HYPERBARIC HEATING (HBH): UNLOCKING THE FULL POTENTIAL OF PCR-READY DIRECT SAMPLE INPUT

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Introduction

When executing nucleic acid amplification tests, intensive preparation procedures are often required to access the detectable nucleic acid within the sample. Patient derived samples, either body fluids or swabs eluted in media, contain various components that inhibit nucleic acid amplification reactions such as PCR. These inhibitors must either be neutralized or separated from the target nucleic acid to conduct an efficient and sensitive nucleic acid amplification reaction. Several techniques can be deployed to accomplish effective sample preparation prior to amplification.

Conventional PCR Sample Preparation Techniques

"To Bead or Not to Bead"

The two major categories of nucleic acid sample preparation techniques are nucleic acid extraction or direct sample heating. **Figure 1** describes the advantages and drawbacks of each sample preparation approach. Extraction leverages adsorption of the nucleic acid to a solid substrate - often comprised of silica - and subsequently washing away other material present in the sample. This technique is particularly effective at eliciting a sensitive subsequent PCR but requires several processing steps and many milliliters of various buffer solutions. Alternatively, heating the nucleic acid sample to near boiling for 5 to 10 minutes enables direct sample input for PCR. The applied heat can lyse most target organisms and denature many protein-based inhibitors, which, when combined with diluting out small molecule inhibitors, provides a simple sample preparation method suitable for some PCR assays. However, while direct sample input is easier to execute than extraction, it does not achieve the same performance for sensitive detection of minute amounts of target nucleic acid.

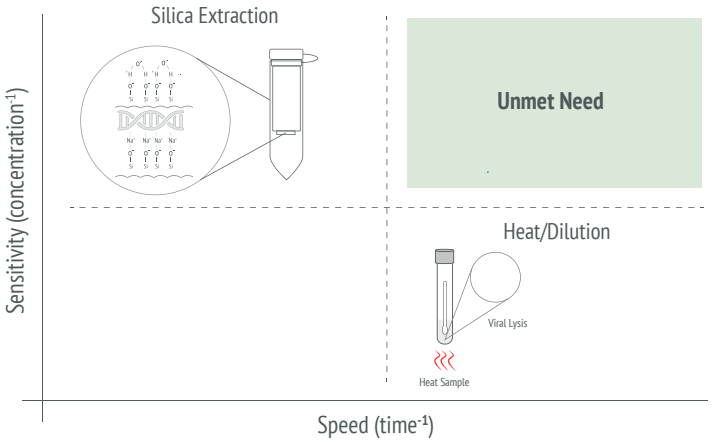

Figure 1: Tradeoffs between contemporary nucleic acid sample preparation techniques. Extraction of nucleic acids from a biological sample often enables the near single molecule sensitivity from a well-designed PCR assay. Yet, extraction is much more time consuming and cumbersome than heating and/or dilution of the sample for direct PCR input. While direct sample heating and dilution offers a simple and distributable sample preparation approach, current deployments of this technique do not elicit the same sensitivity that is achieved by nucleic acid extraction.

