DNA/RNA shield with an accompanying kit from Zymo Research may reveal improved results from the ones reported in this study.

The preliminary qPCR to determine bacterial content did not completely correlate with the total DNA concentration and the shotgun sequencing library efficiency. For example, DNA from mucosal scrape samples stored in ethanol had a much higher total DNA concentration in both the extractions and the shotgun libraries, yet mean Ct values from the amplification of the bacterial 16S target gene, and thus possibly bacterial DNA content, were similar to DNA from frozen gut content samples. A possible explanation could be that the large difference in total DNA concentration is partially influenced by the amount of host DNA being co-extracted. Indeed, a high concentration of host cells is particularly likely in the mucosal scrape samples that were taken along the intestinal host tissue.

There were signs of PCR inhibition in most of the sample types. A possible source of inhibition in the samples stored in ethanol could be insufficient washing or time to dry, as ethanol is known to cause PCR inhibition [24].

DNA from mucosal scrape samples stored in RNAlater exhibited less PCR inhibition than DNA from samples stored in ethanol. Thus, given their similar DNA concentration and fragment length distribution, we propose that RNAlater could be a good alternative option for preserving mucosal scrape samples when high levels of PCR inhibitors are expected.

Library quality

The DNA from two out of three gut content samples stored in RNAlater did not fragment to the target size of 300bp, or within the targeted size region of 100-700bp (Fig. 8), indicating the presence of shearing inhibition. This could be due to leftover salts resulting from RNAlater stabilizing the DNA, making it resilient to ultrasonic shearing. Indeed, we also tried an alternative

library building protocol with an additional purification step and this showed a positive effect on the fragment sizes of the two resilient samples, without any significant loss of DNA, although they were still in the larger range (Fig. S1). Additional purification steps might allow DNA from RNAlater-preserved gut content samples to be consistently sheared to the optimal fragment size for metagenomic sequencing. The DNA from RNAlater-preserved mucosal scrape samples did fragment to within the desired length, and may be considered as a choice of preservation method, especially for samples with high levels of PCR inhibition, as mentioned earlier.

Previous studies have shown ethanol-preserved metagenome samples to be more similar in composition and diversity to the less biased methods of fresh or frozen samples, compared to samples preserved in RNAlater [7, 20]. Ethanol also conserves the microbial composition better over time compared to RNAlater and immediate freezing without a preservation medium, when there is repeated thawing and refreezing [5]. Both preservation in RNAlater and DNA/RNA shield has been observed to have a strong negative effect on the alpha diversity of fecal microbiome samples [25].

Logistic considerations

The cost, labor-intensity and logistics are important aspects to consider when working with large sample sizes, commonly observed in many applied livestock and aquaculture-related studies. Although ethanol does pose some extra cost compared to immediate freezing, which is done in empty tubes, it is still considerably cheaper than RNAlater. Both ethanol and RNAlater have the advantage of making sampling in the field easier, as there is no need for a continuous cold-chain. Although, downstream processing of ethanol preserved samples requires extra work to remove it fully prior to DNA extraction, it is simpler to work with than RNAlater. RNAlater is of similar density to PBS, which caused difficulties in pelleting the sample prior to DNA extraction [25]. We

also speculate that remaining RNAlater may have caused the observed improper DNA shearing required for shotgun sequencing due to leftover salts after extraction.

Conclusions

Although immediate freezing has been seen as the gold standard preservation method for microbiome studies, our findings show that fish gut content samples preserved in 96% ethanol yield higher amounts of total DNA and produced shotgun sequencing libraries of more consistent quality than samples that have been immediately frozen, stored in RNAlater or DNA/RNA shield. RNAlater worked well on mucosal scrape samples, however the fragment length distribution for gut content samples was not optimal. Therefore, unless the samples are further purified, which is time consuming, we conclude that ethanol is the better option. However, RNAlater could be an option for mucosal scrape samples, or for samples intended for 16S rRNA gene sequencing exhibiting high levels of PCR inhibition, as the RNAlater-stored samples in this study showed the lowest levels of inhibition.

In conclusion, two main conclusions emerged from our study. First, optimisation of sample storage method, which is often ignored, is an important part of establishing a successful microbiome research program in a new species or sample type system. Secondly, when jointly considering DNA quality, cost and labor, both in the field and in the lab, we recommend using 96% ethanol for the preservation of rainbow trout gut content and mucosal scrape samples intended for 16S rRNA gene and shotgun sequencing.

Acknowledgements

We would like to thank Lars Birger Nielsen from BISSERUP FISK for providing the live rainbow trout that were sampled. We would also like to thank Jacob Agerbo Rasmussen for the help with the laboratory work.

Declaration of Conflicting Interests

Declaration of interest: none.

Funding

The Norwegian Seafood Research Fund - FHF (Project number: 901436) to MTPG and MTL.

A H2020 Marie Skłodowska-Curie Individual Fellowship (Grant number: 745723) to MTL.

References

1. Vaneechoutte, M., and Van Eldere, J. (1997). The possibilities and limitations of nucleic acid amplification technology in diagnostic microbiology. J Med Microbiol *46*, 188-194.
2. Su, C., Lei, L., Duan, Y., Zhang, K.Q., and Yang, J. (2012). Culture-independent methods for studying environmental microorganisms: methods, application, and perspective. Appl Microbiol Biotechnol *93*, 993-1003.
3. Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol *173*, 697-703.
4. Lindahl, T. (1993). Instability and decay of the primary structure of DNA. Nature *362*, 709-715.
5. Song, S.J., Amir, A., Metcalf, J.L., Amato, K.R., Xu, Z.Z., Humphrey, G., and Knight, R. (2016). Preservation Methods Differ in Fecal Microbiome Stability, Affecting Suitability for Field Studies. mSystems *1*.
6. Fouhy, F., Deane, J., Rea, M.C., O'Sullivan, O., Ross, R.P., O'Callaghan, G., Plant, B.J., and Stanton, C. (2015). The effects of freezing on faecal microbiota as determined using MiSeq sequencing and culture-based investigations. PLoS One *10*, e0119355.

