

16S rRNA Beacons for Bacterial Monitoring During Human Space Missions

MAIA LARIOS-SANZ, KATERINA D. KOURENTZI,
DAVID WARMFLASH, JEFFREY JONES, DUANE L. PIERSON,
RICHARD C. WILLSON, AND GEORGE E. FOX

LARIOS-SANZ M, KOURENTZI KD, WARMFLASH D, JONES J, PIERSON DL, WILLSON RC, FOX GE. 16S rRNA beacons for bacterial monitoring in craft and habitat modules in human space missions. *Aviat Space Environ Med* 2007; 78(4, Suppl.):A43-A47.

Microorganisms are unavoidable in space environments and their presence has, at times, been a source of problems. Concerns about disease during human space missions are particularly important considering the significant changes the immune system incurs during spaceflight and the history of microbial contamination aboard the Mir space station. Additionally, these contaminants may have adverse effects on instrumentation and life-support systems. A sensitive, highly specific system to detect, characterize, and monitor these microbial populations is essential. Herein we describe a monitoring approach that uses 16S rRNA targeted molecular beacons to successfully detect several specific bacterial groupings. This methodology will greatly simplify in-flight monitoring by minimizing sample handling and processing. We also address and provide solutions to target accessibility problems encountered in hybridizations that target 16S rRNA.

Keywords: spacecraft, bacteria, monitoring, 16S rRNA.

SIGNIFICANT TECHNICAL advances have slowly removed some limitations on spaceflight, resulting in longer and increasingly complex space missions. Indeed, many scientists are seriously contemplating the long-held goal of a manned mission to Mars. Important hurdles remain, however, and many factors must be considered before such an endeavor is undertaken. A long-duration manned mission introduces a plethora of problems that might not be significant during short missions. For example, short missions primarily use simple, non-regenerative life support systems that rely on stored supplies. Large cargo for long-duration missions becomes cumbersome and prohibitive, making it essential to recover waste for subsequent use, and regenerate food, air, and water so that resupply from Earth is not necessary (3,11,12). The systems currently being developed for this purpose rely, in part, on plants and microorganisms to produce food and oxygen, recycle waste, and purify air and water. While these regenerative systems decrease the amount of cargo needed, there is some concern that these beneficial organisms might be compromised by the presence of excessive numbers of undesirable microorganisms. Bacteria and fungi can also colonize surfaces, resulting in damage to equipment and instrument malfunction. Integrity of these systems is of paramount importance.

The success of exploration-class human missions also

depends heavily on extended maintenance of a healthy crew. The addition of plants and humans to the space shuttle will undoubtedly increase the overall number and variety of microorganisms in the environment, thereby increasing the potential for unwanted contamination. Additionally, increased microbial burdens on the air and water supplies could lead to disease among crewmembers. The threat of outbreak of disease, particularly a contagious one, is especially serious because means of treatment are limited and isolation of the patient from the rest of the healthy crew is difficult. In addition, the well-documented adverse effects incurred on the immune system as a result of exposure to microgravity (1,5,10,13,20-22) may make a partially immunocompromised crew less efficient at fighting off otherwise harmless organisms (10,17). It has, in fact, been reported that infectious diseases, primarily respiratory infections, occur in about 25% of shuttle flights (22). Many crewmembers have proven to be carriers of potentially pathogenic *Staphylococcus* species (25). Additionally, there is evidence that significant shifts in intestinal microflora occur during extended manned spaceflights (15).

Clearly, multifaceted microbial risks will be heightened due to many unknown variables in the space environment; for example, the effect of reduced gravitational force on bacterial cells is not well understood. Gene expression and other cellular processes may be significantly affected, and increases in bacterial growth, genetic transfer, and resistance to antibiotics have been observed during spaceflight (8,9). Additionally, public (and moral) concerns about forward and backward contamination must be acknowledged (18,19). Microbial survival in simulated deep-space conditions has been established, and many terrestrial microbes, particularly

From the Department of Biology and Biochemistry (M. Larios-Sanz, D. Warmflash, R. C. Willson, G. E. Fox) and the Department of Chemical Engineering (K. D. Kourentzi, R. C. Willson), University of Houston; Universities Space Research Associates (D. Warmflash, D. L. Pierson); and NASA-JSC (J. Jones), Houston, TX.

