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Genetic Inventory Task Final Report

Volume 1 of 2

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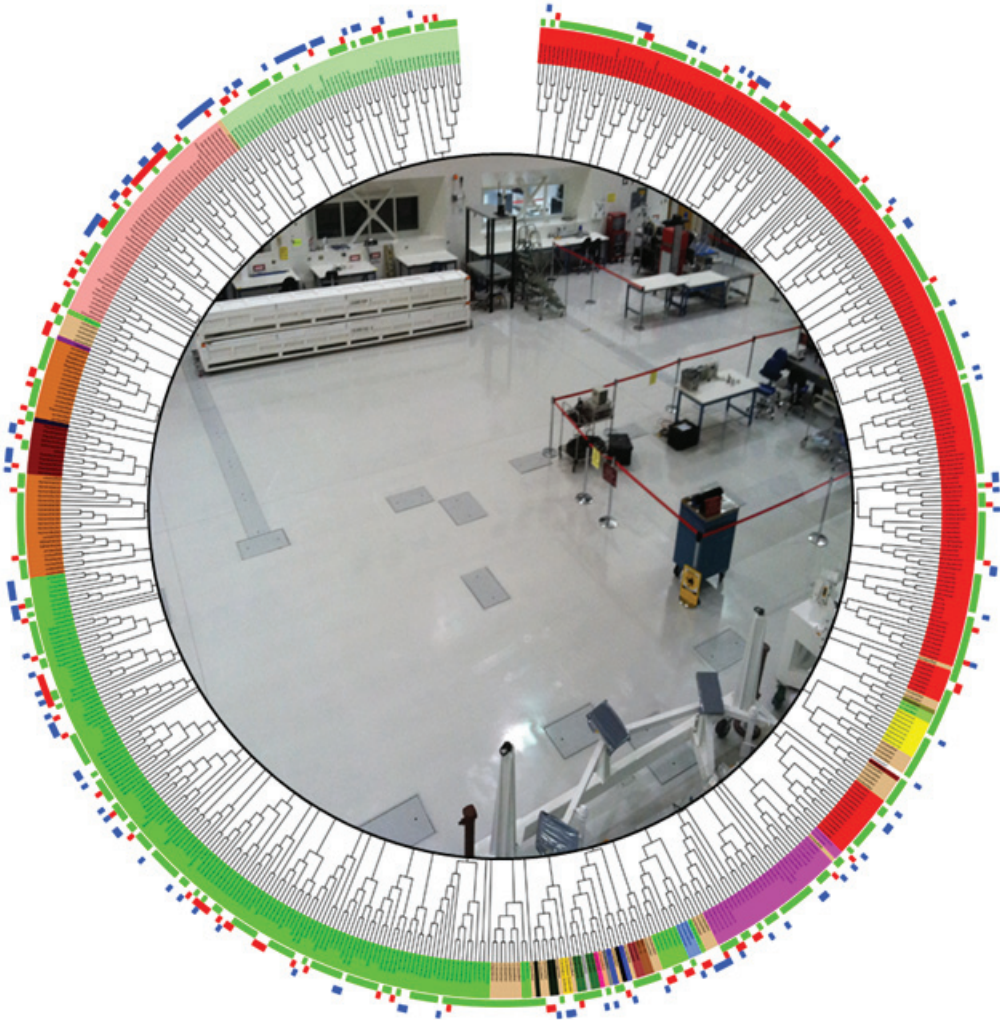

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ABSTRACT

Contaminant terrestrial microbiota could profoundly impact the scientific integrity of extraterrestrial life-detection experiments. It is therefore important to know what organisms persist on spacecraft surfaces so that their presence can be eliminated or discriminated from authentic extraterrestrial biosignatures. Although there is a growing understanding of the biodiversity associated with spacecraft and cleanroom surfaces, it remains challenging to assess the risk of these microbes confounding life-detection or sample-return experiments. A key challenge is to provide a comprehensive inventory of microbes present on spacecraft surfaces. To assess the phylogenetic breadth of microorganisms on spacecraft and associated surfaces, the Genetic Inventory team used three technologies: conventional cloning techniques, PhyloChip DNA microarrays, and 454 tag-encoded pyrosequencing, together with a methodology to systematically collect, process, and archive nucleic acids. These three analysis methods yielded considerably different results: Traditional approaches provided the least comprehensive assessment of microbial diversity, while PhyloChip and pyrosequencing illuminated more diverse microbial populations. The overall results stress the importance of selecting sample collection and processing approaches based on the desired target and required level of detection. The DNA archive generated in this study can be made available to future researchers as genetic-inventory-oriented technologies further mature.

Key Words: planetary protection, genetic inventory, microbiome, microbial diversity, PhyloChip, 454 tag-encoded pyrosequencing

EXECUTIVE SUMMARY

A primary concern of all Mars missions is that contaminant terrestrial organisms could eventually be transferred to Mars or make the round-trip back to Earth, thereby compromising the ability to discriminate a potential Martian organism from a terrestrial contaminant. It was clear from previous studies that culture-based methods would be inadequate to monitor this risk, and that newer molecular (DNA)-based identification techniques would need to be developed for managing the risk of "false positive" detections of life on (or returned from) Mars (NRC 2006).

The Mars Exploration Program made its initial choices defining scope and expectations on how to pursue this challenge based on the findings of a workshop of independent experts held in 2006. The workshop findings included advice *to develop a qualitative inventory capability* (versus quantitative, or a capability to do both) and *to identify both live and dead organisms* (versus live only). These choices, and others to follow, were influenced by practical considerations of time, funding, and the anticipated requirements of upcoming missions. There were modifications over the course of the Genetic Inventory task, but these initial choices remained valid, as endorsed by a second workshop with a different group of independent experts held in 2009.

The genetic target for compiling the inventory was the "hypervariable" regions of the 16S rDNA gene which, for bacterial and archaeal sequences, provides the ability to discriminate among organisms at the operational taxonomic unit (OTU) level—an approach originally proposed by Pace (1997). The analogous target used in this task for fungi is the rDNA internal transcribed spacer (ITS) region. Numerous tools and databases have been developed to assist with the automated analysis of sequence data to determine the taxonomic affiliation of each sequence analyzed.

It is important to note that the end product is a *standardized* approach rather than an optimized one: If the method recommended here is correctly applied, the user will generate a genetic inventory that is free from experimental anomalies, and is (to the best of current knowledge) representative of the DNA types that are actually present on the surfaces sampled. That is not to say that variants of the methodology might not recover higher yields or reduce known biases, only that the Genetic Inventory team did not have the resources to investigate every experimental combination in what is a rapidly evolving field of technology.

Experimental Steps

Sample Collection: Core to any microbial diversity analysis is the ability to collect and analyze a representative sample. For much of the development work, the team used a synthetic aggregation of laboratory organisms, termed a model microbial community (MMC), that they could apply to and recover from test surfaces, then assess the quality of the recovery. The team assessed sampling devices for "large"(~2,500 cm^2) and "small"(~25 cm^2) surface areas, considering parameters such as deposition method, recovery efficiency, biomass, species-specific recovery, contaminant rDNA levels, concentration, and filtration methods, among others. In the final configuration, macrofoam biological sampling kits (BiSKits) (Quicksilver Analytics Inc.; Abingdon, MD) and cotton swabs (Puritan Medical Products; Guilford, ME) were the best performing devices for large- and small-surface-area samplings, respectively.

Sample Processing: In subsequent sample processing studies, the team also used the MMC to address the quantitative, but in particular, the qualitative aspects of sample recovery. The team determined that sample concentration of low-biomass samples is required for efficient recovery and that no single DNA extraction and purification methodology is able to recover all representatives of the MMC. To address this second point, the team split samples into two aliquots, subjecting one half

to a bead-beating step prior to extraction. The study determined that automated extraction (to minimize operator variation) of both aliquots with a Maxwell MDx-16 instrument and consumables (Promega; Madison, WI) gave the best results (qualitative and quantitative yield, lowest DNA contamination from consumables). The two aliquots were then recombined before sample analysis, with DNA from easily lysed MMC members taken from the non-bead-beaten aliquot and DNA from more robust MMC members taken after the bead-beating process. As noted from this step alone, the Genetic Inventory method is a qualitative, and not quantitative, methodology.

Sample Analysis: In the course of the study, the team employed a number of different sample analysis methodologies, both as tools to analyze relative performance of other elements of the end-to-end Genetic Inventory process and as generators of diversity data about the environments sampled. It quickly became apparent that traditional 16S rDNA cloning and Sanger-sequencing approaches were not capable of elucidating an all-inclusive genetic inventory from low-biomass environments. These methods are limited by molecular biases that favor the amplification and/or amplicon ligation of abundant bacterial taxa and hence mask the presence of less abundant taxa. PhyloChip microarray analyses (the Generation 3 [G3] iteration in particular) were found to be superior to conventional 16S rRNA gene cloning and Sanger-sequencing strategies in all aspects of microbial diversity analysis with one limitation: The methodology is based on a detection of hybridization to an array of oligonucleotides from previously described/sequenced organisms. It is therefore unreliable for detecting and identifying previously unreported taxa, particularly at the OTU level. In the course of the Genetic Inventory task, the most comprehensive inventory was obtained using Titanium 454 FLX tag-encoded pyrosequencing. This high-throughput DNA sequencing methodology is capable of detecting the presence of thousands of different microbial DNA sequences (previously reported and novel OTUs) from a single sample. However, key to the integrity of this approach is the need to analyze an appropriate target sequence: Long-read (~350 base pairs) technology yielded good data for genetic inventory purposes, whereas a shorter tag sequence (<80 base pairs) was shown to be susceptible to interference from spurious nonsample DNA and insufficient resolution ability in the downstream data analysis.

Sample Archiving: Ability to store samples without degradation is important in the context of short-term (e.g., for batch processing of samples) or long-term (e.g., to make samples available for analysis by future technologies) storage. The Genetic Inventory team decided that it was insufficient to only store captured data (e.g., from PhyloChip microarray or Titanium 454 FLX tag-encoded pyrosequencing runs), and that the sample DNA itself needs to be preserved. The sample archiving study indicated that archive storage is best done after Amicon filtration, and at −80°C, with pertinent metadata preserved in an appropriate information management system.

Data Analysis: The amount of data produced from the various sequencing methods used for the Genetic Inventory sample analysis is far beyond what can be analyzed manually. Accordingly, the molecular biology community has developed a series of tools and approaches to process the raw rRNA gene sequence data and interpret microbial community structure with statistical confidence; novel tools and strategies are also continually emerging.

The key steps taken in the data analysis of sample-associated microbial diversity over the course of the Genetic Inventory task were thus (1) feed raw sequence data into appropriate bioinformatic and biostatistical pipelines to derive taxonomic affiliation, microbial diversity, community characteristics, and richness; (2) compare community structure and shared membership between samples; and (3) determine the occurrence of various microbial lineages to generate hypotheses regarding those of greatest concern from a planetary protection perspective.

The phylogenetic affiliations and taxonomy calling for the Sanger-sequencing approach is straightforward and well-established. However, while DNA microarray (PhyloChip) and 454 tag-encoded pyrosequencing technologies share a similar goal (the thorough elucidation of biodiversity present in a given sample), each method makes use of its own intrinsic taxonomy calling system for assigning taxonomy to specific sequences. Since these technologies are based on different approaches (probe hybridization to 1.5 kb for PhyloChip vs. sequencing of <350 base pairs [bp] for 454 pyrotags), attempting to directly compare biodiversity profiles resulting from genetic inventory samples is challenging.

Beyond the sequence analysis and taxonomy assignment, a suite of publicly available and in-house-developed bioinformatic and biostatistical tools was used to interpret microbial community structure. Since no single molecular technique or statistical sequence analysis tool can answer all of the questions pertaining to microbial ecology, the Genetic Inventory team applied various approaches and tools for analyzing and interpreting the sequence data.

Molecular Analysis: The end-to-end capability under development was repeatedly tested on relevant spacecraft environment samples of opportunity. Performed over the course of the Genetic Inventory task, tests reflect the deployment of the then state-of-the-art Genetic Inventory methodology. Importantly, the later methods using the PhyloChip G3 and 454 Titanium FLX V1–V3 tag-encoded pyrosequencing reflect the current state of the art at the end of the Genetic Inventory task. In conjunction with the bioinformatics approaches described in Section 9, these latest studies provide the newest data on the occurrence of rDNA sequences (as a surrogate for presence of the organism) on spacecraft and associated surfaces. Performance highlights of the Genetic Inventory capability as applied were the ability to achieve 98.7% coverage (of the detected number of sequences compared to the predicted total diversity) for a spacecraft sample set, in excess of 1,000 distinct bacterial OTUs per individual sample identifiable, and up to 2,686 bacterial OTUs identifiable from a ground-support equipment (GSE) sample set.

Conclusions

In summary, the Genetic Inventory team successfully developed a capability to (1) systematically collect, process, and archive nucleic acids from spacecraft-related environments, and (2) effectively assess, using currently available analysis technologies, the diversity of microorganisms potentially present on spacecraft and associated surfaces. In the process, this capability enabled the Genetic Inventory team to generate the most comprehensive (bacterial, archaeal, and fungal) assessment of spacecraft assembly cleanroom (SAC)–associated biodiversity to date. Lessons learned, conclusions, and recommendations (in Section 11) provide an approach to implementing a genetic inventory if it were needed for a mission today, as well as a path forward toward what could become a NASA Standard Genetic Inventory (NSGI), analogous to the venerable NASA Standard Assay (NSA) for the analysis of mesophilic, heterotrophic, spore-forming organisms.

1. INTRODUCTION

Early in the U.S. space program, scientists realized that interplanetary missions had the potential to jeopardize their investigations because "interplanetary missile(s) … are likely to carry terrestrial organisms to the Moon or other targets."[1] To address this concern, spacefaring nations established international treaties and requirements to protect experiments from such "forward" contamination, i.e., the introduction of terrestrial organisms to other planetary bodies. NASA's policies on planetary protection follow these international agreements (documented by the Committee on Space Research [COSPAR] of the International Council for Science). Planetary protection requirements have evolved over time to meet the needs of increasingly sophisticated missions. However, advances in nucleic acid technologies have not yet found their way into standard planetary protection practice except as research tools; and there is currently an unmet desire to use these technologies to understand more about the potential "passenger list" for certain mission types and destinations.

The current NASA Standard Assay (NSA) method, designed to assess planetary protection risk for spacecraft bound for Mars, is culture-based. Technicians collect samples, allow the organisms in the sample to grow for 3 days, and then count resulting colonies. The standard assay technique is designed to enable a count of organisms that can form endospores, respire aerobically, are resistant to heat processing, and can be cultivated on Tryptic Soy Agar (TSA) media. This technique does not address the issue of microbial diversity of spacecraft.

At the time the Genetic Inventory task was established, available technology was deemed sufficiently advanced to allow NASA to consider developing methods to move toward a DNA-based census of microorganisms about the spacecraft. Over the course of the 6-year Genetic Inventory study, the team evaluated and used three molecular technologies: the well-established vector-based cloning and Sanger-sequencing of polymerase chain reaction (PCR)-amplified 16S rRNA identification genes, the PhyloChip phylogenetic DNA microarray developed jointly by Lawrence Berkeley National Laboratories (LBNL) and Affymetrix, and a 454 tag-encoded PCR-amplicon pyrosequencing method. Each approach has intrinsic strengths and weaknesses when used alone; however, the final success of the Genetic Inventory task resides in the integration of these multiple strategies.

The Genetic Inventory task has advanced sample collection technology sufficiently such that the Mars Program can now validate the capabilities needed to achieve compliance with anticipated planetary protection requirements and to plan for the effective implementation of such mandates at the earliest stages of future missions to Mars and other solar system targets. In particular, the work increases confidence that samples collected from a spacecraft can be used to fully and confidently assess the authenticity (biosignatures of extraterrestrial origin vs. terrestrial contamination) of returned and *in situ* analyzed samples.

This final report is intended to be a comprehensive documentation of the entire 6-year Genetic Inventory task, including the objectives, technical approach, results, recommendations, and lessons learned. A timeline illustrating key milestones and activities throughout the Genetic Inventory task is provided in Figure 1-1. All relevant conference posters, workshop reports, peer-reviewed publications (abstracts and links to full publications online), and miscellaneous supporting materials are included as appendices.

[1] Lederberg, Joshua, and Dean B. Cowie, Moondust: The study of this covering layer by space vehicles may offer clues to the biochemical origin of life, *Science* **127**(3313):1473–1475, 1958.

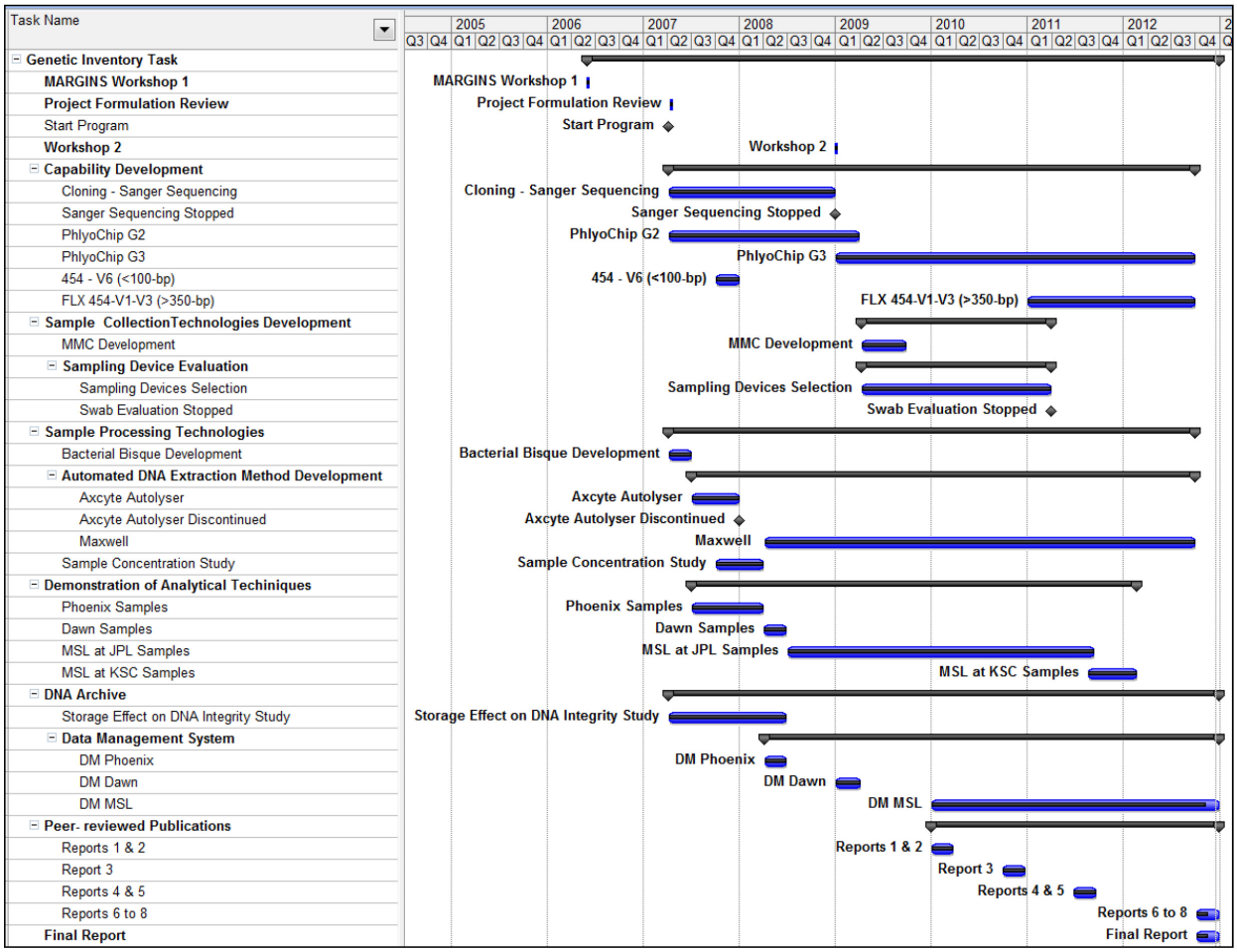


Figure 1-1. Timeline illustrating key milestones (closed diamonds) and concurrent activities of the Genetic Inventory task.

2. STRATEGIC PERSPECTIVE

With serious consideration of an imminent Mars sample return in the late 1990s, it became clear that not enough was known about the diversity and capabilities of organisms inhabiting cleanrooms and present on spacecraft, that could eventually be transferred to Mars or make the round-trip back to Earth. As such, NASA's Planetary Protection Program began a sampling effort that included a number of spacecraft and a variety of cleanrooms around the country. Mars Odyssey, for example, was sampled before it was launched in early 2001 (La Duc *et al.,* 2003). The sampling effort employed methods that were standard practice for acquiring planetary protection samples from spacecraft surfaces; the methods for identification and characterization of organisms were state-of-the-art at the time.

Various threads in the discussion of Mars sample-return missions suggested that assembly of a listing of all organisms on a spacecraft (a "passenger list"), using molecular identification techniques, could provide a great deal of help with "false positive" detections of life on (or returned from) Mars, and might be the most effective way to ensure the goals of forward-contamination control on Mars. That concept became more systematized as time went on, and was the subject of a specific recommendation of the Space Studies Board's PREVCOM report in 2005.[2] Thus, for multiple reasons, the Mars Exploration Program, with endorsement of the NASA Planetary Protection Office, undertook the effort described in this report to develop a genetic inventory capability. It is important to note however that samples are planned to be returned to Earth from many planetary bodies. Those not regarded as posing any biohazard to Earth are given a planetary protection designation of "Unrestricted Earth Return." However, future sample-return missions from Mars and other targets that might potentially harbor life (e.g., Europa and Enceladus) are classified as "Restricted Earth Return." These are subject to quarantine restrictions and require special receiving and curation facilities.

The suggestion to assemble a comprehensive "passenger list" for a planetary spacecraft, once accepted, required important choices to be made to define scope and expectations for the research effort. In its broadest definition, a genetic inventory capability would address the needs of multiple mission types with diverse planetary protection requirements. An idealized genetic inventory capability would identify the passenger list of the spacecraft itself (as opposed to surrogate surfaces) and would distinguish and quantify live/viable organisms from dead/nonviable ones. In its fullest interpretation, functional characterization of the passengers would also be accomplished.

The Mars Exploration Program made its initial choices defining scope and expectations based on a workshop of independent experts held in 2006 (see Appendix F). The workshop findings included advice *to develop a qualitative inventory capability* (versus quantitative or a capability to do both) and *to identify both live and dead organisms* (versus live only). These choices, and others to follow, were influenced by practical considerations of time, funding, and the anticipated requirements of upcoming missions. There were modifications over the course of the task, but these initial choices remained valid, as endorsed by a second workshop held in 2009 (see Appendix F).

This report documents a body of work that delivers recommendations on how to acquire samples from extremely low-biomass environments in the presence of spacecraft hardware, process those samples, and store the resulting DNA. The principal goal of the Genetic Inventory task was to integrate these technologies to demonstrate an end-to-end capability. As part of the demonstration of this capability, the DNA was submitted for analysis by the best molecular methods currently available. These recommendations provide to the Mars Exploration Program and other NASA

[2] J. D. Rummel (then NASA Planetary Protection Officer), email to Karen Buxbaum (Manager, Mars Program Planetary Protection, JPL), December 2005.

stakeholders, a framework for developing a genetic inventory protocol for future missions. The research results and lessons learned also provide a foundation for research to address additional planetary protection and astrobiology research challenges, such as the broad and ambitious recommendations of the PREVCOM report.

3. Objectives and Expected Impact

3.1 Definition of Genetic Inventory

In the context of this report, genetic inventory refers to a census of microorganisms associated with spacecraft and spacecraft assembly cleanroom (SAC) surfaces, achieved by surveys implementing systematic sample collection and processing and cataloging of rRNA gene sequences, which are highly informative with respect to identification of microorganisms.

3.2 Objectives

The overarching objectives of the Genetic Inventory task were to develop the capability to (1) systematically collect, process, and archive nucleic acids, and (2) effectively assess the phylogenetic breadth of microorganisms present on spacecraft and associated surfaces. While these two objectives remained the same throughout the task, the experimental emphasis shifted several times over the course of the effort due to a combination of external and internal factors. Some of these factors are described below.

3.3 Emphasis

While the overall objective remained the same throughout the task, the emphasis shifted over the course of the 6-year effort. In October 2006, the Mars Program Office (MPO) tasked the Genetic Inventory team with identifying technologies that could lead to a capability of fully inventorying the genetic material found on spacecraft and associated surfaces. To ensure that the Genetic Inventory team benefited from the most current expertise of science and technology experts from industry, academia, and government, the Jet Propulsion Laboratory (JPL) hosted two workshops, one in early 2006 and the second in fall 2009. (Workshop descriptions and findings are presented in the text box on the next page.)

Following the first workshop, a task formulation review with key stakeholders was held that advocated the determination of what a 2007 "best available technology" would yield by way of a genetic inventory, then modifying and standardizing it from that point. This was the strategy initially adopted.

In addition, in 2008, the task was asked to support development of a genetic inventory approach for the Mars sample-return cache proposed for the (then) 2009 launch of the Mars Science Laboratory (MSL) mission. The consideration of how a project could implement a genetic inventory on a flight hardware item was instructive in the development of an end-to-end strategy. In the end, the Genetic Inventory team collected samples from the JPL Spacecraft Assembly Facility (SAF) where then MSL components were assembled. This element of the task was instrumental in developing percent coverage as a potentially acceptable analytical endpoint, even though the actual cache hardware was never analyzed.

In the second workshop, participants agreed that standardizing sample collection is critical and that it had not received enough attention. Since the Genetic Inventory team had focused their efforts and energies on the analysis aspect of the project from 2006–2009, the workshop participants advised the team to shift emphasis toward sample collection and processing.

WORKSHOP 1

The Mars Genetic Inventory of Spacecraft Workshop was convened February 28–March 1, 2006, by the JPL Mars Program Office to discuss and recommend the appropriate way forward to implement a genetic inventory for Mars spacecraft (JPL:D-35346 2006).

Workshop 1 Findings

The maturity of existing molecular analysis technology is sufficient to permit the development of a genetic inventory for future missions. However, current sampling methodologies for low-biomass samples may not be adequate and may need further development.

NASA should fund the development of a DNA-based genetic inventory, based on detection of known prokaryotic genetic markers. It is adequate for the technology used to be qualitative (rather than quantitative), as this is sufficient for the requirement, and offers the low-risk route to successful deployment within the anticipated cost and schedule window.

NASA should fund a parallel study of methodologies for the detection, isolation, and archiving of low-abundance genetic material from spacecraft and spacecraft assembly environments.

WORKSHOP 2

The Genetic Inventory of Spacecraft and Associated Environments Workshop, held September 30–October 1, 2009, was hosted by the JPL Mars Program Office to assess JPL Genetic Inventory research team progress and ability to deliver an end-to-end analysis process and, if necessary, to identify other sufficiently mature technologies (JPL:D-65828 2010).

Workshop 2 Findings

Target molecules (e.g., 16S rRNA gene) are not being collected in great enough abundance to render much of the downstream molecular processing and analysis significant and/or reproducible.

Efforts should be made to concentrate samples since polymerase chain reaction (PCR)-dependent techniques generally require ca. 10^3 initial copies of the target per reaction to reproducibly assess microbial diversity. Although theoretical calculations posit the need for as low as one copy of a given target molecule, signal-to-noise ratios demand that target molecules exist in excess of 10^3 copy numbers (due to indigenous DNA in reagents and sampling materials).

Standardizing sample collection is critical and has not received enough attention. Up until now, the Genetic Inventory team has focused its efforts and energies on the analysis aspect of this project. The team is advised to shift emphasis toward sample collection and processing.

A majority of attendees at the second workshop endorse the PhyloChip technology as being the best available technology for planetary protection research and implementation needs, while 454 tag-encoded pyrosequencing is thought to require significant standardization to circumvent a great deal of noise when working with low-biomass samples. However, experts versed in the practices of the Human Microbiome Project, whose samples are always extremely rich in target molecules, lend support to 454 tag-encoded pyrosequencing and suggest that this technology, which is evolving rapidly, holds greater potential to fulfill planetary protection needs than was once thought.

3.4 Expected Impact to Present Knowledge

A reliable means of estimating the total microbial population associated with spacecraft hardware is of value to those tasked with protecting the scientific integrity of *in situ* and sample-return-based, life-detection experiments. At the outset of the Genetic Inventory task, in late 2006, there were a number of attractive molecular methods available for assaying the microbial diversity in a given sample. Reports demonstrating well-established techniques, such as 16S rRNA gene-based cloning and Sanger-sequencing (Giovannoni *et al.,* 1990; Pace 1997), order- and phyla-specific fluorescence *in situ* hybridization (FISH) microscopy (Amann *et al.,* 1990; Bayani and Squire 2004), and terminal restriction fragment-length polymorphism (t-RFLP) analysis (Gruntzig *et al.,* 2002) for rapid and "thorough" assessments of microbial diversity flooded the scientific literature. While each of these techniques had its strengths and merits, in the context of a diverse low-biomass sample, they were also fraught with bias and shortcomings, which limited each of them with regard to achieving a comprehensive genetic census of the diverse populations associated with SAC environments.

When the task commenced in 2007, there were major gaps and unknowns in the technology that precluded the generation of a high-fidelity genetic inventory. It was impossible to probe the presence of numerous microbial lineages without bias; however, the extent of this biasing was poorly understood and overlooked by many investigators. Perhaps the largest confounding factor at this time was the extent of masking of low-abundance and singleton taxa by more dominant microbial lineages. For any given assessment, a given methodology is limited in the amount of macromolecular machinery that is available to the enzymatic and chemical processes involved with the detection of cells and/or biosignatures. Therefore, in a 90:9:1 mixture of three distinct cell types, the cell type constituting 90% of the mixture will sequester the vast majority of the chemical reagents available, oftentimes resulting in the signal from less-abundant cell types (i.e., the 9%) and singleton taxa (i.e., the 1%) being suppressed or going undetected, especially in classical cloning and sequencing approaches.

At about this time, two distinct molecular technologies were becoming part of the mainstream research toolkit. Researchers at Lawrence Berkeley National Laboratories (LBNL) were working with scientists at Affymetrix (Santa Clara, CA) in developing a first-of-its-kind phylogenetic-based DNA microarray, referred to as the PhyloChip. Concurrently, Sogin *et al.* had just reported to the scientific community the first-ever successful use of tag-encoded polymerase chain reaction (PCR) amplicon pyrosequencing to unveil the microbial diversity of the deep-sea and the underexplored "rare biosphere" (Sogin *et al.,* 2006). Though completely different in technological premise, these two advancements, completely independent of one another, opened the door to more sensitive and less biased assessments of sample-associated microbial diversity.

For the LBNL-Affymetrix–devised PhyloChip, declaration of presence or absence of a given bacterial taxa was based on hybridization intensity of predesigned fluorescent probes. For any given bacterial lineage, a number of probes were designed and incorporated onto the PhyloChip. Upon analyzing a sample, researchers measure the fluorescence intensity (which coincides with hybridization strength and thus sequence homology) and number of probes lighting up for each taxon, and only taxa whose signals are detected in statistically significant fashion are deemed present in the sample. In contrast, the tag-encoded pyrosequencing (FLX Titanium) approach is not predicated on *a priori* information; and with proper design and operator expertise, the approach can provide an in-depth evaluation of the microbial diversity present in a given sample based on the amplification of 200- to 350-nt-long hypervariable regions (V1–V3) of the 16S rRNA gene.

These two molecular methodologies allowed the Genetic Inventory task to achieve the most comprehensive assessment of low-biomass sample microbial diversity to date. As a result, future spacecraft projects will be able to use these methodologies to rapidly and economically evaluate both known and undocumented microbial lineages present in their SAC facilities or on co-located spacecraft hardware. Further, in the case that future researchers will develop new methods based on evaluations of emerging technologies, the approaches and lessons learned in this study can inform the development process.

The expected impact of this Genetic Inventory task will be the eventual transition of NASA's planetary protection implementation to methods of bioburden assessment that are more inclusive of all relevant microbes, not just those that are cultivable by a particular method.

1. Of particular impact is the development of a refined sampling methodology for use on surfaces present in low-biomass environments. Nearly all of the reports in current scientific literature focus on determining what microbes are present in a given sample, paying little or no attention to the limitations and/or efficacy/efficiency issues associated with the methods employed to collect and process their samples. In contrast, the Genetic Inventory task allocated a considerable amount of resources to identify and standardize procedures to most-effectively collect biological materials from spacecraft and associated environments.
2. As molecular technologies evolve rapidly, the Genetic Inventory task implemented a sample archiving strategy so as to enable the future processing and analysis of samples collected today with the innovative biotechnologies of tomorrow. The deployment of such a capability will be important in comparing genetic inventories at different stages of a mission (e.g., at launch vs. the sample-return period).
3. This comprehensive Genetic Inventory report details unprecedented work, which is of particularly high value in mitigating the impact of molecular and biological contamination in sampling devices, reagents, and consumable surfaces.
4. In addition, this report documents the numerous procedures and protocols that, when combined, result in an innovative approach for collecting, processing, and analyzing target biomolecules. This report serves as a starting point for those embarking on future genetic inventory–oriented ventures, either using these technologies, or (more likely) standardizing their own technical solutions using the approaches described in this report.
5. Finally, in demonstrating superior means of detecting diverse microbial contaminants from low-biomass environments, the benefits of the work carried out in this task potentially apply not only to space exploration, but also a wide range of scientific, electronic, homeland security, medical, and pharmaceutical ventures.

4. Genetic Inventory Study Methodology

4.1 General Approach

The overarching objectives of the Genetic Inventory task were to develop the capability to (1) systematically collect, process, and archive nucleic acids, and (2) effectively assess the phylogenetic breadth of microorganisms present on spacecraft and associated surfaces.

In order to establish the limitations, and test and demonstrate the rigor of each of the methods employed (from sample collection to analysis), a model microbial community (MMC) of known phylogenetic composition and cellular/endospore density was first synthesized. In addition, methods to obtain "*clean DNA*" were standardized and documented as a standalone compendium of procedures and protocols (Appendix B).

The Genetic Inventory study methodology hinged on developing the *capability* to generate the most comprehensive, high-integrity assessment of spacecraft-associated microbial diversity possible with the technologies available today. This was in large part predicated on the concerted utilization and synergistic standardization of one conventional and two molecular biological approaches.

- PhyloChip (generations 2 and 3) DNA microarrays and 454 tag-encoded pyrosequencing (both traditional and FLX Titanium) assays were critically evaluated using samples collected from a variety of low-biomass spacecraft and SAC surfaces.
- The raw data from these analyses were subjected to appropriate bioinformatic and biostatistic process flows.

Figure 4-1 illustrates the methodological flow of the Genetic Inventory task. The steps taken to achieve Objective 1 centered on standardizing protocols involved with sample collection on through to analysis (see Sections 5–8). Similarly, to accomplish Objective 2, several distinct sample types

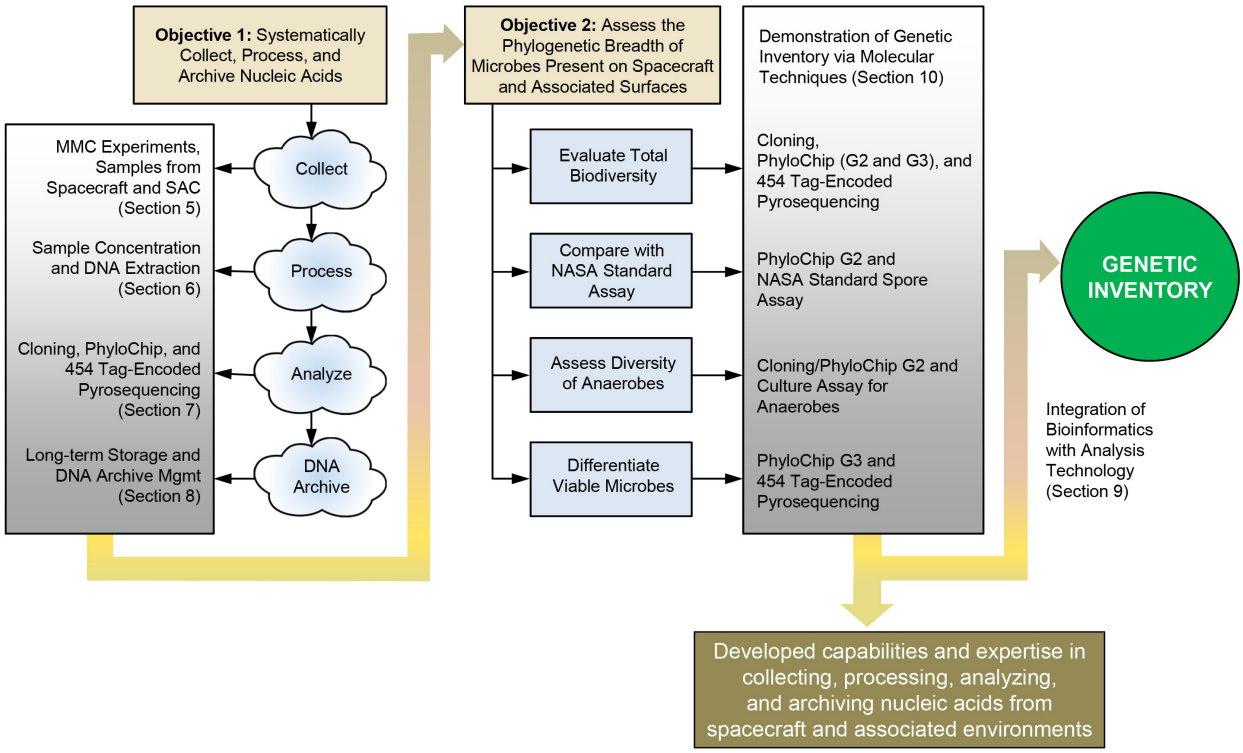


Figure 4-1. Overview of the Genetic Inventory task objectives, activities, and output discussed in this report.

were analyzed to demonstrate the power of molecular techniques for assessing microbial diversity in low-biomass samples (see Section 10).

4.2 Study Methodology to Collect, Process, and Archive Nucleic Acids

The Genetic Inventory team, together with scientists from Lawrence Berkeley National Laboratory (LBNL) and Research and Testing Laboratory (RTL), and input from Marine Biological Laboratory (MBL), developed a test plan for the study based on the recommendations of the Mars Program Office Planetary Protection (MPO-PP) office, MPO-PP review committee, and NASA Headquarters Planetary Protection Office. The plan included both laboratory and field-testing of a statistically significant number of samples. The environmental samples were to be obtained from cleanroom floors, ground-support equipment (GSE), and surfaces of spacecraft hardware from numerous missions.

In order to establish the limitations, and test and validate the rigor of each of the methods employed, an MMC of known phylogenetic composition and cellular/endospore density was first synthesized. Varying concentrations of this MMC were then deposited onto stainless-steel sheets and allowed to dry. These seeded metal sheets of 25 cm^2 and 1 m^2 facilitated a controlled laboratory experiment. From this point forward, numerous experiments were designed and executed to determine the efficacy and efficiency of (a) various materials for sample collection (examining both recovery and retention), and (b) each of several steps involved in sample processing from collection to analysis (e.g., concentration, storage, archiving).

To determine an effective DNA purification regimen for assessing microbial contamination and diversity in low-biomass samples, five distinct DNA purification methods for extracting nucleic acids were tested in parallel: (1) a conventional solvent-based manual DNA extraction method, (2) a commercial MoBio UltraClean Microbial DNA Isolation Kit, (3) a commercial Promega Wizard Genomic DNA Purification Kit, (4) an automated method using an Axcyte AutoLyser (AA), and (5) the AA method with bead-beating agitation (bb-AA), an extraction method devised to more effectively disrupt the cellular structure of hardier cell types than the AA method could lyse alone.

Analysis approaches were then used to gauge the effect of cleanliness of the DNA on products generated, with varying results. PhyloChip and cloning-Sanger sequencing approaches based on 1,500-bp PCR amplicons were indifferent, whereas 454 V6 tag-encoded pyrosequencing, which is based on <100-bp PCR amplicons, was adversely affected by the presence of contaminant DNA molecules arising from gamma-irradiation of reagents and sampling device materials. This problem was resolved with the advent of Titanium 454 FLX V1–V3 tag-encoded pyrosequencing, which allows the sequencing and analysis of much larger amplicons (>350 bp).

In addition, the Genetic Inventory team studied the effect of storage (up to 2 years) and temperature (−80°C and room temperature) on the integrity of DNA. Concurrently, a metadata collection and processing scheme was developed to coincide with the submission of DNA samples collected from spacecraft and associated environments into an archive. In this manner, an appropriate archiving system was established that will enable samples—complete with unique sample names and identification codes—to be easily tracked and identified many years after collection.

