ULTRASENSITIVE DETECTION OF TERRAN DNA FOR PLANETARY PROTECTION. T. A. Stephenson[1], A. S. Burton[2], N. Lehman[3] and J. P. Dworkin[4], [1]Code 541 Materials Engineering Laboratory NASA/GSFC, Greenbelt MD 20771, [2]Code 691 Astrochemistry Laboratory (ORAU) NASA/GSFC, Greenbelt MD 20771, [3]Department of Chemistry, Portland State University, Portland OR 97201, [4]Code 691 Astrochemistry Laboratory NASA/GSFC, Greenbelt MD 20771

Introduction: There is a critical need to rapidly detect, classify, and enumerate the widest possible spectrum of Earth microbes carried by spacecraft. These may inhabit surfaces and bulk materials of spacecraft at low densities before, during and after assembly and launch processing. The assay method used currently is based on colony counts of spore-forming organisms obtained by wiping surfaces with cotton swabs. Isolated organisms are cultured for 72 hours, making this a labor-intensive process with a relatively long turn-around time. It is also limited to culturable bacteria (<1% of bacteria [1]). Two other assays are in development: Limulus Amebocyte Lysate (LAL) and ATP bioluminescence [2]. These methods both offer high sensitivity (10 cells / mL and 5 cells / mL, respectively) but also have significant limitations. In the case of LAL, only gram-negative bacteria and fungi can be assayed, while ATP bioluminescence requires viable cells and must be calibrated for individual organisms [3]. Finally, neither of these assay methods can provide information about the type of organism being detected.

Recent advances in DNA amplification offer a way to further increase the sensitivity of contaminant assays while also providing information about the source of the contamination. Multiple displacement amplification (MDA) is an extremely sensitive technique originally developed to amplify the DNA from a single cell for sequencing. As such, MDA is ~10,000-fold more sensitive than the Polymerase Chain Reaction (PCR) for detecting and amplifying DNA from environmental samples [4]. Furthermore, because MDA copies the entire genome of an organism, MDA does not suffer the same primer restrictions as PCR amplification and can be accomplished on the same time-scale. In addition, products of MDA are templates for PCR, allowing the species of contaminating organisms to be obtained. Through single cell detection, the potential to identify the type(s) of contaminating organism(s), and a processing time of hours, DNA detection by MDA represents a major advance over existing and developing planetary protection technologies.

Background: The driving force for the development of the MDA process was a desire to assess the biodiversity of the microbial world. This was previously done by PCR. However, natural samples often contain only low levels of DNA as well as ions and molecules that inhibit PCR, limiting the success of this approach. Because PCR depends on the use of specific primer pairs, this technique requires that you know what DNA you are trying to amplify. A common target are ribosomal RNA (rRNA) genes; however, these differ between types of organisms and require different primers. (*e.g.*, eukaryotic 18S rRNA or bacterial 16S rRNA genes). The bottom line is that PCR applied directly to soil samples has a detection limit of 10^4 cells [5]. This level of sensitivity will show inherent bias towards the most abundant organisms in a sample, and exclude rare bacteria. Thus, a technique was required that would make multiple copies of any DNA present. Non-specific DNA amplification by the MDA technique was developed at Molecular Staging, Inc., by Lasken [6]. This method can amplify dilute quantities of DNA and can generate over a billion copies starting from a single molecule of DNA [7-9]. In addition, MDA has been shown to be less susceptible than PCR to inhibitors such as humic acids and exopolysaccharides that are commonly found in environmental samples that block DNA amplification reactions [10]. Thus, MDA is now widely accepted in the genomics field as the preferred method to amplify environmental samples including soil [11-13], deep mine environments that have very low biomass [14-15] and clinical samples [16].

In the laboratory this technique has demonstrated detection sensitivity to 1 fg (1×10^{-15} g) of DNA in 1mL water [17]. There are ~2.5 fg of DNA in a typical bacterium, so this technique demonstrates the detection of a DNA fragment and therefore, part of a DNA molecule from a single cell. By enabling the detection of a single organism, MDA theoretically represents the most sensitive method currently available to the Planetary Protection Program. In addition, because MDA is not targeted to specific regions of DNA, it can detect: viable and culturable microbes, viable but non-culturable microbes, non-viable microbes, molecular fragments of microbes, and DNA-based viruses, in addition to the DNA of higher organisms that are involved in spacecraft construction intentionally (engineers) or inadvertently (*e.g.*, mice). We have been successful using MDA to detect rare sequences in environmental samples.

