

## **Evaluation of Sample Preservation Methods for Space Missions**

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For interplanetary spacecraft that will travel to destinations where future life detection experiments may be conducted or samples are to be returned to earth, we should archive and preserve relevant samples from the spacecraft and cleanrooms for evaluation at a future date. Spacecraft and assembly-room derived samples could prove vital as analytical controls, establishing the cleanliness of the spacecraft and the nature and quantities of contaminant levels. We have begun evaluations and experiments to determine which preservation methods should be used for this purpose. Since we cannot predict what detection methods and analytical techniques will be employed in the future, we began by evaluating preservation methods for currently used analytical and detection methods. These detection methods include: the viable growth of microbes, an energetic molecule biomarker adenosine triphosphate (ATP), cell wall constituents known as endotoxins as detected by the Limulus Amebocyte Lysate assay (LAL), direct microscopy, and molecular biology techniques. The basic objective of this study is to understand the best ways to preserve samples for use with future technology. We want to save materials, small parts, coupons, biological samples in water or buffer, DNA, and isolated microbes. This paper will present research data using samples collected from the Mars 2001 orbiter, Odyssey, and environmental samples collected from the cleanroom, during final assembly. We evaluated the changes in the samples by testing before and after freezing at  $-80^{\circ}\text{C}$ . The results show that there are losses in the number of viable microbes after freezing. Two independent studies of pooled cleanroom samples demonstrate good ATP recovery and consistent values after freezing at  $-20^{\circ}\text{C}$ .

# Evaluation of Sample Preservation Methods for Space Missions

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## ABSTRACT

This study of samples collected from Mars 01 Orbiter was conducted to gain a better understanding and practical experience in methods to process and preserve samples intended for planetary protection analysis. Samples were evaluated for the viable growth of microbes, the molecular biomarker adenosine triphosphate (ATP), and the presence of lipopolysaccharide, a bacterial cell wall component. Losses were observed in the number of viable microbes after freezing as well as in the detectable lipopolysaccharide. Two independent studies of pooled cleanroom samples demonstrate good ATP recovery and consistent values after freezing at  $-20^{\circ}\text{C}$ .

## INTRODUCTION

As NASA planetary missions move towards in-situ life detection experiments and the return of interplanetary rock and regolith samples, it has become very important to understand any potential biological contamination originating from earth. The preservation and archiving of samples obtained from spacecraft will be necessary for further analysis of bioburden and biosignatures. We should archive and preserve relevant samples from the spacecraft and assembly environment for later evaluation. This will be especially important for future interplanetary missions, which may travel to destinations where life detection experiments may be conducted or samples may be returned to earth. Spacecraft and assembly-room derived samples could prove vital as analytical controls, establishing the cleanliness of the spacecraft and the quality as well as quantity of contaminant levels.

Since we could not predict which detection methods and analytical techniques may be employed in the future, we began by evaluating preservation methods for our laboratory's currently used analytical and detection methods. We considered the most commonly used methods for preserving the samples attempting to choose a method suitable for all future analyses. We selected freezing at  $-80^{\circ}\text{C}$  because it is one of the most common methods used with presumably the least alteration of the sample. One goal of this work was to

determine if routine freezing at  $-80^{\circ}\text{C}$  could preserve biological samples for further examination using different measurement endpoints. This study was planned for evaluation over 3 to 5 years, but only the initial time points have been obtained and are discussed here. The analysis techniques used for evaluation of the preservation method included detecting microbial growth, and the molecular biomarkers, ATP and lipopolysaccharide. Further sample characterization was accomplished by biochemical identification of individual microbes recovered.

## METHODS

Samples were collected at random locations on the Mars 01 Odyssey Orbiter during the final stages of assembly, at the Kennedy Space Center in February 2001. Additional wipe samples were taken near the orbiter, inside the SAEF-II assembly facility. There are negative controls for both of these sets.

The samples and the preservation method were characterized by three primary techniques, culture-based detection (growth), and the presence of two molecular biomarkers, lipopolysaccharide (LPS) and adenosine triphosphate (ATP). Further characterization of microbes isolated from the samples was done by morphological and biochemical evaluation.