Address reprint requests to: M. Larios-Sanz who is a Research Assistant Professor, Department of Biology and Biochemistry, University of Houston, Houston, TX 77204; maialarios@yahoo.com.

Reprint & Copyright © by Aerospace Medical Association, Alexandria, VA.

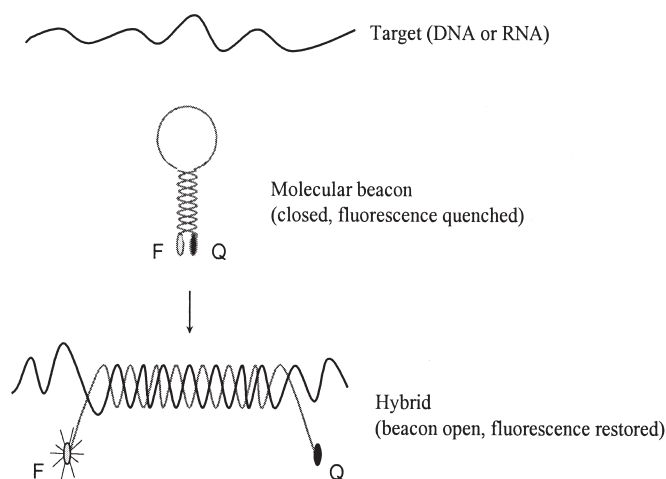


Fig. 1. Mechanism of molecular beacon probes. Fluorescence is only detected on hybrid formation. F = fluorophore, Q = quencher (figure modified from www.genelink.com/products/mbintro.html).

anaerobic spore-formers, readily tolerate simulated Martian environments (7). It is thus our responsibility as explorers of the cosmos to prevent the disruption of natural environments in the places we might visit.

It is, unmistakably, far more desirable to monitor microorganism levels and adapt countermeasures before they become a problem than to try to treat the crewmembers in the event of disease, try to save the plants used for life support after infection by a plant pathogen has occurred, fix faulty or damaged equipment in mid-flight, or attempt to clean up contaminated surfaces in the places we ultimately visit. It is therefore critical that a system that can detect and monitor the presence of microbial communities within the spacecraft environment be developed. A useful monitoring system should have the following characteristics to be ideally suited for the space environment: 1) require minimal effort and limited expertise from the crew with low maintenance needs; 2) be able to analyze samples quickly; 3) be able to detect and correctly identify multiple organisms in one operation; and 4) be highly automated and easily subjected to miniaturization while using little power. Molecular beacon technology offers an attractive format for such a monitoring system.

Molecular beacons are nucleic acid probes that fluoresce only on hybridization to their specific target (23). They are designed to have a stem-loop structure; the loop region is complementary to the target of interest, and the stem carries a fluorophore tag on one end (typically the 5' end) and a quencher molecule on the other. In its closed (unhybridized) state, the fluorescent tag is located close to the quencher molecule, so the probe fluorescence is quenched through energy transfer. On probe hybridization to target, the stem is forced open, separating the fluorophore from the quencher molecule with the result that detectable fluorescence is restored (Fig. 1). Stem-loop probes are more specific than their linear counterparts (2) and washing steps are not necessary since unhybridized probes emit no signal. Additionally, different commercially available fluorescent dyes can be attached to molecular beacons specific for different targets allowing simultaneous detection in

solution. In order to further extend the number of targets that can be detected, and avoid the inherent complications posed by overlapping emission spectra, beacons can be designed for surface-immobilization. Using the well-known biotin-streptavidin interaction, molecular beacons specific for different targets can be attached onto a solid support (such as glass) and be detected based on relative positioning on an array rather than by differences in fluorescence emission wavelength. We show here the applicability of such hybridization technology by demonstrating specificity of molecular beacon probes for their target organisms, and explore two protocols to increase target accessibility in solution assays: chemical fragmentation of the 16S rRNA molecule, and unfolding structurally relevant regions using long "helper" oligonucleotides.