4.3 Study Methodology to Assess the Phylogenetic Breadth of Microorganisms

One of the objectives of the Genetic Inventory task was to examine the microbial diversity contained in low-biomass samples. Three completely distinct modes of assaying the presence or absence of a given microbial phylotype were employed. Each mode was equipped with built-in

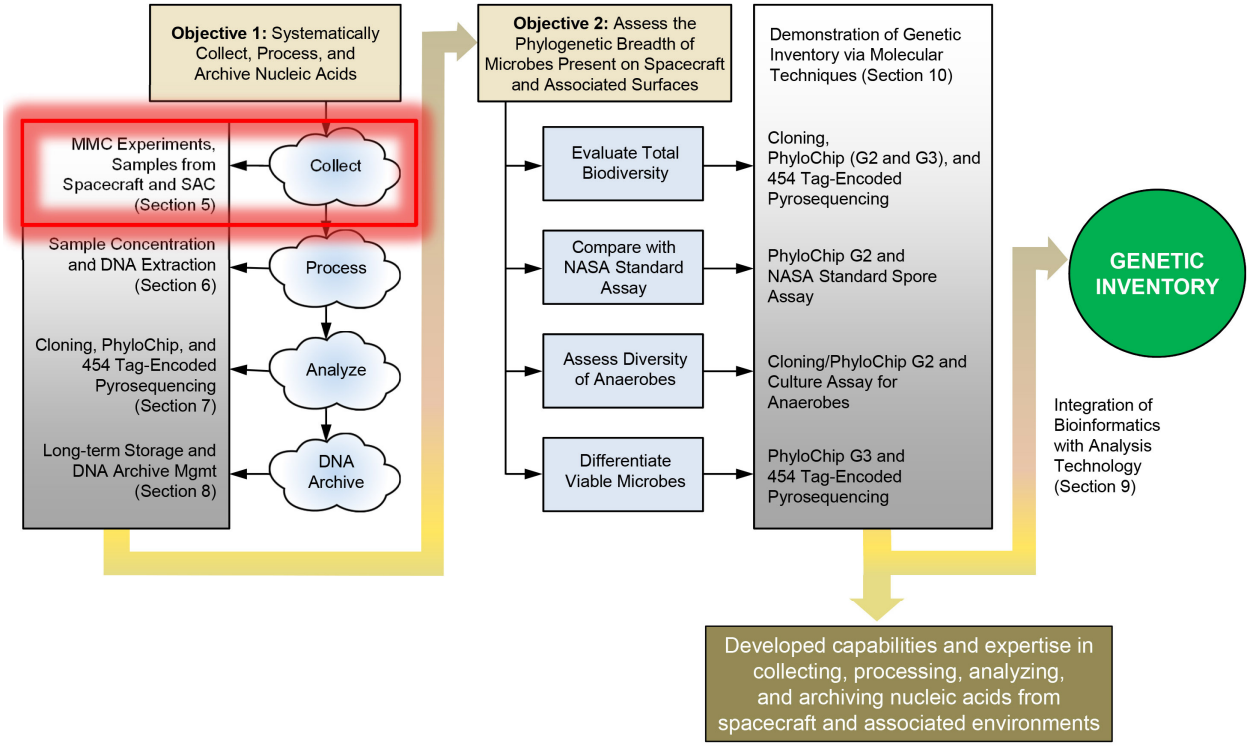
safeguards with respect to data quality control and statistically valid inference. The primary advantage of employing three completely different techniques resided in key differences in their intrinsic analytical basis. Conventional cloning, involving the shuttling of 16S rRNA genes from noncultivable microorganisms into genetically amenable lab strains of *Escherichia coli,* was the established standard, and hitherto the preferred molecular means of elucidating microbial diversity contained in a sample. PhyloChip DNA microarrays were used to identify the presence of previously reported 16S rRNA genes via microarray technology, which was ultimately dependent on fluorescence intensity arising from DNA:DNA hybridization. Finally, the 454 tag-encoded pyrosequencing approach capitalized on high-throughput, massively parallel sequencing, and analysis of subtle variations in the rapidly evolving hypervariable regions of the rRNA genes (or fungal internal transcribed spacer [ITS] regions) sequenced.

Field sampling was devised and carried out to demonstrate the capability of molecular assays by using actual samples from hardware assembly areas and flight hardware and to catalog "the passenger list." The resulting data were used to develop a database and assess microbial diversity.

To obtain the samples, the team developed protocols suited for the molecular assays. Field sampling was planned to sample over a variety of surfaces during the assembly of two missions (Phoenix and Mars Science Laboratory [MSL]). The preferred locations for obtaining samples were spacecraft hardware, spacecraft-related surfaces such as cleanroom facilities (work benches, floors, vents, and walls), and GSE used during spacecraft assembly. Spacecraft hardware was defined as any part of the spacecraft that was included in the launch package, regardless of whether the hardware of interest had specific planetary protection requirements.

5. Sample Collection from Low-Biomass Surfaces

This section describes how the Genetic Inventory team determined the most effective sampling devices for large and small surface areas containing low biomass. Detailed information about sample processing and analysis is provided in subsequent sections. The red box in the figure below highlights this section within the overview of the Genetic Inventory task.



5.1 Objectives

Success criterion: Identification of sampling device for large and small surface areas to collect nucleic acids from low biomass environments

The majority of studies addressing the microbiological sampling of surfaces is limited to cultivation-based approaches, such as the bacterial spore assay (Kirschner and Puleo 1979), or to microscopy-based methods (Brown *et al.,* 2007). Because many microbial species are not cultivable (Amann *et al.,* 1995; Kaprelyants *et al.,* 1993; Kell *et al.,* 1998; Oliver 2010; Vesley *et al.,* 1966), culture-based assays often result in only partial identification of the microbial population present in a given sample. The use of culture-based assays is rapidly being replaced by nucleic acid–based molecular biological detection and enumeration technologies (Breitkopf *et al.,* 2005; Hugenholtz 2002; Vaishampayan *et al.,* 2010; Wilson *et al.,* 2002), and significant advances in the specificity and sensitivity of such technologies are being reported on a seemingly daily basis (Buttner *et al.,* 2004a; Girones *et al.,* 2010; Grindberg *et al.,* 2011; Holman *et al.,* 2009). Despite these advances, however, an efficient and reproducible means of collecting biological materials from low-biomass surfaces remains a challenge.

Therefore, the Genetic Inventory task included a significant effort to develop an efficient and reproducible method for DNA collection from low-biomass surfaces. The objective of the sample collection aspect of the Genetic Inventory task was to

- Identify the most effective sampling devices for low biomass samples on large and small surface areas.

- Acquire sufficient samples to
  - Test the effectiveness of sampling devices in recovering total model microbial community (MMC) biomolecules from surfaces (total recovered yield).
  - Assess the differential recovery of specific members of the MMC by the sampling devices tested.

5.2 General Approach

Time and resources did not permit the Genetic Inventory team to assess every sampling device available to microbiology researchers. Therefore, the team narrowed the field by selecting the sampling tools commonly used by NASA for planetary protection and by surveying relevant literature to select two others. Next, the team used controlled laboratory model–based experiments to compare the performance of the four selected sampling devices. The team synthesized a microbial consortium of known phylogenetic composition and cell/spore density and used it to deliver a known, consistent aliquot of biomass onto numerous stainless-steel surfaces.

5.3 Sampling Device Selection

5.3.1 Sampling Devices Used in Microbial Cultivation Studies

The Genetic Inventory team assessed NASA best practice and sampling protocols for collecting samples from spacecraft surfaces. Since the 1970s Viking missions to Mars, cotton swabs have been the preferred sampling device for collecting samples from spacecraft surfaces, for the purpose of estimating microbial burden. NASA adheres to standard procedures for the microbial examination of spacecraft hardware (NASA 2010); these procedures specify cotton swabs (and polyester wipes for larger surface areas) as the standard device for collecting surface samples. Culture-based studies have shown that cotton swabs, and more recently biological sampling kits (BiSKits), facilitate the greatest recovery of bacterial endospores from small (Rose 2004) and large (Buttner *et al.,* 2004a; Buttner *et al.,* 2004b; Da Silva *et al.,* 2011) surface areas, respectively. However, a recent research communication concluded that nylon-flocked swabs were superior to cotton swabs in collecting *Bacillus* spp. endospores from stainless-steel and other surfaces (Probst *et al.,* 2010).

A limited amount of research on the application of BiSKits to recover DNA from *Bacillus* spores has been reported (Buttner *et al.,* 2004a; Buttner *et al.,* 2004b). The ability to recover total biomolecules (e.g., nucleic acids, lipids, ATP), however, has never been evaluated for cotton swabs, nylon-flocked swabs, or polyester wipes.

5.3.2 Literature Review

Spacecraft and spacecraft assembly cleanrooms (SACs) are unique environments containing low biomass. Substantial efforts are typically made to ensure that spacecraft surfaces are as clean as possible. Unfortunately, reports regarding the collection and analyses of samples from similar low-biomass environments (i.e., industrial and pharmaceutical cleanrooms, hospital operating rooms, etc.) have been almost nonexistent in the primary literature. Available reports primarily employed culture-based methods to set benchmarks for cleanliness and were often contradictory (Buttner *et al.,* 2004b; Favero *et al.,* 1968; Hodges *et al.,* 2010; Hodges *et al.,* 2006; Juozaitis *et al.,* 1994; Sanderson *et al.,* 2002; Sandle 2011). This may be a reflection of differences in methods and/or techniques and may not be reflective of actual sampling performance. For example, it has been demonstrated that sterilization methods do not necessarily remove DNA from an environment (Buttner *et al.,* 2001); therefore, the analyses of sampling performance using culture-based methods would undoubtedly yield inaccurate results with respect to total genomic inventories. It has also been demonstrated that

analyses of samples from low-biomass environments were particularly susceptible to contamination from reagents and user error (Tanner *et al.,* 1998).

The collection of microbial samples from surfaces was historically tailored for the culture-based assessment of target organisms (i.e., coliforms, specific pathogens, etc.). Published reports involving the use of swabs, wipes, sponges, and vacuum-based samplers (Bruckner *et al.,* 2005; Buttner *et al.,* 2004b; Hodges *et al.,* 2006; Kirschner and Puleo 1979; Moore and Griffith 2002; Rose *et al.,* 2004; Sanderson *et al.,* 2002) were evaluated by the Genetic Inventory team with respect to the following variables: material used (cotton, polyester, rayon, macrofoam), area of sample collected (large vs. small), use of mechanical assistance (vacuums), sampling conditions (wet vs. dry), and sample processing (light agitation, vortexing, and/or sonication). The literature review revealed the following findings:

- Published data (Hodges *et al.,* 2006; Rose *et al.,* 2004) indicated that macrofoam was a distinctively better material with respect to both collection and recovery efficiency. Macrofoam swabs were demonstrated to collect 30% more spores than rayon or polyester swabs (Hodges *et al.,* 2006; Rose *et al.,* 2004) even at spore concentrations $<10^1$ spores per preparation (Hodges *et al.,* 2006). When target spores were recovered before drying (within 30 min), recovery efficiencies >90% were achieved from macrofoam swabs during direct inoculation studies. Recovery from rayon and polyester was decidedly less (Rose *et al.,* 2004). Electron micrographs of various swab materials show the more uniform distribution of pores/collection areas within macrofoam, which may be the reason for its superior performance.
- Studies of sample area have clearly demonstrated that the larger the area sampled in low-biomass environments, the more biomass collected (Buttner *et al.,* 2004b; Kirschner and Puleo 1979). The effect of material used was lessened when the sample area was increased (e.g., when sampling areas of 1 m^2, polyester wipes and macrofoam sponges displayed similar collection efficiencies per unit area and collected more biomaterial than smaller swabs) (Buttner *et al.,* 2004b; Kirschner and Puleo 1979). It can be reasoned that the collection of more biomass, particularly from low-biomass environments, would result in a more complete genetic inventory of said environment.
- The use of mechanical aids during sampling (e.g., vacuum/suction sampling) was not shown to have increased collection efficiency when compared to polyester swabs (Buttner *et al.,* 2004b; Sanderson *et al.,* 2002; Whitfield *et al.,* 1969). Although macrofoam swabs were not directly tested against vacuum or suction samplers, it can be inferred from previous material tests (Hodges *et al.,* 2006; Rose *et al.,* 2004) that the macrofoam would outperform these mechanical samplers.
- Regardless of the material used, premoistened samplers always demonstrated higher collection efficiencies than did dry samplers (Bruckner *et al.,* 2005; Hodges *et al.,* 2006; Moore and Griffith 2002; Rose *et al.,* 2004; Sanderson *et al.,* 2002). On average, wet-collection efficiency was shown to be 10–30% higher than dry-collection efficiency; although it should be noted that Buttner *et al.* (2004a) indicated that spore recovery from wet material was decreased due to spores trapped in the wet samplers. This loss of recovery efficiency was estimated to be 5–10%, and was offset by the significantly higher collection efficiency.
- Post-collection processing was shown to affect recovery efficiency. Studies using vortexing, light agitation, and sonication consistently demonstrated that vortexing increased recovery efficiency up to 25% more than the latter two when used alone (Hodges *et al.,* 2006; Rose *et al.,* 2004). Studies using post-collection processing in conjunction (i.e., vortexing followed by sonication) were not found, although it can be inferred that the combination processing would not negatively affect recovery efficiency.
- Based on a European study (Probst *et al.,* 2010), the team determined that nylon-flocked swabs should be evaluated for their efficacy in recovering 16S rRNA genetic material from metal surfaces.

At the start of the Genetic Inventory task and based on the surveyed literature, it was recommended that the best method for collection of surface samples would be to use a pre-moistened macrofoam-based collection apparatus. In addition, any sample collection technology selected would need to standardize sample yield and diversity, especially for low-biomass environments. The ability to collect large sample sizes with high-collection and recovery efficiency would ensure efficient future collection of high-integrity genetic inventories.

5.3.3 Summary of Sample Device Selection

Based on NASA heritage and the relevant literature review, the team selected four sampling device candidates for laboratory model–based controlled study using MMCs: cotton and nylon-flocked swabs for small surface areas (25 cm^2), and BiSKit (macrofoam) and polyester wipes for larger surface areas (2,500 cm^2 to 1 m^2). Some experiments were performed early in the Genetic Inventory task using polyester and macrofoam swabs, but those were not evaluated as extensively as cotton and nylon-flocked swabs. Appendix A discusses these swab studies in greater detail.

5.4 Laboratory Model–Based Study

In order to thoroughly and accurately address the superior, standardized methodologies linking sample collection, processing, and analysis of sample nucleic acids, the team employed an MMC with phylogenetic diversity relevant to low-biomass environments. Using the MMC, the team individually tested several variables, including differences in sampling materials, concentration of target biomolecules after sampling, and the effect of cryostorage on extracted molecules. The Genetic Inventory task is, to authors' knowledge, the first study to employ a complex MMC for testing and standardizing protocols for collecting, processing, and analyzing biomolecular samples from cleanroom surfaces.

5.4.1 MMC Preparation

Extreme care was taken while choosing the MMC constituents to ensure that standardized methodologies developed for the Genetic Inventory task included representatives of all kinds of microbes (e.g., domains, metabolism, spore) present in the relevant low-biomass environment. Representatives from the Jet Propulsion Laboratory (JPL), NASA Headquarters, and the European Space Agency (ESA) had several discussions to identify the most appropriate microbial members to include in the MMC composition. The Genetic Inventory team prepared an MMC comprising 11 distinct microbial species (see Appendix B.3). These 11 MMC constituents—which represent a consensus of the Genetic Inventory team plus input from JPL, NASA, and ESA scientists—spanned all three domains of life, and a variety of metabolisms, modes of respiration, and cellular morphotypes. Most of the MMC constituents were previously isolated from spacecraft and associated cleanroom surfaces (Bruckner *et al.,* 2008; La Duc *et al.,* 2007a; La Duc *et al.,* 2003; Newcombe *et al.,* 2008), and others were procured from various culture collections (Table 5-1). All 11 strains used in this study were placed in the National Center for Agricultural Utilization Research culture collection (NRRL), in Peoria, Illinois. Appendix B.3 provides detailed procedures addressing cultivation conditions, the mixing of various microbial strains, and storage at −80°C.

5.4.2 Experimental Steps

A schematic overview of the deposition of MMC onto stainless-steel surfaces, and subsequent sample collection, sample processing, and sample analyses, is presented in Figure 5-1. The following subsections detail the methods by which MMC was artificially deposited and collected using various sampling devices. (See Section 6 for processing [Step 3 and 4 of Figure 5-1] and Section 7 for analysis [Step 5 of Figure 5-1] methods.)

Table 5-1. Various characteristics of model microbial community constituents; see (Kwan *et al.,* 2011) and Appendix C for greater detail regarding the strains selected.

No.	Microbes	Strain #	Domain: Phylum	Morphology	Cultural Conditions and Reference[1]	Incubation Time (h)	Other Available Sources[2]
1	*Aureobasidium pullulans*	28v1	Eukarya: Ascomycota	Black yeast	TYG agar, 30ºC	86	NRRL 58992
2	*Acinetobacter radioresistens*	50v1	Bacteria: γ–proteobacteria	Short rods	TYG agar, 32ºC	60	NRRL B-59417
3	*Bacillus megaterium*	KL-197	Bacteria: Firmicutes	Rods	TYG agar, 32ºC	38	NRRL B-59415
4	*Bacillus pumilus*	SAFR-032	Bacteria: Firmicutes	Spore	See (Kempf *et al.,* 2005)	N/A[3]	ATCC PTA-7603; NRRL B-30938
5	*Deinococcus radiodurans*	ATCC 13939	Bacteria: Deinococcus-Thermus	Cocci, tetrad	TYG agar, 32ºC	60	NRRL B-59418 and DSM 20539
6	*Microbacterium imperiale*	47v1	Bacteria: Actinobacteria	Short rods	TYG agar, 32ºC	60	NRRL B-59416
7	*Staphylococcus warneri*	82-4	Bacteria: Firmicutes	Cocci	TYG agar, 32ºC	40	NRRL B-59414
8	*Micrococcus luteus*	ATCC 4698	Bacteria: Actinobacteria	Rods	Nutrient agar, 32ºC	60	NRRL B-59413 and DSM 20030
9	*Cupriavidus metallidurans*[4]	CH344	Bacteria: β-proteobacteria	Rods	TYG agar, 32ºC	60	ATCC 43123
10	*Clostridium sporosphaeroides*	DSM 1294	Bacteria: Firmicutes	Anaerobic rods	DSMZ Medium 78, 37ºC, anaerobic	48	ATCC 25781
11	*Methanobacterium formicicum*	DSM 1535	Archaea: Euryarchaeota	Rods	DSMZ Medium 119, 37ºC, anaerobic	72	ATCC 33274

[1]TYG: Tryptone, yeast-extract, glucose (5 g, 3 g, 1 g per liter, respectively).
[2]ATCC: American Type Culture Collection; NRRL: USDA culture collection; DSM: German culture collection; PTA: patented strain.
[3]N/A: Not applicable since the spores were prepared prior to the investigation, and the appropriate concentration was used from the original stock.
[4]Received from Dr. Natalie Leys, Hoofd Onderzoeks Eenheid Microbiologie at SCK-CEN, Antwerp Area, Belgium.

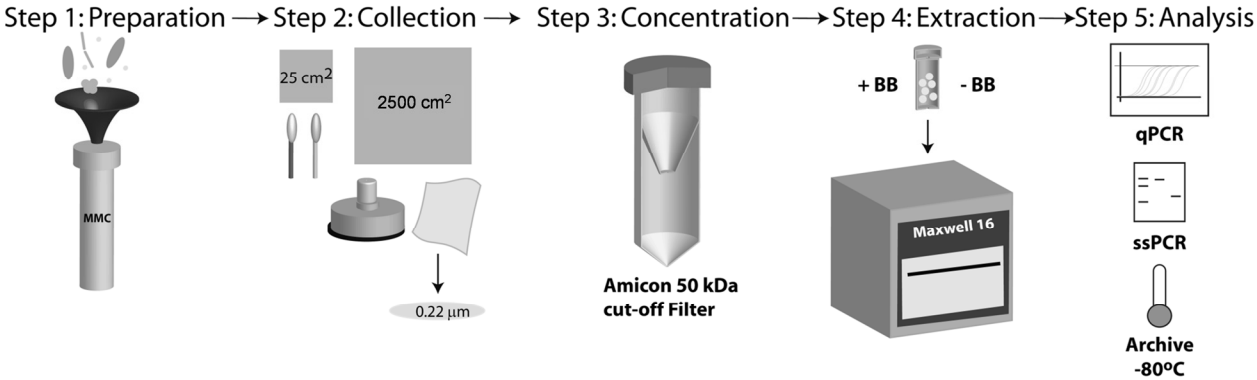


Figure 5-1. Schematic representation of the steps involved in MMC deposition and subsequent sample collection, processing, and analysis.

5.4.2.1 Cleaning and Preparation of Metal Surfaces

All stainless-steel grade 304-020 (304 brushed stainless-steel sheet, 20 gauge, 0.036" thick, unfinished) coupons (25 cm²) and sheets (2,500 cm²) were pre-cleaned with cleanroom-grade polyester wipes (Coventry 6209 c-prime; Tech Spray, L.P., Amarillo, TX). Coupons and sheets were subjected to ultrasonic cleaning with alkaline detergent soap (Brulin 815 GD, Indianapolis, IN) at 160ºF, followed by a deionized water rinse, drying, and a final rinse of 97% ethyl alcohol (Venkateswaran *et al.,* 2004). Subsequently, coupons and sheets were rinsed with deionized water and

dried with clean, dry nitrogen. This precision-cleaning procedure effectively removed any cultivable microbes and genetic materials associated with the metal surfaces. All precision-cleaned coupons and sheets were packaged individually in sterile containers until time of use.

5.4.2.2 Assembling of MMC before Deposition

Aliquots of the MMC (40 × 1.2 mL) were thawed, pooled, and concentrated via centrifugation (15 min, 4000 × g) using the Amicon 50-kDa filtration system (Cat.#UFC905024, Millipore, Billerica, MA). Finally, to circumvent crystal formation resulting from phosphate buffered saline (PBS) precipitation on metal surfaces, the MMC was washed and resuspended in sterile water to a final volume of 48 mL prior to deposition. (The rationale for the decision to use water rather than PBS is described below.)

As anticipated, scanning electron microscopy (SEM) results showed that many MMC cells, when suspended in water, were not intact but rather had degenerated and burst open due to osmotic shock. To compare recovery from metal surfaces of MMC DNA from water-suspended and PBS-suspended MMC cells, one-half of the samples were reconstituted in sterile water and the other with sterile PBS before depositing the samples onto sterile metal coupons. After 24 h of drying, the metal coupons were visualized under SEM to investigate the differences in microstructure resulting from the PBS and water evaporation. The SEM pictures showed a high degree of salt crystal formation on metal coupons inoculated with PBS-suspended MMC, whereas those seeded with water-suspended MMC had no visible crystals. Recovery results stemming from the use of two distinct types of suspension media (water and PBS) for deposition of MMC onto stainless steel revealed a greater recovery (150-fold) of MMC DNA when cells were suspended in water. Since the Genetic Inventory task is focused on recovery of genetic material and not on the recovery of intact cells, prior to deposition, the team decided to remove PBS by washing the MMC in sterile DNA-free water.

5.4.2.3 Deposition of the MMC onto Metal Surfaces

Stainless-steel coupons (25 cm^2) and sheets (2500 cm^2) were aseptically removed from their sterile packaging, and placed into individual sterile petri dishes or trays, respectively. The MMC sample concentrate volume (1 mL) and the amount of DNA (10^6 rRNA gene copies) were the same for both the coupons and the sheets: 20 drops (each comprising 50 µL sample concentrate) were deposited onto each coupon, and 50 drops (each comprising 20 µL sample concentrate) were deposited onto each sheet using a multichannel, multi-dispensing electronic pipette (Rainin E8-200, Oakland, CA). All coupons and sheets, including those serving as sampling blanks, were covered and allowed to dry for 24 h. To minimize bias associated with sample collection, the sampling was carried out "blindly" (i.e., the operator in charge of deposition was not also in charge of sampling).

5.4.2.4 Sampling of MMC-Spiked Metal Surfaces

Small surface areas (25 cm^2): Coupons were sampled with either cotton (Cat. #806-WCL; Puritan Medical Products, Guilford, ME) or nylon-flocked (Cat. #552C.US; Copan Diagnostics Inc., Murrieta, CA) swabs, which were pre-moistened with sterile PBS immediately prior to sampling. Coupons were first sampled in a unidirectional horizontal manner while holding the swab at approximately a 30º angle to the surface. Swabs were rotated (ca. 120º) to present an area of the swab head that had not previously contacted the surface, and coupons were sampled in a unidirectional vertical manner. Finally, swabs were once again rotated (ca. 120º) and coupons were sampled in a unidirectional diagonal manner. Swab heads were aseptically severed from their shafts into individual 50-mL Falcon tubes containing 10 mL of sterile PBS. A negative control, whereby a swab was only pre-moistened with PBS, and a handling control, in which a swab was pre-moistened with PBS and exposed to the sampling environment, were also prepared.

Large surface area (2500 cm²): Stainless-steel sheets were sampled with either ITW Alpha polyester wipes (Cat. #TX1009; Texwipe, Kernersville, NC) or BiSKits (Cat. #BIS-40001A; Quicksilver Analytics Inc., Abingdon, MD). Wipes were rolled and placed into individual 50-mL glass test tubes containing 15 mL of PBS, which were autoclaved and allowed to cool prior to sampling (NASA 2010). Wearing sterile gloves, the researcher removed the wipe from the tube with sterile forceps and unrolled it, leaving it folded in quarters. Sheets were first sampled in a unidirectional horizontal manner, after which wipes were turned over and used to sample the sheets in a unidirectional vertical manner. Finally, the wipe was inverted to expose a fresh surface and the sheet was sampled in a unidirectional diagonal manner. By this method, three-quarters of one side of the wipe was used in sampling the surface. Negative and handling controls were prepared as detailed above for coupons. All wipes were placed into individual sterile 500-mL glass bottles for processing.

BiSKits were moistened with 15 mL of sterile PBS, which was expelled from each unit and pooled to serve as the negative control. The collection bottle of each BiSKit was replenished with 15 mL of sterile PBS; and the entire module was inverted, allowing for the macrofoam sponge to become saturated. The macrofoam sponge sampling portion of the BiSKit module was removed and sheets were first sampled in a unidirectional horizontal manner. The BiSKit was then turned and the sheets were sampled in a unidirectional vertical manner. Finally, the BiSKit was once again turned and sheets were sampled in a unidirectional diagonal manner. A handling control, in which a BiSKit was exposed to the environment but no surfaces, was also prepared.

Key Consideration: PBS would not be used in spacecraft sampling. However, for these laboratory-controlled experiments, PBS was used to pre-moisten the sampling devices. The PBS step was necessary to protect the recovered biological materials from cell death due to osmotic shock. Even though the Genetic Inventory team was targeting biomolecules, in the initial stage of the study, the team also planned to recover cells using cultivability, microscopy (account intact cells), and viability analyses in addition to the quantitative polymerase chain reaction (qPCR) approach. Hence, pre-moistening the sampling devices with PBS was preferred.

5.4.3 Results of Laboratory Model–Based Study

5.4.3.1 Recovery of Total DNA from Metal Surfaces

Cotton swabs were nearly twice as effective (by yield) as nylon-flocked swabs in recovering rDNA from microbes deposited onto metal surfaces; total rDNA recovery efficiencies were 30.2% for cotton and 17.7% for **nylon-flocked swabs** (Figure 5-2). Furthermore, as Table 5-2 shows, cotton swabs collected and released significantly greater amounts of 16S rDNA than their nylon counterparts ($p < 0.05$; CI 95%). The presence of contaminant DNA in the cotton sampling blanks was less than three orders of magnitude compared to

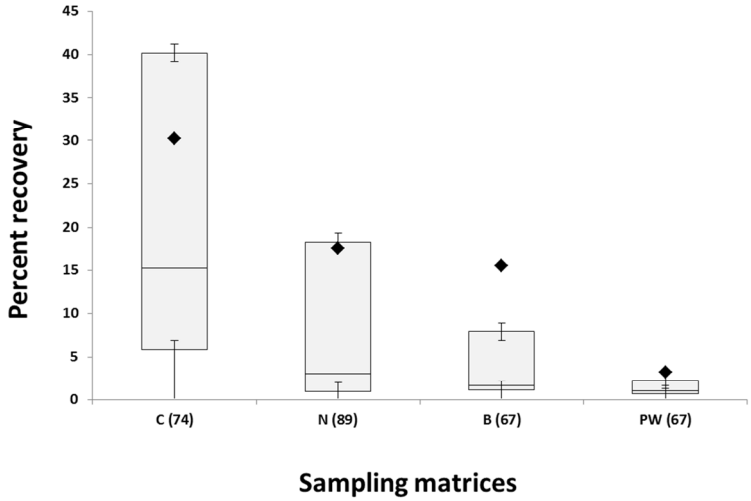


Figure 5-2. Performance and recovery efficiency of various sampling devices. Recovered samples were analyzed via universal qPCR targeting 16S rRNA, and results were summarized with box plots showing the mean (closed diamonds), median, and upper and lower quartiles for cotton (C) and nylon-flocked (N) swabs, BiSKits (B), and polyester wipes (PW). Error bars depict the standard error of the mean placement of each quartile. The number of replicates performed with each sampling device is in parentheses.

Table 5-2. Pairwise assessment of the statistical significance of contaminant 16S rDNA associated with sampling materials in the recovery of microbes from various metal surfaces.

Sample[1]		Description of Traits Compared	t-Test, p-value[2]	Mean ± SD[3]	
a	b		a vs. b	a	b
Smaller Surface Area					
C (74)	N (89)	Recovery of DNA from smaller surfaces	0.026	$1.2 \pm 1.4 \times 10^6$	$7.1 \pm 12.0 \times 10^5$
C (74)	C-SB (36)	Cotton-associated sampling	0.000	$1.2 \pm 1.4 \times 10^6$	$1.2 \pm 4.19 \times 10^3$
N (89)	N-SB (26)	Nylon-associated sampling	0.002	$7.1 \pm 12.0 \times 10^5$	$1.4 \pm 3.8 \times 10^3$
C-HC (17)	**N-HC (17)**	Handling controls	**0.990**	$1.9 \pm 3.9 \times 10^3$	$1.7 \pm 4.0 \times 10^3$
C-SB (36)	**N-SB (26)**	Sampling blanks	**0.879**	$1.2 \pm 4.19 \times 10^3$	$1.4 \pm 3.8 \times 10^3$
C-SD (15)	**N-SD (15)**	Sampling devices	**0.159**	$6.1 \pm 12.7 \times 10^3$	$1.0 \pm 1.3 \times 10^3$
Larger Surface Area					
B (67)	PW (67)	Recovery of DNA from larger surfaces	0.014	$6.0 \pm 15.0 \times 10^5$	$1.4 \pm 2.1 \times 10^5$
B (67)	B-SB (37)	BiSKit-associated sampling	0.011	$6.0 \pm 15.0 \times 10^5$	$1.3 \pm 3.7 \times 10^3$
PW (67)	PW-SB (26)	Polyester wipes–associated sampling	0.000	$1.4 \pm 2.1 \times 10^5$	$8.3 \pm 24.0 \times 10^3$
B-HC (13)	**PW-HC (15)**	Handling controls	**0.283**	$2.4 \pm 5.5 \times 10^3$	$4.7 \pm 9.3 \times 10^3$
B-SB (37)	**PW-SB (26)**	Sampling blanks	**0.199**	$1.3 \pm 3.7 \times 10^3$	$8.3 \pm 24.0 \times 10^3$
B-SD (44)	PW-SD (18)	Sampling devices	0.001	$1.1 \pm 1.54 \times 10^3$	$1.9 \pm 2.41 \times 10^2$

[1]Number of replicates are given in parentheses. Each value is the average of three qPCR measurements.
[2] p-value <0.05 is statistically significant (CI 95%).
[3]Mean total gene copy numbers with standard deviations. Mean values in columns a and b correspond, respectively, to samples a and b of the Sample columns.

C: Cotton swabs
N: Nylon-flocked swabs
B: BiSKit
PW: Polyester wipes

SD: Sampling device control (test device cleanliness; sterile device removed from package, not exposed to sampled surface but subjected to identical extraction procedures as devices used to collect samples)
SB: Sampling blank (test negative-control surface; device used to sample a clean, negative-control metal surface area and then subjected to identical extraction procedures as devices used to collect samples)
HC: Handling control (test fallout; sampling device exposed to the ambient environment but not exposed to the surface sampled)

the actual recovery of sample 16S rDNA by cotton. When nylon-flocked swabs were used to collect samples, 62 out of 89 replicates recovered less than 9.9% (equivalent to ~10^4 16S rRNA gene copies) of the rDNA seeded onto the metal surfaces (Figure 5-3). Cotton swabs proved much more effective in sample recovery, as 48 of the 74 cotton swab sampling replicates yielded recovery greater than 10% (Figure 5-3). With regards to sampling blank negative controls, only one out of every 18 cotton swab replicates exceeded 10^4 contaminant rRNA genes, as opposed to one out of every 13 for nylon-flocked swabs. There were no statistically significant differences in the levels of contaminant rDNA between the handling controls, sampling blanks, and sampling devices of the two swab materials tested (Table 5-2).

BiSKit sampling devices were roughly five times more effective in recovering MMC rDNA than were **polyester wipes** (15.5% and 3.2% recovery, respectively; Figure 5-2). Samples collected via BiSKits and polyester wipes contained significantly more rDNA than did their corresponding sampling blanks ($p<0.05$; Table 5-2). When BiSKits were used for sample collection, 13 of 67 replicates exhibited >20% recovery (equivalent ~10^5 16S rRNA gene copies), whereas only 2 of 67 polyester wipe samplings yielded recovery this high (Figure 5-3).

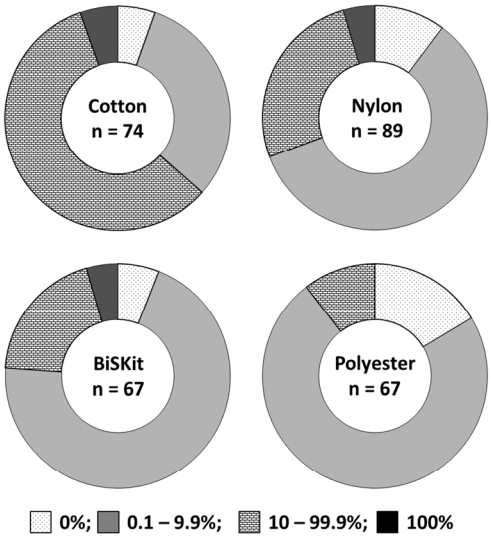


Figure 5-3. Categorical percent recovery efficiency of various sampling matrices.

At first glance, there appears to be no statistically significant difference between the levels of contaminant rDNA present in the polyester wipe sampling blanks (the positive end of the standard mean of error is 8.3×10^4 gene copies) and the

amount of rDNA actually collected with polyester wipe. However, detailed statistical analyses revealed that the rDNA recovery yield typical of polyester wipes ($1.4 \pm 2.1 \times 10^5$ gene copies; 3.3%; Table 5-2) exceeded the levels of contaminant rDNA burden of the sampling blanks (4.1×10^1 to 8.3×10^4 gene copies). Furthermore, no significant differences were observed between the BiSKit handling controls and sampling blanks and those of polyester wipes. The levels of contaminant rDNA associated with the sampling device blanks for each of these fell below the limits of species-specific qPCR (ss-qPCR) detection. The polyester wipe blanks exhibited an average of 1.9×10^2 contaminant gene copies, compared to BiSKit blanks, which averaged 1.1×10^3 contaminant rDNA genes (Table 5-2).

5.4.3.2 Differential Recovery of MMC DNA from Metal Surfaces

The MMC-strain-based recovery efficiencies associated with the various sampling materials tested are provided in Figure 5-4. The initial number of rRNA gene copies of each MMC constituent present in 1 mL of MMC suspension (Figure 5-4A) was calculated and used as the reference point for determining percent recovery. Though not considered in the ss-qPCR–based recovery assays, the purified endospores of *Bacillus pumilus* SAFR-032 and vegetative cells of *Aureobasidium pullulans*, a fungal strain, were included to elucidate the effect of nonspecific interactions and competitive influence of non-targeted microbial taxa on recovery efficiencies. Cotton swabs enabled the recovery of rDNA from all nine MMC constituent microbes, whereas nylon-flocked swabs failed to recover three of the MMC members, including the archaeaon *M. formicicum* (Figure 5-4A). In addition, cotton recovered considerable levels of rDNA of several MMC constituents that had been totally unaccounted for when the samples were

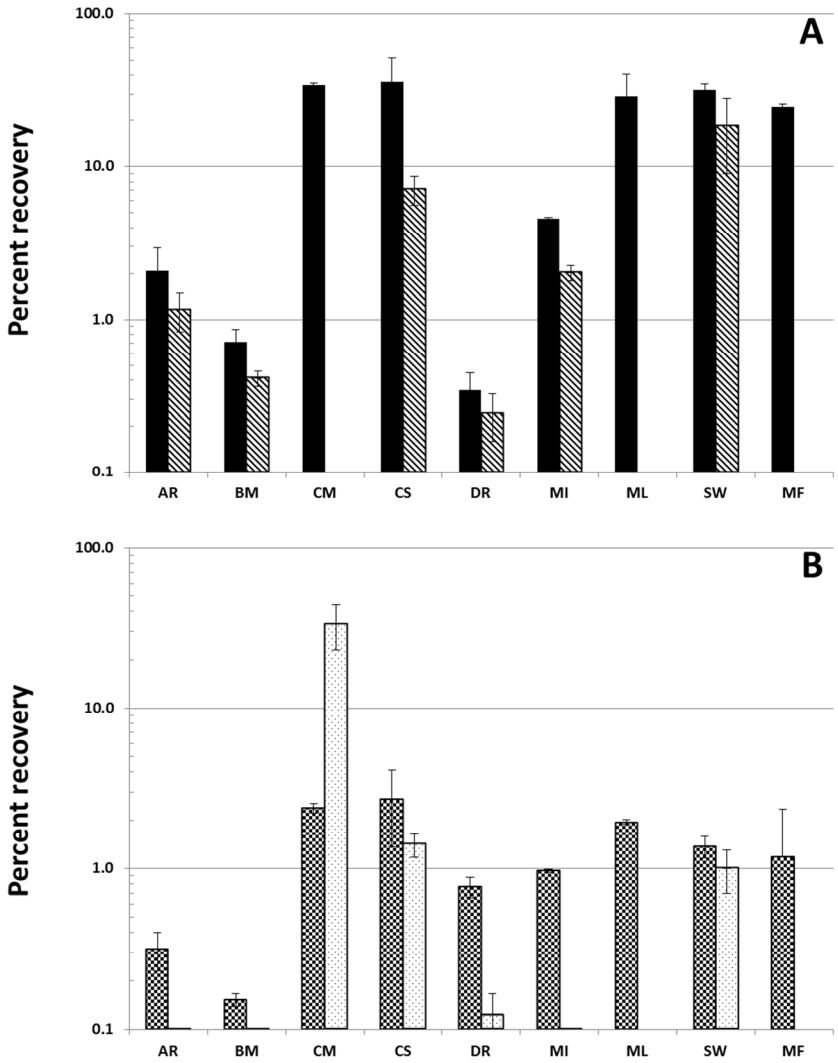


Figure 5-4. Differential recovery of cells and rDNA of MMC constituents from metal surfaces by various sampling devices. (A) Percent recovery of each ss-qPCR-assayed MMC constituent from a small surface area (*dark bars*: cotton swabs; *striped bars*: nylon-flocked swabs). (B) Percent recovery of each ss-qPCR-assayed MMC constituent from a large surface area (*dark checkered bars:* BiSKit; *light spotted bars:* polyester wipe). Error bars depict standard error of the mean.

collected using nylon-flocked swabs. Of the nine MMC constituents assayed, cotton recovered at least 25% of the originally deposited rDNA from *C. metallidurans* (34.5%), *C. sporosphaeroides* (36.7%), *M. luteus* (29.1%), *S. warneri* (31.8%), and *M. formicicum* (24.8%). In contrast, nylon-flocked swabs recovered the rDNA of *S. warneri* at 18.6%, and the rDNA of five additional MMC-strains at 7% or less. Both swab materials had difficulty recovering the rDNA of *D. radiodurans* (<1% each).

Because the surface area of the sheets (2,500 cm^2) was 100 times greater than the surface area of the coupons (25 cm^2), and the sheets and coupons received the same number of MMC cells (10^6 rRNA genes per test surface), the recovery of MMC rDNA from the large surface areas was much lower than that from small surface areas. BiSKit sampling devices consistently collected greater amounts of MMC DNA than did polyester wipes. Furthermore, BiSKits successfully recovered the rDNA of all nine MMC constituents deposited onto the metal surfaces, whereas polyester wipes recovered only four of the MMC strains. BiSKit samplers were up to 14 times more efficient in collecting individual MMC constituent-borne rDNA than polyester wipes. Of the nine MMC microbial constituents assayed, BiSKits were able to recover at least 1% of the rDNA of *C. metallidurans, C. sporosphaeroides, M. luteus, S. warneri,* and *M. formicicum*. Coincidentally, these are the same MMC strains recovered in high abundance by cotton swabs. In contrast, polyester wipes were limited in recovery to the rDNA of *C. metallidurans* (33.3%), and three additional MMC members at less than 1.4%.

5.5 Summary of Sample Collection from Low-Biomass Surfaces

The objective of the sample collection aspect of this study was to determine the most effective sampling device(s) for collecting DNA from low-biomass surfaces. Results demonstrate the intrinsic power of molecular techniques in assessing the differential recovery of rDNA of a mixed microbial consortium deposited and dried on metal surfaces. Measurements of total DNA recovery were directly compared with those corresponding to the specific rRNA gene sequences of each MMC constituent, thereby enabling assessment of differential recovery of the various MMC strains as a function of the device employed for sample collection.

Because swabs have been the NASA standard sampling devices for assessment of small surfaces of spacecraft, the Genetic Inventory team initially focused on the efficacy and efficiency of swab-based nucleic acid sample collection. However, the team identified two shortcomings associated with swab-based sample collection: (1) Pre-existing nucleic acid contamination was concomitant with cotton swabs, due primarily to their organic composition (both cotton matrix and wood shaft), and (2) Due to the limiting surface area able to be collected (~25 cm^2), it was oftentimes not possible to collect the levels of sample material required to accommodate the sensitivity thresholds of downstream molecular analyses. Therefore, the team abandoned the swab investigation in favor of devices capable of collecting samples from much larger surface areas (Appendix A).

Key Consideration: When larger surface areas are required to obtain environmental samples due to the paucity of biomass:

- Use BiSKit samplers to collect samples from SAC and ground-support equipment (GSE) surfaces (Note: BiSKits are not certified [permitted] to sample spacecraft surfaces directly.)
- Use polyester wipes to collect spacecraft samples.

Outcome: Effective sampling devices identified—for optimum nucleic acid recovery, macrofoam BiSKits and cotton swabs were preferred for large- and small-surface-area samplings, respectively.

5.6 Key Considerations

5.6.1 Biomolecular Contamination of Sampling Devices

When dealing with low-biomass samples, even extremely low levels of contaminant DNA in the sampling device can be an important consideration. The rDNA associated with the various sampling devices ranged from 1.8×10^2 to 8.3×10^3 gene copies per device, approximately three orders of magnitude lower than the detected level of MMC positive control rDNA (3.6×10^6 gene copies). Only 10% of the sampling blanks examined exhibited contaminant 16S rRNA genes in excess of 10^3 gene copies. Negative controls corresponding to the media used to culture MMC constituents and the metal surfaces themselves, without MMC deposition, contained 10^1 to 10^2 contaminant rRNA genes (Bargoma *et al.,* 2012). The observed range in pre-sampling rDNA burden was roughly 2 to 3 orders of magnitude lower than that corresponding to the sampling device blanks, handling controls, and sampling blanks, and ~4 orders of magnitude lower than the MMC positive control. Based on this disparity, it seems reasonable to attribute the source of DNA contamination observed in the sampling blanks to the sampling devices and not the metal surfaces (Bargoma *et al.,* 2012).

The following steps were taken to minimize the effects of biomolecular contamination of sampling devices (i.e., for BiSKit samples). Analogous steps should be considered for any other sampling device used in future studies.