7. Blekhman, R., Tang, K., Archie, E.A., Barreiro, L.B., Johnson, Z.P., Wilson, M.E., Kohn, J., Yuan, M.L., Gesquiere, L., Grieneisen, L.E., et al. (2016). Common methods for fecal sample storage in field studies yield consistent signatures of individual identity in microbiome sequencing data. Sci Rep *6*, 31519.
8. Sinha, R., Chen, J., Amir, A., Vogtmann, E., Shi, J., Inman, K.S., Flores, R., Sampson, J., Knight, R., and Chia, N. (2016). Collecting Fecal Samples for Microbiome Analyses in Epidemiology Studies. Cancer Epidemiol Biomarkers Prev *25*, 407-416.
9. Vlckova, K., Mrazek, J., Kopecny, J., and Petrzelkova, K.J. (2012). Evaluation of different storage methods to characterize the fecal bacterial communities of captive western lowland gorillas (Gorilla gorilla gorilla). J Microbiol Methods *91*, 45-51.
10. Choo, J.M., Leong, L.E., and Rogers, G.B. (2015). Sample storage conditions significantly influence faecal microbiome profiles. Sci Rep *5*, 16350.
11. Talwar, C., Nagar, S., Lal, R., and Negi, R.K. (2018). Fish Gut Microbiome: Current Approaches and Future Perspectives. Indian J Microbiol *58*, 397-414.
12. Hofmann, A.F., Hagey, L.R., and Krasowski, M.D. (2010). Bile salts of vertebrates: structural variation and possible evolutionary significance. J Lipid Res *51*, 226-246.
13. Penn, H.J., Chapman, E.G., and Harwood, J.D. (2016). Overcoming PCR Inhibition During DNA-Based Gut Content Analysis of Ants. Environ Entomol *45*, 1255-1261.
14. Lever, M.A., Torti, A., Eickenbusch, P., Michaud, A.B., Santl-Temkiv, T., and Jorgensen, B.B. (2015). A modular method for the extraction of DNA and RNA, and the separation of DNA pools from diverse environmental sample types. Frontiers in Microbiology *6*.
15. Anahtar, M.N., Bowman, B.A., and Kwon, D.S. (2016). Efficient Nucleic Acid Extraction and 16S rRNA Gene Sequencing for Bacterial Community Characterization. Jove-J Vis Exp.
16. Yuan, S., Cohen, D.B., Ravel, J., Abdo, Z., and Forney, L.J. (2012). Evaluation of methods for the extraction and purification of DNA from the human microbiome. PLoS One *7*, e33865.
17. Food and Agriculture Organization of the United Nations. Fisheries Department. The state of world fisheries and aquaculture. (Rome: Food and Agriculture Organization of the United Nations), p. volumes.
18. Hansen, C.H., Krych, L., Nielsen, D.S., Vogensen, F.K., Hansen, L.H., Sorensen, S.J., Buschard, K., and Hansen, A.K. (2012). Early life treatment with vancomycin propagates Akkermansia muciniphila and reduces diabetes incidence in the NOD mouse. Diabetologia *55*, 2285-2294.
19. Caroe, C., Gopalakrishnan, S., Vinner, L., Mak, S.S.T., Sinding, M.H.S., Samaniego, J.A., Wales, N., Sicheritz-Ponten, T., and Gilbert, M.T.P. (2018). Single-tube library preparation for degraded DNA. Methods Ecol Evol *9*, 410-419.
20. Hale, V.L., Tan, C.L., Knight, R., and Amato, K.R. (2015). Effect of preservation method on spider monkey (Ateles geoffroyi) fecal microbiota over 8 weeks. J Microbiol Methods *113*, 16-26.
21. Gill, S.R., Pop, M., DeBoy, R.T., Eckburg, P.B., Turnbaugh, P.J., Samuel, B.S., Gordon, J.I., Relman, D.A., Fraser-Liggett, C.M., and Nelson, K.E. (2006). Metagenomic analysis of the human distal gut microbiome. Science *312*, 1355-1359.
22. Gorzelak, M.A., Gill, S.K., Tasnim, N., Ahmadi-Vand, Z., Jay, M., and Gibson, D.L. (2015). Methods for Improving Human Gut Microbiome Data by Reducing Variability through Sample Processing and Storage of Stool. Plos One *10*.
23. van Eijsden, R.G., Stassen, C., Daenen, L., Van Mulders, S.E., Bapat, P.M., Siewers, V., Goossens, K.V., Nielsen, J., Delvaux, F.R., Van Hummelen, P., et al. (2013). A universal

fixation method based on quaternary ammonium salts (RNAlater) for omics-technologies: Saccharomyces cerevisiae as a case study. Biotechnol Lett *35*, 891-900.
24. Schrader, C., Schielke, A., Ellerbroek, L., and Johne, R. (2012). PCR inhibitors - occurrence, properties and removal. J Appl Microbiol *113*, 1014-1026.
25. Menke, S., Gillingham, M.A., Wilhelm, K., and Sommer, S. (2017). Home-Made Cost Effective Preservation Buffer Is a Better Alternative to Commercial Preservation Methods for Microbiome Research. Front Microbiol *8*, 102.

Figure legends

Figure 1. Gut content extractions. Total DNA concentration (ng/µL) with median, quartiles and minimum/maximum are shown. Lower case letters indicate significant differences. DRS = DNA/RNA shield.

Figure 2. Mucosal scrape extractions. Total DNA concentration (ng/µL) with median, quartiles and minimum/maximum are shown. Lower case letters indicate significant differences.

Figure 3. Gut content extractions. Median, quartiles and minimum/maximum qPCR Ct values of gut content samples amplified with qPCR at 1:20 dilution. Lower case letters indicate significant differences.

Figure 4. Mucosal scrape extractions. Median, quartiles and minimum/maximum Ct value of mucosal scrape samples amplified with qPCR at 1:20 dilution. Lower case letters indicate significant differences.

Figure 5. Difference in Ct value between undiluted DNA and DNA diluted to 1:20, when amplified with qPCR. The dotted green line indicates the optimal difference in Ct value when an uninhibited sample is diluted 1:20. A change in Ct value below 4.3 cycles indicates PCR inhibition.

MS = Mucosal Scrape

Figure 6. Gut content metagenomic libraries. Total DNA concentration (ng/µL) with median, quartiles and minimum/maximum are shown.

Figure 7. Mucosal scrape metagenomic libraries. Total DNA concentration (ng/µL) with median, quartiles and minimum/maximum are shown.

Figure 8. Gel image of metagenomic libraries from gut content samples. The black arrows indicate peaks in concentration.

Figure 9. Gel image of metagenomic libraries from mucosal scrape samples. The black arrows indicate peaks in concentration.

Figure S1. Gel image of metagenomic libraries from gut content samples preserved in RNAlater, built with the alternative library protocol including an additional purification step. The black arrows indicate peaks in concentration.