## SAMPLE COLLECTION AND STORAGE

Various preservation methods were considered, and it was decided that the most practical preservation method was freezing. A sample distribution plan was devised that split the 150 and 250 mL samples into 1 to 10 mL aliquots that could later be independently thawed. The samples were divided according to the assay type, volume needed and the number of replicates for each test. Samples were set aside for each of the assay types to be performed after freezing, at staggered time intervals. The cryoprotectant glycerol was used for a subset of the samples set aside for culture-based growth methods.

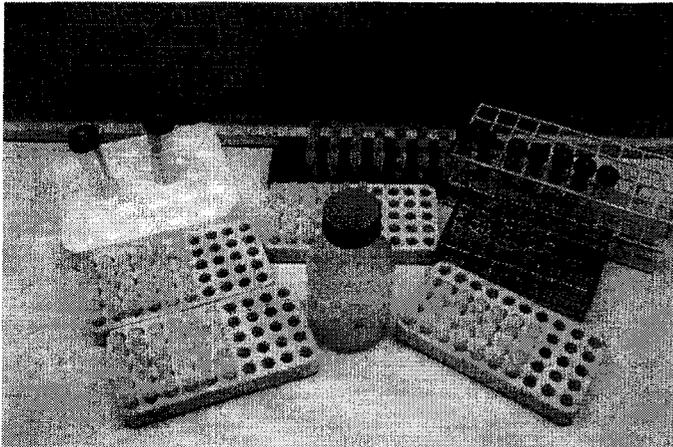


Figure 1. This image shows how one wipe sample was divided into smaller aliquots for freezing and later analysis.

#### GROWTH-BASED ASSAY

The NASA Standard microbiological method for the examination of space hardware was used in this study. The method was quite sensitive, capable of detecting a single microbial cell in 10 mL of water (or better). This fundamental method relies on a microbe's ability to reproduce in the rich nutrient media supplied. There were two relevant variations of the bioassay, with and without heat shock. Only bacterial endospores survive heating to 80 °C. Without the harsh heat-shock, both spores as well as vegetative cells could reproduce, grow and form colonies that could be counted. For this study, only the non-heat shocked method was used for vegetative growth. This allowed a greater portion of the bioburden to be detected and was more suitable for cross comparing to the other techniques used.

For this method, 2.0 mL of the sample was mixed in Tryptic Soy Agar, allowed to solidify in a Petri dish, and then incubated at 32°C, in a normal atmosphere, for 3 days. Samples were tested in quadruplicate. Microbes capable of growing under these conditions, produced visible colonies that were counted. Subsets of these colonies were selected for further characterization.

#### ATP ANALYSIS

ATP or adenosine triphosphate is a universal molecule found in all metabolizing cells and functions as the cell's main energy carrier. Energy that can be used to drive chemical reactions and power cell growth reactions are stored in the phosphate bonds of this molecule. Vegetative cells detectable in the growth assay

described above, have measurable ATP, however dormant spores do not. ATP can also remain on surfaces after cells have died. Therefore, two types of ATP assays are carried out: total ATP and intracellular ATP. Total ATP measures the ATP found inside as well as outside of cells. The intracellular ATP test, measures only ATP within the cell, but only after the removal of any ATP outside of the cell. ATP is determined by measuring the light emitted from the biochemical reaction catalyzed by the firefly enzyme luciferase. ATP provides energy for this biochemical reaction. A sensitive photo-multiplier tube in the Kikkoman Lumitester, an instrument specially designed for ATP analysis, measured photons emitted from the reaction. The results are reported as relative luminescence units (RLU's) per 0.1 mL sample. Samples were analyzed in triplicate, or in quadruplicate for the additional preservation studies.