- Determine the percent occurrence of sampling device negative controls that exceed 10^3 16S rRNA gene copies; assess "false positive risk" associated with BiSKits and polyester wipes.
  - When 20 individual polyester wipes were processed, none of the 20 wipes showed DNA contamination exceeding 10^3 16S rRNA gene copies.
  - When 20 BiSKits were processed, ~35% of the 20 kits showed DNA contamination exceeding 10^3 16S rRNA gene copies.
- Consult vendor and arrange for "clean, nucleic-acid–free" BiSKit samplers to be manufactured.
  - QuickSilver Analytics, Inc., the manufacturer of BiSKit devices, has agreed to take steps toward producing DNA-free kits. QuickSilver Analytics, Inc. followed good manufacturing practices (GMPs) and reduced the contamination level of the BiSKits; all of the 10 "clean" BiSKits tested exhibited $<10^3$ 16S rRNA gene copies (123-bp via qPCR). Before this GMP step, ~35% of the BiSKits (n=20) tested exhibited $>10^3$ 16S rRNA gene copies (123-bp via qPCR).
  - In addition, QuickSilver Analytics, Inc. now has a cleanroom in place to handle designated quantities of macrofoam using sterile gloves and hairnets for packaging. The vendor handled the JPL-designated BiSKits in the same manner before shipping them for gamma irradiation.
- Develop a protocol for cleaning BiSKits and/or other macrofoam sampling tools.
  - The DNA-eliminating enzymatic pre-treatment (DNase) was successful for macrofoam wipes but could not be used for the BiSKit since the integrity of the kit was compromised.
  - The contaminant DNA level in macrofoam wipes was comparable with that found in BiSKits (10^3 copy numbers). When macrofoam wipes are used, a unique sample-processing instrument (such as a stomacher) is required.
  - When these "clean" BiSKits (DNase-treated or GMP-followed) were analyzed using 454 tag-encoded pyrosequencing, no traceable PCR product of long-read (~500 bp length) sequencing was amplified.

The results of these cleanup experiments demonstrate that high-quality lots of BiSKit or macrofoam wipes can be purchased to collect materials from cleanroom surfaces. If, upon pre-use examination, macrofoam-wipe lots exhibit $>10^3$ gene copies, DNase pre-treatment is recommended.

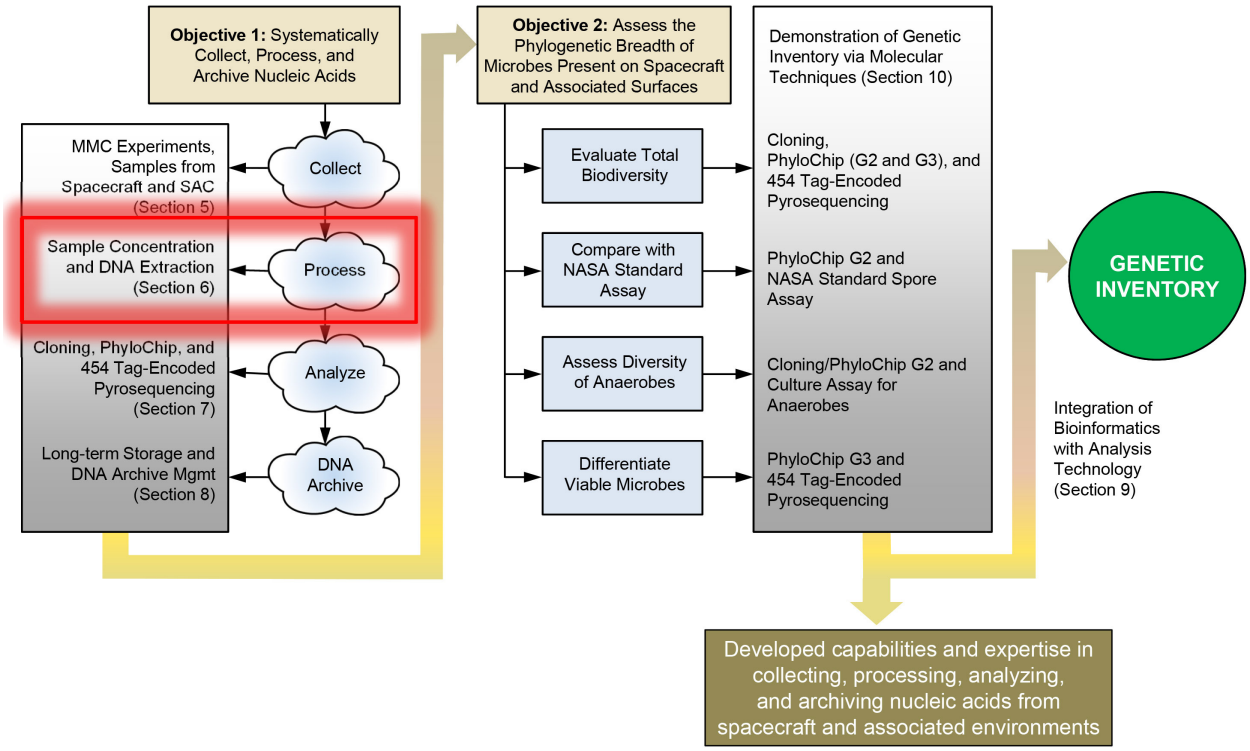
5.6.2 Other Issues

Using the MMC as a positive control, the following steps were taken to evaluate the specific effect of a number of variables, associated with sampling and processing, on the integrity and recovery of the MMC. This evaluation was made before the MMC aliquots were deposited onto stainless-steel surfaces.

- Assess the ramifications of varying concentrations (5–20%) of glycerol used as a cryoprotectant.
  - MMC aliquots (1 mL) were prepared as 0%, 5%, 10%, and 20% glycerol suspensions (total volume: 15 mL) to ascertain whether this cryoprotectant posed problems to the downstream Amicon filter-based sample concentration step. The volumes trapped in the uppermost Amicon filter "boat" were measured and compared pre- and post-centrifugation for each glycerol concentration tested. In addition, measurements of DNA extraction yield helped the team infer whether the presence of glycerol was obstructing sample flow-through. Unfiltered, sterile 100% glycerol was also subjected to DNA extraction procedures to assess the presence of associated contaminant DNA. The results of such studies demonstrated that glycerol concentrations >5% hindered the centrifugal filtration of sample volumes, presumably due to the clogging of filter pores. The sterile glycerol did not harbor detectable levels of 16S rRNA gene copies.
- Assess buffered solutions (PBS vs. water) as suitable liquid media for deposition.
  - Aliquots of MMC (40 × 1.2 mL) were thawed, pooled, and concentrated via centrifugation (15 min, 4000 × g) using the Amicon filtration system (Cat. #UFC905024, Millipore, Billerica, MA). To compare water and PBS, one-half of the samples were reconstituted in sterile water and the other half with sterile PBS before placing the samples onto sterile metal coupons. After 24 h of drying, metal coupons were visualized under SEM to investigate the differences in microstructure resulting from the PBS and water evaporation. Results stemming from the use of two distinct types of suspension media (water and PBS) for deposition of MMC onto stainless steel revealed a greater recovery (150-fold) of MMC DNA when cells were suspended in water. SEM showed a high degree of salt crystal formation on metal coupons laden with PBS-suspended MMC, whereas those seeded with water-suspended MMC had no visible crystals.
- Assess ultrasonic energy to enhance the release of MMC from sampling materials.
  - MMC aliquots (1 mL) were prepared to test the effect of ultrasonic energy (sonication) on cell and spore integrity, and to determine any benefits of this technique with regard to the release of microbes from the metal surface or sampling devices. In this study, ultrasonic energy (25 kHz for 2 min) previously reported to facilitate the release of microbes from sampling materials (Puleo *et al.,* 1967) did not dramatically hinder the cultivation of MMC strains (~80% cultivable after sonication), nor did it compromise the integrity of DNA molecules.

6. Sample Processing

This section describes how the Genetic Inventory team determined the most effective sample processing procedures and protocols suitable for downstream DNA analysis. Appendix B provides a step-by-step manual for the sample processing procedures discussed in this section. The red box in the figure below highlights this section within the overview of the Genetic Inventory task.



6.1 Objectives

Success criterion: Reproducible extraction of representative high-quality DNA from low biomass surfaces

The fields of applied microbiology and molecular biology have undergone considerable technological advancement over the past two decades. Scientific literature teems with reports of microbial diversity from seemingly every niche imaginable (Barton *et al.,* 2006; Giovannoni *et al.,* 1990; Moissl *et al.,* 2007b; Osman *et al.,* 2008; Pace 1997; Priscu *et al.,* 1999), as novel molecular approaches are constantly being reported, providing increasingly accurate measurements of microbial diversity. The onset of DNA-based manipulation and analysis methods has led to sophisticated applications, ranging from quantitative polymerase chain reaction (qPCR), which can be sensitive down to a single cell, to high-throughput sequencing methods that can elucidate the broadest possible spectrum of biosignatures present in a sample (Brodie *et al.,* 2006b; Sogin *et al.,* 2006).

Despite advances in the specificity and sensitivity of molecular biological technologies, the ability to efficiently collect (Bruckner and Venkateswaran 2007) and purify nucleic acids (La Duc *et al.,* 2007a; Moissl *et al.,* 2007b) from low-biomass environments remains a significant challenge, and limits the accuracy of current assessments of microbial diversity in such samples. For any measure of sample bioburden (or biodiversity), a large portion of the actual assayable biomass is unavoidably lost in the act of sample collection (Bruckner and Venkateswaran 2007). Furthermore, a 90% loss of

assayable DNA is typical when extracting nucleic acids from a known titer of bacterial cells (La Duc *et al.,* 2007b). For nutrient-rich environments, the amount of sample loss attributable to sample collection has a negligible impact on DNA-based experiments. However, for the low-biomass samples examined over the course of the Genetic Inventory study, such a loss in total sample biomaterial cannot be merely discounted. With this in mind, the underlying goals of this section were to demonstrate sample-processing protocols to effectively

- Concentrate and uniformly lyse various microbial cell types, thereby enabling more robust, less biased extraction of biomolecules.
- Purify DNA with an automated, commercially available system and compare these results with those of manual extraction methods practiced in industry and academia. Ideal methods to purify DNA from samples should (1) achieve the greatest total yield, and (2) reflect the most accurate account of biodiversity contained in the sample.
  - Perhaps the most meaningful attribute of any nucleic acid extraction regimen is total yield. This is particularly true for low-biomass sample work-up. When processing samples that contain fewer than 10^3 biological entities, it is of utmost importance to ensure that the DNA purification method used to generate the highest possible yield.
  - In addition to total yield, the ability of each DNA purification technique to effectively and uniformly lyse microbiota of varying hardiness is of immense consequence (Karakousis *et al.,* 2006). As environmental samples are typically composed of highly diverse microbiomes, it is important to ensure that the DNA extraction technique used facilitates extraction of the broadest sample biodiversity possible.

6.2 General Approach

Concentration of Sample DNA

To determine the best means of concentrating sample biomass, the Genetic Inventory team evaluated two distinct approaches: traditional vacuum-based filtration and the Amicon Ultra centrifugal filter system (Millipore, Jaffrey, NH, Ultracel-50 membrane Cat #: UFC905096).

Purification of Sample DNA

- **Approach 1:** Initially, the effectiveness of DNA purification from low-biomass SAC samples was tested using five different extraction regimens: (i) a traditional wet-chemistry manual procedure, (ii) MoBio Ultraclean and (iii) Promega Wizard kits, and an automated Axcyte AutoLyser instrument (iv) with and (v) without mechanical (bead-beating) agitation.
  - The mixture of 9 known bacterial cell densities used in this experiment (i.e., the bacterial bisque [BB]) was different from the model microbial community (MMC) used later in the Genetic Inventory study. This BB contained bacterial taxa isolated from low-biomass environments and was prepared exclusively for evaluating DNA extraction in the early part of the Genetic Inventory task. Subsamples of BB were processed in parallel, and DNA extracts arising from each of the methods listed above were subjected to PCR, qPCR, and full-length 16S rRNA gene-based clone library analysis. Results demonstrated that the Axcyte AutoLyser automated DNA purification method consistently yielded both the greatest amount of PCR-amplifiable DNA, and species composition most consistent with the starting suspension; thus, this instrument was adopted as a standard for purifying DNA in the early days of the Genetic Inventory study.
- **Approach 2:** Use of the automated AutoLyser DNA extraction instrument was eventually discontinued due to closure of the Axcyte Genomics, Inc. In response, the Genetic Inventory

team actively sought and tested commercially available automated DNA extraction systems. The Genetic Inventory team placed particular emphasis on ensuring that systems to be considered not only rendered impressive, reproducible results, but also were manufactured and marketed by well-established companies, such as Beckman Coulter, Inc. and Promega.

– Serially diluted MMC subsamples (10^6 to 10^2 cells per mL) were processed in parallel, and DNA extracts resulting from each of several commercially available DNA purification systems were subjected to qPCR. Results indicated that the Promega Maxwell MDx-16 consistently yielded the greatest amount of PCR-amplifiable DNA. The Maxwell MDx-16 instrument also gave rise to the most consistent downstream PCR amplification, even from MMC subsamples of low cell density (10^2–10^3 cells/ml). For these reasons, coupled with considerations relating to cost and continued production, use of this system was adopted as the standard approach for purifying DNA in the later days of the Genetic Inventory study.

6.3 Pooling of Low-Biomass Sample Volumes

Previous studies have demonstrated that spacecraft-associated surfaces are seldom rich enough in biomass to yield PCR-amplifiable DNA following extraction. In light of this, the Genetic Inventory team oftentimes elected to pool several samples together (La Duc *et al.,* 2009a; Moissl *et al.,* 2008; Moissl *et al.,* 2007a; Moissl *et al.,* 2007b; Vaishampayan *et al.,* 2010).

6.4 Concentration of Sample DNA

Each sampling device examined was subjected to three rounds of biomolecule-extraction processing. For example, the macrofoam sponge portion of each BiSKit was mated with its respective sample-extraction module, and with appropriate force, sample volumes were expunged into the collection bottle, per the manufacturer's instructions. Expunged sample volumes were transferred to a 50-mL Falcon tube, and an additional 15 mL of sterile PBS was added to the BiSKit collection bottle, which was then reattached for subsequent reextraction via repetitive expunging (maximum of three 15-ml extractions per sample = 45-ml total volume per sample). Similarly, three rounds of biomolecule-extraction processing were performed for each of the other sampling devices (cotton swabs, nylon-flocked swabs, and 150-cm^2 [12.5 cm × 12.5 cm] polyester wipes). When samples were 45 mL in total volume (the maximum advisable), liquid was aseptically transferred to Amicon Ultra-15 centrifugal filter tubes (Millipore, Jaffrey, NH, Ultracel-50 membrane Cat # UFC905096), which were in turn placed within a Sorvall RC-5B refrigerated centrifuge (Thermo Scientific, Waltham, MA) and spun at 4,000 × *g* for 5 min. Each of these Amicon filter units has a molecular weight cutoff of 50 kDa, which facilitates the concentration of bacterial cells, spores, and nucleic acid fragments larger than 100 bp into a final volume of ~0.5 ml. This volume was aseptically transferred to a sterile microfuge tube.

In contrast to the volumes processed with the aforementioned sampling devices, the heat-shocked planetary protection (PP)–rinse "samples of opportunity" received from the Mars Science Laboratory (MSL) PP implementation team exceeded the volume limitation (45 mL) of the Amicon filtration system. Hence, the total volume of each PP-rinse sample (100 to 600 mL) was first subjected to vacuum filtration through a 0.22-µm pore-size membrane. After filtration, the 0.22-µm membrane filter was aseptically transferred to a sterile 50-mL Falcon tube containing 10 mL of sterile PBS, which was then vigorously agitated via vortex mixing for 5 min. The resulting volume was finally concentrated to ~0.5 ml via Amicon centrifugal filtration (5 min at 4,000 × *g*).

6.5 Bead-Beating prior to DNA Purification

The final Amicon filter-concentrated extract volume (~400 µL) resulting from each sampling device was resuspended in flow-through from the final filtration to a final volume of 1 ml. This 1-ml volume was then divided into two 500 µL fractions, one of which was subjected to bead-beating mechanical abrasion for 1 min at a rate of 5.0 motions/s (MP Bio Lysing Matrix E; using the MP Bio FastPrep-24 Homogenizer). The other 0.5-ml fraction was not subjected to bead-beating. Prior to being loaded into the Promega Maxwell MDx-16 DNA purification instrument, these two 0.5-ml fractions were recombined to a yield a total volume of 1 mL to be subjected to automated DNA extraction (0.5 ml bead-beaten + 0.5 ml untreated = 1 ml total). Processing samples in this manner helped to ensure that the final DNA product sample would contain molecules released from both robust and labile cell types (La Duc *et al.,* 2009b).

6.6 DNA Purification Methods

The basic steps involved in isolating DNA from a cellular suspension are (i) disruption of the cellular structure (i.e., degradation of cell walls and membranes) to create a lysate, (ii) segregation of soluble DNA from cellular debris and other insoluble materials, and (iii) purification of the DNA of interest from soluble proteins and other nucleic acids. Five distinct DNA purification approaches (two kits and three automated systems) were comparatively evaluated in parallel, alongside a traditional wet-chemistry procedure (Sambrook *et al.,* 1989). Each of these DNA purification regimens, detailed in greater extent below, was tested on six replicate 1-ml extracts recovered from 10-ml BB or MMC samples.

6.6.1 Promega Wizard Kit

DNA was purified from samples in accordance with instructions provided in the Wizard Genomic DNA Purification Kit for the isolation of genomic DNA from Gram-positive and Gram-negative bacteria (Catalog No. A1125; Promega, Madison, WI). To maintain consistency and coherently compare results across DNA extraction regimens, the resulting DNA product was rehydrated in 60 µL of manufacturer-supplied rehydration solution. According to the manufacturer, this kit provides a simple, solution-based method for the isolation of DNA from Gram-positive and Gram-negative bacteria. DNA purified with this system is said to be suitable for a variety of downstream applications, including PCR amplification, digestion with restriction endonucleases, and hybridization manipulations.

6.6.2 MoBio UltraClean Kit

An UltraClean Microbial DNA Isolation Kit (MoBio, Carlsbad, CA) was used to purify DNA from BB and/or MMC samples. Briefly, ~1 ml of sample was added to a tube containing a mixture of silicon beads and a proprietary lysis buffer. By combining heat, a chemical detergent, and mechanical shearing with silicon beads, microorganisms were then lysed. Released DNA was then precipitated onto to a silica spin filter, which was washed, and finally subjected to DNA elution. As with all of the extraction methods tested, the final DNA product was eluted from the column in 60 µL of manufacturer-supplied "MD-5" rehydration buffer. According to the manufacturer, this kit has been tested successfully in isolating high-quality genomic DNA from a variety of microorganisms, including bacterial spores and fungi.

6.6.3 Axcyte Genomics, Inc. AutoLyser Automated DNA Extraction System

This approach tested a fully automated DNA-extraction process involving the Axcyte AutoLyser (AA) instrument, which carries out all of the functions involved in DNA purification within a sealed cartridge, with little user interaction. Samples were placed into manufacturer-provided extraction tubes and were processed according to protocols provided by the manufacturer (Axcyte Genomics

Inc., Menlo Park, CA). At the conclusion of a ~30-min processing run, the instrument dispensed purified nucleic acids, suspended in ~60-µL sterile water, into a sterile microcentrifuge tube. Sample volumes of 400 µl were pipetted into manufacturer-provided tubes preloaded with sample buffer and lysis reagents. These tubes were then mated with disposable sample processing cartridges, which attached to the AutoLyser instrument. The "Universal" sample processing program was selected, which is designed to purify DNA from both Gram-positive and Gram-negative bacteria, according to the following protocol: Following an initial pH adjustment to 8.0, the sample solution is first subjected to lysozyme (1 mg/ml) digestion at 45°C for 8 min to assist in breaking down the bacterial cell walls. The sample then encounters a lysis reagent containing guanidinium-chloride, and a nonionic detergent in concert with 400 µg of proteinase-K at 65°C for 8 min to disrupt cell walls and free DNA from protein complexes. Ethanol is then added to the lysate solution, which is then passed through a proprietary membrane that selectively adsorbs all RNA and DNA molecules. This membrane is washed twice with a high molarity salt-detergent solution to remove proteins, carbohydrates, and enzyme inhibitors, followed by three washes with 80% ethanol to remove all traces of salt and detergent. Following a brief spell of heating and drying to remove all traces of alcohol, the membrane is finally flooded with water and heated to 80°C, and purified nucleic acids are eluted in 60 µl of molecular-grade water.

Mid-way through the Genetic Inventory study, the company that manufactured and marketed this instrument (Axcyte Genomics, Inc.) went out of business. Even though the Genetic Inventory team procured a sufficient number of cartridges to complete the task, it was unfortunate that the consumables required for this instrument were no longer available. To this end, the Genetic Inventory team abandoned the use of this system and initiated a search for a market-stable, and yet still effective and reproducible, automated platform.

6.6.4 Beckman Coulter, Inc. Automated DNA Extraction System

Beckman Coulter, Inc. introduced the SPRI-TE Nucleic Acid Extractor for the automated purification of DNA and RNA from culture suspensions and environmental samples. This system processes 1 to 10 samples in approximately 30 min and requires minimal user interaction. Prepackaged, ready-to-use nucleic acid extraction kits are available for preparing genomic DNA or several RNA variants from a variety of samples. The SPRI-TE system incorporates Beckman Coulter's proprietary solid phase reversible immobilization (SPRI) technology, which extracts nucleic acids without the need for centrifugation or a vacuum manifold. The SPRI technology ensures stronger binding and higher quality purification than silica-coated beads, which can shear nucleic acids, leading to lower quality results. The final eluted DNA extract volume was either 50 or 100 µL; an inconsistency the Genetic Inventory team factored into calculations comparing results across the tested DNA purification regimens.

6.6.5 Maxwell MDx-16, a Promega Automated DNA Extraction System

The Maxwell® MDx-16 nucleic acid preparation system offers automation through walk-away extraction, which saves time and labor by eliminating reagent preparation, pipetting, and centrifugation steps. The Maxwell® MDx-16 enables the extraction of DNA, RNA, or protein from up to 16 samples in ~45 min. Using this fully automated nucleic acid extraction system, the manufacturer claims that DNA yield is reproducible across operators and from every sample, and that there is no detectable cross-contamination between samples. The Maxwell® MDx-16 system provides everything needed for nucleic acid extraction, including prefilled reagent cartridges, specialized disposable plastics, and easy-to-understand instructions. This automated system significantly reduces the number of hands-on steps and turnaround time, increasing throughput. In addition, the MDx-16 instrument is a particle processor, not a liquid handler, so there are no clogs or drips. The instrument's unique design enables mixing, capture of paramagnetic particles, binding,

and subsequent purification through a series of capture and release washes. The final purified DNA extract volume was 100 µL.

6.6.6 Standard, Manual DNA Purification Method

According to the well-established procedures of Sambrook *et al.* (1989), each 500-µL Amicon filter-concentrated subsample was first treated with lysozyme (final concentration: 10 mg/ml) for 1 hour at 37°C, to promote the denaturing of Gram-positive cell walls. Samples were then subjected to 0.5% sodium dodecyl-sulfate (SDS) for 30 min at 65°C to lyse cellular phospholipid membranes. This was followed by synergistic Proteinase-K and RNase-A treatments, 20-µg/ml and 5-µg/ml, respectively, for an hour at 37°C. Samples were then mixed with an equal volume of phenol-chloroform-isoamyl alcohol (PCIAA) and centrifuged for 2 min. At this time, the uppermost, aqueous phases were collected via pipette and transferred to new tubes. A 1/10 volume of 3M sodium acetate was then added to each sample, and tubes were mixed vigorously. DNA was then precipitated with two volumes of ice-cold ethanol, washed briefly with 70% ethanol, and resuspended in 10 mM Tris buffer (60-µL) prior to storage at −80°C. Members of the Genetic Inventory team have previously reported the percent of DNA recovery using this extraction method for low-biomass samples to typically be ~10% (Bruckner and Venkateswaran 2007; Bruckner *et al.,* 2005).

6.7 Results of Sample Concentration

As discussed in Section 6.3, the pooling of several low-biomass samples was often required to collect sufficient amounts of template DNA for PCR-amplification of targeted rRNA genes. The results of MMC-dependent lab-controlled experiments showed no significant differences between traditional vacuum-based membrane filtration and Amicon filter centrifugation in the recovery of cultivable microbes and/or 16S rRNA genes. Both of the sample biomass concentration strategies employed in this study facilitated the consistent recovery of ~60% of the initial MMC DNA (in terms of 16S rRNA gene copy number). In a similar vein, known amounts of DNA (10^6 16S rRNA gene copies of *Bacillus pumilus* SAFR-032) were artificially deposited onto swabs (cotton and nylon), BiSKits, and polyester wipes, and total DNA recovery was assayed following the concentration procedure. Considerable variation was observed in the percent recovery of DNA among the sampling devices tested (1 to 30% for swabs and wipes; >90% for BiSKits; Figure 6-1).

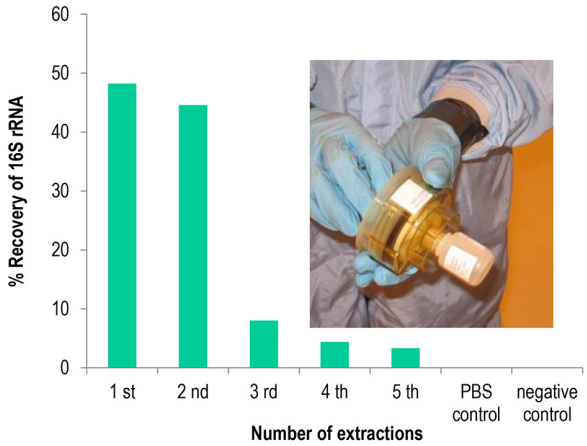


Figure 6-1. Recovery of DNA from biological sampling kits (BiSKits). An increasing concentration (0.1 to 10 µg/BiSKit) of DNA (*B. pumilus* SAFR 032) was added to the BiSKit; DNA was recovered after 1 to 5 extractions using qPCR. Only 45% of the spiked DNA was recovered from one extraction. A minimum of two extractions are required to recover >95% of the spiked DNA (1 µg).

6.8 Results of DNA Purification

6.8.1 Total DNA Yield and Consistency

The efficiencies of various DNA-purification strategies based on post-extraction PCR amplification of rRNA genes are shown in Figure 6-2, and resulting TaqMan qPCR measurements are given in Figure 6-3. As shown, the Axcyte AutoLyser yielded PCR-amplifiable DNA product in all six of its replicate extractions, regardless of the volume of the PCR reaction. When the MoBio UltraClean DNA

extraction kit was used, PCR amplification of the 1.5-kb 16S rRNA gene was successful 67% of the time. Upon employing TaqMan qPCR to quantify the resulting DNA yields (Figure 6-3), the Axcyte AutoLyser again outperformed the other regimens examined, yielding greater than 10^4 16S rRNA gene copies/µL in each of its replicate extractions (Figure 6-4).

As was derived from culture counts, the initial cell density of the BB was 7.2×10^6 CFU/10 ml (Table 6-1). Based on whole genome sequencing of 355 microbial strains, 16S rRNA gene (*rrn*) operon numbers ranged from 1 to 15 for various bacteria and archaea, with 4 *rrn* operon copies standard for α-proteobacteria and 10 for members of the firmicutes (Acinas *et al.,* 2004). It was therefore assumed that members of the BB comprised, on average, 6 *rrn* operons per cell, and that 10 ml of BB was likely to contain 4.3×10^7 *rrn* operons. It was then determined that the best total DNA yield, resulting from the Axcyte AutoLyser, was 4.2×10^4 *rrn*/µl, which equated to 2.5×10^6 *rrn* per 60-µl extraction. The DNA extraction efficiency for the superior AutoLyser approach was therefore $2.5 \times 10^6/7.2 \times 10^6 = 35\%$ without adjusting for multiple *rrn*/cell. After having taken *rrn*/cell into account, the best DNA extraction efficiency routinely achieved was $2.5 \times 10^6/4.3 \times 10^7 = 5.8\%$, rendered by the Axcyte AutoLyser.

The Promega Wizard kit outperformed the MoBio Ultraclean kit and the manual PCIAA-based extraction method in terms of recovered total DNA (Figure 6-3). When prefaced with a bead-beating agitation, the

Method	Extraction replicate	20uL rxn	50uL rxn	100uL rxn
Promega Wizard	A	○	●	◉
	B	●	●	◉
	C	●	◉	●
	D	●	●	●
	E	●	●	●
	F	●	●	●
	(-) control	○	○	○
Axcyte AutoLyser	A	●	●	●
	B	●	●	●
	C	●	●	●
	D	●	●	●
	E	●	●	●
	F	●	●	●
	(-) control	○	○	○
Standard-Manual	A	●	●	●
	B	●	●	●
	C	●	●	●
	D	○	◉	●
	E	○	◉	●
	F	●	◉	●
	(-) control	○	○	○
MoBio Ultraclean	A	●	○	○
	B	●	◉	●
	C	○	○	○
	D	●	●	●
	E	●	◉	●
	F	●	●	●
	(-) control	○	○	○
Igene AutoLyser + Bead-Beating	A	●	●	●
	B	●	◉	●
	C	○	●	●
	D	●	●	●
	E	●	◉	◉
	F	●	●	●
	(-) control	○	○	○

Figure 6-2. Qualitative estimation of total DNA yield. The results of standard PCR amplification are shown following total DNA extraction from each of the methods discussed, in replicates of 6 each (A–F). Sterile, 18 MΩ nanopure water served as a template for the negative control. *Full shaded circles:* positive amplification; *partially shaded circles:* marginal amplification; *unshaded circles:* no amplification.

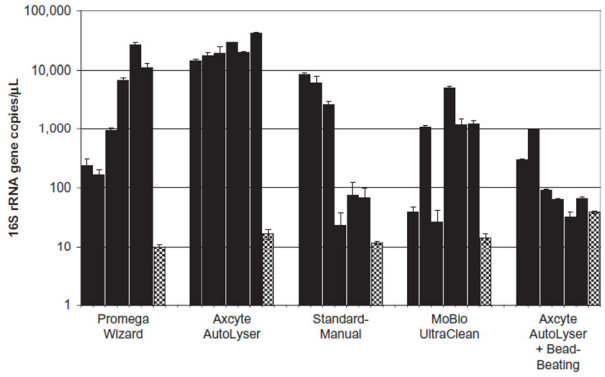


Figure 6-3. Quantitative estimation of total DNA yield. The results of TaqMan qPCR assays on the DNA extract from each of the extraction methods discussed are shown, in replicates of 6 each (A–F). Sterile, 18 MΩ nanopure water served as a template for the negative control. The chart plots the resulting 16S rRNA gene copies per microliter of DNA extract.

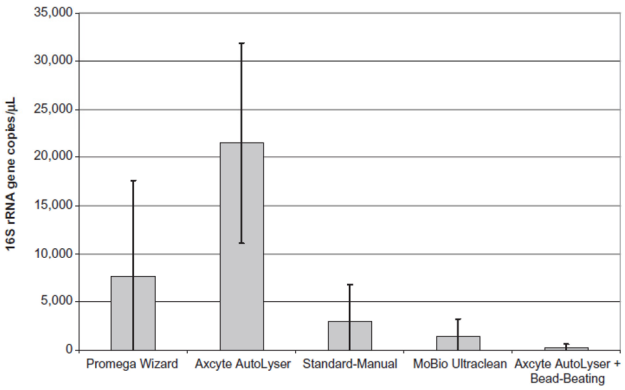


Figure 6-4. Reproducibility of various DNA extraction regimes. Plotted are the mean TaqMan qPCR-based gene copy/µL values arising from the six replicates (A–F) of each DNA extraction regime, with error bars depicting ±1 sigma for each.

Table 6-1. Composition of "bacterial bisque."

Bacteria	Source	CFU Inoculated	Species Richness (% composition)	CFU/mL	*rrn*/mL†
Acinetobacter radioresistens 50v1	Mars Odyssey Spacecraft	7.2×10^7	5	3.6×10^4	2.16×10^5
Bacillus odysseyi 34hs1	Mars Odyssey Spacecraft	4.9×10^7	3	2.4×10^4	1.4×10^5
Bacillus pumilus SAFR-032 spores	JPL Spacecraft Assembly Facility	9.5×10^3	< 1	4.8	2.9×10^1
Curtobacterium flaccumfaciens 48v2	Mars Odyssey Spacecraft	3.2×10^8	22	1.6×10^5	9.6×10^5
Deinococcus radiodurans R1	Nuclear Waste Facility, Hanford, WA	2.7×10^7	2	1.3×10^4	7.8×10^4
Delftia acidovorans 30v3	Mars Odyssey Spacecraft	1.0×10^8	7	5.0×10^4	3.0×10^5
Microbacterium testaceum 47v1	Mars Odyssey Spacecraft	4.6×10^8	32	2.3×10^5	1.4×10^6
Ralstonia picketii 31v3	Mars Odyssey Spacecraft	1.7×10^8	12	8.5×10^4	5.1×10^5
Staphylococcus warneri 82-4	KSC Spacecraft Assembly Facility	2.5×10^8	16	1.2×10^5	7.2×10^5
	Total for 2 L of bacterial bisque		**100**	**7.2×10^5**	**4.3×10^6**

†*Assuming an average of 6 rrn/chromosome*

Axcyte AutoLyser approach (bb-AA) was rather inefficient at yielding high copy numbers of the 16S rRNA gene, which was likely a consequence of DNA adherence to the silicon beads. However, this strategy consistently yielded PCR-amplifiable DNA products (Figure 6-2). The labor-intensive (~6-hr process) PCIAA-based manual extraction required several transfers into various tubes, which coincided with a significant loss of sample biomass (and in particular, DNA) at each transfer.

Consistency can be addressed as it relates to the results of both traditional PCR and TaqMan qPCR. Of the DNA purification methods compared in this study, only the Axcyte AutoLyser delivered PCR-amplifiable DNA product from all 6 replicate extractions (Figure 6-2). If positive PCR reactions are scored +1, weak positive reactions are scored +0.5, and negative reactions are scored 0, then the Axcyte AutoLyser yielded PCR-amplifiable DNA 100% of the time. This consistency diminished a bit when prefacing this technique with bead-beating, as DNA extracted in this manner yielded positive PCR reactions 90% of the time. The extracted DNA product arising from the Promega Wizard kit facilitated 75% positive PCR-amplification, followed by the manual PCIAA-based and MoBio Ultraclean methods at 63% each. As for TaqMan qPCR results, the Axcyte AutoLyser *sans* bead-beating was the only method tested that could statistically be described as consistent (Figure 6-3), yielding a mean 2.2×10^4 copies/µl and a standard deviation of 1×10^4 copies/µL. All of the other extraction approaches yielded standard deviations that exceeded their means.

6.8.2 Conservation of Sample Microbial Diversity

Each of the microbial strains used to test the various DNA extraction motifs was subjected to DNA purification individually, and the DNA product from each strain gave rise to positive PCR amplification. This confirmed that DNA could indeed readily be extracted from each of the microbial constituents of the BB and/or MMC. When these microbes were mixed at densities typical of low-biomass samples, however, the percent recovery of 16S rRNA genes varied. As was observed for total DNA yield, the Axcyte AutoLyser provided the most accurate and most encompassing report of species composition (Table 6-2); these results improved upon prefacing the regimen with bead-beating (bb-AA). The DNA product resulting from Axcyte AutoLyser purification gave rise to clones representing seven of the nine bacterial species comprising the BB. When prefacing this extraction method with bead-beating agitation, PCR-amplifiable DNA was purified from all of the BB constituents, with the exception of *Bacillus odysseyi* 34hs1 which constituted 3% of the BB. It should be

Table 6-2. Comparison of clone-library species richness resulting from various DNA extraction methods.

Bacteria	Known Composition of Bacterial Bisque (%)	Clone library composition (%) resulting from DNA purified via:				
		Standard-Manual Method	MoBio Ultraclean Kit	Promega Wizard Kit	Axcyte AutoLyser	Bead-Beating + Axcyte AutoLyser
***Acinetobacter radioresistens* 50v1** (Gram-negative, g-proteobacteria)	5	–	10	10	5.5	14
Bacillus odysseyi 34hs1 (Gram-positive, spore-forming cells)	3	33	2	30	9.5	–
***Bacillus pumilus* SAFR-032** (spores)	< 1	58	42	54	71	21
***Curtobacterium flaccumfaciens* 48v2** (Gram-positive, actinobacteria)	22	–	–	–	1.5	1.5
***Deinococcus radiodurans* R1** (Gram-positive, non-spore-forming)	2	2.5	–	–	3	3.5
***Delftia acidovorans* 30v3** (Gram-negative, b-proteobacteria)	7	6.5	2	10	12	36
***Microbacterium testaceum* 47v1** (Gram-positive, actinobacteria)	32	–	–	–	–	1.5
***Ralstonia pickettii* 31v3** (Gram-negative, b-proteobacteria)	12	–	–	4	3	14
***Staphylococcus warneri* 82-4** (Gram-positive, non-spore-forming)	16	–	53	2	–	7

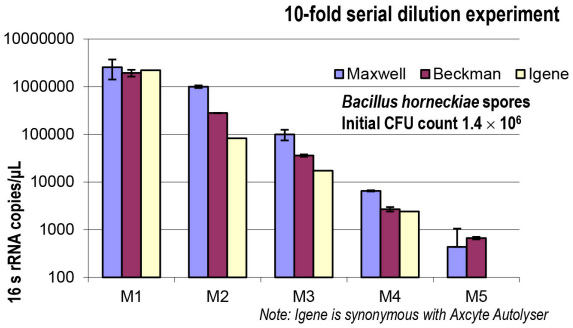
noted that the selective recovery and/or loss of bacterial groups previously has been reported to be a consequence of lengthy bead-beating durations (DeSantis *et al.,* 2005).

The DNA product resulting from the manual PCIAA-based extraction procedure facilitated the cloning of only four of the nine BB constituents. It is likely that the numerous volume transfers required from cell lysis to solvent extraction resulted in the loss of DNA from the five unreported bacterial species. Likewise, the robust cell walls of actinobacteria and *Staphylococcus* spp. may be resistant to the lysozyme treatment used in the manual extraction. The MoBio Ultraclean and Promega Wizard kits elucidated five and six of the nine BB constituents, respectively. The most problematic bacteria, with respect to DNA extraction, were species of *Curtobacterium, Deinococcus,* and *Microbacterium*, all of which were represented in rich abundance in the BB. The elevated recovery of diverse bacterial types resulting from the Axcyte AutoLyser might be due to the purity of the DNA products that it yields. The presence of residual organic solvents in the manually extracted DNA product, silica, or excess SDS in the kit-generated DNA extracts, or any other unforeseen impurities in the final DNA products, cannot be ruled out. Determining this, however, would require extensive study and would have exceeded the scope of the Genetic Inventory investigation.

6.8.3 Evaluation of Commercially Available Automated DNA Purification Systems

Immediately after Axcyte Genomics, Inc. informed the Genetic Inventory team of the imminent closure of the company, Genetic Inventory researchers initiated comparative evaluations of the AutoLyser system alongside two promising commercially available automated DNA extraction systems (Figure 6-5). Spores of *Bacillus horneckiae* were prepared and serially diluted to densities ranging from 10^2 to 10^6 spores per 10 ml, each of which was Amicon filter-concentrated (Section 6.4). As described in the aforementioned protocols, each sample was split into equal halves and one of these fractions was subjected to bead-beating (Section 6.5) prior to being recombined with the

	Maxwell MDx-16	SPRI-TE	Axcyte Autolyser
Weight	19 kg	56 kg	30 kg
Dimensions	33 (w) × 44 (d) × 33 (h) cm	50 (w) × 61.2 (d) × 64.5 (h) cm	43 (w) × 43 (d) × 18 (h) cm
Number of samples	16	10	2
Price of instrument	~$20K	~$30K	~$20K
Price of individual cartridges	<$7–9	$7–9	$17
Time for processing samples	30 min	45 min	40 min
Automation	All methods stored in instrument; all conditions pre-set; one-touch start	Different external memory chips required; need to type method conditions	External computer required



- Maxwell outcompetes other instruments in DNA extraction efficiency
  - High consistency in DNA yield
  - Less lab space, lightweight
  - Less reaction time (30 min)
  - High efficiency (16 samples/run)
  - Complete automation (one-touch protocol)

Figure 6-5. Assessment of various automated systems for DNA extraction efficiency.

untreated fraction. The entire sample volume was then subjected to DNA extraction via each of three automated systems. Unexpectedly, both the Beckman Coulter and Promega systems outperformed the Axcyte system in terms of total DNA yield. Furthermore, the Promega Maxwell MDx-16 instrument consistently yielded greater DNA products than the Beckman Coulter system, especially from low-density (10^3 to 10^5 per 10-ml) spore samples. The DNA yield, in terms of recovered 16S rRNA gene copies, was comparable between these two systems when extracting DNA from samples having densities of 10^6 and 10^2 spores per 10 ml (Figure 6-5). Due to the consistency observed in total DNA yield, relatively high-throughput (16 samples per run), speed (30 min), weight (light), footprint (small), and its field-deployable and completely autonomous nature (Figure 6-5), the Promega Maxwell MDx-16 system was selected for standard DNA purification from samples examined over the remainder of the Genetic Inventory task.

Outcome: The Promega Maxwell MDx-16 automated DNA extraction system effectively extracted high-quality DNA from low-biomass samples and was superior to all other DNA purification regimens examined in this study.

6.9 Key Considerations

Early in the processing and analysis, a decision tree (Figure 6-6) can be applied to determine whether the given sample is of sufficient quality based on initial PCR-amplicon yields (i.e., spiking, PCR product concentration using Bioanalyzer, etc.). Later in the processing, coverage values, rarefaction analysis, and diversity estimations can be conducted to determine how representative the samples are and the thoroughness of the sampling effort.

Figure 6-5 lists the major criteria considered in selecting the Maxwell MDx-16 over other systems. This simple, automated method of nucleic acid extraction consistently yielded (a) the greatest amount of PCR-amplifiable DNA, (b) the most reproducible results throughout the examination, and (c) the most accurate and encompassing assessment of sample species composition (when prefaced with bead-beating agitation). For purifying nucleic acids from low-biomass samples with the intent of elucidating microbial diversity in a highly reproducible manner, as was the case for the Genetic Inventory team, use of the Promega Maxwell MDx-16 yielded the best results.