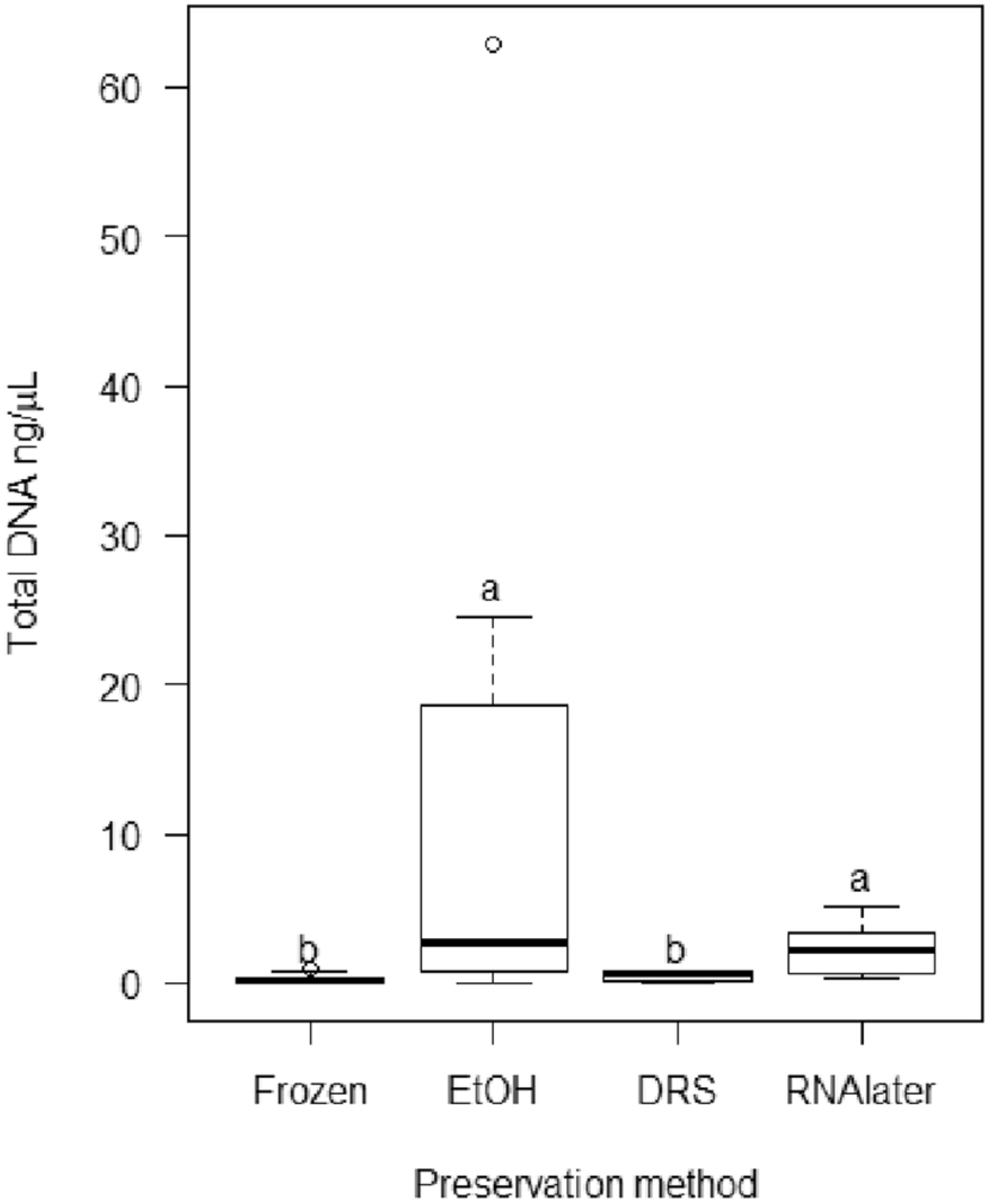
60
50
40
30
20
10
0
Total DNA ng/µL
a
b
b
a
Frozen
EtOH
DRS
RNAlater
Preservation method

Figure 1

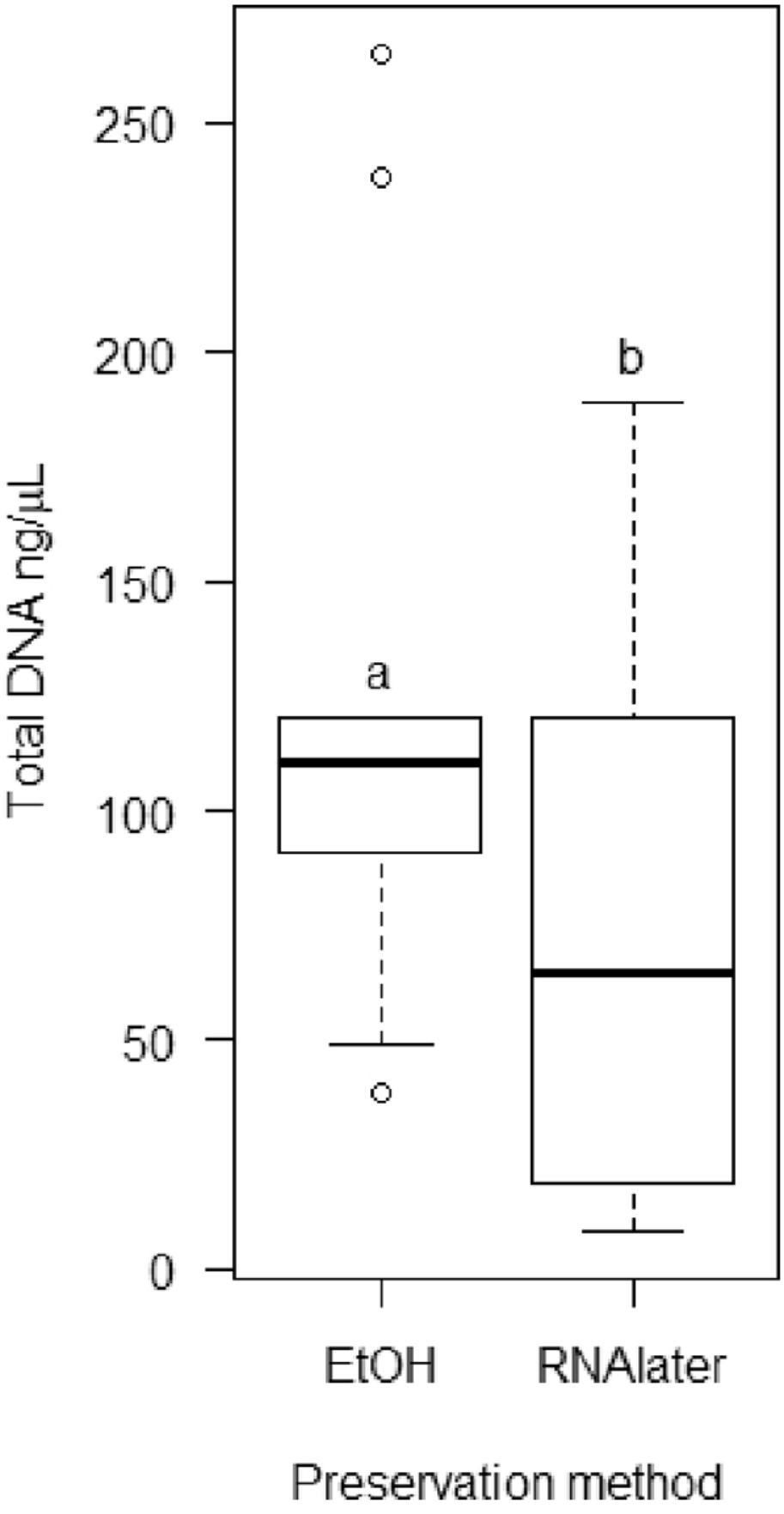
250
200
150
100
50
0
Total DNA ng/µL
a
b
EtOH
RNAlater
Preservation method