#### LIPOPOLYSACCHARIDE ANALYSIS

The Limulus Amebocyte Lysate (LAL) Assay was used to measure lipopolysaccharide (LPS), also known as endotoxin, found in the cell walls of gram-negative bacteria. Because this biomolecule is absent in gram-positive bacteria, only a fraction of the environmental population is capable of producing a signal. Since spore-forming bacteria are gram positive, they do not contribute to the measured values. This procedure used the Charles River Co. Endosafe Chromogenic Assay. This Food and Drug Administration Method is well established, very sensitive, and requires about as much technician set up time as the growth and ATP assays. Values were measured on a Molecular Devices Co. Versamax Microplate Reader. This kinetic assay measured the increase in absorbance at 405 nM and calculated concentration values using the time required to reach a defined threshold. The data analysis software used was Softmax Pro ver. 3.1.1. The results were reported in endotoxin units per 0.1 mL sample. Both fresh and frozen samples were analyzed in duplicate. Sets of controls for each sample were spiked with a known quantity of lipopolysaccharide to check for assay interferences.

#### TRADITIONAL MICROBIOLOGY AND BIOCHEMICAL IDENTIFICATION

The Biolog identification system was based on a bacterial culture's ability to utilize various carbon sources and other enzymatic functions. The identification method was similar to the traditional biochemical approaches to species identification. The Biolog system used a 96 well plate format and the biochemical profile could be obtained in a single day, starting with an overnight culture.

Work done with the environmental isolates led to biochemical utilization pattern generation. The bacteria that grew in the growth assays (both spore formers and non-spore formers) were selected and numbered. The

selection was not random, because an attempt was made to choose colonies that had different morphologies, in an attempt to ensure diversity. These isolates were sub-cultured and frozen in a freezing buffer containing 15% glycerol. The isolates were first worked up by gram stain, microscopic observation, oxidase test, heat shock for spore-former identification and finally a biochemical utilization panel of 96 different tests in the Biolog system. For the Biolog test each 96 well plate was read between 4 to 6 hours and again between 16 to 24 hours

#### ATP STABILITY TESTS

A series of experiments were designed to evaluate the relative stability of both purified ATP and microbe-associated ATP from a special pooled sample collected in a JPL cleanroom. A large environmental sample collected from the JPL Spacecraft Assembly Facility cleanroom was aliquoted into 4000 vials, and stored at  $-20^{\circ}\text{C}$ . Assays of these aliquots over time were carried out to evaluate the stability of the solutions.

### RESULTS AND DISCUSSION

#### SAMPLE PRESERVATION RESULTS

The study consisted of seven primary samples. One sample was taken from the Odyssey spacecraft and consisted of the pooled supernatant from 14 swabs. Four samples of the cleanroom environment were taken by wipes, and the two remaining samples were control blanks. The samples were split into 742 individual containers, and those that were not analyzed fresh, were frozen at  $-80^{\circ}\text{C}$ . Cryogenic freezing vials were found to be suitable for sample storage.

#### GROWTH-BASED ASSAY RESULTS

The NASA Standard Assay for vegetative cells was used. All cells that were capable of growing in the medium were counted. This included both spore formers and the non-spore formers. The growth assay showed that the samples ranged from 1 to 75 bacteria per mL, with the negative control blanks having no detectable colonies as was expected. The samples taken directly from the Odyssey spacecraft and pooled into a single sample, resulted in a quantity that extrapolated to 12,600 vegetative CFU's per  $\text{m}^2$ . This is an unusually large value for a spacecraft sample, despite the fact that spore-formers represent only a fraction of the population. The cleanroom environmental floor samples had counts that ranged from 673 to 50,000 CFU's per  $\text{m}^2$ .

Two conditions for freezing samples were evaluated. The first method preserved the samples directly as collected, in pure water. The second condition used 15% glycerol as a cryoprotectant to protect against freezing damage. The majority of the samples had survival rates between 23 to 48% of the original. The effectiveness of the freezing buffer is not conclusive from this data set alone.

What is apparent in most cases is that more than half of the bacterial population did not survive freezing. Because the samples generally had a small starting population, firm quantitative conclusions could not be made.