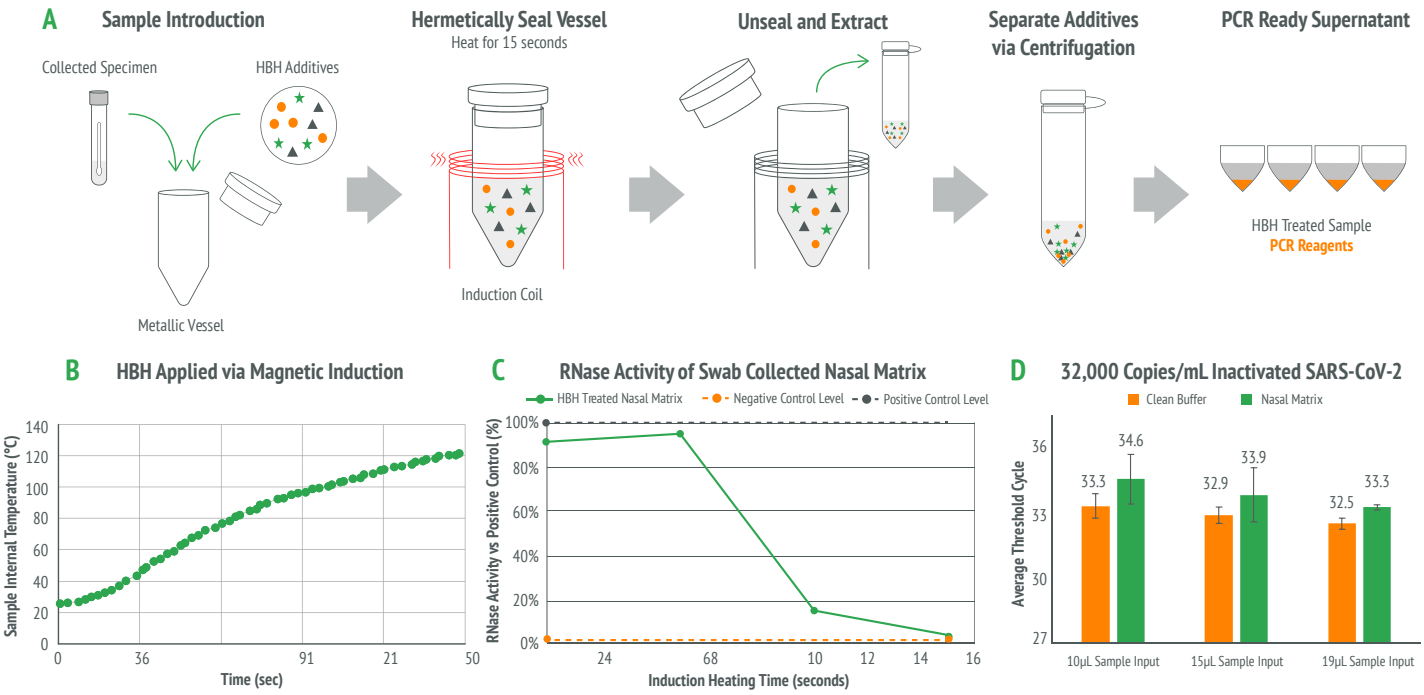

Figure 2: Hyperbaric Heating as a Simple and Effective Sample Preparation Method. **A.** Diagram demonstrating the steps of the hyperbaric heating workflow. HBH additive reagents are combined with the sample of interest in a metallic vessel. Upon capping with an airtight seal, the vessel is heated rapidly via magnetic induction. Once the heating is complete, the sample is transferred to a microcentrifuge tube where the solids are pelleted via centrifugation, and the resulting supernatant is ready for direct PCR input. **B.** Time series of temperature within an HBH sample vessel upon heating with magnetic induction. Temperatures reached the atmospheric boiling point (100°C) within 10 seconds and can surpass 100°C to even higher temperatures because of pressurization within the sealed container. **C.** Ribonuclease activity within a swab collected nasal matrix measured via fluorescence acquisition after 10 minute incubation with a fluorescent RNA probe. After 15 seconds of HBH treatment, nasal matrix elicited equivalent fluorescence intensity as the RNase negative control, indicating nearly complete inactivation of RNases in the nasal specimen. **D.** PCR cycle quantification for increasing amounts of HBH treated samples in 25 µL PCR reactions. Samples treated were contrived SARS-CoV-2 positive specimens both in clean collection media and in swab collected nasal matrix. PCR Ct solely improved as more sample volume was loaded into the PCR reactions, indicating effective neutralization of inhibitors present in nasal matrix by the HBH treatment.

Hyperbaric Heating

Simple and Sensitive PCR-Ready Nucleic Acid Sample Preparation

Hyperbaric heating (HBH) is a direct sample input method that combines reagents that preserve nucleic acids with a heating procedure that heats the sample past its boiling point via pressurization. HBH is accomplished by placing the sample within a sealed container so that, upon sample vaporization from the application of heat, the sample exerts pressure on the container. This additional pressure enables the sample's internal temperature to exceed its atmospheric boiling point (~100°C for aqueous based solutions), efficiently denaturing protein-based inhibitors present in the sample. The workflow we developed for performing HBH is shown in **Figure 2A**.

In combination with this heating method, several reagents are incorporated into the sample that further disrupt inhibitor activity and preserve the target nucleic acids. Chelating resin is incorporated to sequester metal ions in the sample and further remove these ions from solution upon separation of the resin from the sample. Reducing agents are also included to break disulfide bonds present in many protein-based inhibitors, thus hindering any recovery of protein structure and function upon return to ambient temperature. Lastly, nucleic acid binding proteins are included to complex with target nucleic acids and reduce the impact of nucleic acid fragmentation caused by high temperature.