Figure 2

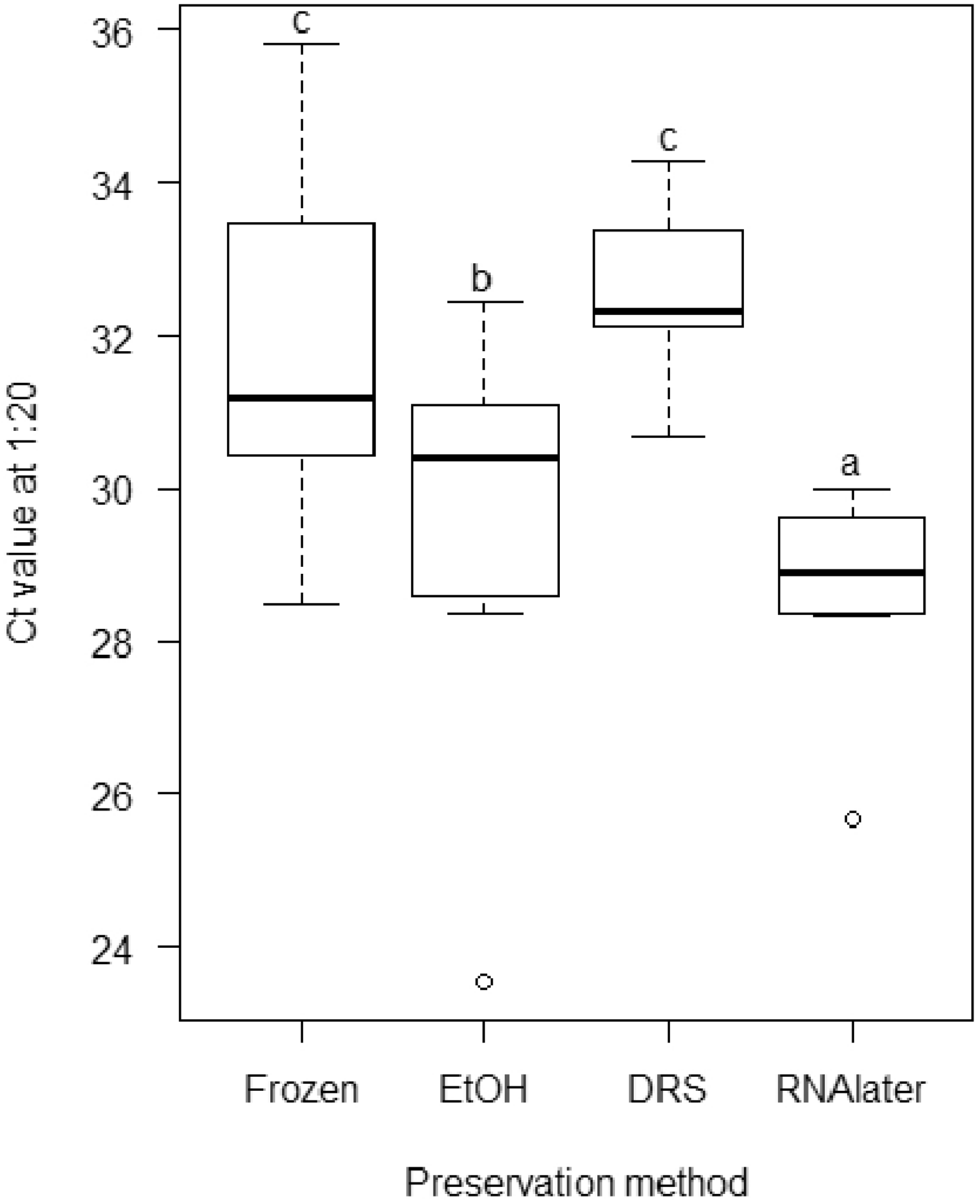
36
34
32
30
28
26
24
Ct value at 1:20
c
b
c
a
Frozen
EtOH
DRS
RNAlater
Preservation method