#### ATP RESULTS

ATP or adenosine triphosphate is the primary energy-carrying molecule of all living cells. Coupling energy transfer to a method of detection, such as in this case, photon production with the bioluminescent firefly assay permitted an estimation of the quantity of ATP. Since all ATP found in the environment should have been associated with or derived from living cells, it was a good molecular biomarker. The samples ranged from 7,800 to 190,000 RLU with the blank negative control at 148 RLU. This provided a good distinction between blanks, and a wide bioload range. The results parallel the NASA growth assay. Freezing in this first study seemed to change the samples, and the trend was toward increased values for both the total and the intracellular ATP. It could have been possible that freezing disrupted the cell membrane integrity and made more ATP available to the assay. However, the effect was not large and could be interpreted similarly to the special ATP studies described below, that showed some sample variability but generally a stable value before and after freezing.

#### LIPOLYACCHARIDE ASSAY RESULTS

The LAL or Limulus Amebocyte Lysate assay detects the lipopolysaccharide cell wall component of gram-negative bacteria. Spores, gram-positive bacteria, and human cells are not detected. Thus, the results do not precisely measure the same thing as the standard growth assay for spores.

The relative ranking of the samples was nearly similar to the standard vegetative growth assay. The highest sample for microbial growth and ATP was also the highest for LPS and the blank controls showed the lowest values, as expected. The highest LPS values were noted in wells A9-B9, (See Figure 2) and corresponded to sample KSC-81, an environmental floor wipe sample. This sample was extremely high for LPS as well as the corresponding ATP and colony forming units of the growth method. The wipe sample from the floor had apparently picked up an insect, an ant, which contributed to the high values. After freezing this sample, was still the highest but had decreased ten-fold. Values are lower after freezing. The molecule used as the standard calibration, bacterial endotoxin (lipopolysaccharide) is prepared and delivered as a freeze dried powder, but according to the supplier, cannot be refrozen in solution without changing the results, because LPS sticks to the container walls.

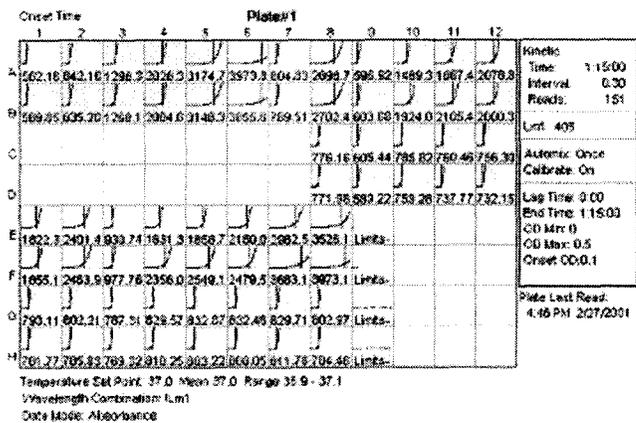


Figure 2. The graphical representations in each small box depict individual test wells for the LAL assay. The LAL assay is kinetic and LPS concentrations are calculated from the time the reaction takes to reach an optical density of 0.1. An upward vertical line can be seen where the threshold is reached. Samples were analyzed in duplicate and are shown on lines A&B and E&F. Spiked samples, which are a control for inhibitory interferences are shown below the samples of the same number, on rows C&D and G&H. The calibration curves are A&B 1 to 5 with column 1 being the most concentrated and decreasing to water in column 6. Blank controls are A&B 6&7.