The Genetic Inventory team advocates concentrating samples to appropriate volumes with centrifugation filters (e.g. Millipore Amicon Ultra) and then dividing this volume into two equal fractions (to meet the optimum loading volume of the Maxwell MDx-16). Continue by subjecting one of these fractions to bead-beating in an MP-Bio instrument (as detailed above), while leaving the other fraction untreated. Finally, recombine these two fractions (see Section 6.5) and load the entire sample volume into the Maxwell MDx-16 purification cartridge and run the process.

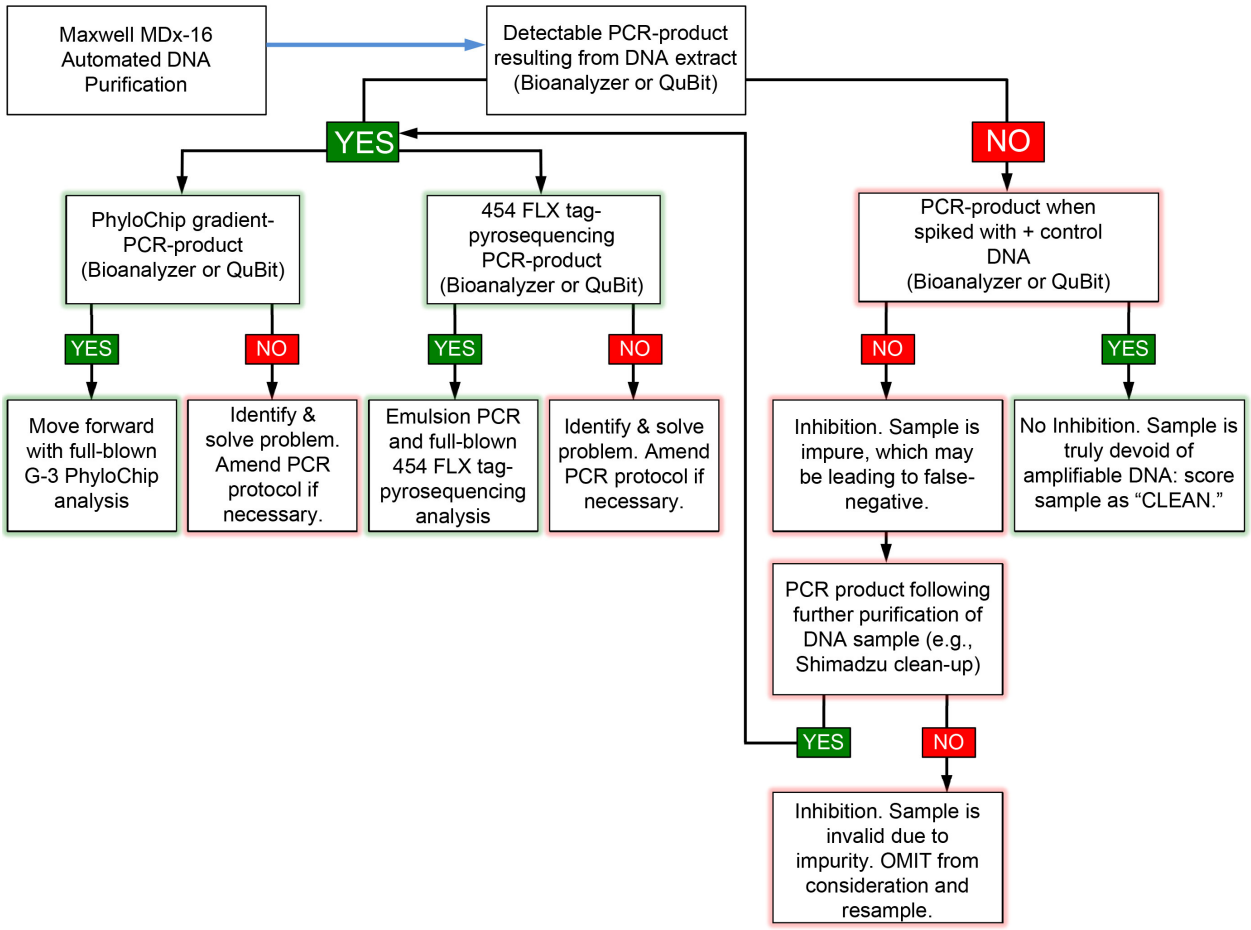
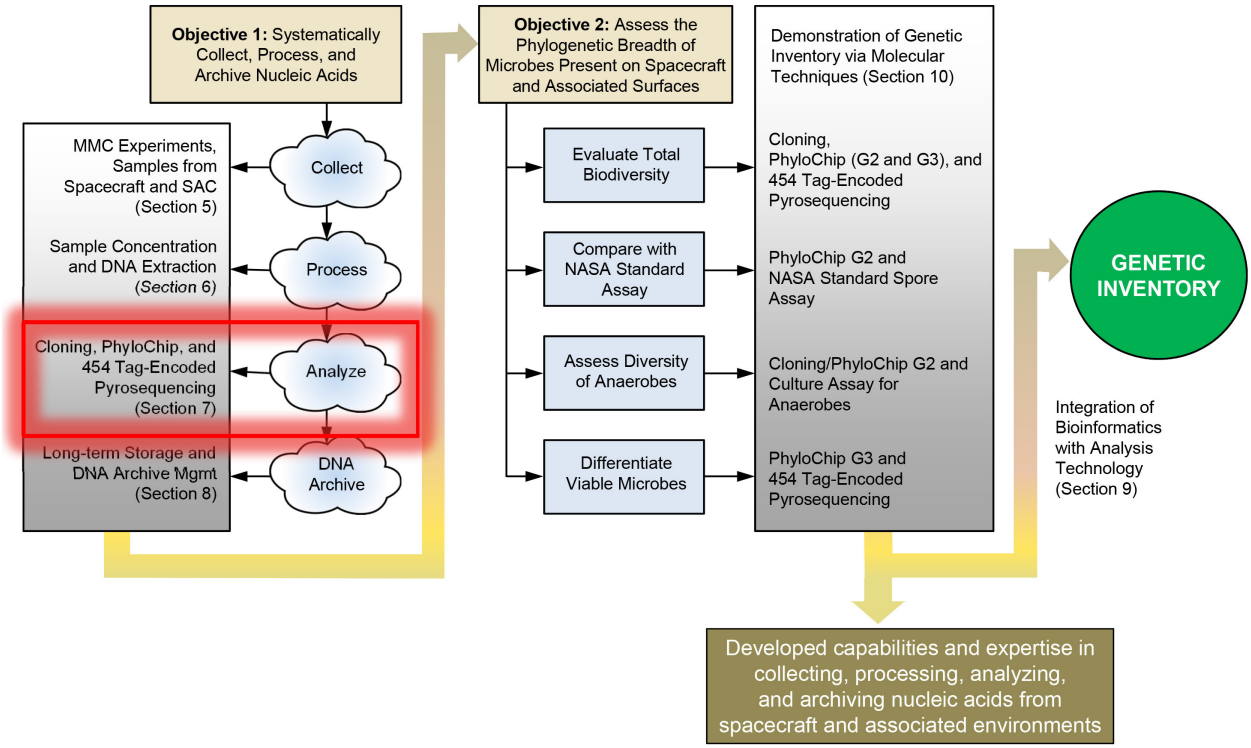


Figure 6-6. Decision-tree for determining sample quality based on PCR-product resulting directly from DNA extraction.

7. Sample Analysis

This section summarizes the materials and methods associated with the numerous molecular analysis techniques that enabled the Genetic Inventory team to (a) select appropriate sampling devices, and (b) conduct a thorough genetic inventory of the surfaces examined. Additional details and scientific results are reported in Sections 5 and 10. The red box in the figure below highlights this section within the overview of the Genetic Inventory task.



7.1 Objectives

Success criterion: Development of end-to-end molecular analysis methods to generate a "passenger list" from low biomass environments.

The majority of studies addressing the microbiological examination of spacecraft surfaces are typically limited to cultivation-based approaches, such as the bacterial spore assay (Kirschner and Puleo 1979), or microscopy-based methods (Brown *et al.,* 2007). Due to the non-cultivable nature of many microbial species (Amann *et al.,* 1995; Kaprelyants *et al.,* 1993; Kell *et al.,* 1998; Oliver 2010; Vesley *et al.,* 1966), culture-based assays often result in only partial identification of the microbial population present in these samples. The key objective of this aspect of the Genetic Inventory study was to capitalize on recent biotechnological advancements and identify and demonstrate innovative modern-day molecular analysis models for (a) quantitatively evaluating the effectiveness and efficiency of various devices in collecting biological samples from low-biomass surfaces, and (b) qualitatively analyzing the most comprehensive genetic inventory of sample microbial diversity to date.

7.2 General Approach

Approach 1: Identify and demonstrate **quantitative** analysis models for evaluating the effectiveness and efficiency of various devices in collecting biological samples from low-biomass surfaces.

At the end of the study, the Genetic Inventory team employed SYBR®-green-based universal quantitative polymerase chain reaction (qPCR) and species-specific qPCR (ss-qPCR) methods of analysis to accomplish the stated objective.

Approach 2: Identify and demonstrate **qualitative** analysis models for generating the most comprehensive genetic inventory of sample microbial diversity to date.

At the end of the study, the Genetic Inventory team demonstrated Generation-3 (G3) PhyloChip DNA microarray and Titanium 454 FLX tag-encoded pyrosequencing analyses to comprehensively inventory the microbial genetic signatures associated with low-biomass surfaces.

7.3 Quantitative-PCR Analysis

Specific 123-bp regions (*E. coli* positions 1369 to 1492) of the bacterial small subunit 16S ribosomal RNA (rRNA) gene (*rrn*) were subjected to SYBR-green-based qPCR (Suzuki *et al.,* 2001) using a BioRad CFX96 thermal-cycling instrument. This method estimated the total number of both live and dead cells and spores present, as it is assumed that all gene copies detected originated from a cell or spore source. All samples were analyzed in triplicate and were exposed to the following thermal cycling conditions: an initial melt at 95°C for 3 min, followed by 40 cycles of melting at 95.0°C for 15 s, primer annealing at 55°C for 30 s, elongation at 72°C for 30 s coupled with an ensuing plate read, and a final melt curve analysis ranging from 60°C to 95°C (increments of 0.2°C every 5 s). Each 25-µl reaction consisted of 12.5 µl of BioRad 2X iQ SYBR Green Supermix (Cat. #: 1708882), 1 µl of each 1369F and 1492R primer (to a final concentration of 700 nM), 9.5 µl of nuclease-free Sigma water, and 1 µl of purified DNA template. Ribosomal RNA gene standards, spanning 10^8–10^2 gene copies/µl, were generated by serially diluting *rrn*::pCR4-TOPO plasmid constructs.

7.4 Species-Specific Quantitative-PCR Analysis

In order to discern differences in the recovery of rRNA genes originating from each of the various microbes present in the model microbial community (MMC) positive control, ss-qPCR assays were designed and carried out targeting each MMC constituent. Table 7-1 lists the species-specific primer sequences, alongside detailed thermal cycling parameters determined for each MMC strain. All gene copy quantifications were performed in triplicate according to previously published protocols for SYBR-green-based qPCR (Kwan *et al.,* 2011). The standards used were serial dilutions of purified, pre-quantified 16S rRNA gene amplicons of the respective MMC target strain. Molecular-grade Gibco water processed through the Promega Maxwell MDx-16 DNA extraction system and qPCR no-template controls (NTC) were included in each run as negative controls for each respective function.

7.5 16S rDNA Cloning and Sanger Sequencing Method

Total genomic DNA was purified directly from each sample, or pooled sample, as described in Section 6.6.3 using an Axcyte AutoLyser instrument. Approximately 400 µl of each Amicon filter-concentrated sample was subjected to MP Bio bead-beating and automated DNA extraction. The total DNA extract volume (~60 µl) arising from any given sample was then split into multiple fractions for downstream PhyloChip DNA microarray and 16S rDNA cloning analyses. To generate PCR amplicons for subsequent cloning manipulations, bacterial *rrn* genes were PCR-amplified with eubacterial-biased primers B27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and B1512R (5'-AAG GAG GTG ATC CAN CCR CA-3'). Two distinct PCR protocols were employed in this study: gradient (see Section 7.6) and conventional. Conventional conditions were as follows: 1-min 95°C denaturation, 2-min 55°C annealing, and 3-min 72°C elongation for 35 cycles using a DNA Engine thermal cycler (MJ Research; Waltham, MA). Following a final 10-min incubation at 72°C,

Table 7-1. Species-specific qPCR primers and reaction parameters.

No.	Microbes	ID	Primer Name	Primer Sequence (5' to 3')	Annealing (°C)	Amplicon Size (bp)
1	*Acinetobacter radioresistens* 50v1	AR	ssARf	GCC TTA TGG TTG TAA AGC	53	94
			ssARr	CTT ATT CTG CGA GTA ACG		
2	*Bacillus megaterium* KL-197	BM	ssBMf	GTG CTA CAA TGG ATG GTA	52	85
			ssBMr	AAT CCG AAC TGA GAA TGG		
3	*Deinococcus radiodurans* ATCC 13939	DR	ssDRf	CCA GAA GTC ACG AAT AAC	54	103
			ssDRr	ATC CAG AAG CGA TAA ATC		
4	*Microbacterium imperiale* 47v1	MI	ssMIf	AAC GGC GTC TAA TAC TGG ATA TGA	54	83
			ssMIr	AGC CCA TCC CAG ACC AAA		
5	*Staphylococcus warneri* 82-4	SW	ssSWf	ATA ACC TAC CTA TAA GAC T	58	124
			ssSWr	ATC CAT CTA TAA GTG ACA		
6	*Micrococcus luteus* ATCC 4698	ML	ssMLf	TAA CCT GCC CTT AAC TCT	55	106
			ssMLr	AAA CCG ATA AAT CTT TCC AA		
7	*Cupriavidus metallidurans* CH34	CM	ssCMf	GGT GGA TGA TGT GGA TTA	64	86
			ssCMr	ATC TCT GCT TCG TTA GTG		
8	*Clostridium sporosphaeroides* DSM 1294	CS	ssCSf	CAA GCA GTG GAG TAT GTG	53	84
			ssCSr	CCT CGT TAG TTG GAT GTC		
9	*Methanobacterium formicicum* DSM 1535	MF	ssMFf	ATT GCT GGA GAT ACT ATT	52	89
			ssMFr	GGG ATT ATA GGA TTT CAC		

1. *Working concentration for all primers: 9 µM*
2. *Reaction mixture (25 µl/sample) consisted of 12.5 µl of BioRad 2x iQ SYBR Green Supermix (Cat. #: 1708882; BioRad, Hercules, CA), 9.5 µL of nuclease-free Sigma water, 0.5 µl of forward primer, 0.5 µl of reverse primer, and 1 µl of purified DNA template*
3. *Cycle parameters for 2-step amplification with melt curve: initial denaturation (95°C, 3 min), denaturation (95°C, 10 s), annealing (see table), melt (60°C to 95°C, 5 s @ 0.2°C increments)*
4. *Total cycles per qPCR run: 40*
5. *Equipment: BioRad CFX96*

the amplification product was purified with a gel excision kit (Qiagen, Chatsworth, CA). Purified PCR amplicons were cloned into the pCR-4 TOPO plasmid vector, and recombinant plasmids were used to transform competent *E. coli* TOP10 cells via TA cloning (Invitrogen, Carlsbad, CA), per the manufacturer's instructions. Approximately 1,000 clones from each sample were selected, subjected to plasmid DNA isolation, and sequenced bi-directionally using M13F and M13R primers at Agencourt Biosciences Corp. (Beverly, MA).

7.6 PhyloChip Microarray Analysis

Due to the paucity of biomass typically associated with spacecraft hardware ($\leq 10^5$ *rrn*/m^2), the concentration of 16S rDNA amplicons loaded into, and analyzed via G3 PhyloChips, varied greatly from sample to sample. Ideally, 350 ng of resulting amplicon product from each sample (or sample set) was added to each corresponding G3 PhyloChip DNA microarray. For samples yielding amplicon product below this threshold, the total amount of amplified product was loaded and analyzed. Ribosomal RNA genes were amplified from DNA extracts via gradient PCR (annealing temperatures of 48.0°C, 48.8°C, 50.1°C, 51.9°C, 54.4°C, 56.3°C, 57.5°C, and 58.0°C) with bacterial-biased primers 27f (5'-AGA GTT TGA TCC TGG CTC AG) and 1492r (GGT TAC CTT GTT ACG ACT T), as described previously (La Duc *et al.,* 2009a). Gradient PCR conditions were as follows: 1 cycle of initial melting for 3 min at 95°C, followed by 35 cycles of 30-s melting at 95°C, and 30-s annealing. Gradient PCR-derived amplicons were then analyzed using G3 PhyloChip (Hazen *et al.,* 2010). To maximize the observed diversity, four separate PCR reactions were

performed for each sample using a gradient of annealing temperatures (48°C, 50.1°C, 54.4°C, and 57.5°C). Whenever possible, a total of 500 ng of PCR amplicon product from each sampling event was used for phylogenetic microarray analysis. Details relating to target fragmentation, biotin labeling, PhyloChip hybridization, scanning, and staining, as well as background subtraction, noise calculation and detection, and quantification criteria are published elsewhere (Flanagan *et al.,* 2007; La Duc *et al.,* 2009a). All G3 PhyloChip microarrays included a spiked mixture of known amounts of non-16S rRNA genes (total 202 ng). Fluorescence intensities of these controls were used to generate standard curves, and thereby normalize total array intensities across samples.

7.7 Titanium 454 FLX Tag-Encoded Pyrosequencing

Bacterial-biased primers 28F (5'-GAG TTT GAT CNT GGC TCA G-3') and 519R (5'-GTN TTA CNG CGG CKG CTG-3') were used to amplify ~500 bp fragments spanning the V1–V3 hypervariable regions of the bacterial 16S rRNA gene. Archaeal-biased 341F (5'-GYG CAS CAG KCG MGA AW-3') and 958R (5'-GGA CTA CVS GGG TAT CTA AT-3') primers were used to amplify ~600 bp fragments spanning the V3–V5 hypervariable regions of the archaeal 16S rRNA gene. A fungal-biased ITS1F (5'- CTT GGT CAT TTA GAG GAA GTA A-3') and ITS4R (5'- TCC TCC GCT TAT TGA TAT GC-3') primer set was employed to amplify ~600 bp fragments of the fungal internal transcribed spacer (ITS) region. These primer pairs were tailored for pyrosequencing by adding a fusion linker and a proprietary 12-nt barcode sequence at the 5' end of the forward primer, and a biotin and fusion linker sequence at the 5' end of the reverse primer (Dowd *et al.,* 2008). A HotStarTaq Plus master mix kit (Qiagen, Valencia, CA) was used to catalyze the PCR under the following thermal cycling conditions: initial denaturing at 95°C for 5 min, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 54°C for 40 s, and extension at 72°C for 1 min, finalized by a 10-min elongation at 72°C. Resulting PCR products were purified via Diffinity Rapid Tip (Diffinity Genomics, Inc., West Henrietta, NY) chemistry, and were then pooled according to the experimental plan. Small fragments were removed with Agencourt Ampure Beads, according to the manufacturer's instructions (Beckman Coulter, Brea, CA).

In preparation for Titanium 454 FLX tag-encoded pyrosequencing (Roche, Nutley, NJ), resulting PCR amplicon size and concentration were measured with DNA 1000 chips using a Bioanalyzer 2100 automated electrophoresis station (Agilent, Santa Clara, CA) and a TBS-380 Fluorometer (Turner Biosystems, Sunnyvale, CA). The total volume of initial PCR product used for subsequent emulsion PCR was 2 µl for strong positives (>10 ng/µl), 5 µl for weak positives (5 to 10 ng/µl), and 20 µl for samples that failed to yield detectable PCR products (<5 ng/µl). This normalization step minimized bias in downstream amplification favoring initially strong PCR products. Approximately 9.6×10^6 molecules of ~600 bp double-stranded DNA were combined with 9.6×10^6 DNA capture beads, and then subjected to emulsion PCR conditions. Following recovery and enrichment, bead-attached DNA molecules were denatured with NaOH and sequencing primers were annealed. A four-region 454 pyrosequencing run was performed on a GS PicoTiterPlate (PTP) using the Genome Sequencer FLX System in accordance with the manufacturer's instructions (Roche, Nutley, NJ). Twenty-four to thirty tagged samples were applied to each quarter region of the PTP. All pyrosequencing procedures were performed at the Research and Testing Laboratory (Lubbock, TX) in accordance with established protocols (Dowd *et al.,* 2008). None of the negative controls, handling controls (sampling devices briefly exposed to the ambient sampling environment), or reagent blanks gave rise to measurable PCR products, and were thus not subjected to pyrosequencing analysis.

7.8 Controls and Lower Detection Limits of Assays Employed

Appropriate environmental controls (which addressed the purity of buffers), molecular reagents, sampling materials, and even fallout facility contaminants, were included at all stages of sample collection, processing, and downstream analysis. Negative controls, handling controls (sampling devices briefly exposed to the ambient sampling environment), and other reagents were also prepared and analyzed. Polyester wipes that were exposed to the sampling environment, but not used for active sampling, were placed (using sterile gloves) directly into sterile rinse solution and processed in blind fashion as handling (negative) controls in all molecular assays. Liquid samples from unopened BiSKit sample cartridges served as negative controls in all molecular assays. In the same manner, ultraclean, molecular-grade sterile water served as a blank (i.e., negative control) to monitor reagent cleanliness. Purified DNA from *B. pumilus* ATCC 7061 was included in all PCR amplification protocols as a positive control. Samples were also intentionally spiked with positive DNA to assess the presence of inhibitory substances, which could lead to false negative results. None of the sampling device materials (e.g., polyester, cotton) used in this study were shown to inhibit PCR, as was evidenced by reliable amplification of internal DNA standards (i.e., 1 pg of *B. pumilus* genomic DNA).

7.9 Key Considerations

By the end of the study, the Genetic Inventory team had successfully designed and established nine distinct ss-qPCR protocols, which aided significantly in quantitatively assessing the molecular integrity and collection efficiency of several sampling devices. A more detailed discussion of results stemming from traditional PCR, universal qPCR, and ss-qPCR validation experiments carried out over the course of the Genetic Inventory study can be found in Section 5 and in previously published reports (Bargoma *et al.,* 2012; Kwan *et al.,* 2011).

After considerable trial-and-error, and a great amount of optimization, the Genetic Inventory team was successful in applying long-read (i.e., Titanium FLX; ~350 bp) 454 tag-encoded pyrosequencing and G3 PhyloChip DNA microarrays, each coupled with a streamlined bioinformatics pipeline, to thoroughly resolve the biodiversity associated with numerous spacecraft and SAC surface samples. At the end of the study, the Genetic Inventory team demonstrated the G3 PhyloChip DNA microarray and Titanium 454 FLX tag-encoded pyrosequencing analyses as the best-available molecular approaches for attempts at comprehensively inventorying the microbial genetic signatures associated with low-biomass surfaces. A more detailed discussion of results stemming from G3 PhyloChip DNA microarray and Titanium 454 FLX tag-encoded pyrosequencing experiments carried out over the course of the Genetic Inventory study can be found in Section 10.

- Traditional 16S rDNA cloning and Sanger-sequencing approaches were not capable of elucidating an all-inclusive genetic inventory from low-biomass environments. These methods are limited by numerical constraints on the number of sequences that can be processed, as well as molecular biases that favor the amplification and/or amplicon ligation of highly represented bacterial taxa, and hence mask the detection of those taxa present in much lower abundance.
- As demonstrated over the course of the Genetic Inventory study, the advent of phylogenetic DNA microarrays and high-throughput pyrosequencing technologies has dramatically increased the resolution and accuracy of detection of specific microbial taxa in mixed microbial assemblages, in spite of the high-background DNA signal associated with many reagents. PhyloChip microarray analyses (the G3 iteration in particular) are superior to conventional

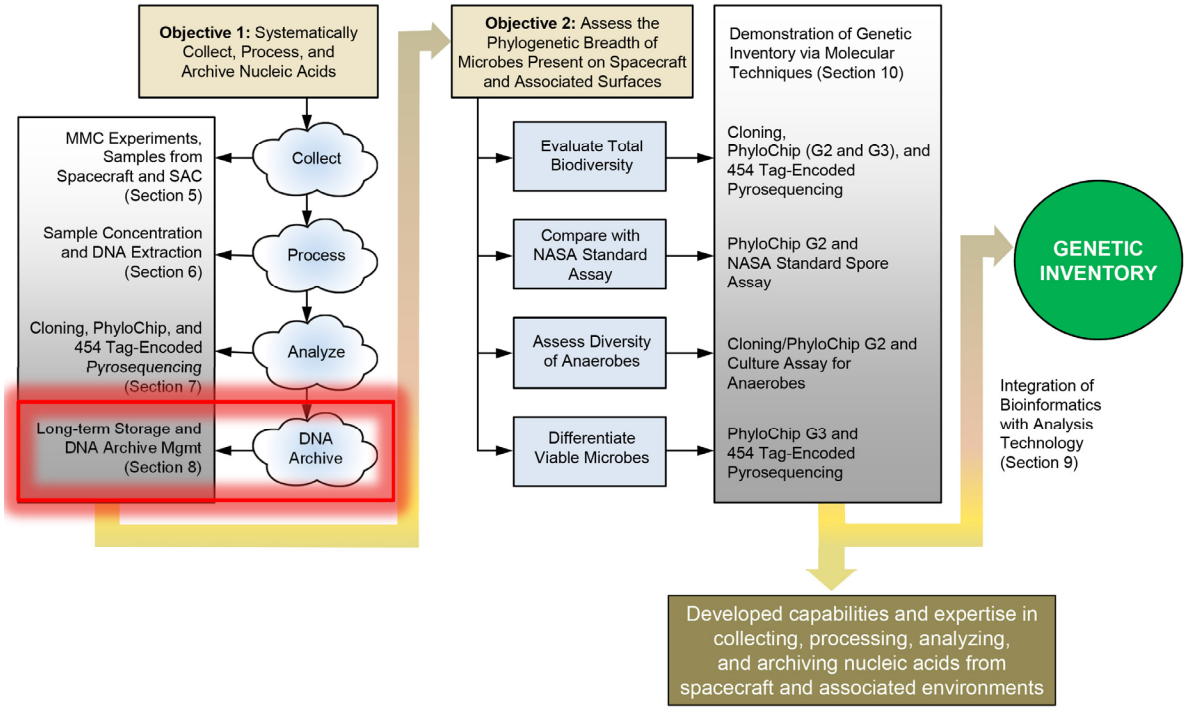
16S rRNA gene cloning and Sanger-sequencing strategies in all aspects of microbial diversity analysis save one: identification of novel, previously unreported taxa.

- An analysis regimen predicated on Titanium 454 FLX tag-encoded pyrosequencing yielded the best data in obtaining a comprehensive genetic inventory, capable of accounting for all reported and unreported microbial lineages.
- If the scope of the study focuses primarily on change comparisons in SAC-associated microbial diversity over time, then G3 PhyloChip DNA microarray approaches would likely suffice.

Outcome: G3 PhyloChip DNA microarrays and Titanium 454 FLX tag-pyrosequencing were shown to be effective analysis regimens, and far superior to traditional 16S rDNA cloning and Sanger-sequencing for generating microbial "passenger lists."

8. Implementation of a DNA Archiving System for the Genetic Inventory Task

This section describes how the Genetic Inventory team determined the most effective system for long-term storage and archiving DNA products purified from low-biomass environmental samples, such as spacecraft and SAC surfaces. The ultimate goal of this aspect of the Genetic Inventory study was to implement a DNA archiving system integrated with effective database management. The red box in the figure below highlights this section within the overview of the Genetic Inventory task.



8.1 Objectives

Success criterion: Demonstration of an effective preservation system for DNA extracted from low biomass environmental samples.

Methods of extracting, amplifying, manipulating, and analyzing sample nucleic acids are widespread in the scientific literature. However, reports on validated strategies for transporting, storing, and archiving samples collected in the field to maintain integrity and ensure subsequent extraction of nucleic acids are scarce (Smith and Burgoyne 2004). To yield nucleic acids of sufficient quality for analysis, standard methods of sample storage and transport generally involve refrigeration or fixation, both of which can prove cumbersome and expensive. Simple refrigeration of samples without first being subjected to typical pre-processing protocols (removal of enzymes, DNA extraction, etc.) is not suitable for long-term storage, as there is no protection from spoilage and/or degradation.

Future success of the Genetic Inventory task hinges on the integrity of the selected method for sample archiving. The Genetic Inventory team set out to ensure that procedures for the purification of DNA from low-biomass samples be developed to (a) achieve the greatest amount of sample DNA yield, (b) enable the most expansive biodiversity assessment possible, and (c) be compatible with envisioned strategies for long-term storage and archiving of sample(s) to ensure that future technologies have access to well-preserved DNA products arising from this Genetic Inventory study.

The Genetic Inventory team studied the effect of long-term storage and temperature (−80ºC or room temperature) on the integrity of DNA. Concurrently, a metadata collection and processing scheme was developed to coincide with the submission of DNA samples collected from spacecraft and associated environments into an archive. In this manner, an appropriate archiving pipeline was established that will enable samples—complete with unique sample names and identification codes—to be easily tracked and identified many years after collection. Key objectives of the DNA archiving aspect of the Genetic Inventory study were to

- Store DNA samples for extended periods of time at room temperature and at −80ºC to determine the best long-term storage strategy.
- Archive DNA samples in accordance with an effective sample information management system (SIMS). This should facilitate not only the long-term storage of purified DNA products, but also the metadata coinciding with each DNA sample.

8.2 General Approach

- *Long-term storage of purified DNA:* A defined bacterial community consisting of eight bacterial species isolated from spacecraft and SAC surfaces was synthesized. DNA was extracted from individual bacterial species and was thoroughly mixed in varying concentrations prior to being subjected to (a) dry storage at room temperature using Biomatrica's SampleMatrix system, and (b) traditional wet storage at −80ºC. The initial microbial DNA mixture was prepared in concentrations ranging from 4 picograms to 40 nanograms, and effect of storage on the integrity of DNA (naked; extracellular) was tested over a ~1-year storage period.
- *Long-term storage of environmental samples:* Immediately following sample collection, samples were concentrated using Amicon filters (see Section 6) and stored at −80ºC for 45 days. The effect of long-term storage on the integrity of DNA (intracellular) was tested.
- *Archival data management:* Metadata collected over the course of sample collection on through to DNA extraction was recorded and managed in accordance with the commercially available SIMS. A user-friendly protocol was developed to store and track the submission of new DNA products.

8.3 Controlled Laboratory–Based Study

The eight bacterial strains (Table 8-1) used in this study had previously been isolated from SACs and co-located spacecraft surfaces (La Duc *et al.,* 2007b). A DNA cocktail (bacterial bisque, BB) was created containing known concentrations of DNA from each of the eight microbial strains.

Table 8-1. Eight bacterial strains used to determine the statistical significance of the effect of storage type (dry sample at room temperature vs. wet sample at –80ºC) on samples.

Site	Microbial Strain	Source Surface
JPL	*Acinetobacter radioresistens 50v1*	Mars Odyssey Spacecraft
	Bacillus odysseyi 34hs1	
	Curtobacterium flaccumfaciens 48v2	
	Delftia acidovorans 30v3	
	Microbacterium testaceum 47v1	
	Ralstonia pickettii 31v3	
KSC	*Staphylococcus warneri* 82-4	Spacecraft Assembly Facility
Hanford, WA	*Deinococcus radiodurans* R1	Nuclear Waste Facility

Each of the eight strains was grown individually in appropriate media and at optimal temperature (Kwan *et al.,* 2011). DNA was extracted from each bacterial culture via manual extraction procedures (Sambrook *et al.,* 1989) and resulting genomic DNA concentrations were determined using a nanodrop instrument. Figure 8-1 provides a workflow schematic, where steps one through four depict the procedures required to prepare both the individual and combined bacterial DNA cocktails. To test the integrity of DNA as a function of the initial concentration, the DNA cocktails were serially diluted in sterile water to the concentrations listed in

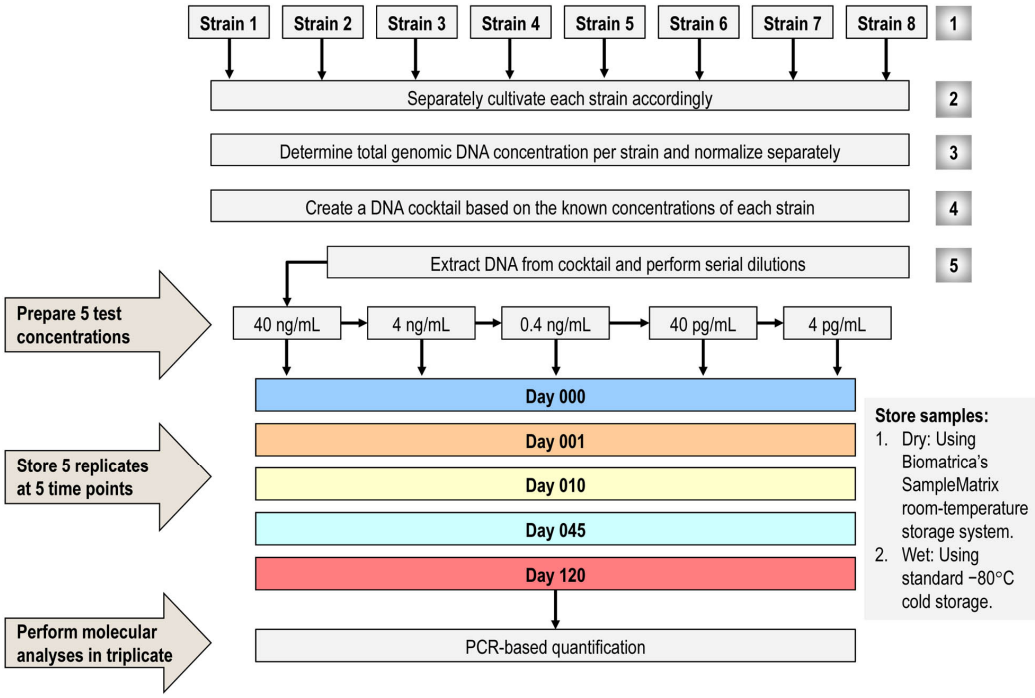


Figure 8-1. Workflow schematic showing steps used to develop the bacterial mix used to study the storage of DNA.

Figure 8-1. Each of the listed concentrations coincides with known 16S rRNA gene copy values, following standardized quantitative polymerase chain reaction (qPCR) analysis. For example, 40 ng is roughly equivalent to 10^6 16S rRNA gene copies, and subsequent serial dilutions each yield one log fewer gene copies. Five replicates of each concentration were prepared.

Each sample was stored under two different storage conditions: (a) dry storage at room temperature using Biomatrica's SampleMatrix system (Figure 8-2a) and (b) traditional wet storage at −80°C (Figure 8-2b). Each sample was also stored for five different time periods: T_0, T_1, T_{10}, T_{45}, and T_{120} (days). Total recovered DNA yield for each sample type was determined via

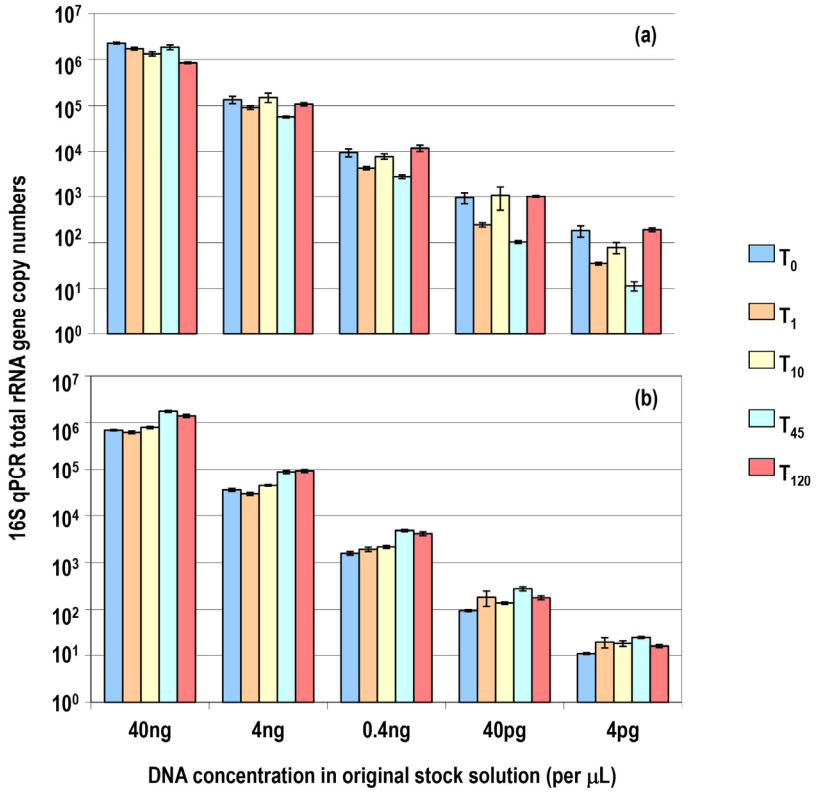


Figure 8-2. DNA recovery efficiency following (a) dry storage at room temperature using Biomatrica's SampleMatrix system, and (b) traditional wet storage at –80°C. Error bars represent standard error of the mean. Bars are color-coded to represent storage time in days (see legend).

TaqMan 16S rRNA gene-directed qPCR chemistry, as described in Section 7.

DNA was recovered from all five concentrations at all five time points. Even the lowest concentration of initial DNA (4 pg) yielded ~10^2 16S rRNA gene copies when stored dry at room temperature. This was one log higher than the number of gene copies recovered from the same initial DNA concentration when stored at −80°C. The dry storage samples consistently yielded greater recoverable gene copy numbers than their frozen counterparts—sometimes at an entire log differential. Although the Biomatrica SampleMatrix system is a superior storage method to storage at −80°C, results stemming from the Biomatrica-stored partitions show greater variability in recovered *rrn* copies between storage times. To determine whether the variability observed in the recovered DNA yields was statistically significant between the two storage conditions, a Student's t-test (Table 8-2) was performed for each concentration condition. While no statistically significant difference ($p < 0.05$) was observed at the highest (40 ng) end of the five-point concentration, concentrations of 4 ng, 0.4 ng, 40 pg, and 4 pg did show a statistically significant difference in the total amount of DNA recovered from dry and wet frozen storage.

Table 8-2. Results of the t-test showing the statistical significance of the effect of storage type (wet sample at –80°C vs. dry sample at room temperature) on samples.

DNA Concentration	p Critical Value
40 ng	0.066
4 ng	0.026
0.4 ng	0.031
40 pg	0.036
4 pg	0.046

Note: The critical alpha value is 0.05 (p <0.05). A p-value less than alpha critical indicates statistical significance. A p-value greater than alpha critical indicates no statistical significance between the two storage systems.

The Genetic Inventory team concluded that even though dry storage facilitated a greater total recovery of DNA from highly concentrated (≥ 40 ng) products, the −80°C storage conditions facilitated more uniform recovery of stored DNA products, even at extremely low concentrations (~4 pg) over an extended period of time. In support of this conclusion, DNA stored at −80°C for 365 days was found to be surprisingly stable, as evidenced by the number of recovered 16S rRNA gene copies (Figure 8-3) for each initial DNA concentration.

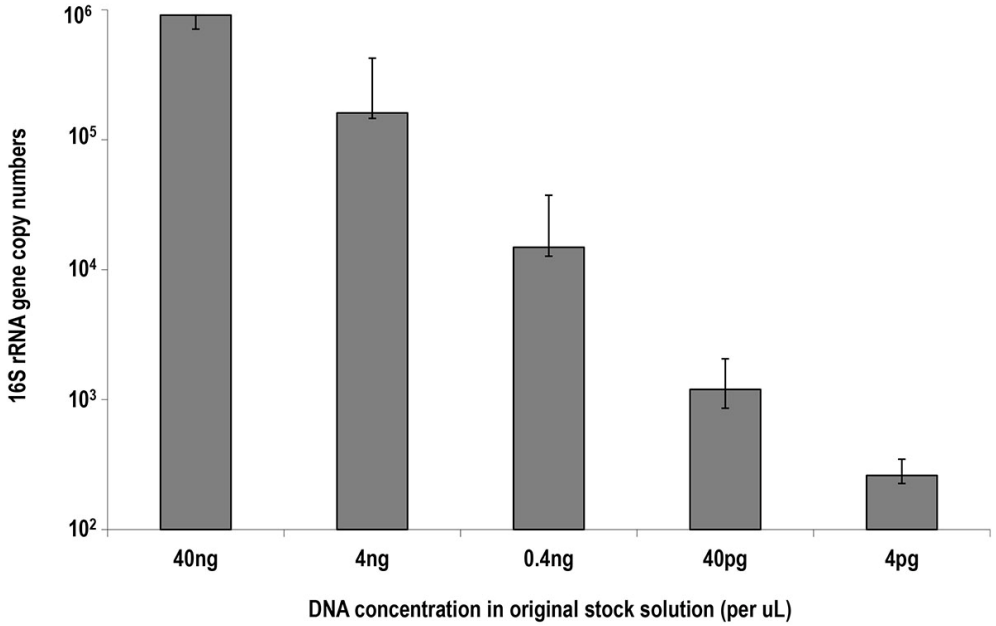


Figure 8-3. DNA recovery efficiency following −80°C liquid DNA sample (in various concentrations) storage for one year. Error bars represent standard error of the mean.

8.4 Environmental-Sample-Based Study

Capitalizing on the knowledge base gained from the laboratory-controlled study, the Genetic Inventory team collected samples from SAC surfaces and studied the effect of storage conditions on the integrity of environmental DNA.

The crux of this investigation was to determine the most effective storage method for samples collected from low-biomass spacecraft associated environments. The results of the laboratory-controlled study led the Genetic Inventory team to conclude that the preferred means of storage is −80°C; but to ascertain the best storage motif for actual environmental samples, a slightly different experimental approach was conducted. The schematic in Figure 8-4 illustrates the experimental workflow, and details how the environmental samples were collected and processed. Two distinct processing techniques were comparatively analyzed to determine the best-suited, most effective method for preserving the integrity of sample DNA.

Twenty environmental-sample replicates were collected from a total sampled surface area of 20 m^2. Each sample was extracted into a volume of 20 mL phosphate buffered saline (PBS) (for a total pooled volume of 400 mL), and replicates of each of the eighteen 15-mL samples were prepared.

Prior to storing at −80°C for up to 42 days (T_{42}), nine of the eighteen sample replicates were concentrated via Amicon filtration (see Section 6). The remaining nine replicates were stored directly at −80°C without the preliminary Amicon filtration step. The purpose of comparing sample storage with and without Amicon filtration was to gauge how significant the filtration step is with respect to DNA recovery, as well as whether any cellular metabolites present in the sample that were <50 kDa adversely affected the integrity of the DNA.