METHODS

RNA Isolation

Total RNA was isolated using trizol (4). Briefly, cells (1.5 ml) were pelleted by centrifugation and resuspended in 50 μ l of 0.5 M NaOAc (pH 5.0). Then 1 ml trizol was added to the cell suspension and gently mixed with 200 μ l cold chloroform. Samples were spun in a microcentrifuge at full speed for 15 min. Total RNA was recovered by isopropanol precipitation at -80°C .

Molecular Beacon Hybridizations

A molecular beacon probe specific for *Vibrio proteolyticus* was designed based on a previously described sequence which targets Variable Region 3 (nucleotides 467–490, *E. coli* numbering) on 16S rRNA (14). This specific probe, termed V3VPR, is labeled with a 5' carboxyfluorescein (FAM) fluorophore, and has the sequence 5'-CATGAG-CGCTAACGTCAAATAATGCATCTA-CTCATG-3' (stem sequence in italics). Another FAM-labeled beacon, targeting all bacteria, was used for the magnesium fragmentation experiments (described below). This beacon, named ECO1050, has the sequence 5'-CCTTGT-TGACGACAGCCATGCAGCACAAGG-3' and targets nucleotides 1050–1067 in 16S rRNA (*E. coli* numbering).

Total RNA was denatured with 5-fold molar excess of beacon in hybridization buffer [20 mmol \cdot L $^{-1}$ Tris-HCl (pH 8.0), 50 mmol \cdot L $^{-1}$ KCl, 10 mmol \cdot L $^{-1}$ MgCl $_2$] at 95°C for 3 min (assay volume 100 μ l). Reactions were incubated at 42°C for a predetermined period of time (usually 2–4 h to overnight). Samples were diluted with 500 μ l of hybridization buffer and fluorescence was measured at 25°C using a SPEX Fluorolog 212 Fluorometer (Instruments SA, Inc., Edison, NJ) with two emission channels in T-format (monochromator and filter, both equipped with Glan-Thompson polarizers) and a 450 W Xenon lamp. Data was collected using DataMax data acquisition and manipulation software and processed using MS Excel.

Increasing Target Accessibility in Solution

To address problems due to extensive tertiary structure of target RNA during solution-phase hybridiza-

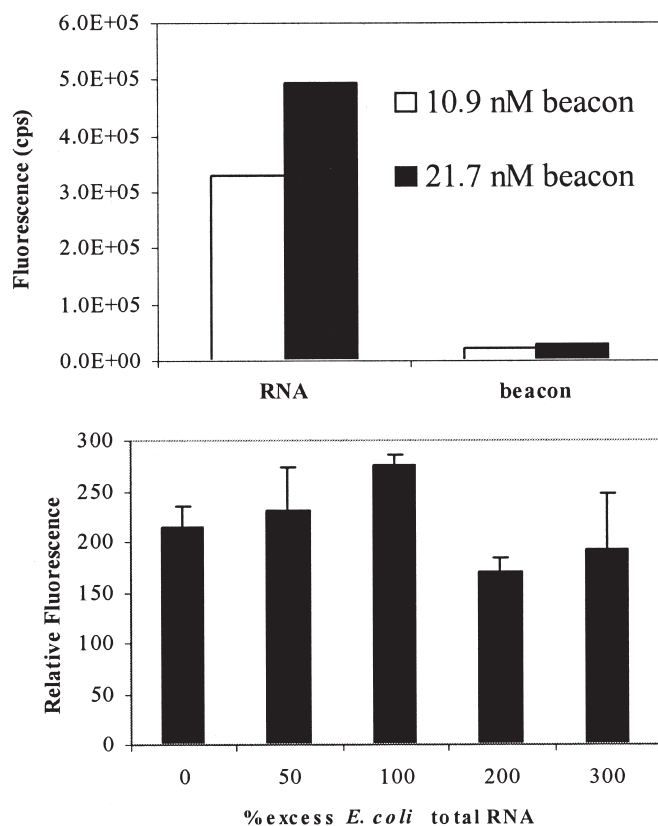


Fig. 2. A (Upper): Detection of *V. proteolyticus* 16S rRNA (12 μg , 8 pmol, total RNA) using the molecular beacon V3VPR (10.9 nmol \cdot L $^{-1}$ or 21.7 nmol \cdot L $^{-1}$). Samples were run in triplicate. Enhancement in fluorescence after an overnight incubation strongly depends on the target to probe ratio used. B (lower): Detection of *V. proteolyticus* 16S rRNA in the presence of excess *E. coli* total RNA. Total RNA (12 μg , 8 pmol) isolated from *V. proteolyticus* was hybridized in the presence of variable amounts of *E. coli* total RNA and 35 nmol \cdot L $^{-1}$ FAM molecular beacon. Fluorescence intensity was recorded after an overnight incubation. The ability to verify the presence of the target was unaffected by the excess non-target *E. coli* RNA.