### TRADITIONAL MICROBIOLOGY AND BIOCHEMICAL IDENTIFICATION RESULTS

Of the 36 colonies that were selected for further study, 75% were found to be gram positive and 25% were gram negative. Fifteen of the 36 colonies (42%) were spore formers. Biochemical based identification has historically been the method for classification of microbes into genera and species. Our lab has recently obtained an instrument system that speeds up this process. The Biolog system has 96 biochemical tests set up in a single plate. The bacterial culture is added to the plate, allowed to interact with the reagents and then the blue reaction color is read at 4 and 24 hours. The cultures were gram-stained and cell morphology was observed. They were grown and then tested by the Biolog system. Although very few isolates were identified and classified down to the species level, the biochemical utilization patterns were saved and used to generate an environmental database. If the same microbe is later reisolated from the environment, the similarity would be observed. Identification of some of the isolates was attempted by sequencing the 16SrDNA. The closest phylogenetic relationships to the isolates were identified. The list included five isolates of the  $\beta$ -proteobacter clade, four of which aligned closely to *Variovorax paradoxus*, and one isolate was not closely matched. *Bacillus thermoamylovorans*, was the only isolate placed in the "low G + C gram positives" clade. Finally, the three matches to the  $\gamma$ -proteobacter group included, *Bradyrhizobium japonicum*, *Methylobacterium extorquens* and *Mesorhizobium loti*.

A subset of the microbes that were obtained from the vegetative growth assay were chosen, subcultured, and then preserved by freezing in 15% glycerol. By preserving a large population by freezing at  $-80^{\circ}\text{C}$ , a fraction will survive, that could be thawed and revived for years to come.

### DATA COMPARISON AND FREEZING EFFECTS

A side-by-side presentation of the data from different analytical techniques is shown in Table 1. It can be seen that sample KSC-81 showed high values consistently for each test. Sample 81 was the environmental wipe of a floor and contained an ant. Similarly, the negative control samples KSC-66 and 85 were the lowest values as expected. Any of the techniques, growth, LAL or ATP can certainly identify a grossly contaminated sample.

Table 1. Freezing Effect Data Comparison

Samples Before Freezing					Samples After Freezing				
Sample	Growth	LPS	ATP- Intra- cellular	ATP- Total	Sample	Growth	LPS	ATP- Intra- cellular	ATP-Total
	CFU /mL	EU /mL	RLU /mL	RLU /mL		CFU /mL	EU /mL	RLU /mL	RLU /mL
KSC-51	3.2	Nd	353	7835	KSC-51	0.8	0.8	Nd	Nd
KSC-66	0.0	0.1	205	148	KSC-66	0.0	0.2	765	633
KSC-81	75	323	73788	1.9*5	KSC-81	19	27	18090	1.0*6
KSC-82	20.4	1.6	385	37965	KSC-82	0.3	0.9	1233	68658
KSC-83	1.0	0.6	195	61090	KSC-83	0.3	0.5	1263	71663
KSC-84	1.9	0.5	288	50738	KSC-84	6.2	0.3	1173	59858
KSC-85	0.0	0.0	Nd	Nd	KSC-85	0.2	0.0	Nd	Nd

In considering the effect of freezing at  $-80^{\circ}\text{C}$ , the growth-based and the LPS methods showed lower values after freezing whereas ATP values are elevated (in this small set only). Samples KSC-66 and KSC-85 are negative blank controls. The growth assay is reported in colony forming units (CFU), lipopolysaccharide in endotoxin units (EU) and ATP given in relative luminescence units (RLU).

We found that all methods used were capable of determining the relative cleanliness of samples and all will detect a dirty sample. The technician time for each assay (growth, ATP and lipopolysaccharide) is from 2-4 hours, provided all reagents and equipment are ready to go. The growth assay could be read at 24 hours but was not official until 72 hours. The ATP and lipopolysaccharide results could be read almost immediately. The greatest amount of technician time was required for the biochemical identification (weeks for a group of 30 samples).

What did we learn from the samples themselves? The cleanroom environmental samples ranged from relatively clean to very dirty. We could tell the difference between blanks and the samples. Blanks were sufficiently clean and did not interfere with any analysis. Freezing did alter the samples, and there was a general trend towards loss of biological signal, with the exception of ATP, which did not decrease after freezing.

### ATP STABILITY TEST RESULTS

A series of special experiments were carried out to determine the effect of storage temperature over time on ATP values derived from both the pure chemical in solution, and microbe-associated ATP values from a large pooled cleanroom sample. The primary sample collected in a JPL cleanroom and had an initial ATP value of 15,000 RLU/mL. The sample was diluted ten-fold and dispensed into 4,000 smaller aliquots so that each individual sample had 1,500 RLU/mL.