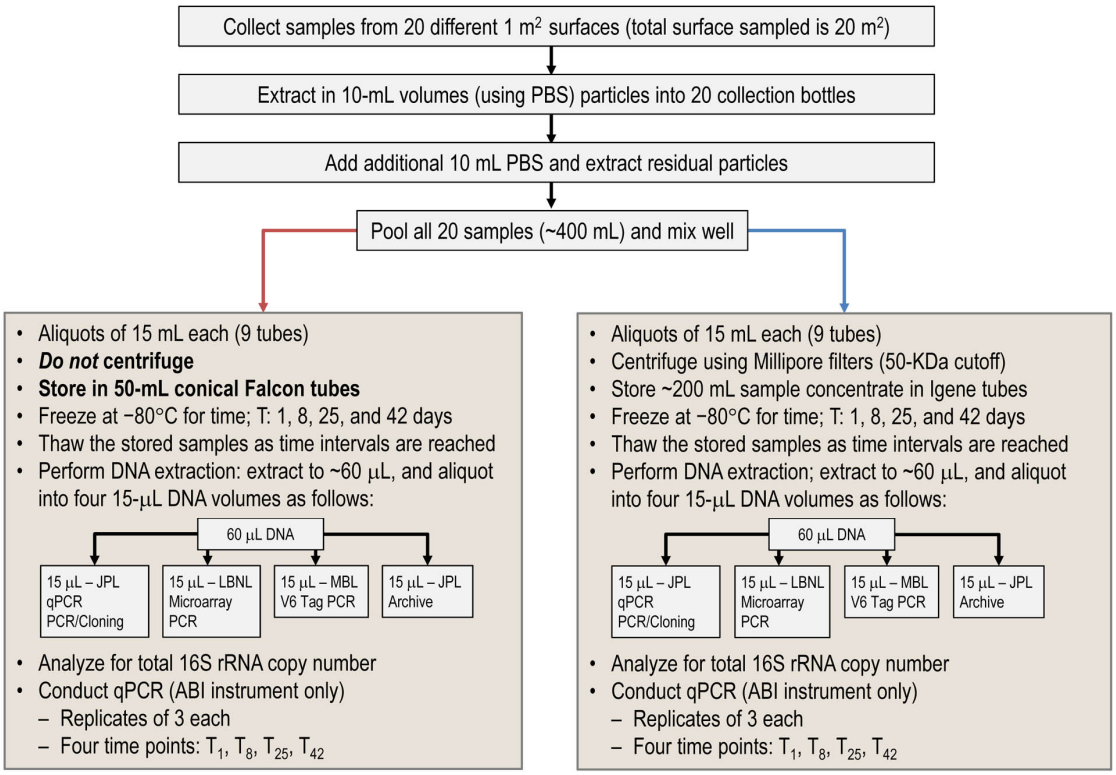


Figure 8-4. Schematic workflow showing the effective collection of environmental samples for the Genetic Inventory task.

For each time period (T_1, T_8, T_{25}, and T_{42}), samples were stored and processed in replicates of two. The ninth sample served as the negative control (T_0). As the allotted storage time was reached for each sample, one-half of the volume of the sample was subjected to bead-beating and DNA was subsequently extracted using an automated extraction system (see Section 6). The final DNA eluate for each sample was 60 µL, which was then aliquoted into four sterile cryovial tubes—three to use for downstream molecular analyses, and the fourth to be archived appropriately. Total DNA yield for each sample type was assessed via TaqMan qPCR analysis, as described in Section 7.

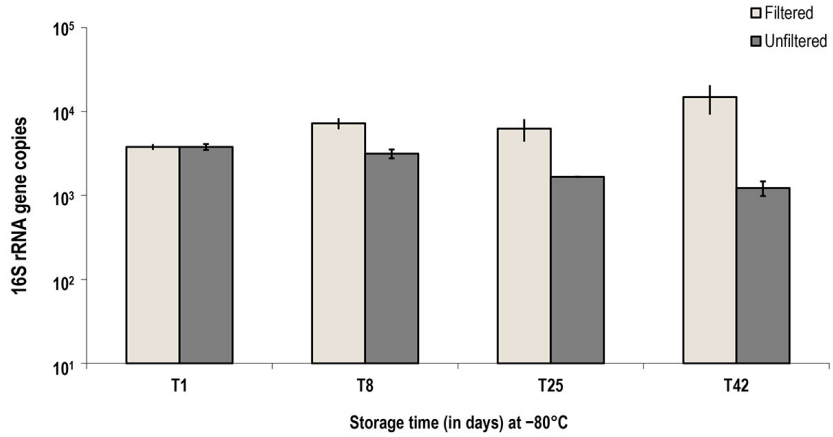


Figure 8-5. Results of 16S rRNA-directed qPCR analysis of environmental samples that had undergone filter-concentration (light tan bars) vs. those that had not (dark gray bars), prior to −80°C storage. Error bars represent standard error of the mean. The p-value of 0.0494 indicates a statistically significant difference between filter-concentrated versus nonfiltered sample fractions that had been stored at −80°C for an extended amount of time.

The fractions of the sample subjected to qPCR showed that by filter-concentrating the samples prior to storage, greater DNA recovery could be achieved after storage. Furthermore, as illustrated in Figure 8-5, the sample fractions that were not filter-concentrated exhibited signs of slow but steady degradation over time—a trend that was not observed for their filter-concentrated counterparts. To assess the statistical significance of this observation, a Student's t-test was performed and the results showed a statistically significant difference between the post-storage DNA recovery from sample fractions that were filter-concentrated and those that were not ($p < 0.05$).

8.5 Key Findings for Archiving Samples

The following are the key findings for archiving samples.

- If the goal of the study is to recover the greatest DNA yield possible following short-term storage of a sample having a high initial concentration, then Biomatrica's SampleMatrix system will yield the best recovery.
- If the goal of the study is to store DNA—of any initial storage concentration—for long-term purposes while minimizing variability in recovery over time, then the more traditional −80°C storage method will yield the best recovery.
- To maximize the recovered DNA yield after storage, it is recommended that environmental samples be filter-concentrated with Amicon filters to remove molecules <50 kDa prior to being archived at −80°C.

The Genetic Inventory task required that precious DNA samples, some of which are unique and/or irreplaceable by virtue of being collected during spacecraft assembly, be preserved for an indefinite amount of time. This is to allow for future research, following the advent of more-advanced analytical biotechnologies. For this reason—and based on the technologies currently available—all Genetic Inventory DNA products archived for indefinite storage were stored at −80°C.

8.6 Perceived Impact and Genetic Inventory–Deployed DNA Storage Practices

Two general rules apply to preventing DNA damage during storage: maintain a low temperature (<−80°C) and use a dry, condensate-free binding matrix (i.e., vial, tube)—both of which minimize the activity of nucleases and general double-strand disassociation. Several biological sample storage manufacturers have developed room-temperature DNA storage capabilities based on anhydrobiosis (dry storage). Qiagen's Qiasafe dry blood storage on Biomatrica's SampleMatrix allows for whole blood to be stored dried at room temperature for extended periods of time. Upon DNA extraction, these samples reportedly yield a much higher DNA recovery than samples stored in conventional (e.g., −20°C and −80°C) storage systems, when stored for 3–6 months. For purified DNA, Gentegra (Genvault/IntegenX) offers storage at room temperature based on an inorganic mineral matrix equipped with protection from oxidation and antimicrobial activity. The Genetic Inventory team tested these two room-temperature storage models and observed that considerable DNA degradation had occurred in the samples after having been stored for 6–12 months. The molecular approaches used during the Genetic Inventory study require that collected samples are amenable to archiving for a prolonged period of time (in excess of 12 months). With all of this in mind, the Genetic Inventory team elected to use the conventional method of storing biological samples in solution at −80°C). Kasper and Lenz (Kasper and Lenz 2004) performed an 8-year study on the effects of DNA stored in water or TE-Buffer (10 mM TrisHCl; 0.5 mM EDTA, pH 9.0). Based on the results of gel electrophoresis and PCR analysis, the team observed no degradation in DNA stored in either water or Tris-EDTA buffer at −20°C or −80°C. This being the case, and until superior dry, ambient storage technologies emerge for the purpose of long-term DNA storage, the Genetic Inventory team remains confident in its decision to archive samples in molecular-grade water at −80°C.

However, liquid storage is not without its own drawbacks. One such drawback is the ubiquitous presence of DNase, which may inadvertently be introduced into the purified DNA sample at any time during sample work-up. To minimize the potential of this occurring, operators are instructed to wear gloves at all times when handling the purified DNA samples. To minimize DNA degradation even further, repeated freeze-thaw cycles should be avoided by partitioning the sample into several smaller volumes. Finally, to minimize the extent of sample loss in the event of an electrical or mechanical failure, aliquots of each DNA sample should be archived and stored in two or more different cryo-freezers, located at distinct geographical locations.

In line with the standard practices implemented by the DNA-Bank network (www.dnabank-network.org/Infrastructure.php), and in accordance with the protocols reported and recommended in much of the related scientific literature, traditional cold storage (−80°C) was preferred and put to practice for long-term archiving of Genetic Inventory–generated DNA products. As such, all DNA samples extracted at the discretion of the Genetic Inventory task were stored in sterile, RNase/DNase-free screw cap tubes (GENEMate, Bioexpress) which were devoid of DNA-damaging polymer additives. The internal shape of these sample storage tubes helps to ensure the lowest possible dead volume, thereby minimizing sample loss. Furthermore, the containers used to store these special tubes have been designed to maximize valuable freezer space, accommodating 81 individual tubes per container. Each DNA sample was assigned a dedicated name and coinciding unique identification number (i.e., barcode)—both of which were printed on a cryo-stable label, which was affixed to the vial.

8.7 Sample Information Management System (SIMS)

The preservation and long term-storage of DNA samples is critical to the integrity of ongoing forensic, epidemiological, clinical, molecular ecology, population, and archeological studies. Millions of nucleic acid samples are actively being processed, analyzed, distributed, and stored worldwide.

The Genetic Inventory task generated a large number of DNA samples (several hundred, representing >2,000 m^2 spacecraft surface area) and generated an immense amount of microbial rRNA gene sequence data. It is in the best interest of the Genetic Inventory team and their affiliates to consolidate and archive these samples in an effective, well-orchestrated manner. Several criteria were considered when selecting the SIMS system, such as user friendliness, cost effectiveness, ease of exporting data, security, ability to accommodate different formats of metadata, customer support, and flexibility in modifying data templates based on user requirements. The commercially available SampleWare (Micronic) program was implemented to provide a database function to help successfully manage the DNA sample archive. The SampleWare system has the following advantages over conventional Microsoft Excel and/or Access–based database management:

1. SampleWare tracks and manages sample inventory not only for samples stored in freezers (−80°C), but also in liquid nitrogen and at room-temperature.
2. With SampleWare, one can easily upload and append files to individual samples (e.g., sequence files, images, PDF reports, and published reference files). The Auto-Attach feature automatically associates new data coinciding with samples. Numerous data files generated daily are cumbersome to upload to a searchable database, and yet associating this data with its corresponding samples will enable one-stop-shopping search events.
3. SampleWare is quick and precise, and provides a simple means of searching and locating specific samples.
4. SampleWare ensures sample security by tracking sample users, roles, and password requirements. It enacts procedures that require users to "check out" samples or mark their location within a workflow. It also itemizes the workflow cycle and assigns ownership to the samples.
5. SampleWare facilitates easy sample tracking. Search results indicate the location of each sample (freezer, rack, slot, container, row, and column); to return samples, individual tubes are scanned and SampleWare indicates the appropriate location to return each sample.

The Genetic Inventory team created a user manual that provides easy-to-follow, step-by-step instructions for submitting sample information into the SampleWare database, and for printing cryo-labels with coinciding barcodes (Appendix B.3.2.3). Two partitions of each and every DNA sample are stored in two different freezers at the Jet Propulsion Laboratory (JPL) (Buildings 89 and 244); it is recommended that two additional fractions be stored in a secure, no-fee, DNA archive. The Genetic Inventory team has not yet identified this third party archiving facility; however, the United States Department of Agriculture Culture Collection might be willing to participate if these samples are made available and distributed openly to the public free of cost.

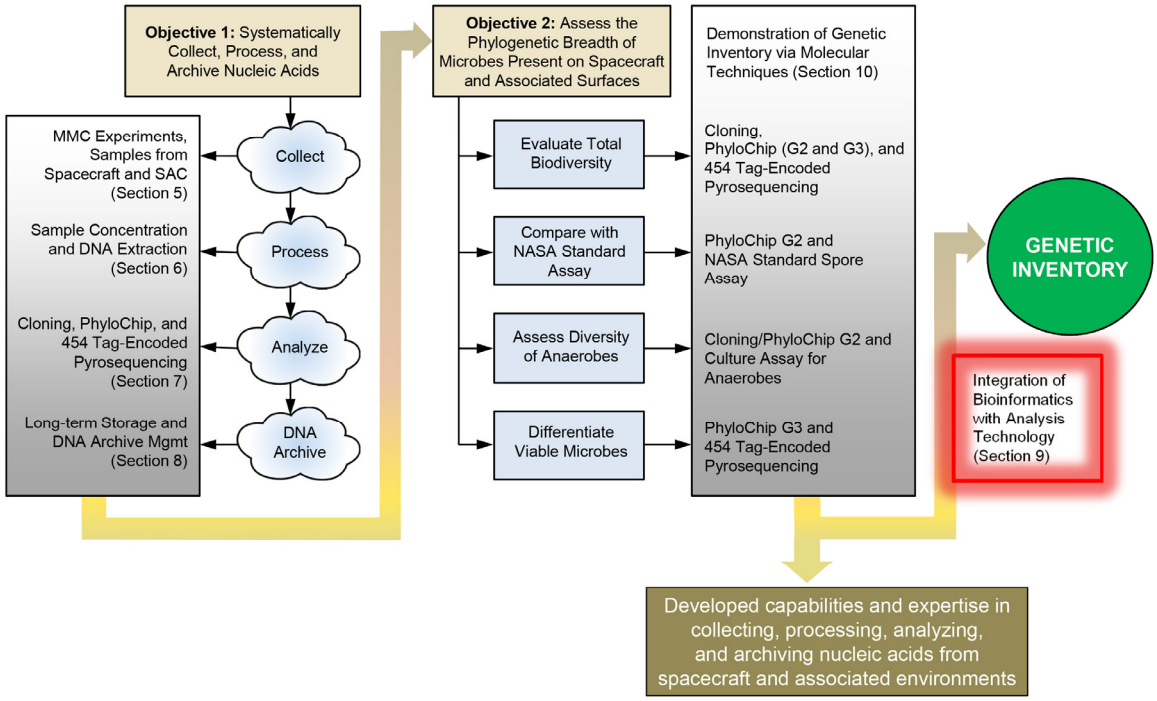
8.8 Summary of DNA Archiving Implementation for the Genetic Inventory Task

Over the course of the 6-year Genetic Inventory study, a total of >2,000 m^2 of surface, pooled into 117 sample sets—directly related to the spacecraft and SAC—were processed, analyzed (using the molecular-based analyses), and subsequently archived as described in this section (and also in greater detail in Appendix B). These unique samples will remain in long-term storage at −80°C to be made available for research studies long after the associated spacecraft are in space, and following the advent of improved biotechnologies equipped with the necessary analytical tools, which will advance further understanding of the samples.

Outcome: The archiving and/or long-term storage of DNA from environmental samples was effectively accomplished at −80°C following Amicon filtration. Purified and concentrated nucleic acids stored better in liquid form at −80°C than at room temperature in dry form. A lab information management system was successfully deployed to manage all corresponding data.

9. Bioinformatic Approaches to Quality Assurance Assessment of Raw Data

This section documents the bioinformatic and biostatistical approaches used by the Genetic Inventory team to interpret the raw data from molecular analysis technologies, and comprehensively describe the microbial diversity present in low-biomass SAC environments. The red box in the figure below highlights this section within the overview of the Genetic Inventory task.



9.1 Objectives

Success criterion: Cogent interpretation of Genetic Inventory raw data using available bioinformatics and biostatistical tools.

Microbial diversity is usually inferred on the basis of variation in the nucleotide sequences of conserved genes (mostly 16S rRNA genes). These measurements can be carried out from within a single community (α-diversity) or between two or more communities (β-diversity), and can be made qualitatively (reporting the presence or absence of distinct community members) or quantitatively (relative abundance of each taxon). It is very important to understand the underlying principles of these measurements and apply rigorous bioinformatic and biostatistical analyses to raw data to bolster confidence in the final assessment.

Molecular microbiology, specifically the application of high-throughput sequencing and bioinformatic and biostatistical data assessment tools, is advancing at a rapid rate. This presents a challenge for researchers striving to generate an exhaustive genetic inventory, as there is no one ideal methodology and keeping pace with rapidly evolving technologies comes at a cost. Moreover, this is exacerbated by the difficulties in cogently comparing the results of numerous available technologies, as the results obtained from any one approach (e.g., Illumina, Ion Torrent, PhyloChip DNA microarray, 454 tag-encoded pyrosequencing) are based on their own intrinsic taxonomy for classifying operational taxonomic units (OTUs).

The Genetic Inventory team used a wide variety of bioinformatic and biostatistical tools to interpret the raw data from molecular analysis technologies, and comprehensively describe the microbial diversity present in low-biomass SAC environments. Several computational tools are available for describing biodiversity, many of which were used by the Genetic Inventory team, in parallel, to interpret and compare the raw data output from differing analysis motifs with a high level of statistical confidence. The objectives of the aspect of the Genetic Inventory task were to

1. Identify relevant bioinformatic pipelines and biostatistical approaches available for interpreting and comparing raw data generated by various innovative molecular analyses of microbial diversity.
2. Select and implement the best bioinformatic and biostatistical methods for interpretation and cross-comparison of raw data generated from the collection, processing, and analysis of SAC environments.

9.2 Available Computational Tools for Assessing Microbial Diversity

There are several available approaches for comparatively assessing the 16S rRNA gene diversity of microbial communities, and novel strategies are emerging at a record pace. This section summarizes the computational tools used over the course of the Genetic Inventory task. Certain strategies were selected and implemented into the standard Genetic Inventory sample processing pipeline primarily for their proficiency in previously reported microbial ecology studies (McDonald *et al.,* 2011; Schloss *et al.,* 2009), specifically for low-biomass samples (La Duc *et al.,* 2012). Discussed herein are factors relating to the applicability and limitations associated with a number of available bioinformatic and biostatistical tools.

9.2.1 Processing of Raw Data

Sanger-sequencing used in this study generated near full-length (~1.5-kb) rRNA gene sequences. Processing these datasets is straightforward. The PhyloChip DNA microarray results were based on the hybridization of multiple probe sets spanning near full-length (~1.5-kb) rRNA gene sequences; the proprietary Lawrence Berkeley National Laboratories (LBNL) software converts these hybridization values into an appropriate OTU calling.

High-throughput sequencing techniques have vastly increased the capacity to understand sample biodiversity (Sogin *et al.,* 2006). However, generating meaningful results from the short DNA sequences (<80 bp) produced by traditional 454 tag-encoded pyrosequencing was not feasible, due to (a) the paucity of biomass contained within the samples, and (b) the unavoidable presence of gamma radiation–induced DNA contamination in reagents and materials. A major technical breakthrough for the Genetic Inventory task—the emergence and application of Titanium 454 FLX tag-encoded pyrosequencing platforms with longer sequence outputs (~350 bp)—provided a viable solution to the gamma radiation–induced contaminant DNA problem. In the end, Genetic Inventory researchers successfully amplified 16S rRNA genes from low-biomass SAC samples and sequenced longer pyrotag reads (~350 bp) using this Titanium 454 FLX tag-encoded pyrosequencing platform. As such, the Genetic Inventory team implemented a systematic approach for ruling out the possibility of non-specific DNA amplification, prior to applying this technique on spacecraft and SAC samples. Adhering to this systematic workflow, the team was unable to amplify sufficient amounts of DNA to perform pyrosequencing from sampling device blanks, reagent blanks, and automated DNA extraction reagent blanks. Such systematic control experiments provided Genetic Inventory researchers with much needed confidence in the applicability and accuracy of this technique with respect to analyzing low-biomass SAC samples.

9.2.2 Quality Control

The raw DNA sequences obtained from automatic sequencing machines are not necessarily of high quality for direct use for downstream sequence comparison methods. Several programs have been designed to specifically solve these problems, and have been tested and implemented by Genetic Inventory researchers (Chou and Holmes 2001; Ewing *et al.,* 1998; Schloss *et al.,* 2009). The importance of initial data quality to the reliability of end results has been repeatedly reported, leading to the following recommendations: (i) filter all raw data according to quality, (ii) remove all chimeric DNA and experimental noise, and (iii) ensure that all sequences correspond to the same region of the 16S rRNA gene. In adhering to these recommendations, the Genetic Inventory team had great success in applying empowering technologies to generate reliable statistically significant data sets (more details in Sections 9.3.1 and 9.3.3).

9.2.3 Reference Sequence Databases

The taxonomic affiliation of a given sequence compared against millions of 16S rRNA gene sequences generated in microbial ecology studies is achieved using a classifier, such as that of the Ribosomal Database Project II (RDP), NCBI, SILVA, and Greengenes. SILVA provides comprehensive, quality-checked and regularly updated datasets of aligned small (16S/18S, SSU) and large subunit (23S/28S, LSU) ribosomal RNA (rRNA) sequences for all three domains of life (Bacteria, Archaea, and Eukarya). The accuracy of the resulting classification depends on the reference sequences and taxonomic hierarchy used to train the model. The influence of the training dataset was recently evaluated (Werner *et al.,* 2012); the outcome of this study indicated that using the largest and most diverse training dataset has numerous advantages. To this end, the Genetic Inventory team used the latest Greengenes bacterial/archaeal 16S rRNA gene sequence database and Greengenes taxonomy (McDonald *et al.,* 2011) for the analysis of PhyloChip data and pyrotag sequences generated in this study. The fungal internal transcribed spacer (ITS) sequences were annotated for taxonomic affiliation during this project using an established ITS TEFAP pipeline (Tedersoo *et al.,* 2010) against the UNITE (Abarenkov *et al.,* 2010) and INSD (Karsch-Mizrachi *et al.,* 2012) databases.

9.2.4 Taxonomy "Calling"

The phylogenetic affiliations and taxonomy calling for the Sanger-sequencing approach is straightforward and well-established (Schloss and Handelsman 2005). However, while DNA microarray (PhyloChip) and high-throughput sequencing technologies share a similar goal (the thorough elucidation of biodiversity present in a given sample), each method makes use of its own intrinsic phylogenetic taxonomy system for assigning environmental targets to taxonomic groups. Since both these technologies are based on different taxonomies, attempts to directly compare biodiversity profiles resulting from SAC samples was rather challenging. In an experiment examining the JPL Spacecraft Assembly Facility (SAF), Sanger-sequencing and PhyloChip, which target the same sequence length and same taxonomy (Greengenes), revealed a highly diverse microbial population. While the Sanger-sequencing was only able to detect 47 OTUs, the G2 PhyloChip detected more than 1,400 unique OTUs (La Duc *et al.,* 2009a). However, such concordance cannot be made between high-throughput sequencing and these two technologies.

High-throughput sequencing results cannot easily or confidently be compared with the results of PhyloChip DNA microarrays because PhyloChip probes (~24-bp) target nucleotide sequences across the entire 16S gene, whereas pyrotag primers target specific hypervariable regions (<450-bp) of the 16S rRNA gene. To directly compare these two distinct data sets would be analogous to comparing apples and oranges, as many OTUs detected via 454 tag-encoded pyrosequencing may have completely lacked sequences complementary to PhyloChip probes in the targeted V6 or V3

regions of the 16S rRNA gene. Conversely, there is also the possibility that regions outside of the targeted V1–V3 hypervariable region lead to detected OTUs by the PhyloChip approach but go undetected via 454 tag-encoded pyrosequencing. Even though the Genetic Inventory task did not compare all of these results directly, an attempt was made on a subset of samples to apply the PhyloChip taxonomy (Greengenes) to the classification of rRNA gene pyrotags (Vaishampayan *et al.,* 2012). However, this strategy most likely precluded the consideration of pyrotag sequences not yet recognized by the published taxonomic system(s). This then results in a significant shortcoming—the reliance on public databases of reported nucleotide sequences (of variable and/or unknown heritage and integrity) for assigning phylogenetic classification to detected pyrotag sequences. Some pyrotags are likely to originate from previously unreported organisms and others from sequencing anomalies (e.g., homopolymers (Haas *et al.,* 2011)]); in these cases, the "nearest match" to the closest microbial neighbor should be substituted as a surrogate.

9.2.5 Sampling Efficiency (Coverage Value)

A variety of statistical approaches have been developed to compare and estimate microbial species richness from samples. From a planetary protection perspective, it is very important to understand the sampling efficiency and extent of diversity captured (i.e., coverage) during sampling exercises. Accounting for the widest possible spectrum of microbial diversity present on and around the spacecraft will allow projects to gauge more accurately the probability of forward-contamination; e.g., a largely proteobacterial population might not be as worrisome as a community dominated by actinobacteria and spore-forming firmicutes. With this in mind, the Genetic Inventory team tested several sample collection devices and techniques with the goal of recovering the greatest number of microorganisms possible from SAC surfaces (see Section 10).

9.2.6 Rarefaction Curves

The results of rarefaction analysis are now reported by most microbial ecology studies. This test compares the observed species richness between samples that have been unequally sampled (i.e., those with disparate coverage values). A rarefaction curve is generated by averaging the randomizations of an observed accumulation curve. This graphic presentation allows one to compare the observed species richness between samples; however, it does not depict a true measure of the actual richness of the communities. A key limitation of these statistical techniques is that diversity comparisons require OTU definitions. Typically, microbial "species" classifications are based on thresholds of percent genetic dissimilarity defined by the researcher. The Genetic Inventory team defined microbial "species (OTU)" at a 3.0% dissimilarity level (i.e., three bp changes in every 100 bp). Another means of determining how well microbial communities have been sampled is to plot their rank-abundance curves. In this analysis, species are ordered from most to least abundant on the x axis, and the abundance of each type observed is plotted on the y axis (Hughes *et al.,* 2001).

9.2.7 Richness Estimation

In contrast to rarefaction analysis, richness estimators approximate the total richness of a community within a given sample, which can then be compared across samples. This approach can be based on either extrapolation from accumulation curves, parametric estimators, or nonparametric estimators. Ultimately, the extrapolation of the true richness contained in a given ecosystem from its calculated estimated richness is possible only when the environment has been sampled repeatedly and exhaustively. Since most microbial ecology projects require comparisons of relative diversity only (as statistically valid exhaustive sampling is seldom achieved), an estimator that is consistent with repeated sampling is immensely valuable.

9.2.8 Diversity Analyses

Over the past 25 years, researchers have deposited more than 10^6 rRNA gene sequences in public repositories, such as the National Center for Biotechnology Information's (NCBI) GenBank. With the increased availability and rapid evolution of sequencing technologies, this number of sequences doubles every 15 to 18 months. At present, a typical microbial diversity study of significant statistical credence generates 10^4 to 10^5 sequences. This massive influx of sequence data from high-throughput sequencing platforms has forced computational microbial ecologists to develop software tools that enable the high-throughput analysis of these complex datasets.

Recent publications have discussed the comparisons between some available sequence data analysis methods and their potential advantages in computing OTU distribution [(Huse *et al.,* 2010; Schloss 2010; White *et al.,* 2010). The first step in most data analysis pipelines is removing primers and omitting low-quality sequence information. Next, a multiple sequence alignment (MSA) is used to calculate evolutionary distance—this is the most demanding step with respect to computational resources, and bears immense impact on downstream OTU classification and abundance estimations.

The application of these techniques has been greatly facilitated by the accessibility of required tools in readily available software suites, which lend themselves to simple, straightforward implementation. Some of these packages include QI-IME (Caporaso *et al.,* 2010), mothur (Schloss *et al.,* 2009), PANGEA (Giongo *et al.,* 2010), (Cole *et al.,* 2009), CROP (Hao *et al.,* 2011), ESPRIT (Sun *et al.,* 2009), and the RDP pipeline. Most researchers commit to using one particular workflow, as it is inconvenient to routinely adapt to the combination of two or more pipelines. Most of these analyses produce consistent results that may be used to gauge changes in biodiversity; however, it is the particulars of each individual dataset that dictate which workflow should be applied (Barriuso *et al.,* 2011).

The mothur suite is an object-oriented, platform-independent, continuously evolving software pipeline that is free to the public (Schloss *et al.,* 2009). Mothur software integrates the algorithms of previous tools, such as DOTUR, SONS, TreeClimber, LIBSHUFF, and UniFrac, in addition to (i) more than 25 calculators for quantifying key ecological parameters for measuring α and β diversity; (ii) visualization tools including Venn diagrams, heat maps, and dendrograms; (iii) functions for screening sequence collections based on data quality; (iv) a NAST-based sequence aligner; (v) a pairwise sequence distance calculator; and (vi) the ability to call individual commands either from within mothur, using files with lists of commands (i.e., batch files), or directly from the command line, providing greater flexibility in setting up analysis pipelines.

Early in the task, the Genetic Inventory team used a handful of these algorithms to process data generated via cloning and Sanger-sequencing (described in detail in Section 9.4). Dr. Schloss (the developer of mothur software), who participated in the second Genetic Inventory workshop, was well aware of the goals of this task and continuously supported Genetic Inventory researchers in analyzing and interpreting raw data. Section 9.3.3 describes in detail the implementation of the mothur software–based bioinformatic pipeline for the Genetic Inventory task.

9.3 Implementation of Bioinformatic and Biostatistical Methods for Raw Data Generated over the Course of the Genetic Inventory Task

The Genetic Inventory team adapted and applied advanced molecular approaches to more accurately assess the widest possible spectrum of biodiversity present on SAC surfaces. A suite of publically available and in-house developed bioinformatic and biostatistical tools were used to

process the raw sequence data and interpret microbial community structure with statistical confidence. Since no single molecular technique or statistical sequence analysis tool can answer all of the various questions pertaining to microbial ecology, the Genetic Inventory team applied various approaches and tools for analyzing and interpreting raw sequence data, which are described here in detail. The key steps taken in studying SAC-associated microbial ecology over the course of the Genetic Inventory task were as follows:

1. Generate raw Sanger sequence, pyrotag sequence, and microarray hybridization data.
2. Feed raw data into appropriate bioinformatic and biostatistical pipelines to derive microbial diversity, community characteristics, and richness.
3. Compare community structure and shared membership between samples.
4. Establish the core microbiome and determine the frequency at which various microbial lineages are encountered to generate hypotheses regarding those of greatest interest from a planetary protection perspective.

9.3.1 Cloning and Sequencing

Over the past 25 years, sequence analysis of polymerase chain reaction (PCR)-amplified and vector-cloned rRNA genes has become the well-established standard for characterizing relationships between microbes and identifying the phylogenetic affiliation of unknowns. As a result, the total resolvable microbial diversity is now estimated to be threefold greater than that based solely on cultivation (Pace 1997). The Genetic Inventory team explored this approach for the assessment of biodiversity-associated SAC surfaces.

Sequence Quality Assessment

Phred: Phred is a base-calling program widely used by large academic and commercial sequencing laboratories for DNA sequence traces. Phred reads DNA sequence chromatogram files and analyzes the peaks to call bases, assigning quality scores ("Phred scores") to each base call. Quality scores range from 4 to around 60, with higher values corresponding to higher quality. The quality scores are logarithmically linked to error probabilities. The high accuracy of Phred quality scores makes them an effective tool to assess the quality of sequences. The most commonly used method is to count the bases with a quality score of 20 and above (sometimes called "high-quality bases"); the resulting number is often called the "Phred20 score." The Genetic Inventory team retained only high-quality (PHRED >20) sequences for further consideration.

STITCH: STICH is an algorithm used to splice, trim, identify, track, and capture the uniqueness of 16S rRNA gene sequence pairs using public or in-house databases. Until only recently, the PCR amplification of 16S rRNA genes followed by cloning and the sequencing of numerous individual clones was an extensively used molecular approach for elucidating microbial diversity. This Sanger-sequencing process typically uses a forward and reverse primer pair to produce two partial reads (~700 to 800 bp each) that overlap and cover a large region of the 16S rRNA sequence (~1.5 kb). In a typical application, this approach generates large numbers of 16S rRNA sequence datasets that can overwhelm manual processing efforts—leading to both delays and errors. The approach presents two computational challenges: (1) the assembly of an error-free composite sequence from the two partial reads, and (2) the subsequent appropriate identification of the organism represented by the sequenced clone. In collaboration with the University of Houston, the Genetic Inventory team developed a software package to search, trim, identify, track, and capture the uniqueness of 16S rRNAs using public and in-house database [STITCH; (Zhu *et al.,* 2011)]. This publically available tool offers automated sequence pair splicing and genetic identification, thus simplifying the

computationally intensive analysis of large sequencing libraries. It is a fully automated pipeline to process paired 16S rRNA sequence reads and to determine the genetic affinity of the underlying organism. STITCH software, accessible at http://prion.bchs.uh.edu/stitch/, is able to handle high-throughput datasets and generate intermediate information that the user can access for other purposes. It creates a spreadsheet with information relating to the processed sequences, allowing the user to obtain further information by redirecting the user to the related online resources.

Multiple Sequence Alignment: Multiple sequence alignment (MSA) typically refers to the alignment of three or more nucleotide sequences of similar length. From the output of this computational analysis, homology can be inferred and evolutionary relationships between the sequences studied. Over the course of the Genetic Inventory task, nucleotide sequences were aligned using Clustal W2 (Larkin), and a Jukes-Cantor-corrected distance matrix was constructed using the DNADIST program from PHYLIP (PHYLIP). The DNADIST program exploits dissimilarity in nucleotide sequences to compute a distance matrix, in accordance with three different models of nucleotide substitution. The Jukes and Cantor model assumes that there is independent change at all sites, with equal probability.

Rarefaction Analysis: By accounting for distance matrices, which describe the genetic distance between DNA sequence data, the DOTUR program bins sequences into OTUs according to either the furthest, average, or nearest neighbor algorithm for all possible distances. Using OTU composition data, DOTUR constructs rarefaction curves for sampling intensity, richness estimators, and diversity indices. Rarefaction curves were generated by plotting the number of OTUs observed against the number of clones screened. The coverage of clone libraries was calculated according to Good's (Good 1953) equation: C= [1-(n1/N)]*100, where C is the homologous coverage, n1 is the number of OTUs appearing only once in the library, and N is the total number of clones examined. In a given environmental sample, the number of different types of organisms observed increases with sampling effort until all types are observed. The correlation between the number of types observed and sampling effort provides information pertaining to the total diversity of the sampled community. This pattern can be visualized by plotting the number of OTUs observed against the number of clones screened (sampling effort). The Genetic Inventory team applied rarefaction analysis to estimate sampling intensity and generate coverage values for sampling efforts (Figure 9-1). Based on these results, it can be said that macrofoam (C=>90%) collected the majority of the microbes associated with the surfaces compared to swabs (C=72 to 88%)

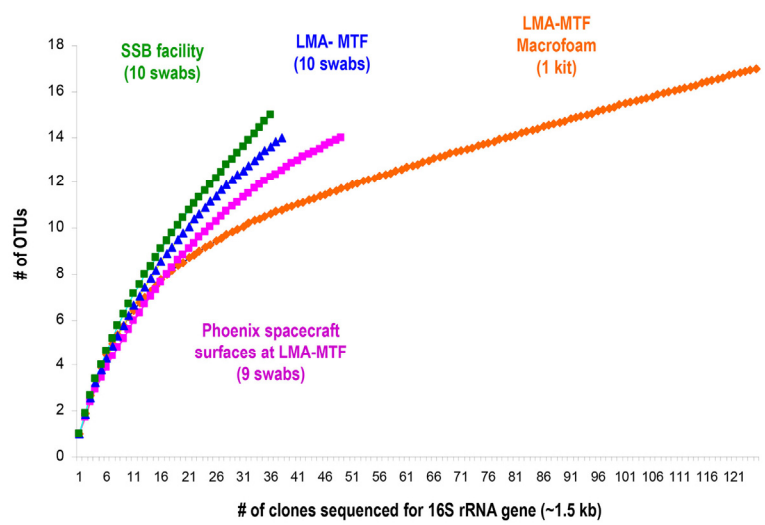


Figure 9-1. Rarefaction curves analyzing the sampling efficiencies of different sampling materials. Macrofoam exhibits much higher coverage values (>90%) than does cotton swab sampling (72% to 88%).

Microbial Diversity Assessments: The assessment of microbial diversity in a natural environment involves two fundamental aspects, species richness (number of species present in a sample) and

species evenness (distribution of relative abundance of species) (Magurran 2005). Various statistical approaches for determining the richness and evenness of SAC-associated microbial communities were used during the Genetic Inventory study. In order to estimate species richness, the Genetic Inventory team binned resulting 16S rRNA gene sequences into OTU or phylotype clusters using DOTUR (Schloss and Handelsman 2005). The criterion used to define an OTU at the species level was a 3% nucleotide sequence divergence. Section 10 provides details of the microbial diversity assessment of the SAC surfaces based on the Sanger-sequencing method.

Community Membership: Two microbial communities may share the same membership, but not necessarily the same structure. Likewise, if two communities have the same richness, they may not necessarily have the same membership. With this in mind, the Genetic Inventory team used the SONS (shared OTUs and similarity) program to determine the phylogenetic resolution of the communities' memberships and structures. This program compares the memberships and structures in communities by accounting for the abundance distributions of OTUs that are either endemic to one community or shared by two communities. It measures the fraction of OTUs shared by two communities as a function of genetic distance. During the Genetic Inventory study, comparisons between clone libraries with the SONS program (Schloss and Handelsman 2006)—which thereby considered the OTUs described by DOTUR and mapped their occurrence in each clone library—enabled the identification of exclusive and overlapping populations. Similarly, the LIBSHUFF program (Schloss and Handelsman 2004), designed to compare and determine significant differences between two 16S rRNA gene sequence libraries, was used to understand differences in bacterial populations between different environments with statistical confidence (Figure 9-2).

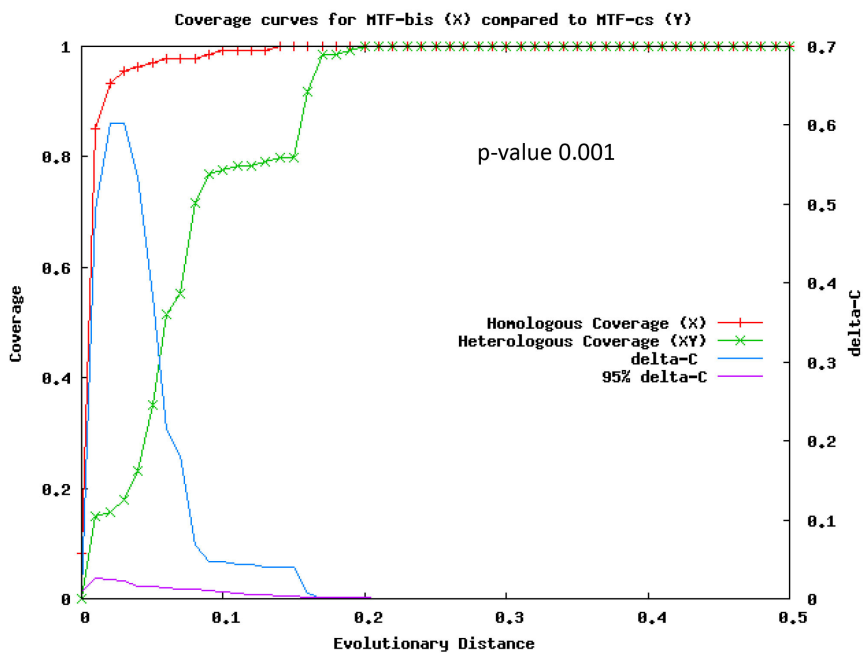


Figure 9-2. LIBSHUFF analysis calculating the statistical difference in microbial population of MTF-macrofoam and MTF-cotton swabs.

Environmental Clustering: The UniFrac software suite (Lozupone *et al.,* 2006; Lozupone and Knight 2005) enabled the comparison of environmental clustering of microbial communities using phylogenetic information from the clone libraries under investigation. In this analysis, each sequence was assigned with an environment name based on the sample from which it was derived. The UniFrac metric can be used to determine whether the microbial communities of two or more distinct environments are significantly different. Communities are significantly different if the UniFrac value for the real tree is greater than that expected if the sequences were randomly distributed between the environments. A P-test was performed to test how significantly each pair of environments differs from one another. Jackknifing is a statistical resampling technique that determines if the same results would occur using only a random sample of part of the data. A jackknife environment cluster test, which

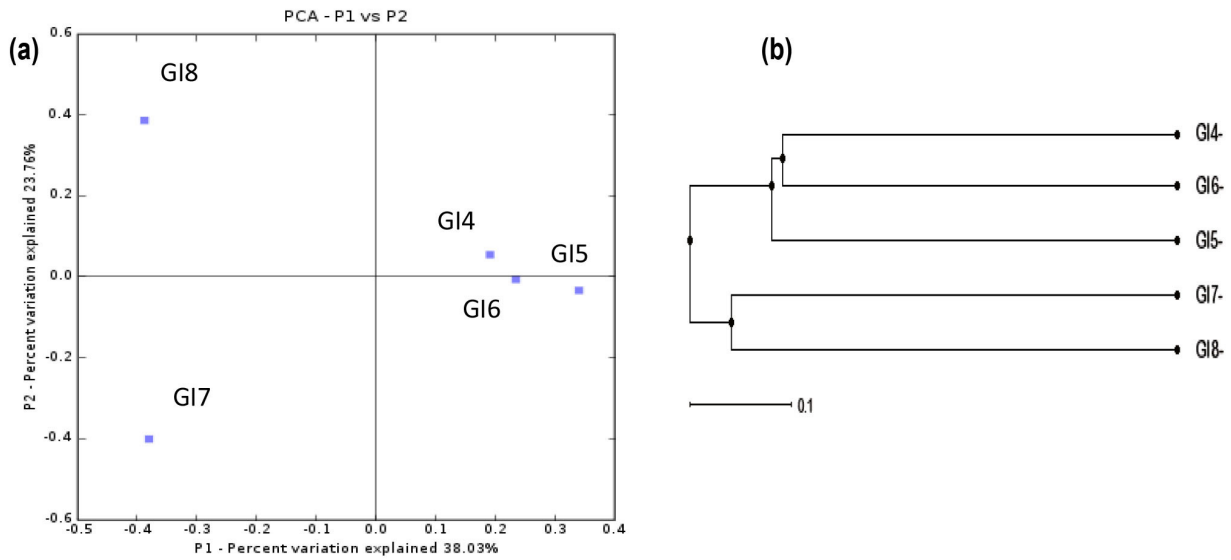


Figure 9-3. Statistical analysis using the UniFrac tool to compare significant similarities or differences between bacterial diversity of different sampling events. (a) Principal component analysis; (b) environmental clustering.

samples a smaller number of sequences from each environment and determines whether the clusters are well-supported, was also carried out. Venn diagrams were also constructed to illustrate the recurrent and overlapping detection of bacterial species.