tion, total RNA was fragmented by heating in a high magnesium buffer or unfolded using helper oligonucleotides. For chemical fragmentation, 10 μl total RNA (1 $\mu\text{g} \cdot \mu\text{l}^{-1}$) was incubated in 1X fragmentation buffer (FB) at 85°C and 95°C for 15, 30, 45, 60, 75, and 90 min. 5X FB is 200 mmol \cdot L $^{-1}$ Tris acetate (pH 8.1), 500 mmol \cdot L $^{-1}$ potassium acetate and 150 mmol \cdot L $^{-1}$ magnesium acetate. Fragmented RNA was used in solution hybridization assays using molecular beacon probes as described above. For the helper oligonucleotide protocol, equimolar amounts of long oligonucleotides targeting particular secondary structures in the 16S rRNA molecule (termed "unfold oligos") were prehybridized with target RNA and then incubated with molecular beacon probes as described above.

RESULTS AND DISCUSSION

Molecular Beacon Specificity

Fig. 2 shows results obtained using V3VPR, a FAM-labeled molecular beacon specific for *Vibrio proteolyticus*. This particular beacon was designed based on a previously characterized sequence (14). An enhancement in fluorescence is observed on hybridization to

target RNA (Fig. 2A). No signal was detected with non-target RNA (data not shown). The specificity of this beacon for its target is exceptional even at 37°C, a relatively low temperature for hybridizations, especially for such a complex target as 16S rRNA. This can be attributed to the structural constraints of the probe's hairpin conformation, which lead to enhanced specificity and broaden the range of temperatures over which a clear differentiation between similar targets is possible (2). This extraordinary specificity for target is one of the most attractive attributes of molecular beacons and makes them invaluable tools in sequence-specific nucleic acid detection. Single mismatches in the target sequence contribute to a decrease in signal, making them extremely efficient in distinguishing even closely related organisms whose target sequences might differ by just a few bases. Notably, a contaminated environmental (air, water, surface) sample will rarely contain only a single type of organism. It is thus important that a particular organism of interest be readily detected even in the presence of many other species in a sample. Our results demonstrate that the integrity of fluorescence signal for a specific microorganism was maintained even when mixtures of target RNA and excess non-target RNA were used in the hybridization reactions (Fig. 2B).

Enhancing Target Accessibility in Solution Assays

Although all beacons gave an increased fluorescence signal in the presence of target, most were much lower than would have been expected from positive control values (fluorescence enhancement observed from hybridization of the molecular beacon to a complementary DNA oligonucleotide). This discrepancy is not due to a preference or increased stability with a DNA target over an RNA one. Hybridizations with a molecular beacon and its DNA and RNA complementary oligonucleotides showed that the kinetics of hybridization are very similar for both (data not shown). These results could instead be explained by the hindering effect of the 16S rRNA folding on target accessibility. The 16S rRNA molecule has significant tertiary structure, making many potential target sites inaccessible. An initial 1–3-min heat treatment is typically sufficient to denature small targets such as 5S rRNA for hybridization in solution. With larger targets, like 16S rRNA, chemical fragmentation or extended physical unfolding of the target is necessary since the molecule quickly snaps back to its native (and inaccessible) shape even after extended heating. The presence of the hairpin probe prohibits the use of traditional RNA denaturing agents such as formamide and urea. Heat treatment of RNA in high magnesium-containing buffers induces random strand scission and produces fragments whose length decreases with increasing duration of heat treatment. Fragmentation of 16S rRNA was achieved with the use of a high magnesium (30 mmol \cdot L $^{-1}$) buffer and heat. Fragment length in this case strongly depends on time of incubation as well as temperature, and improvement on hybridization signal due to Mg $^{2+}$ -induced fragmentation is also temperature dependant, as can be seen in **Fig. 3**. At 90°C, the maximum fluorescence enhance-