Approach: This approach represents a major improvement over existing Planetary Protection (PP)

techniques summarized in Table 1. Using optimized extraction techniques together with MDA and PCR methods enables evaluation of the biological cleanliness of NASA spacecraft and the biological cleanliness of returned samples.

Table 1 – Comparison of PP Detection Techniques

Technique	Limit of detection (cells/mL)	Notes
NASA Standard Assay (Spore Culture) [18]	Single cell on agar	Heritage use; applicable only to culturable cells (<1% of currently known bacteria)
LAL assay [19]	~10	Sensitive to living and dead cells. Limited to Gram negative bacteria and fungi.
ATP bioluminescence [20]	~5	Sensitive to living and dead cells. However, ATP in dead cells quickly degrades.
PCR [21]	~10,000	Amplification of target DNA sequences only. Reaction easily inhibited in environmental samples.
MDA[22]	<1	General amplification, detection of ALL Terran DNA. Enables PCR of low biomass environmental samples.

By combining the high sensitivity and general amplification abilities of MDA with the specificity obtained with specific primers in PCR, we meet the Planetary Protection goals of enhanced sensitivity and broadened diversity in the detection of Earth microbes.

In summary, we will be able to detect, classify and enumerate both outbound biological contamination and also returned biological contamination for the Earth-like bioburdan that makes the round-trip in sample-return missions.

References: [1] The New Science of Metagenomics: Revealing the Secrets of Our Microbial Planet (2007), National Academies Press., www.nap.edu/catalog/11902.html. [2] Conley, C., (2010) *http://planetaryprotection.nasa.gov/methods*, accessed 9/102011. [3] Stanley, P. E. (1989) *Biolumin Chemilumin*, 4(1), 375-80. [4] Gonzales, J. M., et al. (2005) *Environ. Microbiol.*, 7, 1024-28. [5] *Ibit*. [6] Lasken, R. S. and Egholm, M., *Trends Biotechnol*, 21(12), 531-535. [7] Lasken, R. S. (2007) *Current Opinion in Microbiology*, 10, 1-7. [8] Raghunathan, A. et al. (2005) *Appl Environ Microbiol* 71(6), 3342-3347. [9] Lasken, R. S. (2009) *Biochemical Society Transactions*, 37(2), 450-453. [10] Kiesling, T. et al. (2007) *Nucleic Acids Res* 35(18) e117 doi:10.1093/nar/gkm654. [11] Lasken, R. S. et al. *Whole Genome Amplification:Methods Express*, 119-147. [12] Gonzales, J. M., et al. (2005) *Environ. Microbiol.*, 7, 1024-28. [13] Ishoey, T. et al. (2008) *Trends Biotechnol.*, 21, 531-535. [14] Edwards, R. A. et al. (2006) *BMC Genomics* 7, 57. [15] Abulencia, C. B. et al. (2006) *Appl Environ Microbiol.* 72, 3291-3301. [16] Kiesling, T. et al. (2007) *Nucleic Acids Res* 35(18) e117, doi:10.1093/nar/gkm654. [17] Blainey, P. C. and Quake, S. R. (2010) *Nucleic Acids Res* 1-9, doi:10.1093/nar/gkq1074. [18] NASA Standard Proc for Microbiological Examination of Space Hardware, NHB 5340.1 REV B (1980). [19] Sample Analysis at mars (SAM) Planetary Protection Implementation Document, SAM-PLTENV-PLAN-0067, Effective Date: March 6, 2007, Expiration March 6, 2012. [20] ATP Biomass Kit HS, Biothema Luminescent Assays, www.biothema.com. [21] Gonzales, J. M., et al. (2005) *Environ. Microbiol.*, 7, 1024-28. [22] Blainey, P. C. and Quake, S. R. (2010) *Nucleic Acids Res* 1-9, doi:10.1093/nar/gkq1074.