Ordination Plots: Principal coordinates analysis (PCA) was performed to determine which environments were most similar to each other. PCA is a multivariate statistical technique for finding the most important axes along which samples vary and identifying clusters accordingly. Based on the UniFrac metric calculated for "environmental clustering" (see above), PCA calculates the distance matrix for each pair of environments. These distances are then transformed into points in space, where they are subjected to one fewer dimension than the number of samples. The principal components, in descending order, describe how much of the variation each of the axes in this new space explains. The first principal component segregates the most impactful determinant. Genetic Inventory researchers performed statistical analysis using the UniFrac tool and observed significant differences between bacterial diversity of different sampling events based on PCA and environmental clustering (Figure 9-3).

9.3.2 Bioinformatic Processing of Raw PhyloChip Data

In parallel to the cloning and sequencing approach, advancements in DNA microarray technologies have facilitated the detection of almost all known bacterial taxa. A recently updated version (Generation 3 [G3] PhyloChip) is able to categorize prokaryotes (known bacteria and archaeal OTUs) into 31,000 taxonomic bins via hybridization of 1,100,000 25-mer probes targeting sequence variations in the 16S rRNA gene. Stringent computational approaches were used to interpret the raw PhyloChip data generated during this study. PhyloChip data processing was performed using the latest version of the Greengenes taxonomy (McDonald *et al.,* 2011). For a PhyloChip taxonomic unit (PTU) to pass Stage 1 quality control (QC), the following criteria had to be fulfilled: rQ1 ≥ 0.05, rQ2 ≥ 0.93, and rQ3 ≥ 0.98. PTUs that passed this test were considered for Stage 2 QC analysis, whose defined cutoffs (cross-hybridization-adjusted response scores) were set to rxQ1 ≥ 0.30, rxQ2 ≥ 0.40, rxQ3 ≥ 0.60. At least 18 positive probes were considered for each PTU. For measuring microbial richness, PTUs were phylogenetically grouped at the genus level. All statistical procedures

were carried out using the R programming environment (R-project 2011) with diverse packages (vegan, ape, MASS, stats).

Microbial Richness: The Genetic Inventory team calculated correlation coefficients of microbial richness measurements of different taxonomic levels using both Pearson's correlation and the Spearman rank correlation. Pearson's correlation reflects the degree of linear relationship between two variables, and ranges from +1 to −1. A correlation of +1 represents a perfect positive linear relationship between variables. The Genetic Inventory team applied these statistical tools to calculate correlation coefficients for samples collected from SAC floors, ground-support equipment (GSE), and spacecraft surfaces. In total, 13,930 distinct PTUs were detected in these samples. Table 9-1 provides the numbers of distinct taxa detected per sample.

- For all samples, Pearson's "R" correlation coefficient was 0.91 for PTU vs. genus richness, 0.86 for PTU vs. family richness, and 0.99 for genus vs. family richness.
- For all samples, Spearmans "rho" rank correlation was 0.96 for PTU vs. genus richness, 0.95 for PTU vs. family richness, and 0.99 for genus vs. family richness.

These correlation coefficients indicate that richness measurements can be displayed appropriately at different taxonomic levels.

Ordination Analysis: Nonmetric multidimensional scaling (NMDS) was used to display the relative relatedness of samples based on the presence or absence (P/A) of detected PTUs (Figure 9-4). The relatedness in P/A ordination analysis is dependent on the microbial richness (number of different PTUs per sample)

Table 9-1. Numbers of distinct taxa detected using PhyloChip analysis.

Sample-ID	PTUs	Genera	Families
GI-16	317	16	14
GI-17	2198	142	94
GI-18	33	4	4
GI-19	1788	102	67
GI-20	1548	90	67
GI-21	4193	146	92
GI-22	1033	69	51
GI-24	1905	124	83
GI-25	366	40	29
GI-26	1355	82	57
GI-27	101	24	21
GI-28	277	24	19
GI-29	1328	109	83
GI-30	67	15	14
GI-32	124	9	8
GI-38	87	13	12
GI-39	0	0	0
GI-40	19	9	8
GI-41	129	20	15
GI-36-3	12005	278	164
GI-36-4	2790	134	100

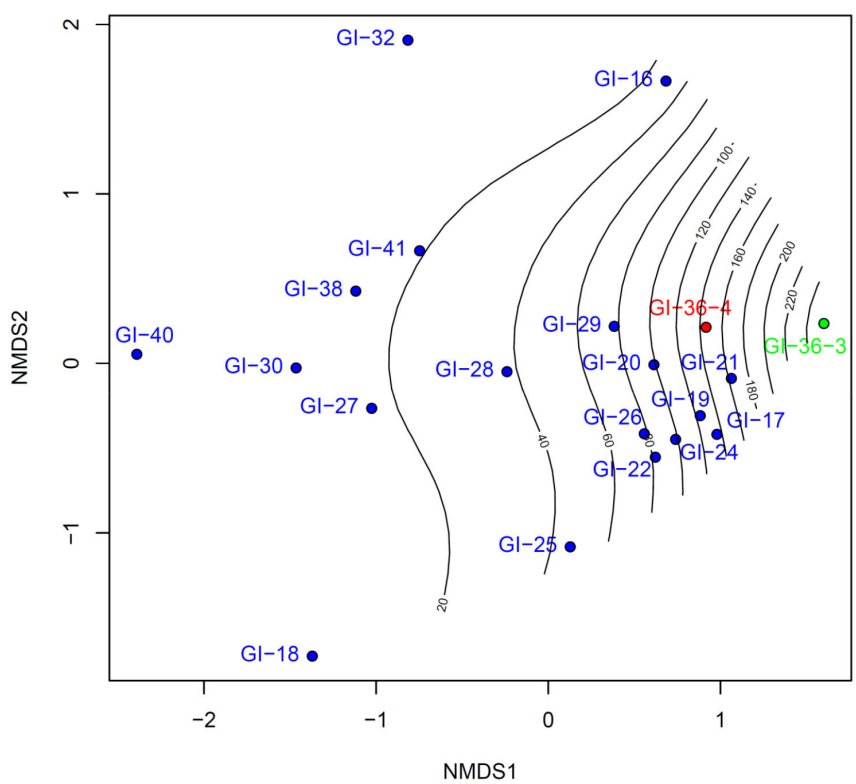


Figure 9-4. Nonmetric multidimensional scaling (NMDS) to display the relative relatedness of various samples based on PhyloChip taxonomy. NMDS is based on Bray-Curtis distance of presence and absence scores of PTUs (stress = 14.23). Curve-fitting model describes the gradient of the number of genera detected in each sample.

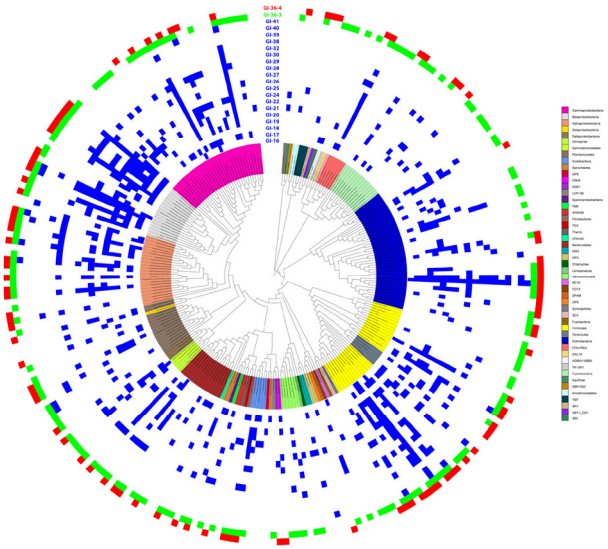


Figure 9-5. An iTOL phylogenetic tree to display the microbial richness based on presence and absence of families detected by PhyloChip analysis. Different families (258) were depicted using a color-coding at higher taxa level.

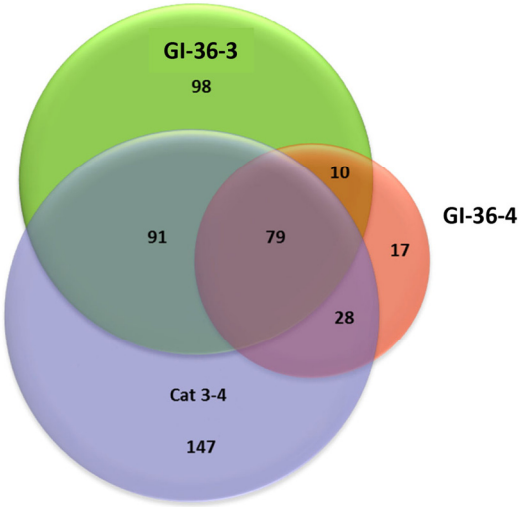


Figure 9-6. Venn diagram depicting the number of genera detected on spacecraft (cat 3–4), SAC floor (GI-36-4), and GSE (GI-36-3) samples using PhyloChip. All spacecraft samples were grouped into one category (cat 3-4). 79 genera were present in all three samples.

and number of common PTUs between samples at a given time. In this study, many samples (7 out of 21) clustered close to sample GI-19, which fell into place near the center of these particular samples. A curve-fitting model was used to display the relation of microbial richness measurements at the genus level with the results of environmental clustering. As mentioned above, the total number of different PTUs and different genera had a high correlation coefficient; consequently, the number of detected genera from several spacecraft samples appeared to coincide with a gradient from low microbial richness (spacecraft surfaces; left side of Figure 9-4) to non-spacecraft samples (floor and GSE; right side of Figure 9-4). GI-36-3 (GSE), having the highest microbial richness, placed somewhat apart from the spacecraft surfaces samples, while GI-36-4 (SAC floor sample) was more related to spacecraft samples GI-21, GI-20, and GI-29. In the end, 7 of the 19 spacecraft samples contained fewer than 20 distinct genera and each was spread apart from one another (left side of Figure 9-4), indicating that they had very few taxa in common.

Phylogenetic interactive trees of life (iTOLs): Phylogenetic trees were calculated based on the neighbor-joining algorithm SINA (Pruesse *et al.,* 2012), which aligns 16S rRNA gene sequences of representative PTUs. Interactive trees of life (iTOLs) were rendered as previously described (Letunic and Bork 2011). A phylogenetic iTOL was used to display the microbial richness of PTUs at the family level (Figure 9-5). The figure depicts 258 different families, which were color-coded at a higher taxonomic level (49 different taxa, mainly phyla level or in case of Proteobacteria, class level). Displaying the array of microbial families present in each sample in this manner enables a simple, rapid visual assessment of community patterns. For instance, it is easy to see that Corynebacteriaceae (Actinobacteria) were present in all samples. In order to identify the total spectrum of biodiversity present on SAC floor, GSE, and spacecraft samples, all samples were grouped into one category (cat3). A genus level–based Venn diagram was constructed to delineate common and exclusive microbial populations present in these sample categories (Figure 9-6). In total, 79 bacterial genera, spanning more than 12 distinct bacterial phyla, were detected in all three sample categories (SAC floor, GSE, and spacecraft surfaces). Spore-formers, such as *Bacillus* spp., *Clostridium* spp., and *Paenibacillus* spp., and human-associated *Pseudomonas* spp., *Spahylococcus* spp., *Streptococcus* spp., and unclassified *Ruminococcaceae* spp. and *Lachnospiraceae* spp., were common in all three sample types (Figure 9-7).

9.3.3 Bioinformatic Processing of Pyrotag Sequences

The Genetic Inventory team adopted a stand-alone, platform-independent, open-source, community-supported software package—mothur—for the analysis of raw 454 tag-encoded pyrosequencing data. Building upon previous tools, this package affords users a single, seamless software suite to analyze community sequence data with immense power and flexibility.

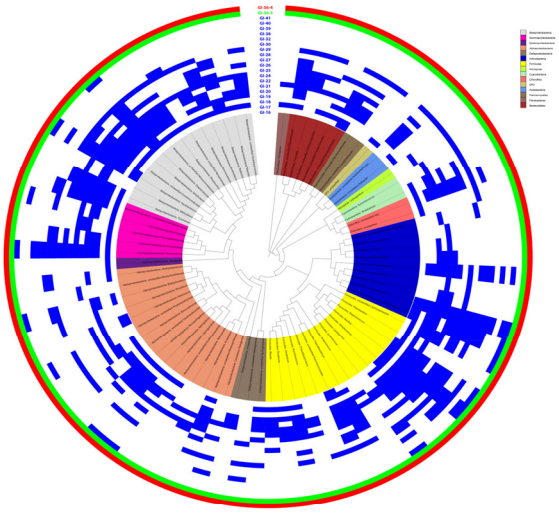


Figure 9-7. An iTOL phylogenetic tree displaying the core microbiome detected using PhyloChip: common genera present on spacecraft samples, GSE (GI-36-3), and floor (GI-36-4) samples.

Bioinformatic Analysis of Raw 454 Tag-Encoded Pyrosequencing Data

Resulting bacterial and archaeal pyrotag sequences generated by the Genetic Inventory team were processed and analyzed using the mothur software package (Schloss *et al.,* 2009), with the AmpliconNoise algorithm implemented. Previously described standard operating procedures were followed for the analysis of sequence data in this study (Schloss *et al.,* 2011). Sequences were removed from consideration if they (a) did not contain the primer sequence, (b) contained an uncorrectable barcode, (c) were < 200-nt in length, (d) had homopolymers longer than 8 nt, or (e) had a quality score of < 25. Unique sequences were aligned using the Greengenes reference alignment (McDonald *et al.,* 2012; Schloss *et al.,* 2009) and trimmed such that only regions of conserved overlapping sequence data were considered (~85% of the overlapping sequence length). Filtered sequences were assigned to samples according to their 12-nt barcode. Recently, researchers demonstrated that training on the correct segment is important (Werner *et al.,* 2012); therefore, the Genetic Inventory team created a custom Greengenes reference sequence file based on the primers used in this study, and used for alignments and classification (McDonald *et al.,* 2012; Schloss *et al.,* 2009). Unique sequences were aligned using the custom reference alignment file and trimmed such that all sequences overlapped in the same alignment space. After removing chimeras, sequences were classified in accordance with the new Greengenes training set and taxonomy (McDonald *et al.,* 2012; Werner *et al.,* 2012) and clustered into OTUs at the 0.03 level (i.e., at 97% similarity (Hazen *et al.,* 2010)]).

For fungi, the full-length ITS1 sub-region was extracted from the fungal ITS dataset using ITS Extractor v. 1.1 (Nilsson *et al.,* 2010), dismissing entries of which both the 3' end of the SSU gene and the 5' end of the 5.8S gene were not found. The ITS1 sequences were clustered in CrunchClust v. 43 (CrunchClust ; Hartmann *et al.,* 2012) with pyrosequencing homopolymer correction enabled and at a Levenshtein distance of 7 [approximately 97% sequence similarity, a threshold value known to perform well for a wide range of fungi in terms of ITS distances (Nilsson *et al.,* 2008)]. A representative sequence from each OTU, as designated by CrunchClust, was annotated for taxonomic affiliation using an established ITS pyrosequence analysis pipeline (Tedersoo *et al.,* 2010) against the UNITE (Abarenkov *et al.,* 2010) and INSD (Karsch-Mizrachi *et al.,* 2012) databases. The results were verified manually for each OTU. An entry was deemed as belonging to a given OTU if it produced a ≥97% match over the full length of the sequence to a fully identified reference sequence, whose name was not contradicted by other equally good matches. The corresponding values for tentative genus and order phylogenetic affiliation were 85% and 70%, respectively, which

were deliberately set to be high to avoid false-positive inclusions. In the case of competing names for which neither synonymy nor anamorph-telemorph relationships could be established through MycoBank (Crous *et al.,* 2004), the least inclusive parent nomenclatural level was used (e.g., *Penicillium* sp.).

Rarefaction Analysis: Section 10 documents the extent of coverage achieved in the sampling and pyrosequencing effort.

Venn Diagram: Venn diagrams were constructed to compare the richness shared among samples (Figure 9-8). The Genetic Inventory team applied this visualization tool to better understand the "core" microbial population present in all three SAC floors, GSE, and spacecraft surface samples. From a planetary protection perspective, it may be this core microbiome that is of greatest relevance as it persists in all samples despite variations in cleaning regimens. Venn diagrams clearly illustrated "exclusive" microbial constituents present in each sample, which might imply different heritage.

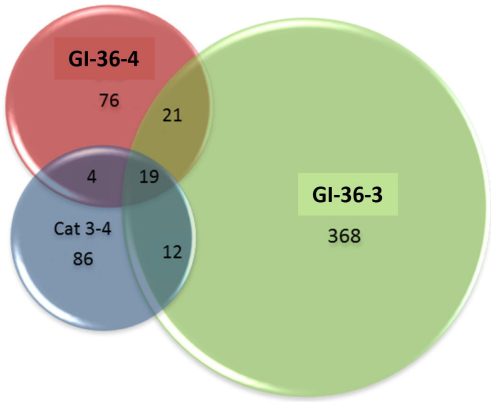


Figure 9-8. Venn diagram depicting the number of genera detected on spacecraft (cat 3-4), SAC floor (GI-36-4), and GSE (GI-36-3) samples using PhyloChip. All spacecraft samples were grouped into one category (cat 3-4). 19 genera were present in all the three samples.

Environmental Clustering:
The Genetic Inventory team generated a dendrogram to illustrate the observed similarity among samples (Figure 9-9). To determine whether the clustering within the dendrogram was statistically significant, parsimony- and UnifFrac-weighted algorithms were applied. The UnifFrac-weighted analysis for bacterial and fungal data clearly indicated a statistical difference among all four groups (Wsig <0.001).

Ordination Plots: In the scientific literature, the most popular means of visualizing beta-diversity information is via ordination plotting, principal coordinate analysis (PCoA), and NMDS. Statistical significance in the spatial separation among the distinct clouds observed in the ordination plots (NMDS) was tested using the analysis of molecular variance (AMOVA) test. This test determines whether the centers of the clouds representing a given category exhibit more variation than that exhibited from those across sample categories (Figure 9-10).

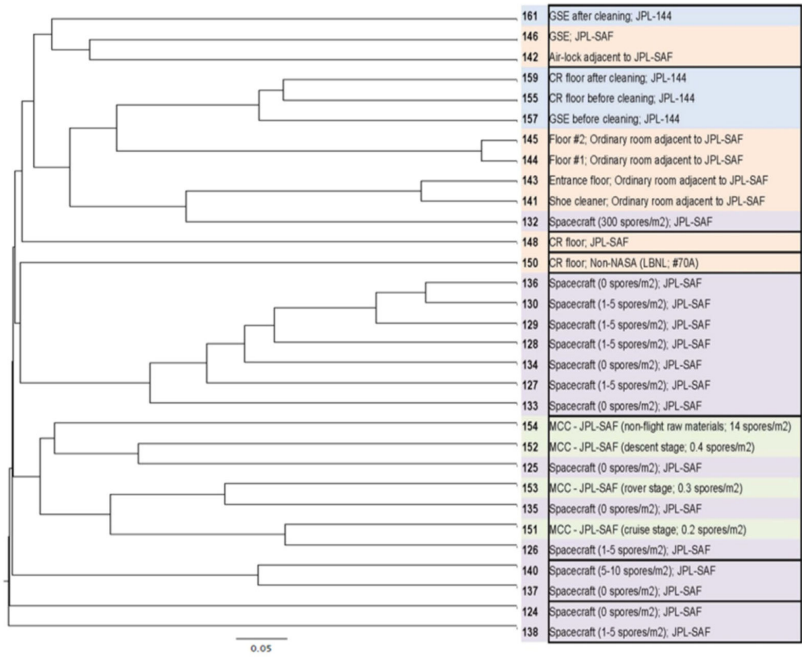


Figure 9-9. Environmental cluster analyses showing the relationship of different samples. MCC: mission critical componentry, CR: cleanroom, JPL-SAF: Jet Propulsion Laboratory-Spacecraft Assembly Facility, GSE: ground support equipment.

Phylogenetic interactive trees of life (iTOLs): An phylogenetic iTOL was constructed by selecting one representative 16S rRNA gene sequence from each OTU. This was accomplished by applying the neighbor-joining algorithm, and compiling results with the mothur software suite's clearcut program. The iTOL was rendered in circular fashion with Letunic's tree viewing program (Letunic *et al.* 2011). Peripheral rings indicate the detected presence of the coinciding microbial class in the sample(s) under investigation (Figure 9-11). In the end, 594 distinct OTUs were selected for the construction of this tree and were color-coded at "class" level (24 classes). Displaying the array of detected OTUs across all samples under investigation enables a simple, rapid, visual assessment of community patterns. Each concentric ring on the periphery of the circular phylogenetic tree represents one sample; sample categories were color-coded as follows: SAC floors—*red*, GSE—*green*, spacecraft surfaces—*blue*. The Genetic Inventory team also constructed sub-trees illustrating the presence of OTUs belonging to a specific class (e.g., actinobacteria, alphaproteobacteria, firmicutes, betaproteobacteria and gammaproteobacteria). This graphic representation allowed the Genetic Inventory team to comparatively assess the presence of OTUs belonging to a specific "class" across all study samples. The bioinformatics and biostatistics approaches adopted in this task allowed interpretation of the results. Some key observations (not exhaustive) are as follows:

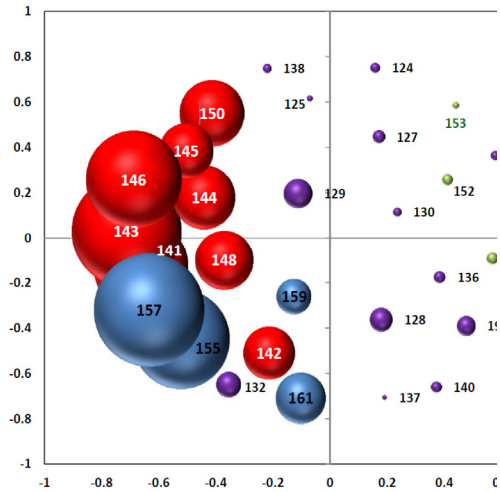


Figure 9-10. NMDS analysis depicting variations in bacterial diversity using pyrosequences across samples (stress = 0.38). Spheres are labeled according to numerical sample identification, and sized according to relative OTU abundance. *Red:* cleanroom types; *blue:* prior to vs. after cleaning; *purple:* general spacecraft hardware surfaces; *green:* mission-critical componentry samples.

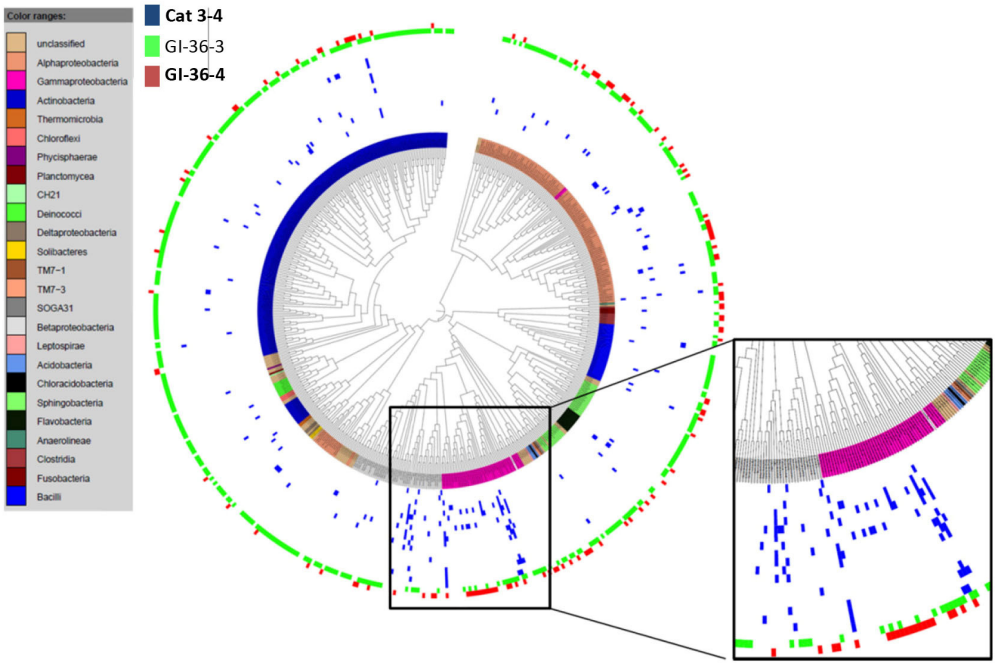


Figure 9-11. An iTOL phylogenetic tree displaying the microbial richness, based on presence and absence of OTUs detected by pyrosequence analysis.

1. SAC floors alone do not encompass the entire spectrum of microbial diversity present on GSE and spacecraft surfaces. In other words, SAC floors alone cannot be used reliably as a proxy for interpolating the biodiversity associated with co-located spacecraft surfaces.
2. Some of the OTUs belonging to betaproteobacteria and firmicutes were exclusively detected in spacecraft surface samples. This observation underscores the need for repeated sampling and sufficient sample collection from actual spacecraft hardware or from proxy surfaces determined to be more representative of the flight hardware itself.

Outcome: Bioinformatics and biostatistical tools applied to raw Genetic Inventory data rendered the most in-depth analysis of microbial diversity associated with spacecraft and related environments to date.

9.4 Key Considerations

There are numerous factors to consider in choosing an appropriate methodology for elucidating microbial diversity in environmental samples. While factors of cost, time, labor intensity, and reproducibility weigh heavily individually, the bias and accuracy of a given approach are the most important aspects in considering the goal of planetary protection endeavors. Potential pitfalls in the application of cloning and sequencing, 454 tag-encoded sequencing, and PhyloChip DNA microarray techniques for assessing microbial diversity are summarized as follows:

1. Selection of appropriate primers and PCR amplification parameters is a key factor in any biodiversity assessment.
2. Intrinsic sequencing error rate is an important caveat with respect to the 454 tag-encoded pyrosequencing approach. Homopolymers and other sequencing artifacts could inadvertently inflate diversity estimates.
3. Pyrosequencing-based diversity estimates can be influenced by the formation of chimeric 16S amplification products. Chimeras are hybrid products between multiple parent sequences that can be incorrectly interpreted as novel organisms, thus inflating apparent diversity.
4. 454 tag-encoded pyrosequencing is not a phylogenetic tool. Due to the short read length resulting from traditional 454 tag-encoded pyrosequencing (~100 bp), comparing 16S rRNA gene sequence data and assigning phylogenetic affiliation and/or identification is a significant challenge (despite the fact that hypervariable regions of the gene are analyzed). This is less of a problem for later Titanium FLX technologies with longer (~350 bp) read lengths.
5. In order to maximize the specificity of DNA amplification prior to 454 tag-encoded pyrosequencing, it is imperative to use high-fidelity, proofreading thermostable DNA polymerases and apply temperature gradients to PCR amplification regimens.
6. Inadequate reference database material is currently a limiting factor in the context of analyzing archaeal and fungal diversity with high-throughput sequencing approaches.
7. DNA microarray (PhyloChip) analysis is not a particularly effective approach for determining the presence and absence of microbial species in an environmental sample, but is a powerful tool to compare community dynamics and elucidate β-diversity.
8. Because PhyloChip and tag-encoded pyrosequencing technologies are based on different taxonomies (Greengenes vs. Hugenholtz), directly comparing biodiversity profiles resulting from SAC samples is extremely challenging. Even though a single taxonomy can be applied

to the raw data of these two analysis techniques, directly comparing the biodiversity profiles obtained from these techniques may be difficult, if not impossible.

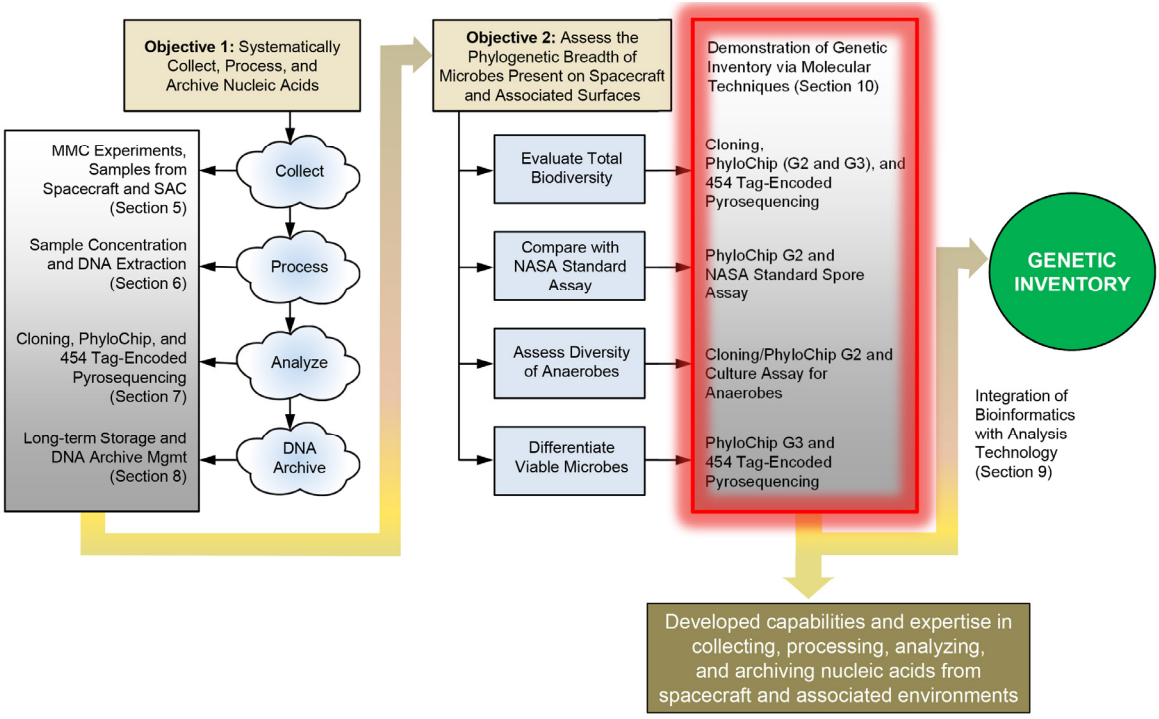
9. Since DNA microarrays are dependent on the hybridization of environmental oligonucleotides to known probes of specific sequence, an enormous amount of *a priori* sequence information is required.
10. Currently, many particularly helpful bioinformatic and biostatistical tools used to analyze and interpret G3 PhyloChip raw data are proprietary and require collaboration with LBNL or their commercial affiliate Second Genome Inc. In contrast, several tools are publically available for processing raw high-throughput sequencing data, such as mothur, QI-IME, PANGEA, ESPRIT, and RDP.

10. Demonstration of the Use of Molecular Techniques to Assess Microbial Diversity in Low-Biomass Samples

This section summarizes the results of Genetic Inventory team studies to demonstrate the use of two molecular technologies, PhyloChip DNA microarray and 454 tag-encoded pyrosequencing, to assess the microbial population present in low-biomass environmental samples. It also describes the team's comparison of the PhyloChip method with the conventional cloning and Sanger-sequencing method and cultivation-based methods.

Note that these studies were performed over the course of the Genetic Inventory task on an opportunity basis, and thus reflect the deployment of the then state-of-the-art Genetic Inventory methodology. This means that earlier and later studies may not be directly comparable, reflecting different sampling preferences or protocols at different times (see Figure 1-1, Timeline). Importantly, the later methods using the PhyloChip G3 and 454 Titanium FLX V1–V3 tag-encoded pyrosequencing reflect the current state-of-the-art at the end of the Genetic Inventory task.

The red box in the figure below highlights this section within the overview of the Genetic Inventory task.



10.1 Objectives

Success criterion: (1) Demonstration of an end-to-end Genetic Inventory capability; (2) Comprehensive "passenger list" of microbes associated with spacecraft-associated environments generated via current molecular methods.

The vast majority of microorganisms cannot be cultivated, and many that can require long cultivation times (Amann *et al.,* 1995; Ghosh *et al.,* 2010; La Duc *et al.,* 2007a). Advanced molecular approaches targeting DNA have been used to assess microbial diversity (DeAngelis *et al.,* 2011; Pace 1997). Several methods are also available to scientists to create a genetic microbial inventory. Factors considered in selecting an appropriate methodology include cost, time, labor intensity, and reproducibility; however, to meet planetary protection goals, lack of bias and accuracy of a given approach are perhaps the most important considerations.

In theory, cloning and sequencing 16S ribosomal genes from every cell present in a SAC would be an acceptable method; however, the full-length sequencing of all 16S ribosomal ribonucleic acid (rRNA) genes from environmental samples is prohibitively expensive. Therefore, the approach is not feasible for generating comprehensive profiles of complex microbial communities found in SACs. In addition, classic cloning methods are time-intensive and have proven to be insufficient for detecting organisms that may be present in cleanrooms in very small quantities. A second approach is to use DNA microarray PhyloChip methodologies, which are highly sensitive and can quickly detect organisms present in very small amounts (below 10^{-4} abundance of the total sample) (DeSantis *et al.,* 2007). Furthermore, the development of high-throughput pyrosequencing, which has enabled the analysis of longer DNA sequence reads (>400 bp), has dramatically increased the resolution and detectable spectrum of diverse microbial lineages from environmental samples (Mendes *et al.,* 2011; Sogin *et al.,* 2006).

This section discusses the Genetic Inventory task objective to demonstrate the feasibility of molecular methods to effectively assess the phylogenetic breadth of microorganisms present on spacecraft and associated surfaces. In doing so, the team also (a) established the use of the preferred sampling tool (BiSKits) for the cleanroom environment, and (b) demonstrated that even with other sampling approaches (e.g., polyester wipes used to collect spacecraft surface samples), it is possible to catalog genetic inventory of low-biomass samples using molecular techniques.

10.2 General Approach

Using samples collected from SAC surfaces and from the Mars Science Laboratory (MSL) spacecraft, the Genetic Inventory team demonstrated the use of two innovative molecular technologies (PhyloChip DNA microarray and 454 tag-encoded pyrosequencing)—in addition to the conventional cloning and Sanger sequencing method—to assess the microbial population present in low-biomass environmental samples. Specifically, the approach was to

- Demonstrate PhyloChip DNA microarray by conducting an extensive census of the bacterial communities on surfaces in three NASA SACs supporting two distinct missions (Phoenix and MSL).
- Ensure that the maximum diversity of resident bacteria was uncovered by subjecting subsamples from each cleanroom surface sampling to conventional cloning and Sanger sequencing of 16S rRNA genes (in addition to the PhyloChip DNA microarray protocols).
- Demonstrate the 454 tag-encoded pyrosequencing method by cataloging the genetic inventories of all three microbial domains (bacterial, archaeal, and fungal [*Eukarya*]) associated with the MSL spacecraft and associated environmental samples.

10.3 Sample Collection for Demonstrations

10.3.1 Samples Collected for PhyloChip Demonstration

10.3.1.1 Cleanroom Characteristics and Maintenance

Each of the facilities examined was a certified Class 100k cleanroom (100,000 particles >0.5 μm ft^{-3} air), which is equivalent to ISO 8 (3,520,000 particles >0.5 μm m^{-3}). All of these cleanroom facilities were operated at a positive pressure, with temperatures in the range of 20 ± 4°C, and relative humidity ranging from 30 to 50%. In all instances, the total hydrocarbon content of the facility air (gases and vapors) was below 15 ppm (calculated as methane). All facilities used High Efficiency Particle Air (HEPA) filters for continuous air filtration. Air was volumetrically exchanged a minimum of four times per hour, with positive pressure maintained at all times. For all facilities,

temperature, relative humidity, and airborne particle concentration were continuously monitored and recorded. Surface particulate matter, nonvolatile residue, and volatile hydrocarbons were monitored using conventional methods (NASA-KSC 1999).

Anyone entering the cleanrooms was required to take prescribed actions to minimize the influx of particulate matter. Specific entry procedures varied depending on the presence or absence of mission hardware. All spacecraft assembly facilities examined in this study were maintained with a cleaning regimen that (depending on the presence or absence of mission hardware) included replacing tacky mats, wiping surfaces and support hardware fixtures, and vacuuming and mopping floors with cleanroom-certified sanitizing agents (disinfectants, alcohol, and/or ultrapure water). To minimize cleanroom floor surface contamination (resulting from deposition of moisture, particles, dirt, grease, oil, scale, corrosion, and nonvolatile residues introduced during fabrication, assembly, integration, testing, storage, and shipping operations), the surface was manually cleaned with detergent. At JPL, a protocol using an all-purpose cleaning and degreasing agent (Kleenol 30, Accurate Industrial Supply, Inc., Cerritos, CA, Cat #: J-CC-00040) was used to maintain floor cleanliness. (Other organizations have their own protocols, which are proprietary.) The cleaning schedule varied depending on the presence or absence of mission hardware. Typically, cleanroom surface cleaning was performed twice a day when spacecraft were actively undergoing assembly.

The ground-support equipment (GSE) consisted of non-flight hardware items associated with the project, employed prior to or during hardware receipt, assembly, integration, test, storage, shipment, and pre-launch activities. All GSE materials used in direct contact with flight hardware or inside the cleanroom were inspected for compliance to visible cleanliness levels. Inspection of surfaces for visible contamination was conducted under white light (100 to 125 foot candles), held at an oblique angle, at a distance of 15.2–45.7 cm. Initial cleaning for this level was mandatory. Ultraviolet light inspection was used for GSE that had been transferred from an uncontrolled area to the controlled clean area. To ensure that contamination was not transferred to flight hardware, GSE such as mating gas/liquid lines and electrical connectors were also pre-cleaned according to established in-house protocols. In contrast to cleanroom floors, where detergents were routinely applied, GSE surfaces were cleaned solely by wiping with 70% alcohol (typically 70–100%).

10.3.1.2 Cleanroom and GSE Surface Sampling

Surface sampling of SAC floors (1 m^2) and GSE was performed using BiSKits pre-moistened with phosphate-buffered saline (PBS), as previously described (Bruckner and Venkateswaran 2007). Controls specific for each BiSKit were prepared in a class II biological hood immediately prior to sampling. Fifteen milliliters of sterile PBS was then added to the macrofoam sponge component of the BiSKit. The PBS was recovered from the sponge by screwing the sponge casing against the BiSKit cover several times, allowing samples to be collected into the attached sample bottle. The resulting volume of PBS (~15 mL) was removed from the sample bottle and stored in a 50-mL centrifuge tube as a sample negative control. A new, sterile, 15-mL aliquot of PBS was used for sample collection. Once the PBS had adequately absorbed to the macrofoam sponge, the sampler was unscrewed from the module and was traversed about the surface area of interest (ca. 1 m^2), first in a horizontal manner, then in a vertical manner, and finally in a diagonal sweeping pattern. Immediately following the collection of a sample from a surface, the macrofoam sponge sampler was forcefully screwed back into the BiSKit module, to expunge as much sample as possible from the sponge into the collection tube. The module and attached collection tube was then transported in a sealed bag back to the laboratory, where it was immediately processed in a biological hood.

Key Consideration: The Genetic Inventory team collected samples from the SACs at the convenience of the missions using the facilities. Therefore, the team was typically not able to control

sample collection relative to facility maintenance. It is known that the bioburden and biodiversity contained in samples collected before SAC cleaning vary from samples taken after SAC cleaning.

10.3.1.3 Categorization of Samples

Over a period of 18 months, the Genetic Inventory team collected 107 individual surface samples from three SACs: the Kennedy Space Center Payload Hazardous Servicing Facility (KSC-PHSF), the Lockheed Martin Aeronautics Multiple Testing Facility (LMA-MTF), and the Jet Propulsion Laboratory Spacecraft Assembly Facility (JPL-SAF). Due to the limitations and challenges associated with processing samples of such extremely low biomass, several individual samples from a given sampling event/locale were pooled, which resulted in 10 distinct pooled samples for further analysis (Table 10-1). These pooled samples were grouped into 3 categories, based on the presence/absence of spacecraft and the mission it was supporting in a given SAC, as is shown in Table 10-1.

- Category A: 37 samples collected from KSC-PHSF and LMA-MTF in the presence of Phoenix spacecraft hardware.
- Category B: 30 samples collected from KSC-PHSF and JPL-SAF, both without spacecraft.
  Category C: 40 samples collected from JPL-SAF during assembly of the MSL spacecraft.

10.3.2 Samples Collected for 454 Tag-Encoded Pyrosequencing Demonstration

To develop the genetic inventory for all three microbial domains associated with the MSL spacecraft, the Genetic Inventory team used 454 tag-encoded pyrosequencing, a high-throughput sequencing method capable of rendering an all-inclusive account of the "rare biosphere" (i.e., singleton and low-abundance taxa) within a given sample. The 454 tag-encoded pyrosequencing method is used to ascertain the phylogenetic affiliations of members of complex microbial communities found on spacecraft hardware and associated surfaces, without the need for *a priori* knowledge of gene sequence data pertaining to the diverse microbes present. The massively parallel capability of 454 tag-encoded pyrosequencing (Margulies *et al.,* 2005) can generate 300,000–450,000 "tag" sequences (~500 bp) from one or more of nine known bacterial and archaeal ribosomal small-subunit 16S rRNA gene hypervariable regions (Neefs *et al.,* 1990; Sogin *et al.,* 2006) in a single run without requiring the construction of clone libraries.

The Genetic Inventory team and MSL Planetary Protection implementation team collected 31 sets of 233 samples total from a surface area of 233 m^2. These sets were categorized into four groups, which were analyzed for pyrosequencing-derived microbial diversity (Table 10-2).

10.3.2.1 Samples Collected from Various Indoor Environments (Group I and II)

Over a period of 3 years, the Genetic Inventory team collected 71 individual surface samples associated with various rooms, including floor samples from SACs (JPL-SAF and JPL-144), an ordinary room adjacent to JPL-SAF, and a non-NASA cleanroom. Samples were also collected from GSE and cleanroom floors. The team used the same BiSKit collection procedure as described in Section 10.3.1.2 for samples collected for PhyloChip analyses.