Effect of Storage Temperature for Purified ATP

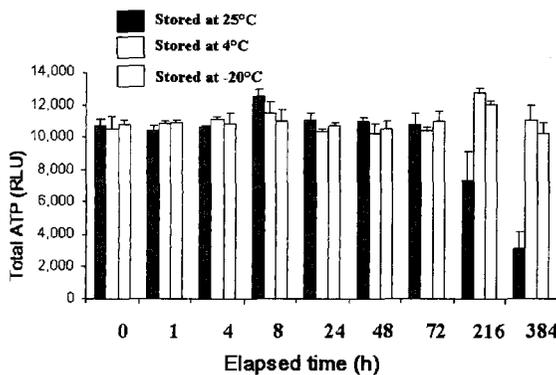


Figure 3. The Effect of Storage Temperature for Purified ATP shows good preservation for refrigerated and frozen conditions, and a substantial decrease for room temperature storage after 72 hours.

In Figure 3., it can be seen that purified ATP can be held at room temperature for 72 hours before substantial changes occur. This finding gives confidence that calibration standards do not change so rapidly as to cause concern about changes while performing assays during a single day. Stability of a pure chemical in solution implies that any hydrolysis reactions are very slow. The stability of ATP where there may be enzymatic and metabolic activity (i.e. in the presence of microbes) cannot be predicted from this graph and may not be as stable. The refrigerated and frozen samples showed no

degradation trends over the 16-day experiment. A single freeze-thaw cycle did not substantially alter the ATP levels of a pure solution.

Influence of storage temperature (4°C) in the microbial ATP of environmental samples collected from SAF

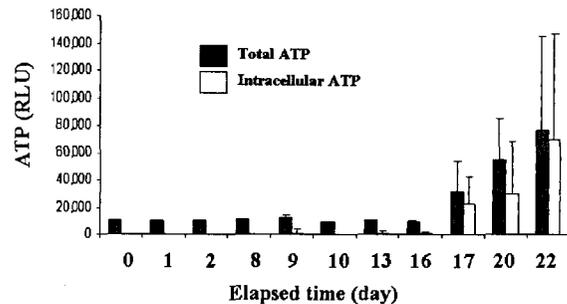


Figure 4. The influence of refrigerator temperature on the microbial ATP of environmental samples collected from a cleanroom.

Simply refrigerating the samples can permit delayed analysis. It can be seen in Figure 4., that the ATP values remained reasonably constant for the first 16 days. However, gross changes were first observed on day 17. This is likely to be due to the growth of a microbe at refrigeration temperatures, following a prolonged growth lag. Although it is preferable to process samples promptly, this data suggests that even a two-week holding time is acceptable.

The data seems to show an increase in ATP levels presumably due to the growth of a psychrophile (cold-loving bacteria). Psychrophiles typically have long growth lag times. This presumptive evidence of a psychrophile is another example where the standard 3-day growth assay would not detect the presence of a viable microbe. Another organism not detected was the heat tolerant *Bacillus xerothermodurans* shown by Bond, et al. to have a long growth lag, and was first observed only after a one-month incubation. Several other groups of microbes may not be detectable in the growth assay notably, anaerobes, phototrophs, and chemoautotrophs. The increasing ATP values after several weeks could be a useful technique to identify which tubes to screen for psychrophiles.

### Hold time study

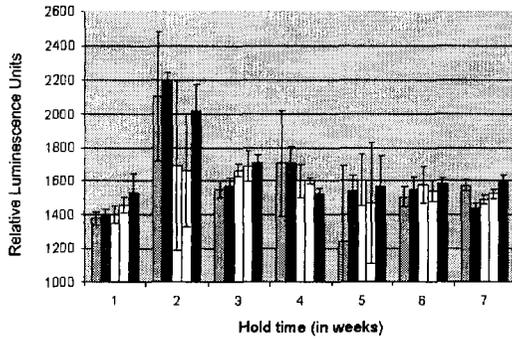


Figure 5. The effect of  $-20^{\circ}\text{C}$  storage on cleanroom samples can be seen in this graph. Each bar is the average of 5 determinations of a single frozen sample.