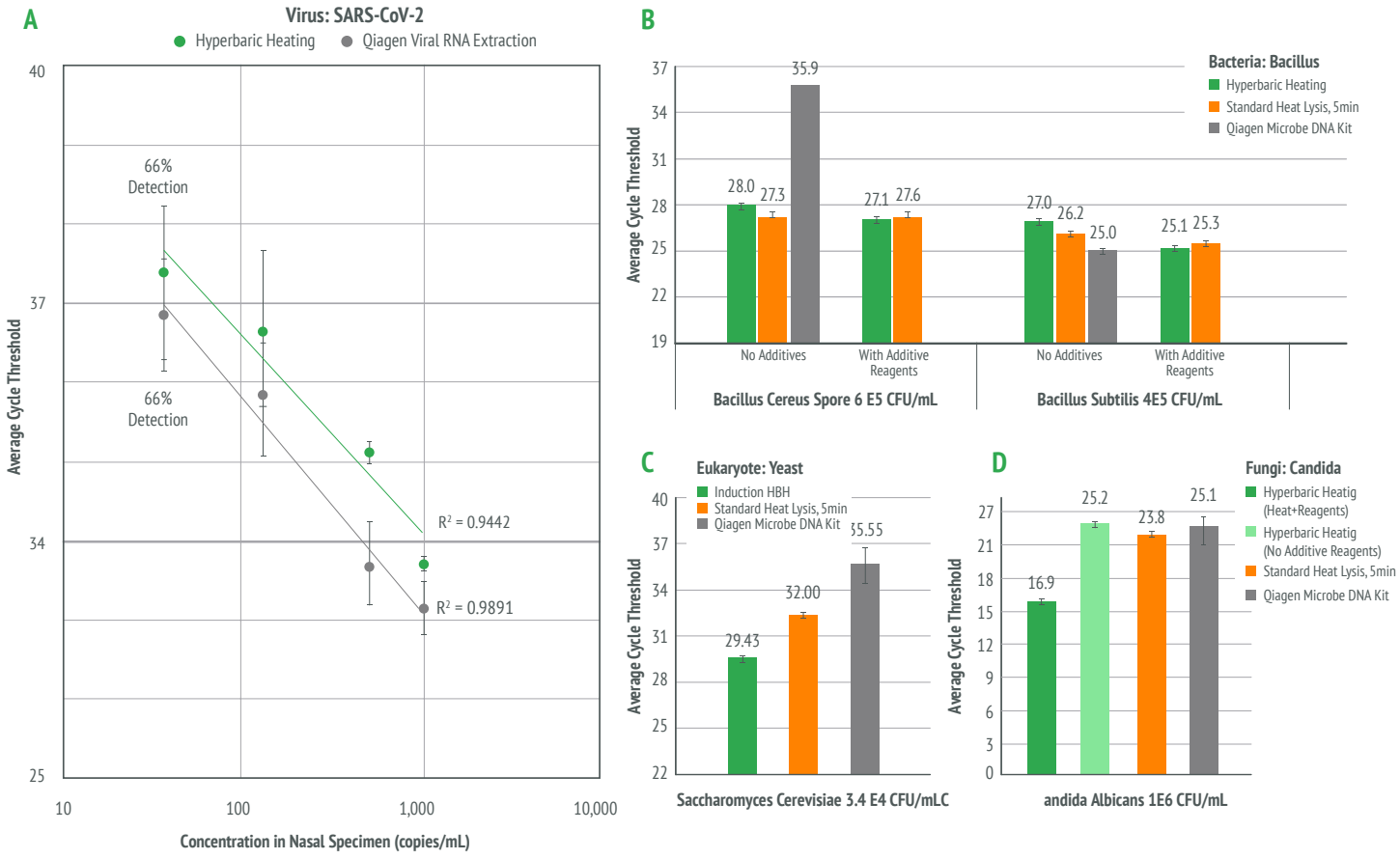

Figure 3: HBH Enables Highly Sensitive PCR Detection of Multiple Organisms Across Diverse Organism Types. **A.** PCR cycle quantification at multiple levels of contrived SARS-CoV-2 samples in nasal specimen. HBH treated sample Ct values, shown in green, performed equivalently to samples that were processed via Qiagen Viral RNA extraction, shown in brown. Detectable PCR amplification was achieved in samples with as little as 125 copies/milliliter of SARS-CoV-2 inactivated virus. **B.-D.** PCR cycle quantification for organisms frequently targeted in molecular assays prepared for PCR via HBH. Samples of Yeast (S. cerevisiae), Fungus (C. albicans), or Bacteria (B. subtilis and B. cereus spore) were contrived in collection media and separated into aliquots. Each aliquot was prepared for PCR via different methods: either HBH, standard 100°C heat treatment for 5 minutes, or nucleic acid extraction. In all cases, HBH treated samples elicited a PCR Ct that was equivalent or faster than the other two preparation methods. PCR Ct was delayed in samples in which the HBH additive reagents were withheld, demonstrating the importance of the additive reagents when conducting HBH in conjunction with the intense heating to ensure optimal PCR results.