- Group I (8 sets; 33 samples) samples were clustered together to examine the effect of varying cleanroom certification on resulting pyrosequencing data. This group consisted of floor and GSE samples from JPL-SAF, an ordinary room adjacent to JPL-SAF, and a non-NASA cleanroom.
- Group II samples (4 sets; 38 samples) were collected to assess the impact of cleaning procedures on pyrosequencing profiles. This group comprised floor and GSE samples gathered from the JPL-144 facility that was expected to receive spacecraft hardware both (a) prior to cleaning, and (b) from identical locations one day after cleaning.

Table 10-1. Changes in bacterial diversity of cleanrooms during various spacecraft assembly phases of two different missions.

Category/ Sample ID	Functional Relevance of the Facility (Date of Collection)	Facility	No. of Samples Pooled	No. of Clones Sequenced	No. of High-Quality Sequences (N)[a]	No. of OTUs Detected Once (*n*1)	Coverage Value C= [1−(*n*1/N)]*100	No. of OTUs Detected by Cloning	No. of OTUs Detected by PhyloChip	Fold Increase in OTUs by PhyloChip
(A) Phoenix Spacecraft Facility Surfaces										
MTF-PHX-D1	During-Phoenix (Jan '07)	LMA-MTF	7	672	448	15	96.7	33	1222	37
PHSF-PHX-D1	During-Phoenix (Jun '07)	KSC-PHSF	10	160	134	10	92.5	18	1172	65
PHSF-PHX-D2	During-Phoenix (Jul '07)	KSC-PHSF	20	768	637	39	93.9	48	1519	32
(B) Facility Surfaces with No Spacecraft										
PHSF-PHX-B	Before-Phoenix (Apr '07)	KSC-PHSF	10	1152	959	129	86.5	166	1491	9
PHSF-PHX-A	After-Phoenix (Aug '07)	KSC-PHSF	10	608	468	7	98.5	18	728	40
SAF-MSL-B	Before-MSL (Oct '07)	JPL-SAF	10	396	224	59	73.7	91	1492	16
(C) MSL Spacecraft Facility Surfaces										
SAF-MSL-D1	During-MSL (Jan '08)	JPL-SAF	10	96	68	5	92.6	7	491	70
SAF-MSL-D2	During-MSL (Feb '08)	JPL-SAF	10	None[b]	0	0	0	0	924	-[c]
SAF-MSL-D3	During-MSL (Jun '08)	JPL-SAF	10	None	0	0	0	0	697	-
SAF-MSL-D4	During-MSL (Aug '08)	JPL-SAF	10	None	0	0	0	0	468	-

[a]*Number of clones after eliminating low quality reads and chimeras.*
[b]*The amplification of 1.5-kb fragment of 16S rRNA gene is failed.*
[c]*Due to the absence of clones, this value could not be determined.*

Table 10-2. Sample characteristics of various spacecraft and associated surfaces.

GI Sampling Event	Sample ID	Sampling Devices	Sample Type	Total Area Sampled (m^2)	Mission	Cleanroom Type	Description
Group I: Cleanroom Types (33 m^2)							
GI-37	**150**	BisKit	Floor-70A	10	None	Non-NASA Cleanroom	Non-NASA cleanroom (#70A)
GI-35-6	**143**	BisKit	Entrance floor	1	None	Ordinary room	Ordinary room adjacent to JPL-SAF
GI-35-4	**141**	BisKit	Shoe Cleaner	1	None	Ordinary room	Ordinary room adjacent to JPL-SAF
GI-35-7	**144**	BisKit	Floor 1	1	None	Ordinary room	Ordinary room adjacent to JPL-SAF
GI-35-8	**145**	BisKit	Floor 2	1	None	Ordinary room	Ordinary room adjacent to JPL-SAF
GI-35-5	**142**	BisKit	Air-lock	1	None	Ordinary room	Ordinary room adjacent to JPL-SAF
GI-36-3	**146**	BisKit	JPL-SAF GSE	9	Mars	Class 100k	During spacecraft assembly (JPL-SAF)
GI-36-4	**148**	BisKit	JPL-SAF Floor	9	Mars	Class 100k	During spacecraft assembly (JPL-SAF)
Group II: Cleaning vs. Prior to Cleaning (38 m^2)							
GI-42-1	**155**	BisKit	Floor	9	None	Class 100k	JPL-144 cleanroom prior to cleaning
GI-42-2	**157**	BisKit	GSE	9	None	Class 100k	JPL-144 cleanroom prior to cleaning
GI-43-1	**159**	Polyester wipe	Floor	10	Mars	Class 100k	JPL-144 cleanroom after cleaning
GI-43-2	**161**	Polyester wipe	GSE	10	Mars	Class 100k	JPL-144 cleanroom after cleaning
Group III: Spacecraft Surfaces (spore count-based; 110 m^2)							
GI-16	**124**	Polyester wipe	Spacecraft	6	Mars	Class 100k	No spore count
GI-17	**125**	Polyester wipe	Spacecraft	10	Mars	Class 100k	No spore count
GI-25	**133**	Polyester wipe	Spacecraft	8	Mars	Class 100k	No spore count
GI-26	**134**	Polyester wipe	Spacecraft	7	Mars	Class 100k	No spore count
GI-27	**135**	Polyester wipe	Spacecraft	4	Mars	Class 100k	No spore count
GI-28	**136**	Polyester wipe	Spacecraft	6	Mars	Class 100k	No spore count
GI-29	**137**	Polyester wipe	Spacecraft	18	Mars	Class 100k	No spore count
GI-18	**126**	Polyester wipe	Spacecraft	10	Mars	Class 100k	1 to 5 spore per m^2
GI-19	**127**	Polyester wipe	Spacecraft	14	Mars	Class 100k	1 to 5 spore per m^2
GI-20	**128**	Polyester wipe	Spacecraft	5	Mars	Class 100k	1 to 5 spore per m^2
GI-21	**129**	Polyester wipe	Spacecraft	4	Mars	Class 100k	1 to 5 spore per m^2
GI-22	**130**	Polyester wipe	Spacecraft	1	Mars	Class 100k	1 to 5 spore per m^2
GI-30	**138**	Polyester wipe	Spacecraft	13	Mars	Class 100k	1 to 5 spore per m^2
GI-32	**140**	Polyester wipe	Spacecraft	3	Mars	Class 100k	5 to 10 spore per m^2
GI-24	**132**	Polyester wipe	Spacecraft	1	Mars	Class 100k	300 spores per m^2
Group IV: Spacecraft Surfaces (mission component-based; 52 m^2)							
GI-38	**151**	Polyester wipe	Spacecraft	26	Mars	Class 100k	Cruise stage (0.2 spores per m^2)
GI-39	**152**	Polyester wipe	Spacecraft	9	Mars	Class 100k	Descent stage (0.4 spores per m^2)
GI-40	**153**	Polyester wipe	Spacecraft	16	Mars	Class 100k	Rover (0.3 spores per m^2)
GI-41	**154**	Polyester wipe	Spacecraft	1	None	Class 100k	Non-flight samples (14 spores per m^2)

GI: Genetic Inventory; JPL-SAF: Jet Propulsion Laboratory Spacecraft Assembly Facility

10.3.2.2 Samples Collected from MSL Flight Hardware (Group III and IV)

All 162 spacecraft hardware samples examined over the course of this study were truly "samples of opportunity"; that is, no spacecraft hardware samples were collected exclusively for the Genetic Inventory task. The MSL Planetary Protection implementation team collected samples from the MSL spacecraft flight hardware (housed in the JPL–SAF and KSC–PHSF) specifically for the purpose of accomplishing bioburden assessments for planetary protection compliance. In doing so, the team's standard process left residual rinse fluid that was useful to the Genetic Inventory team but no longer needed by the MSL Planetary Protection team.

The sampling method was to wipe ~1 m^2 surface areas with sterile, water-moistened polyester wipes (9"× 9" Catalog #TX761; Texwipe, Upper Saddle River, NJ) using the NASA sampling protocol (NASA 2010). In preparation for sampling, all wipes were folded, rolled, and placed in glass test tubes containing 15 mL of sterile water; the tubes were then autoclaved. Immediately prior to sampling, wipes were aseptically removed from tubes with sterile forceps and handled with sterile-gloved hands. Immediately prior to sampling, the wipe was unrolled and successively folded into quarters such that the sampling surface was approximately 1/8 the total surface area of the wipe. Spacecraft surfaces of 0.7 m^2 were then sampled via successive wiping, first in a unidirectional horizontal, then in a unidirectional vertical, and finally in a unidirectional diagonal manner, each time with an unused quarter of the wipe.

Sample-laden wipes were then placed in sterile 500-mL glass bottles, which were immediately transported to the laboratory where the standard NASA spore assay was carried out within 2 h of sample collection by the Planetary Protection implementation team. The results of these spore assays were not documented in this study. Following sample collection from spacecraft hardware, 200 mL of sterile rinse solution (85 mg/l potassium dihydrogen phosphate, 200 mg/l Tween 80; pH 7.2) were added to each wipe-containing bottle, which was then subjected to vortex mixing at maximum power for 5 s and sonication at 19–27 kHz for 2 min ± 5 s. After the spacecraft surface samples were processed for analysis by the Planetary Protection implementation team via the NASA spore assay (NASA 2010), the remaining heat-shocked Planetary Protection–rinse fluid (~152 ml)—which was no longer needed by the Planetary Protection implementation team—was frozen at –20°C for subsequent downstream DNA-based analyses by the Genetic Inventory team.

- Group III samples (15 sets; 110 samples) were collected when MSL was undergoing assembly and test at JPL-SAF and were grouped together to evaluate the extent of correlation between endospore burden associated with spacecraft hardware surfaces and resulting OTU abundance. This group comprised spacecraft hardware surface samples whose bacterial endospore burden had been previously determined (Cooper *et al.,* 2011).
- Group IV samples (4 sets; 52 samples) were collected when MSL was undergoing assembly and test at KSC-PHSF and were analyzed together to investigate how pyrosequencing results differ across various mission subsystem components (e.g., cruise stage, rover). This group consisted of spacecraft hardware surface samples collected from the various components of the flight system.

10.4 Results of Comparison of Conventional Cloning and PhyloChip DNA Microarray Analyses

10.4.1 Clone Library–Derived Bacterial Diversity

Subsamples from each sampling event were subjected to conventional cloning and sequencing of 16S rRNA genes as well as to PhyloChip DNA microarray protocols. Table 10-1 lists the number of distinct OTUs detected by conventional cloning and/or PhyloChip DNA microarray analyses in each sample. This table also provides the number of high-quality sequence reads, number of detected OTU, coverage values, and fold-increase in detection via PhyloChip DNA microarray, per sample. After chimeras were disregarded, 76% of the total 3,852 full-length 16S rRNA sequences generated were deemed of sufficient quality to be included in this study (GenBank accession # FJ191310 to FJ194034; GQ129843 to GQ130128). Table 10-3 provides a detailed breakdown of the clones including relative abundance, members assigned to each bacterial family or genera based on the Ribosomal Database Project (RDP) classifier. The PhyloChip detected biosignatures in more than 140 known bacterial families, almost 100 of which had never been observed in any of the clone libraries (Figure 10-1).

The team observed that coverage values (see Section 9 for definition) were linked to the presence or absence of spacecraft hardware in the SAC. Based on clone library–derived coverage values, sampling efforts were incomplete for category B samples (those collected prior to the arrival of spacecraft hardware), as was evident in two different facilities (74% to 87%; Table 10-1). However, category A and C samples (collected in the presence of spacecraft hardware) yielded much higher coverage values (>94%). While PHSF-PHX-A (category B) may seem to contradict such a trend (98.5% coverage), it must be noted that the KSC-PHSF SAC in which this sample was collected was kept under stringent maintenance (as that of categories A and C) in the unlikely event of launch delay and consequential return of the Phoenix spacecraft. A similar trend was noted in the occurrence of singleton OTUs (taxa whose unique sequence is retrieved only once), suggestive of broader bacterial diversity prior to the arrival of spacecraft hardware in SACs (59 in JPL-SAF and 129 in KSC-PHSF). Of the SACs sampled during the spacecraft assembly, JPL-SAF (0 to 7 OTUs) exhibited significantly less diversity than either the LMA-MTF (33 OTUs) or KSC-PHSF (18 and 48 OTUs), as assessed by the cloning and Sanger sequencing method (Table 10-1).

Table 10-3. PhyloChip-based bacterial OTUs occurrence as a function of geographical sampling locations.

Family[a]	Number of OTUs that Can Be Resolved by PhyloChip	Number of OTUs Observed in Cleanroom Floors of:								p Value[b]
		PHSF				SAF				
		PHX-B	PHX-D1	PHX-A		MSL-B	MSL-D1	MSL-D2	MSL-D3	
Syntrophobacteraceae	35	8	7	8		7	4	3	2	0.04
Sphingomonadaceae	98	41	36	40		31	33	28	16	0.04
Sphingobacteriaceae	39	5	7	8		4	2	4	3	0.04
Shewanellaceae	5	5	5	5		3	1	2	0	0.01
Piscirickettsiaceae	28	4	4	4		3	2	3	1	0.04
Helicobacteraceae	64	23	21	25		22	14	11	9	0.04
Enterobacteriaceae	183	52	65	65		54	10	6	3	0.03
Coxiellaceae	15	5	4	5		3	4	2	1	0.04
Chromatiaceae	44	7	7	6		2	3	1	0	0.00
Caulobacteraceae	30	12	14	17		7	12	10	7	0.04
Burkholderiaceae	38	11	13	9		8	9	4	5	0.04

[a] *Bacteria associated with aquatic environments*
[b] *p-value derived from student t-test. Denotes statistically significant difference between KSC and JPL facilities (a = 0.05)*

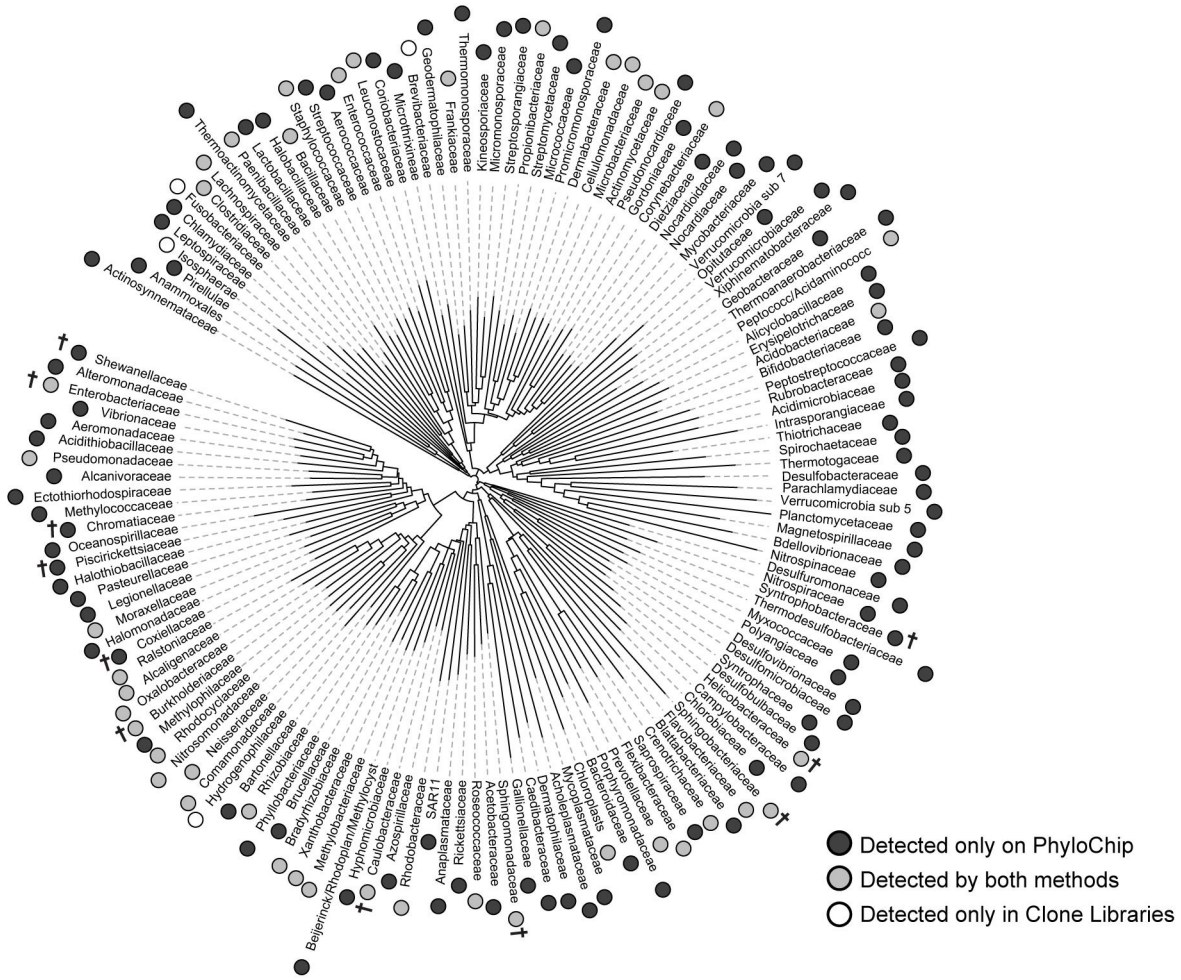


Figure 10-1. Family-level phylogenetic tree of bacterial taxa detected across all sampling events using PhyloChip technologies, clone library sequencing, or both. The unclassified bacterial groups (~30) were not included while constructing this phylogenetic tree. Bacterial groups in which the PhyloChip detected significant differences in biodiversity between JPL-SAF and KSC-PHSF are noted (†) and described further in Table 10-3.

There were very few bacterial families observed to be truly cosmopolitan when assessed via cloning. Species likely to be novel were encountered within 10 bacterial families, as these had earlier been detected via cloning yet their detection remained elusive to the PhyloChip. A lower bacterial diversity was detected in the presence of the MSL spacecraft compared to the Phoenix spacecraft. While not a single novel family was encountered solely in the presence of MSL hardware (category C), 23 families of bacteria were detected while either in the presence of Phoenix (category A) or in the empty SAC (category B).

Perhaps the most striking of all cloning-based results was the correlation between the incidence of streptococci and the presence of humans during MSL hardware assembly in JPL-SAF. When MSL hardware was present in JPL-SAF, the bacterial diversity was drastically reduced to streptococci only, devoid of even the cosmopolitan *Acinetobacter*, *Bradyrhizobium*, and *Ralstonia* species (Table 10-3). Also evident via cloning approaches was a considerable disparity in bacterial diversity between the MSL and Phoenix SACs. As Table 10-1 illustrates, the microbial DNA presence in the SAC housing Phoenix hardware was significantly more diverse than those associated with the MSL spacecraft.

10.5 Results of PhyloChip DNA Microarrays

When analyzed with PhyloChip DNA microarrays, samples obtained from one sampling event (MTF-PHSF-D1) at LMA-MTF yielded 1,222 distinct OTUs (Table 10-1). Samples obtained from four sampling events at KSC-PHSF yielded between 728 and 1,519 OTUs, and samples collected from five samplings at JPL-SAF yielded between 468 and 1,492 OTUs.

The team observed correlations between the detected presence of certain bacterial families and the presence or absence of Phoenix or MSL hardware in SAC facilities. The PhyloChip exclusively detected the presence of *Dictoglomaceae* and *Leuconostocaceae* members, and cloning exclusively detected members of *Burkholderiaceae*, *Pseudomonodaceae*, and *Aurantimonadaceae*, in spacecraft-devoid SACs (category B) but neither was able to detect such microbes when spacecraft were present (both category A and C) (see Figure 2; (La Duc *et al.,* 2009a)]). Certain bacterial families appeared to be associated with a given spacecraft, as is evident in the PhyloChip-derived detection of *Actinosynnemataceae*, *Halothiobacillaceae*, *Hyphomonadaceae*, *Intrasporangiaceae*, and *Vibrionaceae* solely in samples collected in the presence of the Phoenix spacecraft (category A). Similarly, *Procabacteriaceae* species were uncovered via PhyloChip in the presence of the Phoenix (category A) and MSL (category C) spacecraft, but not encountered at all when such hardware was absent (category B; see Figure 2; (La Duc *et al.,* 2009a)]). Only 3 (*Flexibacteraceae*, *Oxalobacteraceae*, and *Streptococcaceae*) of 173 families were detected in all three categories by both PhyloChip and cloning approaches (see Figure 2; (La Duc *et al.,* 2009a)]). There were, however, a great many bacterial families (130) whose presence was revealed in all three categories, exclusively by PhyloChip (see Figure 2; (La Duc *et al.,* 2009a)]).

Category C samples were collected from a SAC located in an arid, desert-like location (JPL, Pasadena, California; RH 40%–45%), whereas category A samples were collected from a brackish, swamp-like location (KSC, Cape Canaveral, Florida; RH 60%–85%). Changes in relative abundance at the OTU level, as inferred from PhyloChip DNA microarray, of category A and C samples are depicted in Figure 10-2. This analysis can readily be determined between samples based on the fluorescence intensity of OTU probe sets, where a change in 500 relative fluorescence units

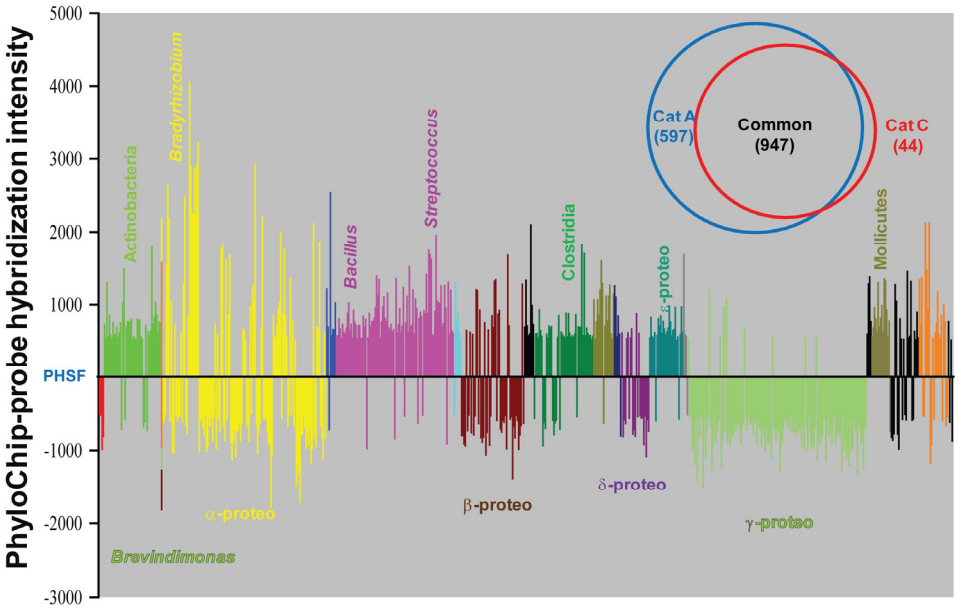


Figure 10-2. PhyloChip analysis of complete bacterial communities as a function of SAC categorization. (A) Bacteria are ordered alphabetically from left to right according to taxonomic affiliation. Bars above the zero line represent bacteria that increased in abundance relative to JPL-SAF during the MSL mission; bars below represent bacteria that declined in abundance. (B) Venn diagrams demonstrate the number of bacterial subfamilies detected in each SAC category.

corresponds to an approximate five-fold change in 16S rRNA gene copy number (Brodie *et al.,* 2006a). Members of genera known to thrive in dry conditions exhibited high fluorescent intensities in category C samples, and included *Bacillus, Clostridium, Streptococcus,* and some actinobacteria, ε-proteobacteria, and mollicutes. Conversely, α–, β–, γ–, and δ–proteobacteria yielded significantly elevated fluorescence intensities in category A samples. An expanded, statistically significant (p-value >95%) biodiversity was observed in aquatic bacterial families in samples collected at the more humid KSC, in comparison to those collected at JPL (Table 10-3).

10.6 Reason for Discontinuing Conventional Cloning and Sequencing-Based Approaches

There are numerous factors to consider when choosing an appropriate methodology for elucidating microbial diversity in environmental samples. While cost, time, labor intensity, and reproducibility each weigh quite heavily in their own right, the bias and accuracy of a given approach are perhaps the most important aspects when considering the goal of planetary protection endeavors, including the Genetic Inventory task. Due in large part to biases in the generation and picking of transformant colonies, PhyloChip DNA microarrays detected a much broader biodiversity than clone libraries, even at very high taxonomic levels (DeSantis *et al.,* 2007; Wilson *et al.,* 2002). Cloning and Sanger sequencing is time- and labor-intensive, and there was an appreciable difference in the level at which the PhyloChip "out-detected" cloning approaches based on the presence or absence of spacecraft hardware at the time of sample collection.

The PhyloChip detected biosignatures in more than 140 known bacterial families, almost 100 of which had never been observed in any of the clone libraries (Figure 10-1). Furthermore, meaningful data were retrieved from three samples with PhyloChip arrays even after several attempts at polymerase chain reaction (PCR) and subsequent cloning regimes had failed (Table 10-1). PhyloChip DNA microarrays were able to detect the presence of anywhere from 9- to 70-fold more bacterial taxa than 16S rRNA-based cloning approaches. The superior detection capabilities of the PhyloChip were far more pronounced when the facility was sampled while housing spacecraft hardware (32- to 70-fold), as opposed to when sampled facilities were vacant (9- to 16-fold). This was a reasonable correlation, since the bacterial diversity associated with any given SAC should be a combination of the bacterial diversities associated with that facility plus that associated with foreign spacecraft components fabricated from countless geographic locations. Therefore, the Genetic Inventory team discontinued the use of conventional cloning and sequencing approaches in favor of PhyloChip DNA microarrays and high-throughput pyrosequencing technologies.

10.7 Results of 454 Tag-Encoded Pyrosequencing

The 454 tag-encoded pyrosequencing methods are a valuable tool for ascertaining the phylogenetic affiliations of members of the complex microbial communities found on spacecraft hardware. The massively parallel capability of 454 tag-encoded pyrosequencing technology (Margulies *et al.,* 2005) implemented on the Roche Genome Sequencer FLX (GS-FLX) can generate 300,000–450,000 (~500 bp) "tag" sequences from one or more of nine known rRNA gene hypervariable regions (V1 to V9; Neefs *et al.* 1990) in a single run without requiring the construction of clone libraries or preparation of DNA templates.

A constraint of competing phylogenetic microarray (PhyloChip) technology is that the detection of a given organism requires *a priori* knowledge of sequences specific to its rRNA coding regions to be printed on the chip. In contrast, while 454 tag-encoded pyrosequencing methods are currently more costly for processing a single sample, they do not require previous information about DNA sequences that might be recovered from a microbial population. There was a striking difference in

the ability to address rRNA gene-based microbial diversity between standard 454 tag-encoded pyrosequencing (which is based on ~100-bp reads) and long-read (~350-bp) FLX Titanium pyrosequencing. The former routinely generated false-positives that appear to be associated with the remnants of contaminant biomaterials from consumables and reagents after gamma-irradiation. However, such fragmentation products were small enough not to interfere with longer read FLX chemistry. As a result, Titanium long-read pyrosequencing proved effective in discerning true sample-derived microbe sequences from contamination remnants due to gamma-radiation sterilization processing.

Before the start of the Genetic Inventory task, although not comprehensive, biological diversity surveys of the spacecraft and associated surfaces were generally focused on a census of bacteria (La Duc *et al.,* 2004; La Duc *et al.,* 2003; Moissl *et al.,* 2007b; Venkateswaran *et al.,* 2001) and there were few reports on archaea (Moissl-Eichinger 2010; Moissl *et al.,* 2008). This made sense, since historically, bacterial endospores have been the logical candidates when considering life forms potentially capable of surviving interplanetary transport (Favero 1971; La Duc *et al.,* 2004; Puleo *et al.,* 1977). However, the Genetic Inventory team considered that no census of terrestrial microbiology is complete without a consideration of Eukarya and Archaea; and for avoidance of false-positive detection on life detection and returned sample missions, all terrestrial life forms are of concern. The success of the 454 Titanium FLX tag-encoded pyrosequencing technology for Bacteria encouraged the application to relevant eukaryotic and archaeal rDNA tags.

10.7.1 Pyrosequencing-Derived Bacterial Diversity

In total, the pyrosequencing procedures carried out in this study yielded ~200,000 bacterial 16S rRNA gene sequences >350 bp in length from the 31 sample sets examined. When these sequences were processed in mothur, ~70% of the sequences were omitted from consideration due to quality control criteria designed to select-out incomplete or spurious sequences. The remaining 30% of sequences, with a minimum length of 250 bp, were aligned and subjected to cluster analyses to reveal their phylogenetic affiliations. Resulting pyrosequencing-derived sequence abundance and coinciding numbers of OTUs associated with the various surface samples examined are given in Table 10-4, respectively.

Collectively, Group I and II samples, which had been collected from floor and GSE surfaces gave rise to a greater number of OTUs (2,686 OTUs from 71 m^2) compared to the spacecraft hardware surface samples of Group III and IV (194 OTUs from 162 m^2). The Group I floor samples, collected from a surface area of ~10 m^2 from a non-NASA cleanroom floor (sample #150), yielded two times the number of OTUs than the NASA cleanroom floor (sample #148; JPL-SAF). The JPL-SAF cleanroom GSE surfaces (sample # 146) gave rise to 2.5 times more bacterial OTU diversity than the floors that they rested on (sample #148; JPL-SAF). Cleanroom floors yielded 100 to 200 different bacterial OTUs, whereas the number of OTUs detected on cleanroom GSE surfaces was ~500.

The extremely clean nature of the JPL-SAF cleanroom floor samples required that ten 1 m^2 surface areas be sampled and pooled together to generate sufficient amounts of PCR product for downstream pyrosequencing analysis. However, the floors of the ordinary room adjacent to the JPL-SAF cleanroom required only 1 m^2 be sampled and processed to amplify sufficient amounts of template DNA. With respect to the floors of this ordinary room, the entrance floor (sample #143) where staff ingress/egress in street clothes, gave rise to ~5.3 times more OTUs (654 OTUs) than the adjacent JPL-SAF cleanroom floor. The central floors of this ordinary room (sample #144 and #145), and the surfaces of a co-located shoe cleaner instrument (sample #141), yielded between 159 and 486 OTUs, many of which were of proteobacterial and actinobacterial lineage (Table 10-4). The floors of the air-lock chamber (sample #142) that connect the ordinary room to the JPL-SAF cleanroom gave rise to 145 distinct bacterial OTUs.

Table 10-4. Pyrosequencing-based bacterial OTUs retrieved from various spacecraft and associated surfaces.

Bacterial Taxa	Number of OTUs retrieved from:																															
	Group I								Group II				Group III															Group IV			
	150	141	142	143	144	145	146	148	155	157	159	161	124	125	133	134	135	136	137	126	127	128	129	130	138	140	132	151	152	153	154
	Floor-70A	Shoe Cleaner	Air-lock	Entrance Floor	Floor 1	Floor 2	JPL-SAF GSE	JPL-SAF Floor	Floor	GSE	Floor	GSE	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft
Actinobacteria	31	122	54	169	50	31	185	25	51	186	3	30			1	6		1	1		3	10	15			5	1	1			
Armatimonadetes	1	4		6	1				3	1					1																
Bacteroidetes	6	45	10	65	9	21	43	8	39	75	4	22						1				1					4				
Chlorobi										1																					
Verrucomicrobia				1					4		2																				
Chloroflexi	1	6	1	4	2	2	5	1		8													3								
Deinococcus-Thermus	2	1		3	3	2	8		5	15		3											1								
Acidobacteria	2	7	1	6	2	1	4	1	1	5		1			2																
Firmicutes	7	50	3	49	15	4	27	11	10	33		2				1					1		4		2	1					
Fusobacteria		2		2	3	1	1	1		2													1								
Gemmatimonadetes	1	3		3			3		3	2		1																			
Nitrospirae									1	1																					
Planctomycetes		1	2	4			1		2	2		1																			
Proteobacteria																															
Alpha	113	113	33	156	49	50	124	41	233	188	38	22		1	3	3						7	9		1		14		2		
Beta	38	47	13	61	23	17	35	9	48	61	7	23	4	1	2	5	2	3		4	1	3	5	2	1		9	2	1	1	
Delta		3		1	1		1	1	3	3					1																
Gamma	11	39	15	67	38	23	29	19	76	40	12	16			2	4	2	2		2	3	8	5	2			6	3	3	1	3
Unidentified		4	1	3	4	3			4	3			1																		
Spirochaetes								1																							
Tenericutes	2	1																													
Unidentified division																															
SC4							3			3																					
TM7		3		2			1																					1			
WPS-2				2					2																						
Unclassified bacteria	12	35	12	50	12	4	27	4	28	33		15			1								2								
Total # of OTUs	**227**	**486**	**145**	**654**	**212**	**159**	**497**	**122**	**513**	**662**	**66**	**136**	**5**	**2**	**13**	**19**	**4**	**7**	**1**	**6**	**8**	**29**	**45**	**4**	**4**	**6**	**34**	**7**	**6**	**2**	**3**

The cleaning and maintenance involved in preparing the JPL-144 cleanroom to receive mission-critical spacecraft hardware successfully reduced and even eliminated many bacterial lineages, as evident by OTUs not detected Prior to cleaning, floor sample #155 gave rise to 513 OTUs, whereas after cleaning the same location (sample #159) showed a complete loss of 447 previously detected OTUs. In general, the incidence of all bacterial lineages declined after cleaning of these cleanroom floors. In similar fashion, prior to cleaning procedures, 662 OTUs were observed from the JPL-144 GSE surfaces (sample #157). This was ~5 times more than the number of OTUs detected after cleaning (136 OTUs; sample #161). Several bacterial groups reported to be capable of surviving desiccation and UV radiation (e.g., actinobacteria, deinococci, firmicutes) were not detected after the cleaning of the floors, whereas the same bacterial types persisted even after cleaning on GSE surfaces. In contrast, the vast majority of purported human-associated bacteria (e.g., proteobacteria) was eliminated from both the cleanroom floors and co-located GSE surfaces after cleaning.

The results of this investigation predominantly showed pyrosequences typical of human skin–associated bacterial commensals in the Group III and IV spacecraft hardware samples, whereas cleanroom floor samples yielded pyrosequences from a greater variety of microbial sources. Even though firmicute pyrosequences were generated from spacecraft hardware surface samples early in assembly, test, and launch operations (ATLO) (Group III), late ATLO component surfaces (Group IV) were devoid of these, as well as pyrosequences representing desiccation-resistant deinococci and actinobacteria.

10.7.2 Pyrosequencing-Derived Archaeal Diversity

Pyrosequencing generated significantly fewer high-quality archaeal sequences than bacterial sequences for the very same samples. Although ~30,000 sequences spanning >350 bp of the V3–V5 region of archaeal 16S rRNA gene were generated, bioinformatic quality control measures demonstrated that only 151 sequences were truly of archaeal lineage. The remainder of these sequences was related to bacterial taxa, predominantly *Verrucomicrobia*. Resulting archaeal sequence and OTU abundances from the various samples examined in this study are given in Table 10-5.

None of the 31 sample sets examined yielded 1.1-kb archaeal 16S rRNA gene amplicons via traditional PCR techniques (Moissl *et al.,* 2007b). In most cases, even following nested PCR amplification, there was insufficient DNA to perform successful pyrosequencing analysis. However, three samples (#142 to #144) collected from the ordinary room adjacent to the JPL-SAF cleanroom gave rise to measurable levels of archaeal 16S rRNA gene nested-PCR product. Of the 12 samples composing Groups I and II, no long-read (~600-bp) archaeal pyrosequences could be generated from any of the five cleanroom floor (~10 m^2 area) samples tested. In contrast, archaeal

Table 10-5. Archaeal pyrosequences and OTUs retrieved from various spacecraft and associated surfaces.

Archaeal taxa	Number of archaeal pyrosequences/OTUs from:													
	Group I					Group II		Group III						
	150	143	141	142	148	157	161	124	126	127	128	130	132
	Floor	Entrance Floor	Shoe Cleaner	Air-lock	JPL-SAF GSE	GSE	GSE	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft
Nitrosphaeraceae – SCA114-1	17		35	47		5	2						
Nitrosphaeraceae – SCA114-2			1										
Nitrosphaeraceae – SCA117		6											
Methanobacteriaceae – 1								1	2	7	3	16	1
Methanobacteriaceae – 3												1	
Number of archaeal OTUs	1	1	2	1		1	1	1	1	1	1	2	1

pyrosequences were successfully obtained from all five of the ordinary room samples (1 m^2 area), and two of the Group II GSE samples (Table 10-5). In addition, archaeal pyrosequences were generated from six of the 15 spacecraft hardware (Group III) samples, whereas all four sets of mission subsystem component surface (Group IV) samples failed to yield amplifiable archaeal DNA. Group I and II samples exclusively gave rise to pyrosequences representing members of *Nitrososphaeraceae* (120 sequences; 3 OTUs), while Group III spacecraft hardware samples generated only *Methanobacteriaceae* OTUs (31 sequences; 2 OTUs).

10.7.3 Pyrosequencing-Derived Fungal Diversity

The number of fungal OTUs present in the various surface samples examined is given in Table 10-6. In total, 143,557 high-quality fungal sequences (~50% of the total number generated, and more than twice that of high-quality bacterial sequences) comprising 456 distinct fungal OTUs were generated in this investigation. Unlike the case with bacterial pyrosequence, where at least some sequences were generated from each of the 31 sample sets examined, only 18 of these sample sets gave rise to fungal pyrosequences. Fungal pyrosequences were not generated from many of the spacecraft hardware samples (11 out of 19 sample sets), despite the fact that a large surface area was sampled. Group I samples gave rise to a high abundance of fungal OTUs, compared to the samples of all other categories. Of these, the JPL-SAF cleanroom floor samples yielded only three OTUs, whereas the non-NASA cleanroom floors (sample #150) gave rise to the most fungal OTUs, followed by the entrance floor of the ordinary room adjacent to the JPL-SAF cleanroom (#141), and co-located shoe cleaner surfaces (#142). The central floors of this ordinary room did not present as much fungal diversity (12 and 3 OTUs) as the entrance floor (133 OTUs). Fungal communities were more diverse (44 OTUs) on the floors of the airlock (#142), which connect this ordinary room to the JPL-SAF cleanroom.

Results show that the cleaning procedures applied to both the JPL-SAF and JPL-144 cleanroom floors effectively reduced a significant proportion of the fungal population, for 0 to 3 fungal OTUs were observed in these samples after cleaning. In contrast, GSE surfaces that were sampled after cleaning still gave rise to 30 fungal OTUs (compared to 186 fungal OTUs observed in these samples before cleaning). The observed abundance of fungal OTUs arising from GSE surfaces (#146) residing in the JPL-SAF cleanroom far exceeded (51 OTUs) that corresponding to their floor counterparts (#148; 3 OTUs). Fewer than four fungal OTUs were observed in several (6 out of 15) of the Group III spacecraft hardware samples, and even fewer OTUs (0 to 2) were detected in the Group IV subsystem component samples.

With respect to diversity, the total fungal population encountered in this study spanned four distinct phyla (Table 10-6), and the majority of detected fungal pyrosequences (99.9%) represented either Ascomycota (42.7%) or Basidiomycota (57.2%). With regard to OTU incidence and diversity, 71% and 28% of the 831 OTUs observed in this study belonged to the phylum Ascomycota, and Basidiomycota, respectively. Pyrosequences representative of members of the *Dothideomycetes* were observed in the Group III spacecraft hardware samples. Members of this class were encountered in high abundance (241 OTUs) in most of the samples examined. In addition, OTUs representing species of the genus *Penicillium* (*Eurotiomycetes*) were found in the JPL-SAF cleanroom floor, spacecraft hardware, and mission subsystem component samples (Table 10-6).

Table 10-6. Pyrosequencing-based fungal OTUs associated with spacecraft surfaces and assembly environments.

	Number of OTUs from:																			
	Group I								Group II		Group III						Group IV	
Fungal taxa* that are:	**150**	**142**	**143**	**144**	**145**	**146**	**148**	**141**	**157**	**161**	**125**	**126**	**127**	**128**	**129**	**130**	**151**	**154**
Phylum / ***Sub-Phylum*** / ***Class***	**Floor-70A**	**Air-lock**	**Entrance Floor**	**Floor 1**	**Floor 2**	**JPL-SAF GSE**	**JPL-SAF Floor**	**Shoe Cleaner**	**GSE**	**GSE**	**Spacecraft**	**Spacecraft**	**Spacecraft**	**Spacecraft**	**Spacecraft**	**Spacecraft**	**Spacecraft**	**Spacecraft**
Ascomycota	12	4	7		1	4		3	3	1								
Pezizomycotina																		
Unclassified Pezizomycotina	23	6	17	2		7		14	28	2								
Dothideomycetes	59	13	36	3	1	17		35	56	16	2	1	1	1				
Eurotiomycetes	27	3	11		1	2	2	5	12	1					1	1	1	
Lecanoromycetes	9	1	1			1		1	1	1								
Leotiomycetes	16	2	6			4		5	9									
Pezizomycetes	1								2									
Sordariomycetes	21	3	10	4		3		10	19	2					1	1		
Saccharomycetes	1	1	6			1		4	2									
Basidiomycota	1																	
Agaricomycotina																		
Unclassified Agaricomycotina									1									
Agaricomycetes	11	2	4			1		3	3					1	1			
Tremellomycetes	28	5	17			9	1	16	23	3								
Pucciniomycotina			1															
Agaricostilbomycetes			1					1										
Cystobasidiomycetes	4	1	5	1				4	3	1								
Microbotryomycetes	1		2															
Pucciniomycetes	16	3	8			1		12	14	2	1							2
Ustilaginomycotina																		
Exobasidiomycetes	4		1	2		1		1	3									
Ustilaginomycetes	1							1	4									
Chytridiomycota																		
Chytridiomycetes								1	3	1								
Former Zygomycota																		
Mortierellomycotina	1																	
Total	**236**	**44**	**133**	**12**	**3**	**51**	**3**	**116**	**186**	**30**	**3**	**1**	**1**	**2**	**3**	**2**	**1**	**2**

*Note: *NCBI Taxonomy*

10.8 Additional Applications of Genetic Inventory Methods

10.8.1 Comparison of NASA Standard Spore Assay and PhyloChip Method

A cultivation-dependent NASA standard spore assay and a molecular DNA microarray method were compared for their ability to assess the relative cleanliness, with regard to microbial abundance and diversity, of spacecraft surfaces. Colony counts resulting from NASA standard spore assays of spacecraft surfaces were extremely low, whereas PhyloChip Generation 3 (G3) DNA microarray analyses of the same sample set resolved the genetic signatures of a highly diverse suite of microorganisms. Samples completely devoid of cultivable spores were shown to harbor the DNA of more than 100 distinct microbial phylotypes. Furthermore, samples more highly laden with cultivable spores did not necessarily give rise to a greater microbial diversity upon analysis with the DNA microarray.