Figure 3

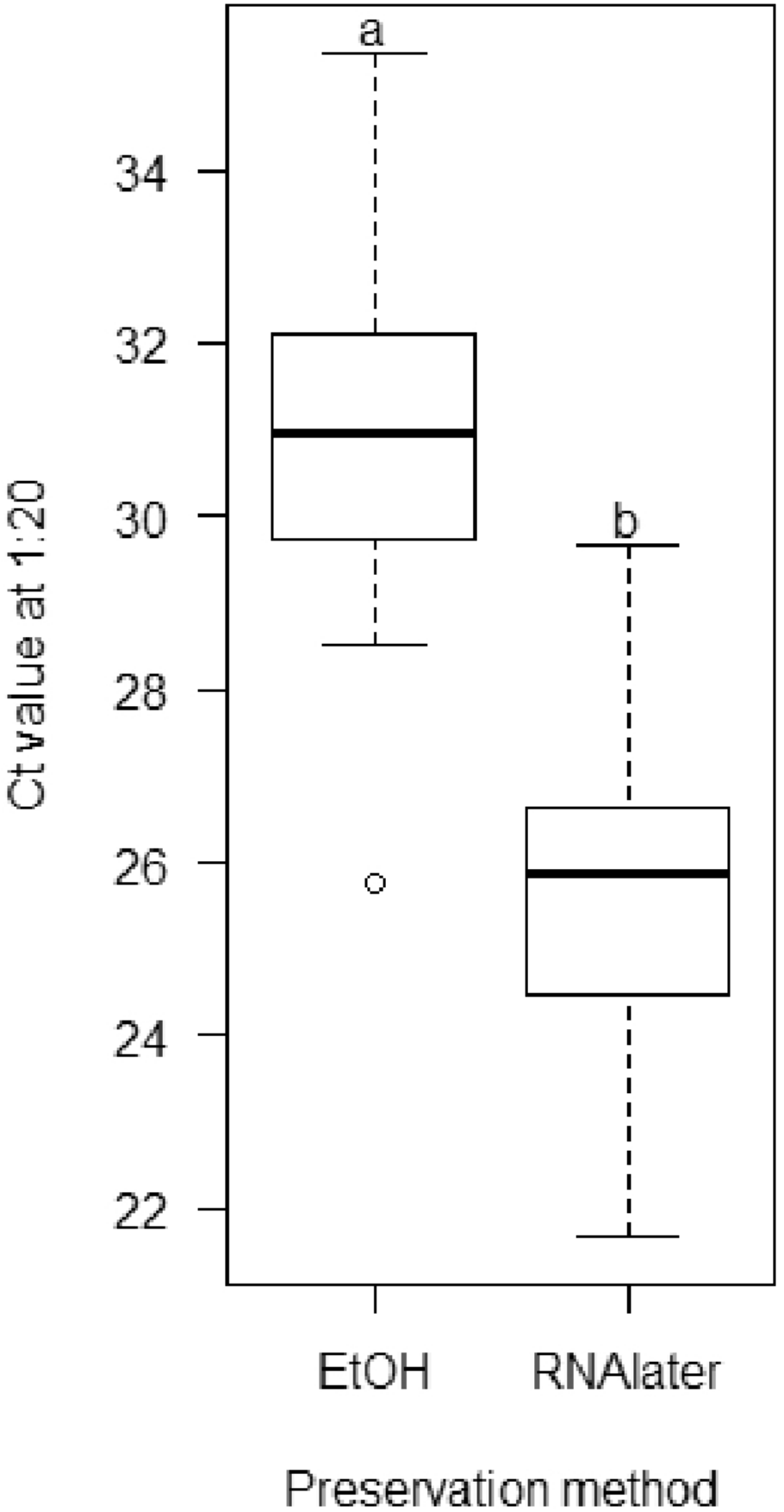
a
b
34
32
30
28
26
24
22
Ct value at 1:20
EtOH
RNAlater
Preservation method

Figure 4

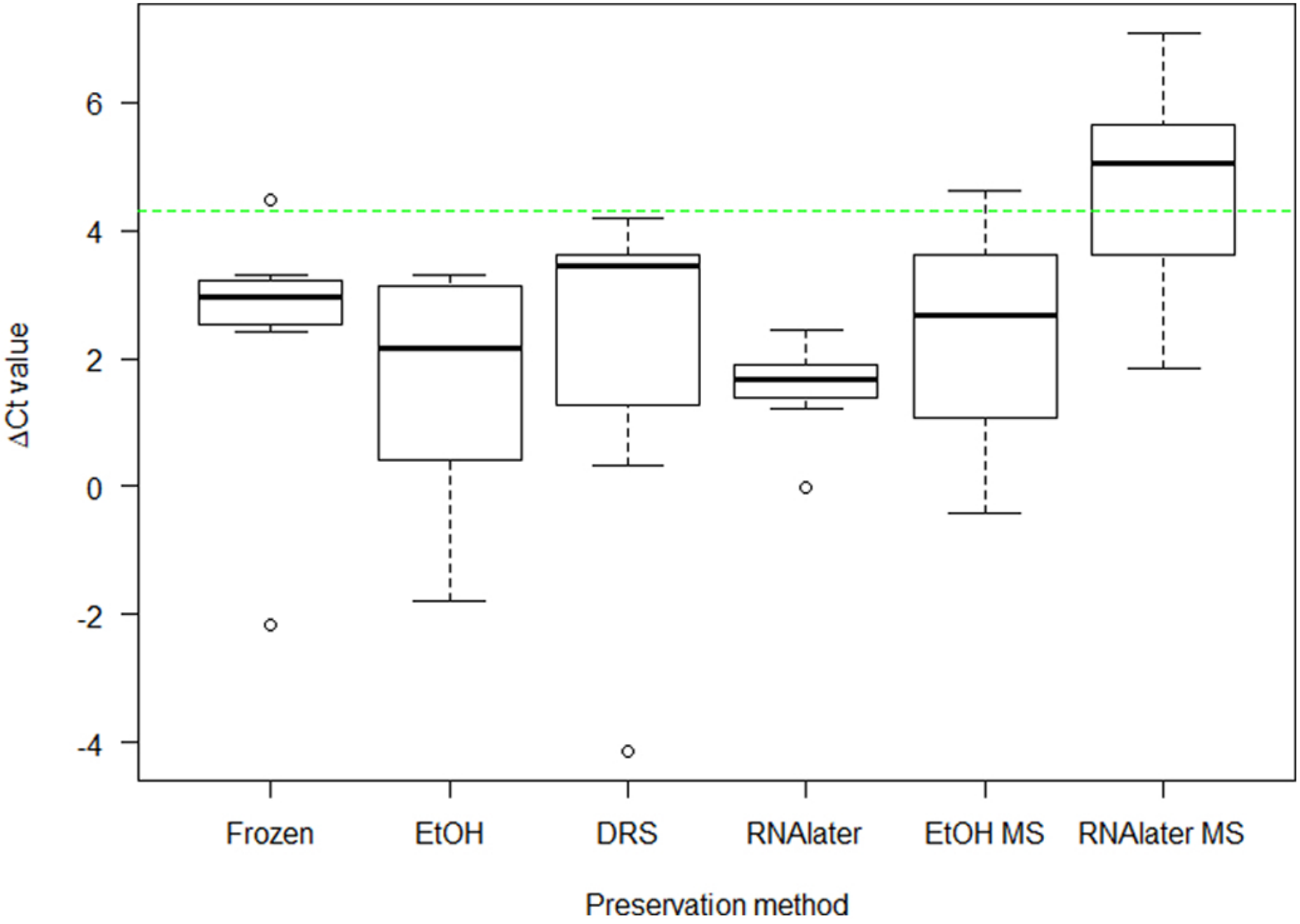
6
4
2
0
-2
-4
ΔCt value
Frozen
EtOH
DRS
RNAlater
EtOH MS
RNAlater MS
Preservation method