Nucleic Acid Samples Direct to PCR in 15 Seconds

Induction heating maximizes the efficiency of HBH through rapid temperature change to the high temperatures that induce pressurization. Using a stainless-steel sample container, contact-free heat can be applied rapidly by placing the container within a coil-generated inductive magnetic field. This heating approach can bring the internal temperature to over 100°C in mere seconds, demonstrated in **Figure 2B**. This rapid temperature change shortens the required heating time to only 15 seconds, a major improvement over other preparation techniques that apply heat for up to tens of minutes.

Upon this flash heating and brief centrifugation to remove solids in the sample, HBH renders a sample "PCR clean": ensuring template preservation while neutralizing PCR inhibitors. When using patient derived samples for nucleic acid detection, a key group of inhibitors that require inactivation are nucleases, which degrade RNA and DNA to undetectable fragments. We demonstrated in **Figure 2C** that only 15 seconds of HBH effectively eliminates nuclease activity, wherein the HBH treated sample exhibits the same RNA degradation as that of the no nuclease control.

Another measure of PCR cleanliness is the impact of additional sample volume within a PCR reaction on amplification output. Sample preparation strategies that do not sufficiently neutralize inhibitors induce a loss of sensitivity when utilizing more sample in the PCR, i.e., more inhibitors outweigh more starting template. We showed that nearly an entire 25 µL PCR reaction can be comprised of an HBH treated sample. Shown in **Figure 2D**, improvement in cycle quantification was observed as the HBH treated sample volume was increased and PCR reagent volume was minimized.

Achieving High Sensitivity and Ensuring Broad Applicability

We found that HBH enabled near single-copy detection when targeting SARS-CoV-2 in nasal matrix, on par with time consuming and cumbersome nucleic acid extraction methods. We compared contrived SARS-CoV-2 samples in a nasal matrix treated via HBH to samples processed with the Qiagen Viral RNA extraction kit (Hilden, Germany). Shown in **Figure 3A**, our results show that HBH exhibits the same limit of detection of 125 copies/milliliter. Factoring in the dilution of the sample by the PCR reagents (buffer, polymerase, etc.), this LOD concentration corresponds to approximately 2 template copies in the PCR reaction. This demonstrated sensitivity therefore enables users of HBH to achieve the same PCR performance as accomplished previously with extraction-based methods but requires only a minimal fraction of the time and resources for extraction.

We demonstrated the broad applicability of HBH by testing it on several different organisms, including some organisms known to be difficult to lyse. Focusing on the main classes of organisms commonly assayed via PCR, we successfully amplified genetic material from at least one organism from each class, shown in **Figures 3B-3D**. For eukaryotes, we applied HBH to two different fungal organisms (C. albicans, S. cerevisiae). For prokaryotes, we applied HBH to Bacillus bacteria, both in active form and spore form. In all cases examined, we achieved detectable amplification up to 9 cycles earlier than that of the most relevant extraction method - some of which took over 70 minutes. We additionally demonstrated in **Figures 3B and 3D** the essential importance of the additive reagents in the HBH workflow, in which inclusion of these reagents elicited improvements in cycle quantification over heating alone.

Figure 4: AMDI Hyperbaric Heating Instrument. Model of compact benchtop instrument designed for ease-of-use when performing PCR sample preparation. Fit for use with AMDI HBH consumable and will heat sample to approximately 130°C in 15 seconds. Samples treated by the HBH method have exhibited the same high PCR performance as extraction-based preparation methods.

Conclusions

With HBH, we aim to enable the newest discoveries in molecular biology by leveraging an easy-to-use sample preparation method that maintains the high sensitivity standards required for cutting edge molecular techniques. Shown in **Figure 4**, AMDI's HBH sample preparation system, complete with functionalized metallic containers for loading any sample and a simple compact instrument serves as a comprehensive solution for several user applications. As demonstrated by our findings, HBH truly unlocks the full potential of direct sample input for PCR. Further, we foresee the expansion of HBH applications extending beyond molecular amplification assays. Other nucleic acid focused molecular biology techniques, such as next-generation sequencing or nucleic acid therapeutics, could be aided by pre-treatment with HBH. Additional applications for HBH are being actively pursued to expand its use with the goal of being at the forefront of the next generation of molecular biology methods.

References

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