

# **Standardization of Spore Inactivation method for PMA-PhyloChip Analysis**

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## **Introduction:**

In compliance with the Committee on Space Research (COSPAR) planetary protection policy, National Aeronautics and Space Administration (NASA) monitors the total microbial burden of spacecraft as a means for minimizing the inadvertent transfer of viable contaminant microorganisms to extraterrestrial environments (forward contamination). NASA standard assay-based counts are used both as a proxy for relative surface cleanliness and to estimate overall microbial burden as well as to assess whether forward planetary protection risk criteria are met for a given mission, which vary by the planetary body to be explored and whether or not life detection missions are present. Despite efforts to reduce presence of microorganisms from spacecraft prior to launch, microbes have been isolated from spacecraft and associated surfaces within the extreme conditions of clean room facilities (La Duc et al. 2004) using state of the art molecular technologies. Development of a more sensitive method that will better enumerate all viable microorganisms from spacecraft and associated surfaces could support future life-detection missions. Current culture-based (NASA standard spore assay) and nucleic-acid-based polymerase chain reaction (PCR) methods have significant shortcomings in this type of analysis.

The overall goal of this project is to evaluate and validate a new molecular method based on the use of a deoxyribonucleic acid (DNA) intercalating agent propidium monoazide (PMA). This is used in combination with DNA microarray (PhyloChip) which has been shown to identify very low levels of organisms on spacecraft associated surfaces. PMA can only penetrate the membrane of dead cells. Once penetrated, it intercalates the DNA and, upon photolysis using visible light it produces stable DNA monoadducts. This allows DNA to be unavailable for further PCR analysis. The specific aim of this study is to standardize the spore inactivation method for PMA-PhyloChip analysis. We have used the bacterial spores *Bacillus subtilis* 168 (standard laboratory isolate) as a test organism.

## **Background:**

Previous work with PMA and ethidium monoazide (EMA) dyes has demonstrated that they can only penetrate the membrane of dead cells (Nogva et al. 2003, Rudi et al. 2005) once penetrated, they intercalate the DNA and, upon photolysis using visible light, produce stable DNA monoadducts (Rudi et al. 2005, Rueckert et al. 2005). Once the DNA is cross-linked, it becomes insoluble and is lost during genomic DNA preparation (Nocker et al. 2006). In theory,

if PMA is used to treat bacterial cells prior to DNA extraction, only the DNA of viable cells should be available for PCR amplification (Nocker et al. 2007).

Nogva et al. (2003) provided proof-of-concept when they demonstrated that EMA-bound DNA cannot be amplified by PCR, and therefore the compound can be used for the selective removal of DNA from mixtures of both live and dead bacterial cells. Since that time, numerous investigators have confirmed these findings, having applied PMA and EMA-PCR methodology for the selective detection, differentiation, and enumeration of living cells. Studies have not however explored the use of PMA on spore forming bacteria. Despite the promise of the method described above, the use of DNA intercalating agents has not been applied for the discrimination of live and dead bacterial endospores.

### **Objective:**

The objective of this project is to standardize the spore inactivation method with no detrimental effect on DNA for downstream PMA-PhyloChip analysis.

### **Approach:**

A wet heat was used in this study because it is a method to inactivate the spore. A water bath at 90°C was used, samples incubated in wet heat were taken out at 30, 60, 90, 120 and 150 minutes. After exposure to wet heat treatment samples were placed in ice for 15 minutes. Samples not exposed to heat are the control. Autoclaved samples (121 °c 15lbs for 30 min) were used to differentiate DNA damage that occurred in autoclaving versus wet heat. Samples were centrifuged down, supernatant removed and re-suspended in 1mL of PBS for downstream analysis. 100µl was used for plating on tryptic soy agar (TSA) and incubated at 32<sup>0</sup>C for 48 hr. By plate count technique colony forming units(cfu) were established for each sample. 800µl was used in DNA extraction Maxwell-16 (Promega, Automated DNA extraction) and the resulting DNA was used in q-PCR Bio-RAD CFX-96 (real time q-PCR).

### **Results:**

Results from CFU counts indicate that 90°C was sufficient to inactivate the *B. subtilis* spores. A 3 to 4 log reduction in cultivable counts was observed for 30 to 60 min wet heat and complete reduction after 60 min (Fig 1). Very little reductions were seen in the q-PCR results throughout the heat shock treatment. A 2 log reduction was seen in the 16s rRNA copy number for autoclave spore samples. These Results show that autoclaving inactivates spores but has significant damage on DNA. Final results indicate that at 90°C for 120 minutes there is no growth and very little damage occurred to the DNA. This method will be used as the standardization of spore inactivation.