Figure 5

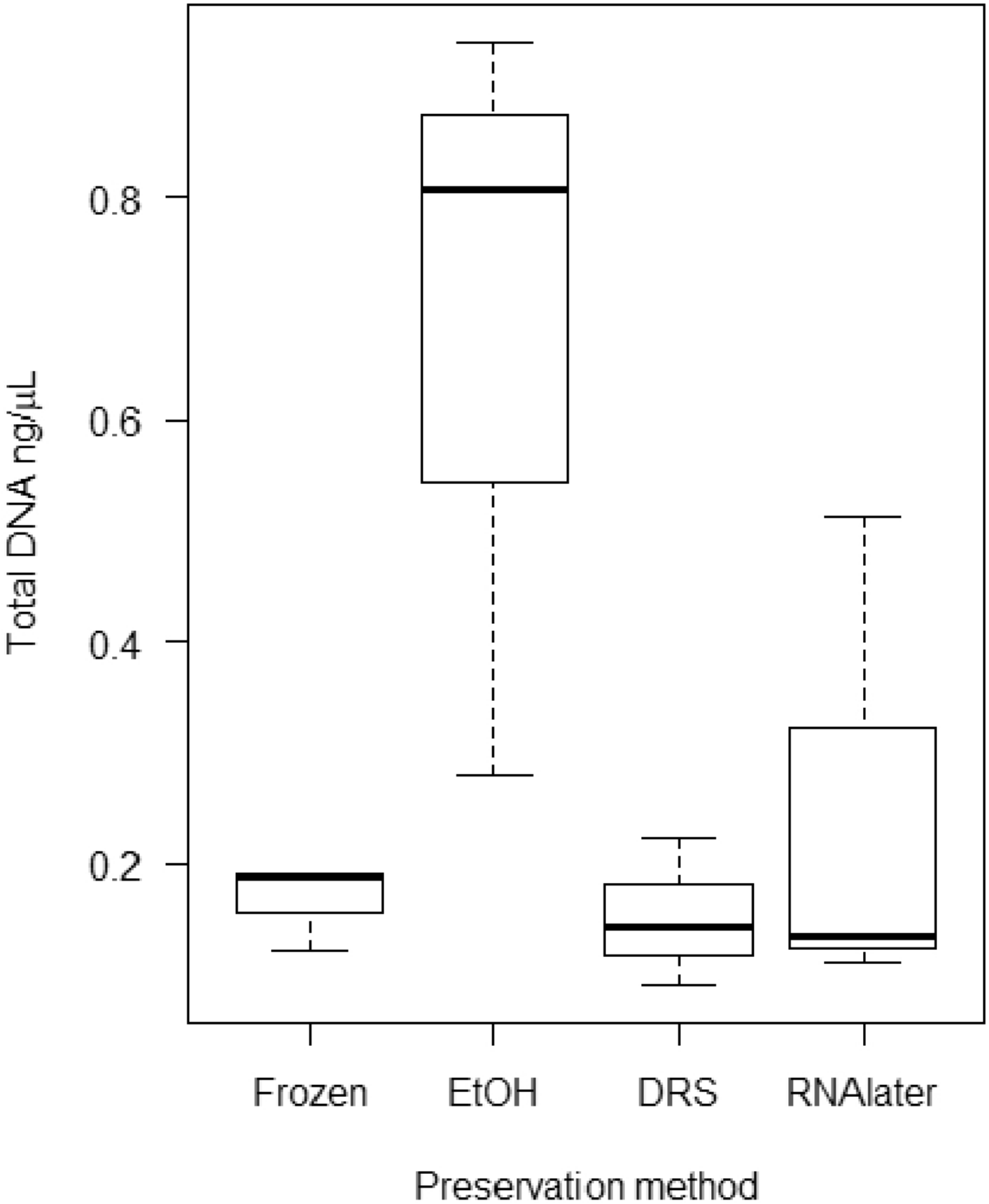
0.8
0.6
0.4
0.2
Total DNA ng/µL
Frozen
EtOH
DRS
RNAlater
Preservation method

