2746 Faker Avenue West, Carlsbad, CA 92010 Toll Free in US: 800-606-6246, Outside US: 760-929-9911, Fax: 760-929-0104

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Quantitative Assessment of the Removal of Humic Acid from Purified DNA and Environmental Samples Using Inhibitor Removal Technology

H. A. Callahan, S. J. Kennedy, M. N. Brolaski
MO BIO Laboratories Inc., Carlsbad, CA 92010

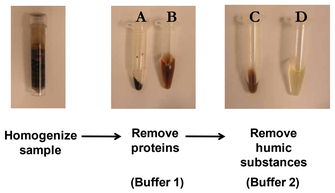
INTRODUCTION

Humic Substances (HSs) are large heterogeneous macromolecules that are the byproducts of organic decomposition of plants and microbes. HSs are primary components of soils, sediments, and biofilms. They are also major components of dissolved organic matter in water. The carryover of these inhibitors during nucleic acid purification can reduce yields and inhibit applications such as PCR. Inhibitor Removal Technology® (IRT) is a patented method for removal of humic substances as well as polyphenolics and polysaccharides which are building blocks of HSs. Here, we quantitatively demonstrate the importance of IRT for treatment of samples during nucleic acid extraction and purification by incorporating IRT into various DNA and RNA extraction kit protocols using environmental samples.

MATERIALS AND METHODS

Inhibitor Removal Technology® (IRT): IRT consists of two buffers. The first buffer solubilizes the DNA and precipitates proteins, while the second buffer binds and precipitates the large macromolecules (*i.e.* HSs, polysaccharides, polyphenols) separating them from the nucleic acid (**Fig. 1**).

Figure 1. Visualization of Inhibitor Removal Technology. Buffer 1 is added to the homogenized sample, incubated and the protein pelleted under centrifugation (A). Buffer 2 is then added to the supernatant (B), incubated and the macromolecules are pelleted (C) resulting in a clear supernatant (D) that is ready for spin column binding.



Evaluation of Inhibitor Removal Technology: 1.2 µg of *Escherichia coli* DNA was combined with humic acid (Sigma-Aldrich) serially diluted in sterile water. Humic acid was removed from samples using the PowerClean® DNA Clean-up kit protocol which contains IRT (MO BIO Laboratories). Humic acid was detected at A_{320} using a Nanodrop® (Thermo Scientific). All samples were subjected to end-point PCR using universal *Streptomyces* 16S rRNA primers with the Kapa2G Fast HotStart Readymix (MO BIO Laboratories).

DNA was isolated from 0.1 g of compost using either the PowerSoil® DNA Isolation Kit (MO BIO Laboratories) or a competitors kit that does not contain inhibitor removal. PCR was done as described previously.

Application of Inhibitor Removal Technology®:
Elliot Bay, Seattle, WA: 50 ml of water was filtered through 0.45 µm MCE membranes in duplicate. Extraction was with either the PowerWater® or RapidWater® DNA Isolation kit protocol which only differ by the incorporation of IRT. Real-time PCR was used to evaluate the DNA using Kapa SYBR Fast qPCR Kit (MO BIO Laboratories) with universal 16S rRNA primers (RNADirect). Purified and serially diluted *Enterococcus faecalis* DNA was used for the standard curve. Reactions were run and analyzed using a StepOne Real-Time PCR System (Applied Biosystems).

Buena Vista Lagoon, Carlsbad, CA: RNA from 0.15 g of rock surface biofilm was extracted following the PowerBiofilm™ RNA Isolation Kit protocol. Duplicates were treated with the same amount of IRT buffer 1 but with either 100 µl or 200 µl of IRT buffer 2. Reverse transcription was done (Qiagen) followed by end-point PCR as described previously. A modified, PowerClean® DNA Clean-up kit protocol was used for additional RNA sample clean-up.

RESULTS

Evaluation of Inhibitor Removal Technology: Regardless of concentration, IRT was able to reduce humic acid levels to those of the unspiked control (**Fig. 2A**). Sample clean-up also resulted in the expected amplification product while untreated samples were inhibited down to 7.5 ng (0.75 ng/µl) of humic acid (**Fig. 2B**).

Figure 2A - A_{320} readings of untreated (red) and IRT treated (green) *E. coli* DNA including a humic acid dilution series in water (blue) as a baseline.

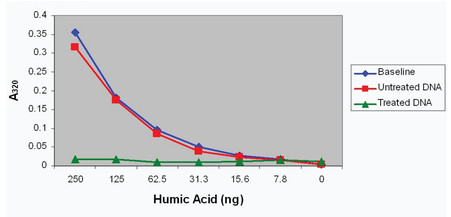
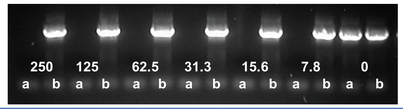


Figure 2B - PCR results of untreated (**a**) and IRT treated (**b**) *E. coli* DNA. PCR product size was 1.2 kb.