The detailed results are presented in Appendix C (Cooper *et al.,* 2011). The findings of this study clearly demonstrate that there is not a statistically significant correlation between the cultivable spore counts obtained from a sample and the phylogenetic breadth enveloped therein. Based on these results, it is clear that innovative molecular techniques, such as DNA microarrays, should be coupled with classical culture-based methods to more thoroughly assess the relative cleanliness of spacecraft surfaces.

10.8.2 Anaerobic Bacterial Diversity of NASA Cleanrooms Using Cultivation, Cloning and Sanger Sequencing, and PhyloChip Assays

Although the cultivable and non-cultivable microbial diversity of SACs has been previously documented using conventional and state-of-the art molecular techniques, the occurrence of obligate anaerobes within these cleanrooms is still uncertain. Therefore, anaerobic bacterial communities of three cleanroom facilities were analyzed during assembly of the MSL rover. Anaerobic bacteria were cultured on several media, and DNA was extracted from suitable anaerobic enrichments and examined with conventional 16S rRNA gene clone library as well as high-density phylogenetic 16S rRNA gene microarray (PhyloChip) technologies. The culture-dependent analyses predominantly showed the presence of clostridia and propionibacteria strains. The 16S rRNA gene sequences retrieved from clone libraries revealed distinct microbial populations associated with each cleanroom facility, clustered exclusively within Gram-positive organisms. PhyloChip analysis detected a greater microbial diversity, spanning many phyla of bacteria, and provided a deeper insight into the microbial community structure of the cleanroom facilities.

The detailed results are presented in Appendix C (Probst *et al.,* 2010). This study presents an integrated approach for assessing the anaerobic microbial population within cleanroom facilities, using both molecular and cultivation-based analyses. The results reveal that highly diverse anaerobic bacterial populations persist in cleanrooms even after imposition of rigorous maintenance programs and will pose a challenge to planetary protection implementation activities.

10.8.3 Utilization of Molecular Assays to Measure "Viable" Bacterial Population of NASA Cleanrooms using PhyloChip and 454 Tag-Encoded Pyrosequencing Methods

The advent of phylogenetic DNA microarrays and high-throughput pyrosequencing technologies has dramatically increased the resolution and accuracy of detection of distinct microbial lineages in mixed microbial assemblages. Despite an expanding array of approaches for detecting microbes in a given sample, rapid and robust means of assessing the differential viability of these cells, as a function of phylogenetic lineage, remain elusive. In this study, pre-PCR propidium monoazide

(PMA) treatment was coupled with downstream pyrosequencing and PhyloChip DNA microarray analyses to better understand the frequency, diversity, and distribution of viable bacteria in SACs. Sample fractions not treated with PMA, which were indicative of the presence of both live and dead cells, yielded a great abundance of highly diverse bacterial pyrosequences. In contrast, only 1 to 10% of all of the pyrosequencing reads, arising from a few robust bacterial lineages, originated from sample fractions that had been pretreated with PMA. The results of PhyloChip analyses of PMA-treated and untreated sample fractions were in agreement with those of pyrosequencing. The viable bacterial population detected in the cleanrooms devoid of spacecraft hardware was far more diverse than that observed in the cleanrooms that housed mission-critical spacecraft hardware. The latter was dominated by hardy, robust organisms previously reported to survive in oligotrophic cleanroom environments.

More details are presented in Appendix C (Vaishampayan *et al.,* 2012) about the findings of the first-ever effort to assess the viability of cells in low-biomass environmental samples, and correlate differential viability with phylogenetic affiliation. In this paper, the Genetic Inventory team describes the studies that enabled the first ever statistically significant differentiation between the total and viable-only portion of microbial communities in cleanroom environments.

10.9 Key Considerations

10.9.1 Limitations of the Molecular Methods for Low-Biomass Samples

All methods tested required the PCR step prior to analysis. As for limitations with the cloning and mass-sequencing approaches, there was likely a molecular bias that favored the PCR amplification and/or amplicon ligation of certain microbial lineages and henceforth masked the detection of taxa that were present in much lower abundance.

10.9.2 Conventional Cloning and Sanger Sequencing

- Even though all 10 pooled samples yielded full-length 16S rRNA gene amplicons (1.5-kb), only 7 gave rise to clone libraries following transformation of competent *E. coli*. Despite the faint visibility of some bands in agarose gels, the PCR products from all 10 of these samples were sufficient to be analyzed via PhyloChip DNA microarray.
- Due to the superior detection capabilities of both PhyloChip and 454 tag-encoded pyrosequencing, the Genetic Inventory team elected to abandon cloning and Sanger sequencing–based approaches in favor of the following more sensitive molecular techniques.

10.9.3 PhyloChip DNA Microarray

- Data regarding the bacterial diversity of NASA cleanrooms, in which spacecraft are assembled or prepared for launch, indicate that PhyloChip analysis was capable of detecting over 9- to 70-fold more OTUs and 4 times as many bacterial families as conventional cloning.
- Although each sample analysis by the PhyloChip provides detailed information on microbial composition, the highly parallel and reproducible nature of this array allows tracking community dynamics over time and treatment. With no prior knowledge, specific microbial interactions may be identified that are key to particular changing environments.
- Over the course of the Genetic Inventory task, many PhyloChips were run to assess the microbial diversity present in SAC samples. During the process, the Genetic Inventory team questioned at exactly what point (i.e., after what number of PhyloChip analyses) could confidence levels be drawn to ensure that (a) what was being detected was truly present, and (b) the extent to which a given amount of sampling was representative of the true environmental population. To this end, the Genetic Inventory team carried out an experiment where the datasets from 53 individual PhyloChip

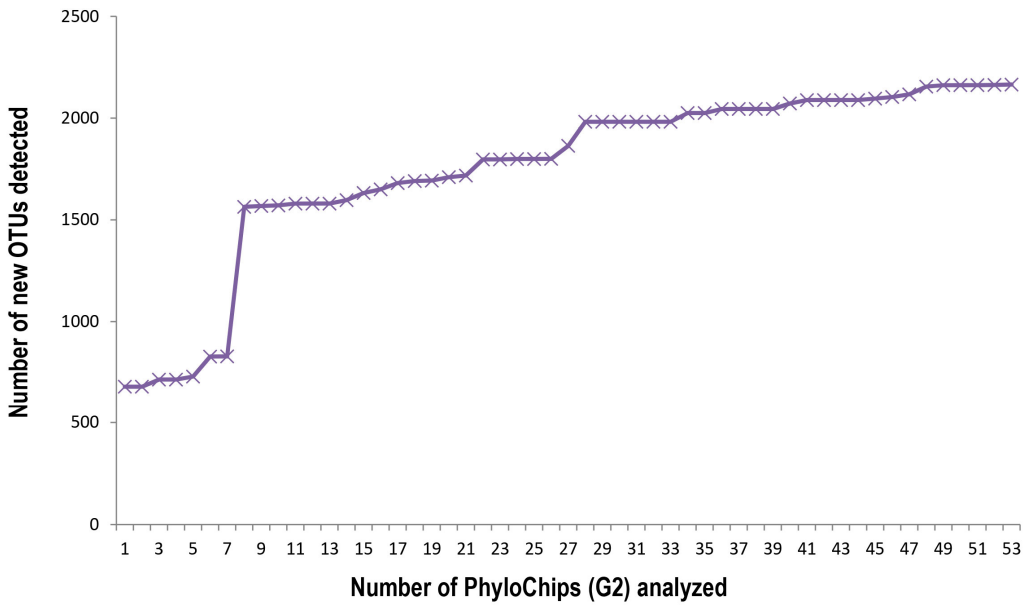


Figure 10-3. A rarefaction curve was plotted for the number of new OTUs observed as a function of PhyloChips (G2) analyzed. The plateau effect observed after the ~30th PhyloChip suggests occurrence of fewer number of new OTUs despite continued PhyloChip analysis.

runs were randomized and results were visualized in the manner of rarefaction curves (i.e., the number of new OTUs were plotted vertically against the number of samplings plotted horizontally). A "plateau effect" was observed in the rarefaction curve around the 30th PhyloChip, implying that after ~30 PhyloChip analyses, the sampled biodiversity had finally begun to mirror the true environmental population diversity (Figure 10-3). From the onset of this plateau (the 30th chip analyzed) on through to the last chip analyzed (the 53rd), there was a 0% to 2.17% increase in new OTUs. This confirmed that after having sampled and analyzed a given environment with 30 PhyloChips, greater than 95% of the biodiversity had been detected.

- Upon comparing JPL-SAF and KSC-PHSF diversity, ~6.7% of the total number of combined OTUs were unique to JPL-SAF (122 OTUs); KSC-PHSF yielded 318 OTUs not detected in JPL-SAF.

10.9.4 454 Tag-Encoded Pyrosequencing PhyloChip DNA Microarray

- The findings of this study provide new and important insights into the benefits and limitations of innovative molecular approaches for assessing the microbial diversity associated with samples extremely low in total biomass.
- Coupling the innovative pyrosequencing techniques employed in this study with other innovative molecular methodologies, such as PhyloChip G3 DNA microarrays etc., could lead to significantly improved approaches for monitoring and mitigating the diverse microbial populations associated with ultra-clean environments, without the biases that plague culture-dependent techniques.
- The greatest microbial diversity was encountered in ordinary room facilities, followed next by GSE surfaces, then cleanroom floors, and finally spacecraft components.
- The results of this study underscore that, based on mission need, (a) continued monitoring and bio-signature detection should be extended throughout all three domains of life, and that (b) a shift toward high-throughput, data-rich molecular assays with significant bioinformatic analysis is inevitable in microbial community analyses.

Bacterial Pyrosequence Analysis

- The floors of both NASA and non-NASA cleanrooms yielded 100 to 200 bacterial OTUs, whereas cleanroom GSE surfaces yielded 2 to 5 times more OTUs.
- The extremely clean nature of the cleanroom floor samples required that ten 1 m^2 surface areas be sampled to generate sufficient amounts of PCR product for downstream pyrosequencing analysis whereas the floors of the ordinary room required only 1 m^2 be sampled and processed.
- The bacterial OTUs of the cleanroom floors declined (~5 times reduction) after cleaning them using Kleenol.
- Cleanroom floor surfaces yielded sequences from a greater variety of microbial sources.
- Pyrosequences typical of human skin-associated bacterial commensals were observed in spacecraft hardware samples

Archaeal Pyrosequence Analysis

- None of the 31 samples sets examined yielded 1.1-kb archaeal 16S rRNA gene amplicons via traditional PCR techniques and hence cloning was not possible.
- There were no archaeal sequences generated from any of the samples collected from the cleanroom floors.
- Archaeal pyrosequences were generated from some spacecraft hardware samples.

Fungal Pyrosequence Analysis

- Only 18 of 31 sample sets gave rise to fungal pyrosequences.
- As observed for bacteria, the fungal OTUs of the cleanroom floors also declined after cleaning using Kleenol.

Outcome: The Genetic Inventory capability was demonstrated, as the application of PhyloChip DNA microarrays and Titanium 454 FLX tag-pyrosequencing technologies rendered the most in depth analysis of spacecraft-associated microbial diversity to date.

11. CONCLUSIONS

This section summarizes the key conclusions and recommendations resulting from the Genetic Inventory task. Pertinent research challenges and lessons learned are discussed.

The purpose of the Genetic Inventory task was to develop an end-to-end capability, with the precautionary principle assumed that if the DNA of an organism is detected in a spacecraft sample, a live representative of that species might be present elsewhere on the spacecraft. Initial studies in 2007 using non-standardized procedures and low-biomass samples from a spacecraft environment demonstrated that it was possible to perform an end-to-end analysis and obtain meaningful genetic inventory data. The remainder of the work was characterizing and optimizing the multiple experimental steps (sample collection, sample processing, sample analysis, sample archiving and data analysis) in the end-to-end process to ensure the final analysis output was representative of the surface analyzed. **By the end of the 6-year task, the Genetic Inventory team had successfully developed a capability to (a) systematically collect, process, and archive nucleic acids, and (b) effectively assess the phylogenetic breadth of microorganisms present on spacecraft and associated surfaces. This capability enabled the Genetic Inventory team to generate the most comprehensive (bacterial, archaeal, and fungal) assessment of spacecraft assembly cleanroom (SAC)-associated biodiversity to date.**

While the overall objective remained the same throughout the task, the experimental emphasis shifted over the course of the 6-year effort, based on recommendations resulting from two workshops engaging science and technology experts from industry, academia, and government (Appendix F). As a result, the team shifted emphasis toward sample collection and processing of low-biomass samples, while balancing efforts to test the applicability and optimize the effectiveness of molecular analysis techniques.

The results of the Genetic Inventory task collectively stress the importance of aligning sample collection and processing approaches with the level of detection and/or resolution of diversity required. The study found cotton swabs and BisKits to be the preferred sampling instruments when surveying the entire spectrum of microbial diversity on a given surface. Laboratory-controlled experiments found BiSKits to be qualitatively and quantitatively superior to NASA-certified polyester wipes for retrieving DNA molecules from large surface areas. Results indicated that BiSKit samplers were able to recover all microbial constituents of the model microbial community (MMC), whereas polyester wipes only recovered four of the nine MMC strains assayed. Similarly, cotton swabs were found to be nearly twice as effective (by yield) as nylon-flocked swabs in recovering microbial rDNA from surfaces. Cotton swabs enabled the recovery of rDNA from all microbial constituents, whereas nylon-flocked swabs recovered only six of the nine strains assayed.

As demonstrated over the course of the Genetic Inventory study, the advent of phylogenetic DNA microarrays and high-throughput pyrosequencing technologies has dramatically increased the resolution and accuracy of detection of specific microbial taxa in mixed microbial assemblages. At the start of the Genetic Inventory study, G2 PhyloChip microarrays could only resolve a small fraction (~7,800 OTUs) of the microbial diversity found in public databases. Midway through the study, the availability of G3 PhyloChips enabled almost complete coverage (~59,000 OTUs) of the microbial lineages known and available in public databases. Moreover, the bioinformatic analysis of G3 PhyloChip data statistically addressed potential cross-hybridizations between OTUs (a capability not available with G2). The studies conducted by the Genetic Inventory team also yielded the first-ever 454 FLX Titanium tag-encoded pyrosequencing-derived survey of microbial diversity from all

three domains of life (bacteria, archaea, and eukarya) from low-biomass SAC and spacecraft surfaces (La Duc *et al.,* 2012)

11.1 Conclusions: General Principles of Current Genetic Inventory Capability

The following recommendations highlight the current best materials, methods, and modes of analysis for generating a genetic inventory.

11.1.1 Sample Collection

Quantity of Samples

The Genetic Inventory team set out to determine the extent of sampling effort required to yield a full assessment of the biodiversity present in low-biomass SAC samples. As a conservative estimate, and based on the results stemming from rarefaction analysis of randomized PhyloChip microarrays (where a plateau effect was observed in the rarefaction curve at approximately the 30th sample analyzed), the Genetic Inventory team advocates collecting, processing, and analyzing no fewer than 35 samples, in parallel (as though they were replicates), from each distinct system of interest (e.g., cleanroom floors, ground-support equipment [GSE], spacecraft hardware).

Sample-Collection Periodicity

Determining the ideal periodicity of sample collection is based largely on the scope and primary objective of the study. For studies addressing a single hardware system, or the cleanliness of a given SAC at a given time and date, all samples should be collected at once. For studies more interested in changes in SAC-associated biodiversity as a function of seasonal change, or spacecraft hardware-associated biodiversity fluctuations over the course of ATLO, samples should be collected incrementally over a given period of time.

1. It is recommended that sample collection for genetic inventories be performed in accordance with the procedures and protocols found in Appendix B. The Genetic Inventory team recommends the use of BiSKit samplers and cotton swabs to collect materials from large surface areas and small surfaces, respectively. As time passes, new technologies may be developed that would improve on the present capability, in which case the procedures and protocols found in Appendix B can be used as a reference point for evaluating the new technology.
2. Investigators are encouraged to exercise caution when assuming that any given amount of sampled area from a low-biomass surface will yield sufficient biomass for downstream analyses; some surfaces may be just too clean. The Genetic Inventory team recommends collecting samples from as many relevant sources (e.g., cleanroom floors and GSE in addition to spacecraft surfaces), and sampling as much surface area from each source as is practical (no less than 10 m^2) to ensure that the total amount of DNA collected will be sufficient (in excess of 4.4×10^{-12} μg = ~4×10^3 microbial chromosomes) to yield reproducible downstream results representative of the environment being studied.
3. Prior to experimenting with actual, oftentimes irreplaceable samples, the level of background contamination (i.e., cells, nucleic acids, etc.) associated with the reagents, consumables, and devices used in the study should be determined to understand how contaminants affect the outcome and statistical significance of the investigation.
4. Investigators are encouraged to collect as much metadata throughout the course of the investigation as is practical. Many easily overlooked variables pertaining to sample collection

(i.e., periodicity of sampling, cleaning regimen(s) applied, geographic location, particle counts, relative humidity of the cleanroom, human activity, etc.) factor into the analysis.

11.1.2 Sample Processing

1. Early in the processing and analysis, a decision tree (Figure 6-6) can be applied to determine whether the given sample is of sufficient quality based on initial PCR-amplicon yields (i.e., spiking, PCR product concentration using Bioanalyzer, etc.). Later in the processing, coverage values, rarefaction analysis, and diversity estimations can be conducted to determine how representative the samples are and the thoroughness of the sampling effort.
2. Millipore Amicon Ultra 50-kDa molecular weight cut-off centrifugal filters are the recommended approach for concentrating samples. No more than 3 centrifugations (equating to a 45-ml volume able to be concentrated) can be applied to any one filter unit, or the integrity of the filter unit will be compromised. Pooled sample volumes in excess of 45 ml should be pre-filtered down to 45 ml with a vacuum filtration system through a 0.22-µm membrane.
3. To promote detection of organisms with cell walls and outer membranes that are difficult to lyse, it is suggested that automated DNA purification procedures be augmented with some form of mechanical agitation. For example, a simple bead-beating step prior to nucleic acid extraction protocols will lyse extremely resilient actinobacterial cells and even bacterial spores from environmental samples. However, it should be noted that such mechanical agitation can shear and/or significantly degrade large nucleic acids from less robust organisms. Therefore, it is suggested that environmental samples be divided into two equivalent fractions, with only one fraction being subjected to bead-beating. Both fractions should then be recombined and subjected to automated DNA extraction.
4. The automated Promega Maxwell MDx-16 instrument performs better than traditional manual phenol-chloroform-isoamyl alcohol (PCIAA)-based techniques for purifying DNA from samples. This automated approach maximizes reproducibility and avoids technician-based variability.
5. With regard to storing and archiving DNA samples:
   a. If the goal is to recover the greatest DNA yield following short-term storage of a sample rich in initial DNA concentration, then Biomatrica's SampleMatrix will yield the best recovery.
   b. If the goal of the study is to store and archive DNA—of any initial concentration—for long-term purposes while minimizing variability in recovery over time, then the more traditional −80°C storage will yield the best recovery.
   c. To maximize the recovered DNA yield after storage, it is recommended that environmental samples be filter-concentrated with Amicon filters to remove molecules <50 kDa prior to being archived at −80°C.

11.1.3 Molecular Analysis and Bioinformatic Interpretation

1. Traditional cloning and Sanger-sequencing approaches are not capable of generating an all-inclusive genetic inventory from low-biomass environments. These methods are limited by numerical constraints on the number of sequences that can be processed, as well as molecular biases that favor the amplification and/or amplicon ligation of highly represented bacterial taxa and hence mask the detection of those present in lower abundance.

a. PhyloChip microarray analyses (the G3 iteration in particular) are superior to conventional 16S rRNA gene cloning and Sanger-sequencing strategies in all aspects of microbial diversity analysis save one: the detection of novel microbial taxa. Since DNA microarrays are dependent on the hybridization of environmental oligonucleotides to known probes of specific sequence, an enormous amount of *a priori* sequence information is required. This need for previously inferred probe sequence data precludes the ability of this technique to detect the presence of DNA arising from novel microorganisms.

b. The Genetic Inventory team recommends that future inventories of biodiversity abandon traditional cloning and Sanger-sequencing approaches in favor of high-throughput, data-rich molecular strategies such as G3 PhyloChip and Titanium 454 FLX tag-encoded pyrosequencing (which will require considerable bioinformatics analysis).

2. The combined application of advanced molecular analysis approaches, such as G3 PhyloChip DNA microarrays and Titanium 454 FLX tag-encoded pyrosequencing can be employed to increase the thoroughness of the genetic inventory obtained. By capitalizing on the unique strengths of each of these innovative methods, the cross-comparison of resulting data sets works to expand the resolvable spectrum of biodiversity contained in a given sample, while bolstering the statistical confidence of such measurements.

3. PhyloChip DNA microarray analysis is excellent for assigning taxonomy at the subfamily level, whereas Titanium 454 FLX tag-encoded pyrosequencing can confidently resolve taxonomy down to the genus level. In order to cogently compare between the microbial diversities resulting from these two distinct approaches, the Genetic Inventory team recommends setting the level of analysis to the subfamily-level and working toward more sensitive resolution as the study warrants. It is important to keep in mind that PhyloChip analysis is suitable only for beta-diversity estimations—not for total diversity assessment. PhyloChip analysis is more robust at comparing two or more samples at the subfamily level, while high-throughput sequencing approaches, like Titanium FLX 454 tag-encoded pyrosequencing, are more appropriate for listing total microbial diversity in a given sample(s).

4. To achieve a comprehensive genetic inventory and account for all reported and previously unreported microbial lineages (as in the case of a spacecraft "passenger list"), a molecular method based largely on Titanium 454 FLX tag-encoded pyrosequencing is recommended, with use of the PhyloChip as a quality/reality check. Should the scope of the study focus primarily on changes in SAC-associated microbial diversity over time, then PhyloChip DNA microarray analysis alone could be considered. In summary, 454 FLX Titanium tag-encoded pyrosequencing is recommended for studies primarily interested in alpha-diversity while PhyloChip microarray technologies are recommended for studies interested in beta-diversity and beyond.

5. Of the numerous bioinformatics approaches available, the Genetic Inventory team recommends the publically available mothur software packages to process the enormous amounts of raw data generated by 454 FLX tag-encoded pyrosequencing. As for PhyloChip G3 post-processing, proprietary bioinformatics packages can be accessed via fee-for-service contracts with either Lawrence Berkeley National Laboratory (LBNL) or its commercial entity, Second Genome.

11.1.4 Other Considerations

1. Investigators planning or implementing their own genetic inventory are, in the first instance, encouraged to follow the protocols and procedures that accompany this report. However, should the decision be made to pursue alternative approaches, it is of utmost importance to evaluate such techniques with appropriate controls. There are numerous variables associated with any given method that factor into such an analysis; the Genetic Inventory team recommends that these be understood in full, and that the approaches favored over those detailed here be demonstrated with a controlled microbial consortium (such as the MMC) prior to testing them on actual spacecraft-associated samples.
2. The Genetic Inventory team broadly recommends that NASA work with appropriate experts to develop practical standard methods [such as those described for other techniques in the NASA 6022 handbook (NASA 2010)] for future projects.
3. There are numerous aspects of a "comprehensive" genetic inventory that cannot be inferred from the (phylo-) genetic inventory presented here, including but not limited to a functional gene- and/or genomics-based survey, and an account of viruses, prions, and Eukaryotic taxa other than Fungi (e.g., paramecia, amoebae). This may or may not be relevant for any given future mission, but should be considered.

11.1.5 Lessons Learned

1. It is of utmost importance to choose the proper tools for the task at hand. Elect to use the best materials possible for the given entity (i.e., nucleic acids, cultivable cells/spores, etc.) targeted.
    a. In the Genetic Inventory task, differences in the ability to resolve rRNA gene-based microbial diversity was striking between 454 V6 tag-encoded pyrosequencing (<100-bp reads) and Titanium 454 FLX V1-V3 tag-encoded pyrosequencing (long-read; >350-bp). The former was confounded by false-positives that arose from the remnants of contaminant biomaterials associated with reagents post-gamma irradiation. However, the fragmentation products of gamma-irradiation were small enough to go undetected by longer read FLX chemistry, and thus Titanium long-read pyrosequencing proved compatible and effective in discerning true sample-borne nucleic acids from the remnants of contaminants after having been sterilized via gamma-radiation.
2. An adequate amount of time should be spent not only in relevant experimentation, but also in (a) the design and logistics of experimental procedures, and (b) interpretation and comprehensive analysis involved in transforming raw data into meaningful information. Investigations can rapidly and easily become too data-rich and analysis-poor without proper planning and logistics.
3. Prior to embarking on a research endeavor heavily dependent on high-throughput sequencing, such as 454 tag-encoded pyrosequencing, future investigators should take the time to understand the amount of time, resources, and effort that will go into tailoring and optimizing the chemistry, bioinformatics, and biostatistics aspects of this technique for their particular objective(s). Though it may seem simple and easily augmented into current experimental designs, investigators should be wary of unforeseen complications and shortcomings.

11.2 Considerations for Future Development

1. Future investigators are encouraged to monitor metadata and apply biostatistics and bioinformatics approaches early and often, in order to detect potential changes in data (i.e., over time or as a consequence of another external factor such as a reagent batch change), in order to preserve the consistency and validity of the collected data set.
2. Though not feasible at the commencement of the Genetic Inventory study, future investigators are encouraged to consider emerging methodologies that now seem plausible. These include the InnovaPrep sample concentration system (for concentration from as high as two liters to few hundred microliters), and; the coupling of propidium monoazide treatment with G3 PhyloChip and Titanium 454 FLX tag-encoded pyrosequencing to differentiate the viable and non-viable fractions of the microbiomes detected.
3. A genetic inventory need not be based solely on rRNA gene fragments. The Genetic Inventory team encourages future investigators to consider other high-throughput sequencing technologies (not only 454 but also other platforms such as Illumina, Ion Torrent, etc.) that will facilitate whole-genome, and even functional genomics-based inventories, according to the need of the mission.
4. The scope of future spacecraft-associated biodiversity assessments should consider whether attention should also be given to Eukarya and Archaea. Based on the success of the Genetic Inventory study, the team considers that future investigators could continue to expand the limits of resolvable biodiversity across the three domains of life, to achieve an even more comprehensive biodiversity census, as required by the individual mission.

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14. ACRONYMS

AA	Axcyte AutoLyser
ABI	Applied Biosystems
AMOVA	analysis of molecular variance
ATCC	American Type Culture Collection
ATLO	assembly, test, and launch operations
ATP	adenosine triphosphate
BB	bacterial bisque
bb-AA	Axcyte AutoLyser with bead-beating agitation
BiSKit	biological sampling kit
bTEFAP	bacterial tag-encoded FLX amplicon pyrosequencing
COSPAR	Committee on Space Research of the International Council for Science
CR	cleanroom
DNA	deoxyribonucleic acid
DNADIST	DNA Distance (program to compute a distance matrix)
DNase	enzyme that catalyzes the cleavage of phosphodiester linkages in the DNA.
DSM(Z)	Deutsche Sammlung von Mikroorganismen und Zellkulturen, a human and animal cell culture collection library in Germany.
ESA	European Space Agency
ESPRIT	algorithm for analyzing species richness of large volumes of 16S rRNA pyrosequences
FISH	fluorescence *in situ* hybridization
FLX	trademark for genome sequencer system manufactured by 454 Life Sciences (a Roche Diagnostics Applied Science company)
G2	Generation 2
G3	Generation 3
GI	Genetic Inventory
GMP	good manufacturing practice
GSE	ground support equipment
GS-FLX	Genome Sequencer FLX
HEPA	High-Efficiency Particle Air
INSD	International Nucleotide Sequence Database
ISO	International Organization for Standardization (a set of cleanliness codes to quantify particulate contamination levels per milliliter of given sample)
iTOL	Interactive Tree of Life
ITS	internal transcribed spacer (region for fungi amplification)
ITW	Illinois Tool Works (makers of industrial polyester wipes used in cleanrooms)
JPL	Jet Propulsion Laboratory
JPL-144	Building 144 cleanroom
JPL-SAF	Jet Propulsion Laboratory Spacecraft Assembly Facility
KSC	Kennedy Space Center
KSC-PHSF	Kennedy Space Center Payload Hazardous Servicing Facility
LBNL	Lawrence Berkeley National Laboratory
LIBSHUFF	a computer program designed to compare two libraries of 16S rRNA gene sequences and determine if they are significantly different
LMA	Lockheed Martin Aeronautics
LMA-MTF	Lockheed Martin Aeronautics Multiple Testing Facility
LSU	large subunit
MASS	modern applied statistics with S ("R" programming package)
MBL	Marine Biological Laboratory

MCC	mission subsystem component surface samples
MD-5	MoBio proprietary DNA elution buffer
MDx-16	Maxwell-16 automated DNA extraction system
MMC	model microbial community
MoBio	UltraClean® Microbial DNA Isolation Kit
MPO	Mars Program Office
MPO-PP	Mars Program Office Planetary Protection
MSA	multiple sequence alignment
MSL	Mars Science Laboratory
MSL-PP	Mars Science Laboratory Planetary Protection
MTF	Multiple Testing Facility (LMA)
NASA	National Aeronautics and Space Administration
NASA HQ PP	NASA HQ Planetary Protection
NAST	Nearest Alignment Space Termination (a multiple sequence alignment server for comparative analysis of 16S rRNA)
NCBI	National Center for Biotechnology Information
NMDS	non-metric multidimensional scaling
NRC	National Research Council
NRRL	National Center for Agricultural Utilization Research culture collection (USDA culture collection)
NSGI	NASA Standard Genetic Inventory
OTU	operational taxonomic unit
P/A	presence or absence
PANGEA	pipeline for analysis of next generation amplicons
PBS	phosphate buffered saline
PCA	principal component analysis
PCIAA	phenol-chloroform-isoamyl alcohol
PCoA	principal coordinates analysis
PCR	polymerase chain reaction
PHSF	Payload Hazardous Servicing Facility (KSC)
PHX	Phoenix
PHYLIP	PHYLogeny Inference Package (computational phylogenetics package of programs for inferring evolutionary trees [phylogenies])
PMA	propidium monoazide
PREVCOM	*Preventing the Forward Contamination of Mars* (NRC document)
PTA	patented strain
PTP	PicoTiterPlate
PTU	PhyloChip taxonomic unit
PW	polyester wipe
QC	quality control
QI-IME	Quantitative Insights into Microbial Ecology (open-source software package for comparison and analysis of microbial communities, primarily based on high-throughput amplicon sequencing data)
qPCR	quantitative PCR
rDNA	recombinant DNA
RDP	Ribosomal Database Project
RH	relative humidity
RNase	ribonuclease
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid

RTL	Research and Testing Laboratory
SAC	spacecraft assembly cleanroom
SAF	Spacecraft Assembly Facility
SAFR-032	*Bacillus pumilus* strain isolated from spacecraft assembly facility
SCK-CEN	The Belgian Nuclear Research Centre
SDS	sodium dodecyl-sulfate
SEM	scanning electron microscopy
SILVA	rRNA database
SIMS	Sample Information Management System
SPRI	solid-phase reversible immobilization
SPRI-TE	automated DNA extraction system by Beckman Coulter
ssPCR	species-specific PCR
ss-qPCR	species-specific quantitative PCR
SSU	small subunit of the RNA
STITCH	algorithm used to *splice, trim, identify, track, and capture* the uniqueness of 16S rRNA gene sequence pairs
TA	cloning kit supplied by Invitrogen.
TEFAP	tag-encoded FLX amplicon pyrosequencing
TOP10	chemically competent *E. coli* cells
t-RFLP	terminal restriction fragment-length polymorphism
TSA	Tryptic Soy Agar
TYG	tryptone yeast extract glucose
U.S.	United States
UNITE	a database providing Web-based methods for the molecular identification of fungal ribosomal DNA sequences

15. Glossary of Terms

16S rRNA gene
The 16S ribosomal RNA (16S rRNA) is a component of the 30S small subunit of prokaryotic ribosomes, and is commonly used in phylogenetic studies as it is highly conserved between different species of bacteria and archaea.

454 tag-encoded pyrosequencing
A method of molecular analysis that provides an in-depth evaluation of the microbial diversity present in samples by examining the highly informative hypervariable regions on the 16S ribosomal RNA gene. 454 refers to 454 Life Science, the company that developed this technology.

Actinobacteria
A dominant group of Gram-positive bacteria having high guanine and cytosine content in their DNA. These bacteria can be terrestrial or aquatic, and typically have hardy cell walls that help them persist in harsh environments.

Analysis of molecular variance (AMOVA)
A statistical model for assessing the molecular variation in a single species, typically biological. AMOVA is a nonparametric analog of the traditional analysis of variance, and is widely used in population genetics.

bead-beating
A process used to promote cell lysis via mechanical agitation.

Betaproteobacteria
A class of proteobacteria consisting of several groups of aerobic or facultative bacteria that are often highly versatile in their degradation capacities, but also contain chemolithotrophic genera (e.g., the ammonia-oxidizing genus Nitrosomonas) and some phototrophs (members of the genera Rhodocyclus and Rubrivivax). Betaproteobacteria play a role in nitrogen fixation in various types of plants, oxidizing ammonium to produce nitrite—an important chemical for plant function. Many of them are found in environmental samples, such as waste water, soil, and even cleanroom environments.

biodiversity
The degree of variation of life forms within a given species, ecosystem, or microbiome.

biological sampling kit (BiSKit)
A macrofoam-based sampling device, which is protectively encased in a sterile plastic unit meant to minimize potential risk of sampling device contamination. In the Genetic Inventory experiment, it serves as one of two materials used for sample collection of large surface areas (i.e., stainless-steel sheets).

bioinformatics
A branch of biological science that deals with the study of methods for storing, retrieving and analyzing biological data, such as biochemical pathways, genetic interactions, and nucleic acid (DNA/RNA) and protein sequence, structure, and function..

biostatistics
The application of statistics for designing biological experiments, summarization, and analysis of data from those experiments and the interpretation of these results.

cloning and Sanger-sequencing
A somewhat conventional approach to elucidating sample microbial diversity via clone libraries and subsequent DNA sequencing. Cloning refers to the shuttling of PCR-amplified ribosomal RNA genes

from noncultivable microorganisms into genetically amenable lab strains of *E. coli* via plasmid vectors. Sanger was the scientist that devised the regimen of DNA sequencing used in this approach, wherein 4 reservoirs each containing a different radiolabeled dNTP are used in concert to render a DNA sequence read of approximately 600-bp.

Committee on Space Research (COSPAR)
The COSPAR was established by the International Council for Science in 1958. Among COSPAR's objectives are the promotion of scientific research in space on an international level, with emphasis on the free exchange of results, information, and opinions, and providing a forum, open to all scientists, for the discussion of problems that may affect space research. These objectives are achieved through the organization of symposia, publication, and other means. COSPAR has created a number of research programs on different topics, a few in cooperation with other scientific Unions.

environmental clustering
A statistical technique for determining the extent of dissimilarity in microbial diversity among different samples.

cotton swab
A sampling device consisting of an organic, cellulose-rich (>95%) material that is wrapped around one end of a wooden stick. In the Genetic Inventory task, cotton swabs served as one of two materials used to collect samples from small surface areas (i.e., stainless-steel coupons).

DNase
Deoxyribonuclease I (usually called DNase I), is an endonuclease coded by the human gene DNASE1. DNase I is a nuclease that cleaves DNA preferentially at phosphodiester linkages adjacent to a pyrimidine nucleotide, yielding 5'-phosphate-terminated polynucleotides with a free hydroxyl group on position 3', on average producing tetranucleotides. It acts on single-stranded DNA, double-stranded DNA, and chromatin.

genetic inventory
An extensive census, a.k.a. "passenger list," of microorganisms associated with a given sample, achieved by numerous surveys implementing systematic sample collection, processing, analysis, and cataloging of rRNA gene sequences.

Greengenes
A web application developed by Lawrence Berkeley National Laboratory (LBNL) that enables access to comprehensive 16S rRNA gene sequence alignment tools.

handling control (HC)
An experimental negative control in which a sampling device is pre-moistened with PBS and exposed to the ambient sampling environment (without any contact with the surface of interest).

homopolymers
Short intervals in DNA sequence where the same base is tandemly repeated, often as a result of sequencing error. For example: G**A**TCGATC → G**AAA**TCGATC.

Interactive Tree of Life (iTOL)
An online tool that aids in the generation of interactive phylogenetic trees.

low-biomass
A characteristic of a sample that contains very few living or dead microorganisms (i.e., bacteria, archaea, and fungi).

macrofoam
A sponge material provided as part of the BisKit. In the Genetic Inventory experiment, the macrofoam sponge is used to sample large surface areas such as 2500-cm^2 stainless-steel sheets.

model microbial community (MMC)
A mixed microbial assemblage of known phylogenetic composition and cellular/endospore density. In the Genetic Inventory experiment, MMC is synthesized to serve as the positive control.

Mars Exploration Program
Mars Exploration Program (MEP) is a long-term effort to explore Mars, funded and led by NASA. MEP oversees the Mars Exploration Program Analysis Group (MEPAG), which primarily provides input for planning future Mars missions. The program is managed by NASA's Science Mission Directorate of the Planetary Science Division. In early 2012, the Mars Program Planning Group (MPPG) was formed to help reformulate the agency's Mars Exploration Program. This brought together leaders of the administration's technology, science, human operations, and science missions.

mothur
Open-source, expandable software tool—developed by the Department of Microbiology & Imunnology at The University of Michigan—used to analyze and interpret raw sequencing data.

multiple sequence alignment
The alignment of three or more nucleotide sequences of similar length; homology and evolutionary relationships can be inferred between the sequences studied based on multiple sequence alignment.

negative control
A group in a scientific experiment in which the results are known to be negative; used to isolate any variables from the experiment results.

National Center for Biotechnology Information's (NCBI) GenBank
A comprehensive database that contains publically available nucleotide sequences.

non-metric multidimensional scaling (NMDS)
A tool for the general task of assigning Euclidean coordinates to a set of objects, such that a given set of dissimilarity, similarity or ordinal relations between the points are obeyed.

nylon-flocked swab
A sampling device consisting of a plastic stick—with a scored neck for easier breakage—with liquid nylon sprayed perpendicular to one end of the stick and allowed to dry onto the stick. In the Genetic Inventory experiment, nylon-flocked swabs served as one of two materials used to collect samples from small surface areas (i.e., stainless-steel coupons).

operational taxonomic units (OTUs)
User-defined taxonomic bins that are based on throsholds in dissimilarity in DNA sequence.

ordination
The arrangement or "ordering" of species and/or sample units along gradients. A popular way of visualizing beta-diversity information is through ordination plots.

Phred
A base-calling program widely used by large academic and commercial sequencing laboratories for DNA sequence traces.

PhyloChip DNA microarray
A DNA microarray method of molecular analysis that targets specific DNA sequences of all previously reported microorganisms present in a given sample, irrespective of their cultivability. G2 PhyloChip refers to Generation 2, and G3 to Generation 3. The G3 PhyloChip version is able to categorize prokaryotes (known bacteria and archaeal OTUs) into 31,000 taxonomic bins via hybridization of 1,100,000 25-mer probes targeting sequence variations in the 16S rRNA gene.

PhyloChip taxonomic unit (PTU)
User defined binning of DNA sequences into taxonomic units based on their hybridization intensities with probe sets on the DNA microarray (PhyloChip).

phylogenetics
The study and taxonomical classification of organisms based on of their evolutionary relationships.

polyester wipe
A sampling device of continuous tightly interwoven long polyester fibers . In the Genetic Inventory experiment, it serves as one of two materials used for sample collection of large surface areas (i.e., stainless-steel sheets).

polymerase chain reaction
An enzyme-catalyzed molecular reaction used to amplify DNA molecules. This reaction transforms unusable, insignificant numbers of DNA molecules into highly robust and overly abundant template DNA concentrations, which are required for many downstream molecular analyses.

principal coordinates analysis (PCA)
A multivariate statistical technique used to determine the most important axes along which samples vary; after which clusters can be identified and interpreted accordingly.

proteobacteria
A major group (phylum) of bacteria that includes a wide variety of pathogens, such as *Escherichia, Salmonella, Vibrio, Helicobacter*, and many other notable genera. Other proteobacteria are free-living, and include many of the bacteria responsible for nitrogen fixation.

rarefaction analysis
A technique used to assess species richness resulting from a given sampling effort. Rarefaction curves plot the number of species or OTUs observed against the number of samples (or sequences) analyzed.

RNase
Ribonuclease A (RNase A) is an enzyme that cleaves single-stranded RNA. Bovine pancreatic RNase A is a classic model systems in enzymology and protein science.

spacecraft assembly cleanroom (SAC)
A cleanroom is an environment, typically used in manufacturing (in this case spacecraft assembly) or scientific research, that has a low level of environmental pollutants such as dust, airborne microbes, aerosol particles and chemical vapors. More accurately, a cleanroom has a controlled level of contamination that is specified by the number of particles per cubic meter at a specified particle size. To give perspective, the ambient air in a typical class 100k cleanroom environment contains 3,520,000 particles per cubic meter in the size range 0.5 μm and larger in diameter, corresponding to an ISO 8 cleanroom.

Spacecraft Assembly Facility (SAF)
A spacecraft assembly cleanroom situated at JPL, Pasadena, CA..

species evenness
A quantitative measure of the extent of equality (i.e., evenness) in species abundance among samples.

species richness
The number of distinct species present in a sample set.

standard assay
The NASA Standard Assay (NSA) method, designed to assess planetary protection risk for spacecraft bound for Mars, is culture-based. Technicians collect samples, allow the organisms in the sample to grow for 3 days, and then count resulting colonies. The standard assay technique is designed to enable a count of organisms that are capable of forming endospores, respire aerobically, are resistant to heat processing, and are able to be cultivated on Tryptic Soy Agar media. This technique does not address the issue of microbial diversity of spacecraft.

stainless-steel coupons
A medium used to test the efficacy of the sampling devices in collecting seeded MMC; 25 cm^2 in surface area.

stainless-steel sheets
A medium used to test the efficacy of the sampling devices in collecting seeded MMC; 2,500 cm^2 in surface area.

STITCH
An in-house bioinformatic tool used to splice, trim, identify, track, and capture the uniqueness of 16S rRNA gene sequence pairs using public or in-house databases

taxonomy "calling"
Applying a given set of taxonomic criteria to assign taxonomic affiliations to observed DNA sequences.

UniFrac tool
A software suite used to perform environmental clustering and principal coordinates analysis. Phylogenetic information is used to statistically compare microbial community diversity between samples.