Figure 6

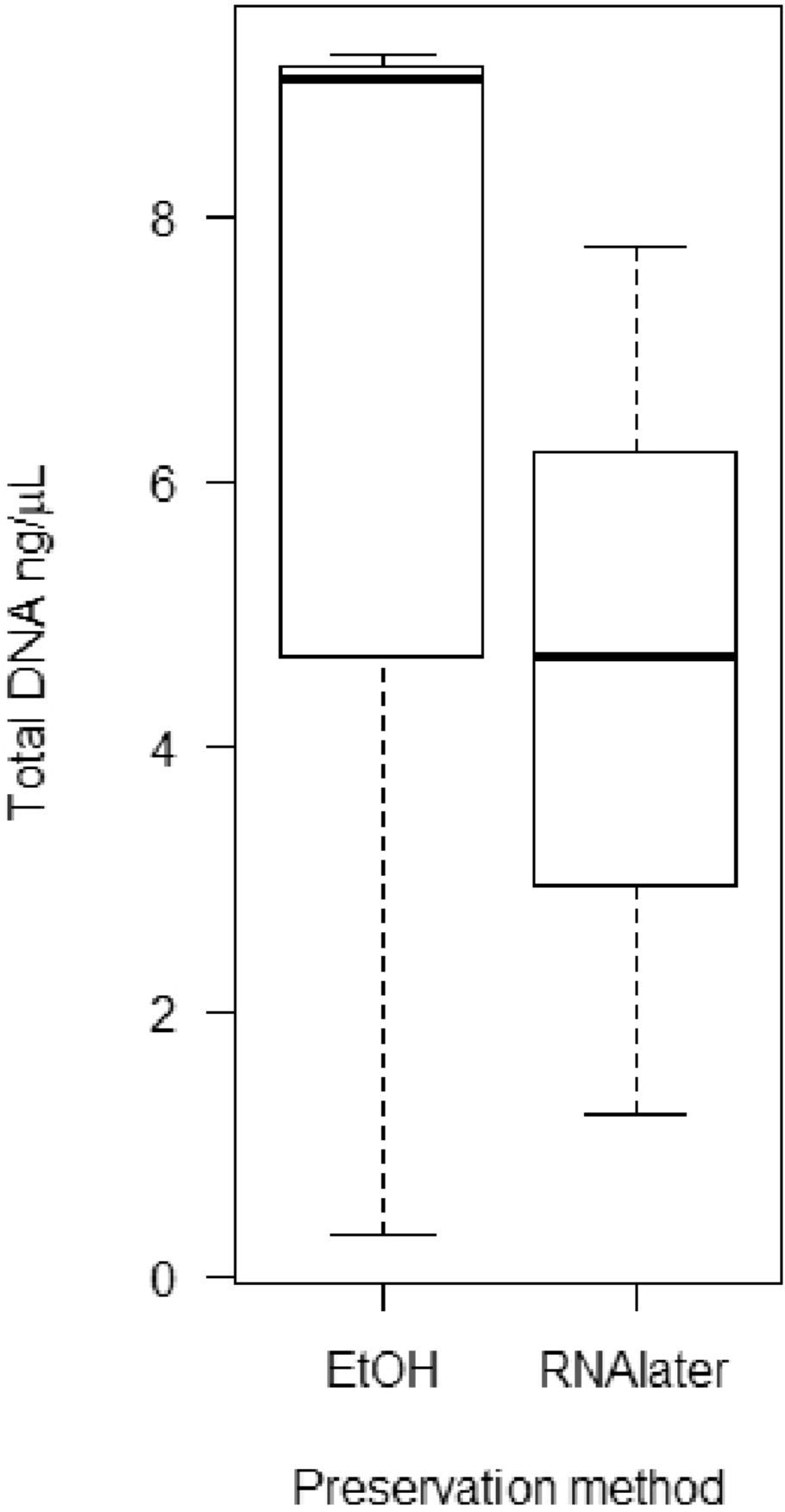

Figure 7

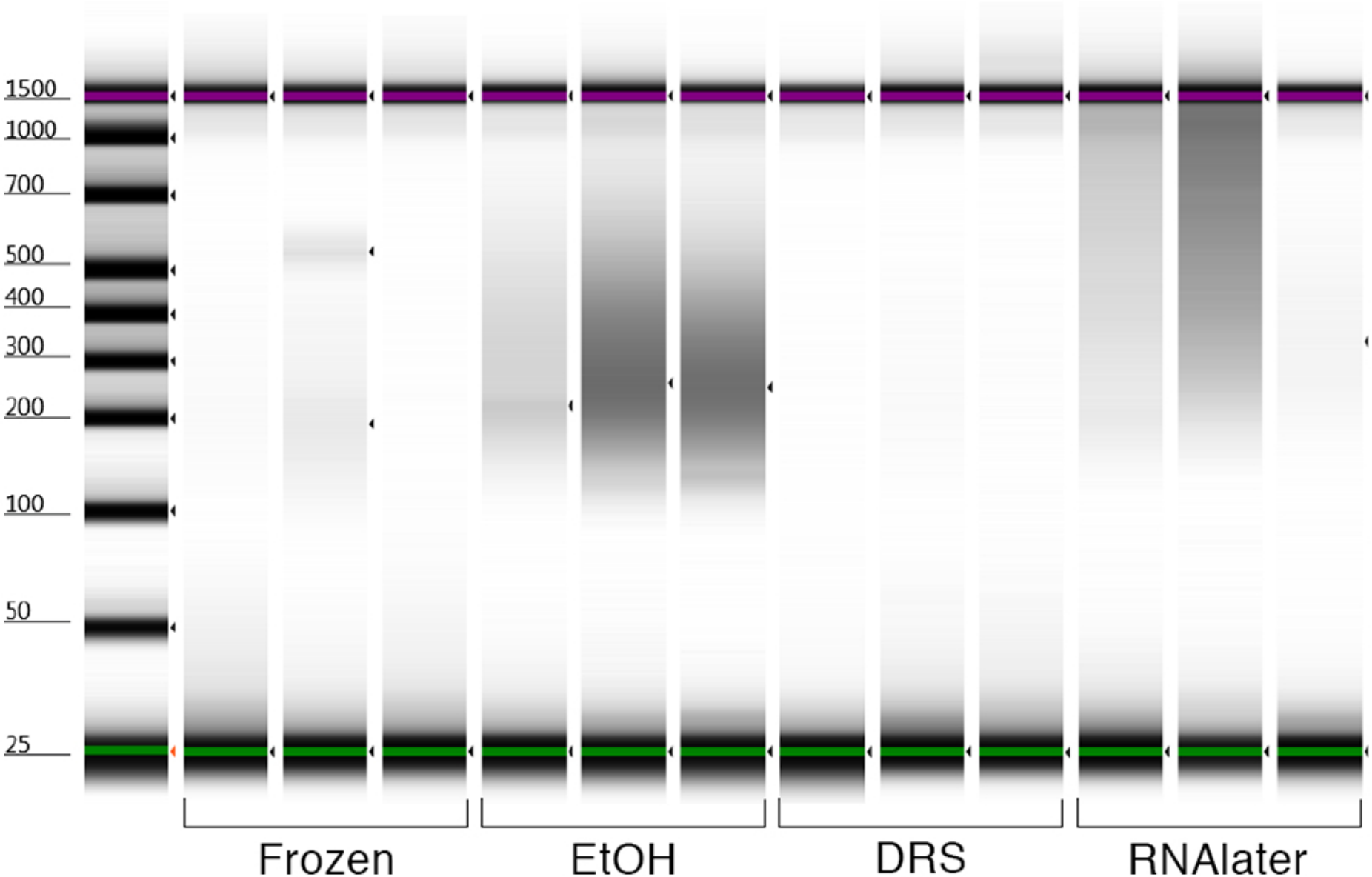
1500
1000
700
500
400
300
200
100
50
25
Frozen
EtOH
DRS
RNAlater