To assess the effect of frozen storage on JPL Spacecraft Assembly Facility environmental samples over time, ATP assays on random samples were performed for six consecutive weeks (Figure 5.) The samples were collected by pooling wipes from a cleanroom where spacecraft are assembled. This mixture was pooled and then distributed in small aliquots and frozen at  $-20^{\circ}\text{C}$ . Each sample was tested 5 times and the standard deviation is shown. There did not seem to be a consistent trend over the course of the study, at least there was no dramatic reduction in ATP levels over time.

Influence of storage temperature ( $-20^{\circ}\text{C}$ ) in the microbial ATP of environmental samples collected from SAF

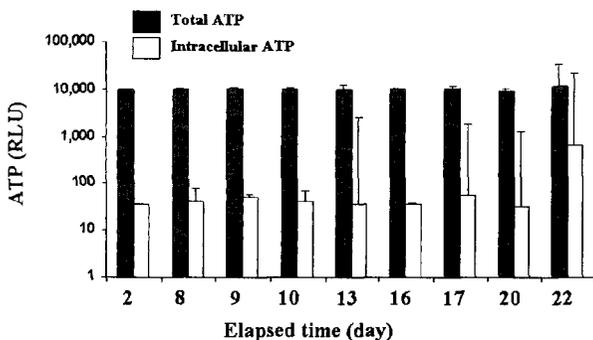


Figure 6. Storage of cleanroom environmental samples at  $-20^{\circ}\text{C}$  shows good preservation of ATP for the length of the study.

There seems to be no degradation of either intracellular or total ATP levels over a 3 week period when stored at  $-20^{\circ}\text{C}$  as seen in Figure 6.

## CONCLUSIONS

This study of samples collected from Mars 01 Orbiter was conducted to evaluate certain methods that could be used to process and preserve samples intended for later analysis. We evaluated freezing as a commonly used method for the preservation of liquid samples by taking actual specimens from a spacecraft and assembly environment. These kinds of samples generally have low quantities of bacteria and are relatively clean. Both the number of viable organisms and the detectable lipopolysaccharide levels decrease after freezing. The use of the cryoprotectant (glycerol) did not substantially or uniformly increase bacterial recovery yields in the growth assay for these low bacterial population samples. A controlled study showed that pure free ATP, as well as the ATP content in environmental samples could be preserved for weeks. Storage of the samples at refrigeration temperatures permitted the growth and detection of psychrophiles. Freezing the samples at  $-80^{\circ}\text{C}$  is perhaps the best current preservation method, despite some changes to the samples. It should be realized, for the techniques used here, biological changes do occur on freezing, and it should be anticipated that frozen or otherwise archived specimens will be different than the fresh pristine material.

## ACKNOWLEDGMENTS

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## DEFINITIONS, ACRONYMS, ABBREVIATIONS

**ATP:** Adenosine triphosphate, a key energy transfer biomolecule found in all actively metabolizing cells.

**CFU:** Colony Forming Units are visible microbial colonies growing on solid agar medium and generally arising from a single cell.

**Endotoxin:** A lipopolysaccharide found in the walls of gram-negative bacteria.

**EU:** Endotoxin Units. A measure of the lipopolysaccharide found in the gram-negative bacterial cell wall.

**LAL:** Limulus Amebocyte Lysate Assay. An enzyme cascade method for the determination of cell wall components found in gram-negative bacteria.

**LPS:** abbreviation for lipopolysaccharide, a biomolecule that is found in the walls of gram-negative bacteria, also known as endotoxin.

**Psychrophile:** A cold-loving organism that prefers or requires cold temperatures for growth and metabolism. A type of extremophile.

**RLU:** Relative Luminescence Units.

**Spore or Endospore:** A cellular structure produced by a few bacterial genera, characterized by substantial resistance to environmental factors such as desiccation, heat, cold, vacuum and many chemicals.