A competitor's kit (C) appeared to have more than double the yield of the PowerSoil® kit (P) but the increased yield was due to DNA/RNA degradation (**Fig. 3A**). In addition, $A_{260/230}$ values were low and the A_{340} value high due to inhibitor carryover (**Fig. 3B**). Inhibition was further confirmed by PCR (**Fig. 3C**).

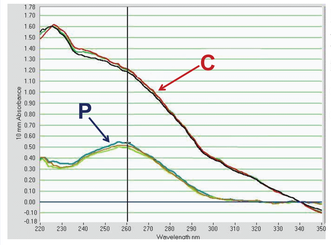


Figure 3A – Compost DNA curves and gel results for PowerSoil® (**P**) and a competitor's (**C**) kit. Arrow denotes degradation.

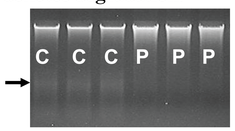
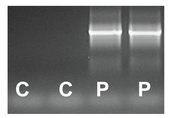


Figure 3B – Absorbance values of purified DNA from compost using PowerSoil® (**P**) and a competitor's (**C**) kit.

	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
C	59.38	1.188	0.807	1.47	0.78	50.00	230	1.527	0.696
C	60.71	1.214	0.818	1.48	0.78	50.00	230	1.565	0.852
C	60.30	1.206	0.828	1.46	0.78	50.00	230	1.539	0.768
P	25.58	0.512	0.268	1.91	1.63	50.00	230	0.314	0.056
P	24.60	0.492	0.262	1.88	1.60	50.00	230	0.308	0.043
P	26.97	0.539	0.287	1.88	1.55	50.00	230	0.349	0.052

Figure 3C – PCR results from compost using PowerSoil® (**P**) and a competitor's (**C**) kit.



Elliot Bay: DNA was obtained using both the PowerWater® and RapidWater® protocols (**Fig. 4A**), but Cq values were lower and more consistent when IRT was used (**Fig. 4B**). Assay efficiency was 104%.

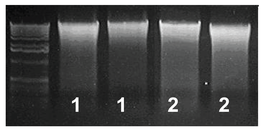


Figure 4A - Genomic DNA from Elliot Bay samples. PowerWater® DNA Isolation Kit (**1**); RapidWater® DNA Isolation Kit (**2**)

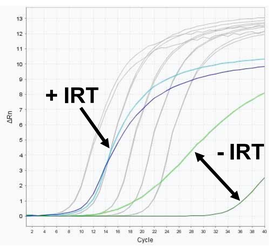


Figure 4B - Real-time PCR results from Elliot Bay. IRT containing PowerWater® DNA Isolation Kit (Blue); RapidWater® DNA Isolation Kit (Green); standard curve (Gray).

Buena Vista Lagoon: Slightly less RNA was obtained when 200 µl of IRT buffer 2 was used compared to 100 µl of buffer (**Fig. 5A**). However, PCR amplification was not as robust or consistent for these samples (**Fig. 5B**). When a modified PowerClean® DNA Clean-up kit protocol was applied to sample 1a, inhibitors were removed and PCR amplification products were obtained (**Fig. 5C**).

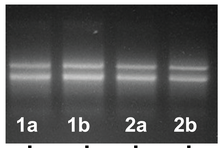


Figure 5A - RNA from Buena Vista Lagoon biofilm. 100 µl of IRT buffer 2 (**1**); 200 µl of IRT buffer 2 (**2**). All samples were in duplicate.

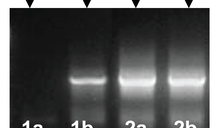


Figure 5B - Two-step RT-PCR amplification products from samples in Figure 5A. PCR product size was 1.2 kb.

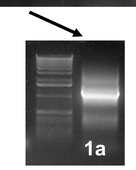


Figure 5C - Two-step RT-PCR amplification product for sample **1a** after additional clean-up of the original RNA sample.

CONCLUSIONS

The abundance of HSs in environmental samples poses significant problems for DNA/RNA isolation and analysis.

IRT is a two buffer system that removes HSs and is easily incorporated into nucleic acid purification protocols regardless of sample type.

IRT completely removes humic acid (even at high levels) to restore the ability of *Taq* to amplify the DNA.

IRT is necessary for removal of inhibitors from complex samples such as compost.

IRT- treated DNA yields consistent and sensitive results in qPCR

Samples with concentrated inhibitors, such as biofilms, may require more IRT for consistent PCR amplification

ACKNOWLEDGEMENTS

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