Figure 8

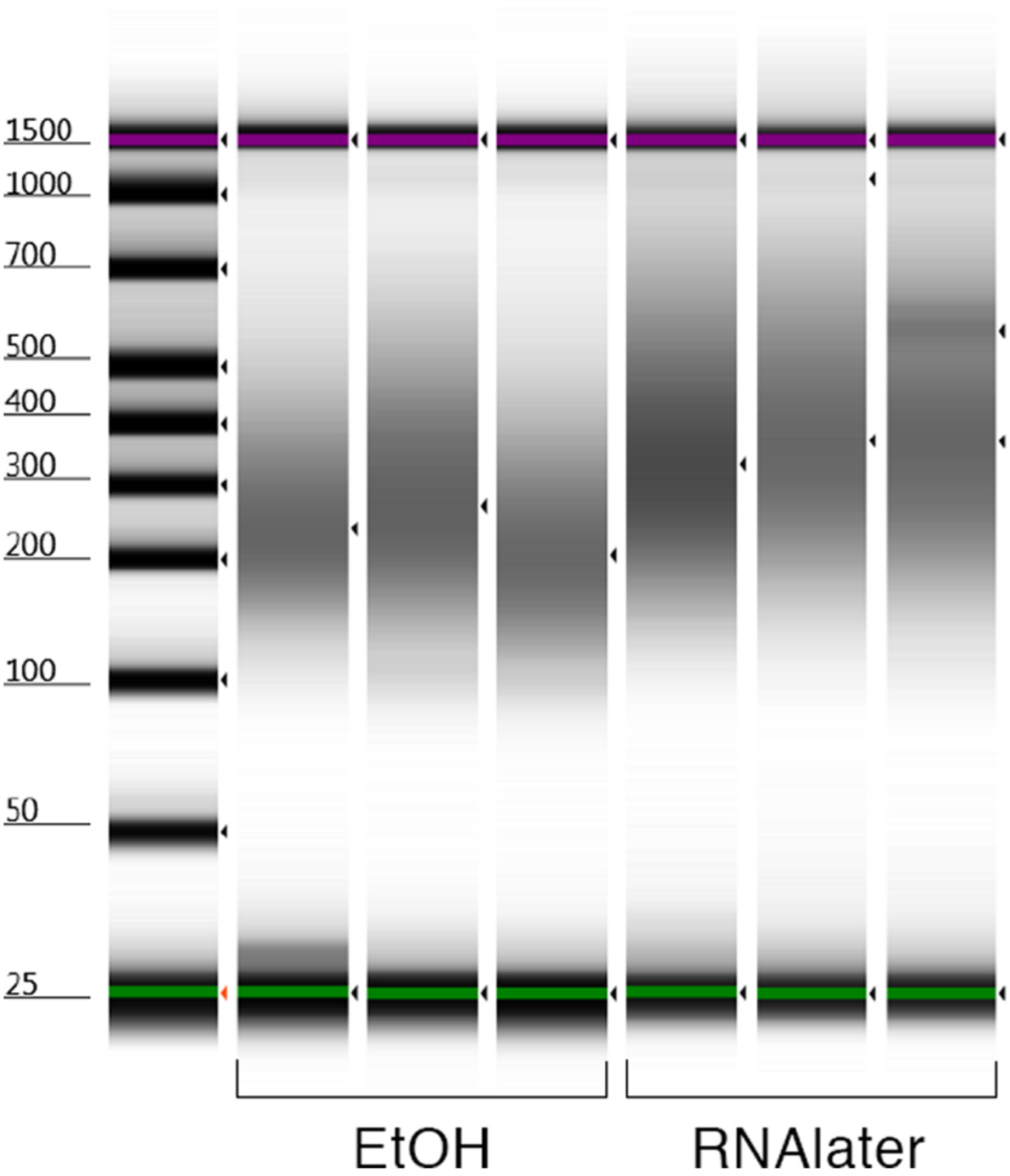

Figure 9

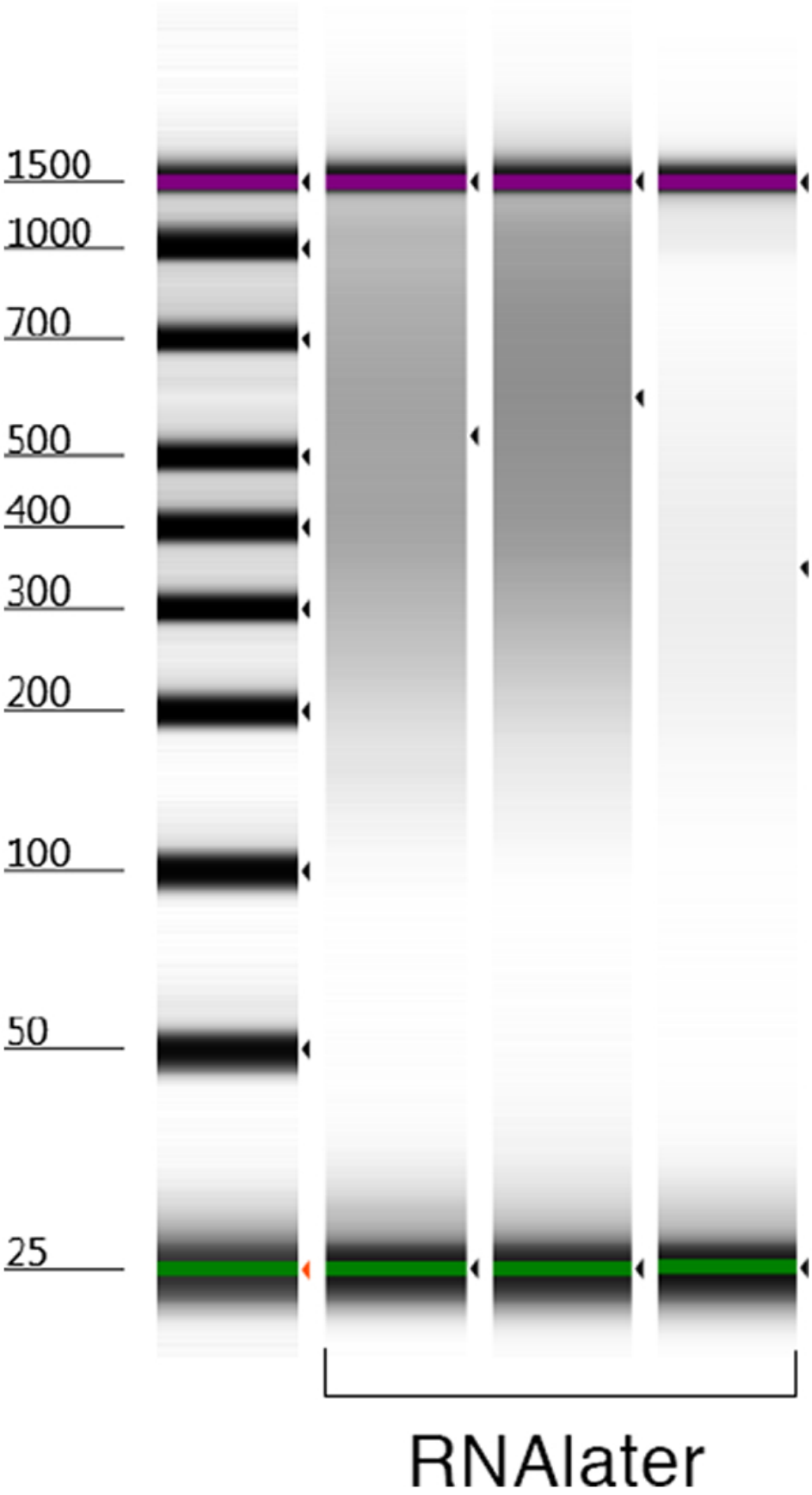

Figure 10