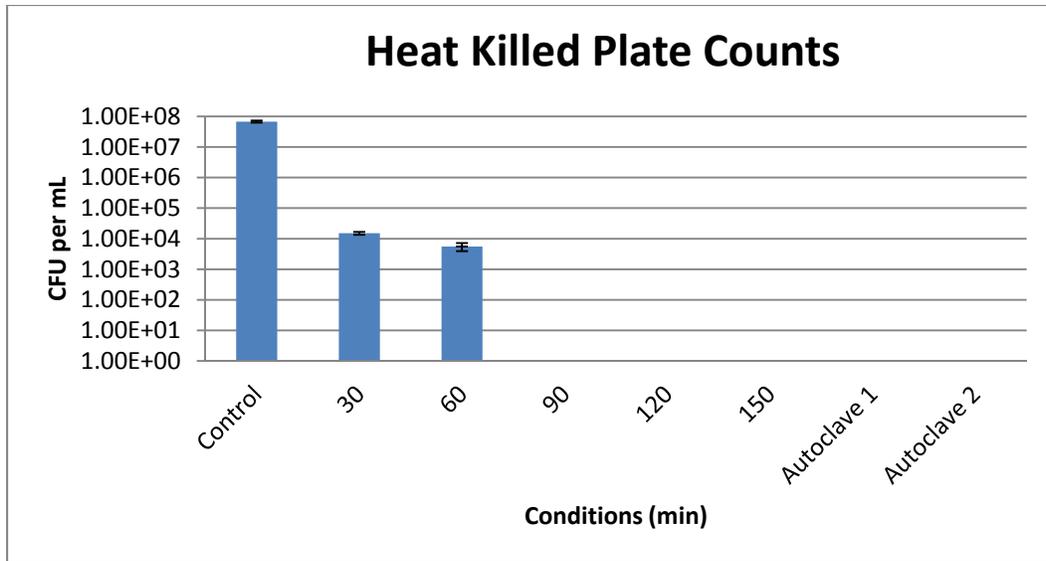


Fig 1: Samples (30-150) were exposed to 90°C Wet heat; Autoclaved samples ran on 30 min liquid cycle

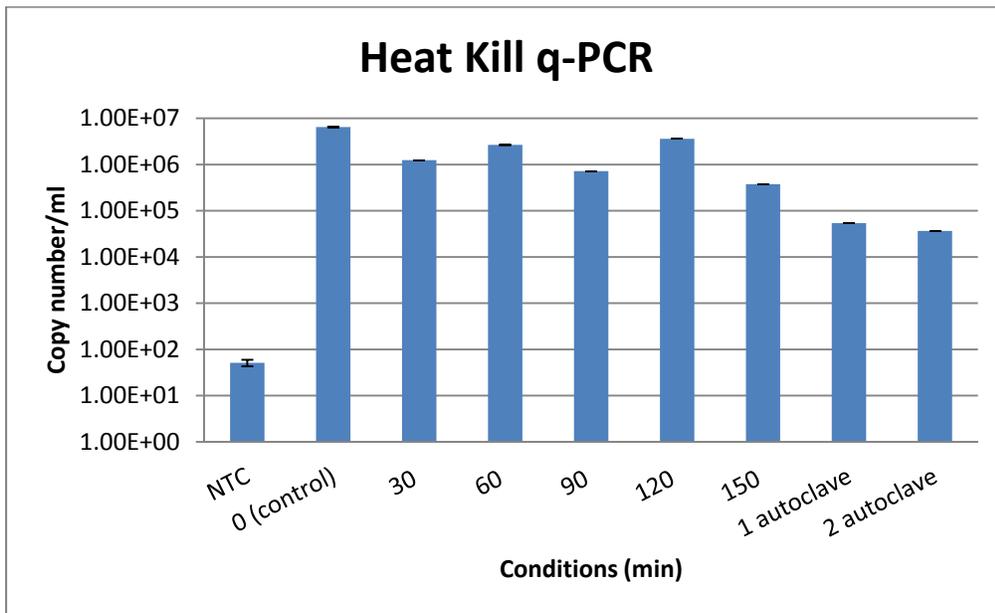


Fig 2: Results of q-PCR in copy number counts

### Discussion:

Results were as expected, by using wet heat the spore is inactivated and DNA is not significantly affected. A wet heat of 90°C for 120 minutes will be used to properly inactivate the spores for future projects. Since spore inactivation has been successfully established PMA-PhyloChip analysis experiments can now proceed. NASA's current standard spore assay to measure microbial life within spacecraft associated clean rooms only detects specified cultivable condition and fails to differentiate live and dead microbes. PMA coupled with PhyloChip

analysis could detect a larger variety of viable life since the PhyloChip is a powerful tool in measuring microbial diversity.

### **Conclusion:**

Since a method has been established to inactivate spores further research can be performed in the use of PMA in live/dead cultures. Once PMA is established the use of the PhyloChip analysis can then be explored to enumerate solutions. The hope is that this can serve as an additional tool for planetary protection “tool-box kit” to detect viable life in spacecraft associated environments. This research may also help develop or improve methods for reducing spacecraft bioburden.

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