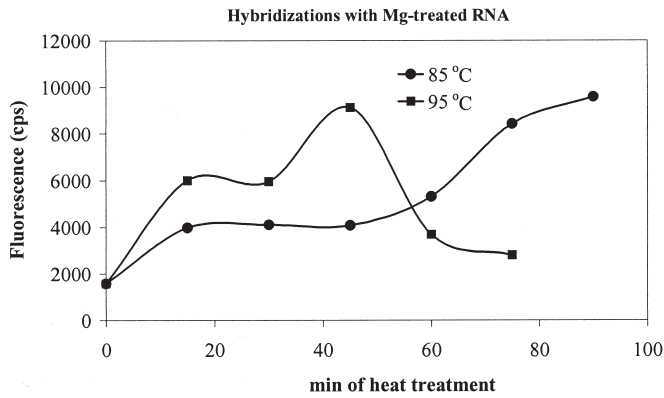


Fig. 3. Hybridization results obtained with the molecular beacon ECO1050 (20 pmol), targeting all bacteria. Target RNA (40.2 pmol) was heated for different time intervals at 85 and 95°C in a high magnesium fragmentation buffer.

ment (about 5-fold) is observed after 40 min of incubation, and then quickly decreases as the RNA is completely hydrolyzed and no target remains available for hybridization. After 40 min, the enhancement in fluorescence is only two-fold at 85°C. The increase in signal is more gradual for the lower temperature, so that a comparable 5-fold intensification in fluorescence is achieved after 80 min, with a decrease well beyond 100 min.

Attempts to minimize target secondary structure and enhance site accessibility also include the use of helper oligonucleotides. This approach is an attractive alternative to chemical hydrolysis, because cleavage of the molecule might not be the most practical or even applicable approach for a specific target, and the target RNA is not ultimately destroyed, leaving it available for further analysis if desired. Previous reports have shown that the use of unlabeled “helper” or “capture” oligonucleotides, short stretches which typically target regions immediately upstream of a target of interest, greatly increase recovery (16) or detection (6) of a particular RNA. Helper oligonucleotides are designed to bind adjacent to the probe target site, thus disrupting RNA secondary structure and keeping the probe target site available (6). The most significant signal enhancement has been achieved by directly adjacent helper oligonucleotides and by helpers targeting the region that is complementary to the probe target site. While it has been shown to be effective, this method forcibly requires additional complications in the design of probe sets, namely, the need for previous knowledge of sequence information, which might not be practical for high through-put testing assays that involve multiple target regions, or the targeting of unknown organisms or multiple organisms with varying sequences. A more general approach to unfolding the RNA molecule is thus desirable. To this end, we have designed long oligonucleotides targeting the longest helices in 16S rRNA and the central pseudoknot (four long stretches of highly conserved sequence) in an effort to unfold the molecule, making different areas more accessible to probing in solution. The four oligos, termed Unfold oligos I-IV, range in length from 36 to 70 nucleotides, and are thus long enough that even some slight mis-

matches across genera will result in hybridization, ensuring that the RNA will become unfolded regardless of the target organism. There was more than a two-fold enhancement in signal when a mixture of Unfold I, II, and III were used (Fig. 4). This increase is probably exclusively due to the effect of Unfold II, since the same hybridization signal is observed when Unfold II is used alone (data not shown). This unfold oligonucleotide targets the central pseudoknot of 16S rRNA (Fig. 4), a region that contributes to the complex structure of the rRNA and is essential for ribosomal function (24). The pseudoknot is a fundamental player in 16S rRNA folding and its disruption contributes to a significant enhancement in the accessibility of target areas that were hidden to potential probes in the native state.

Conclusions

Before we embark on long-duration human missions, a robust microbial monitoring system is needed to ensure both crew safety and system integrity. It is clear that to be successful this monitoring system should not rely solely on culturing methods. Ribosomal RNA hybridization using molecular beacon probes is a promis-

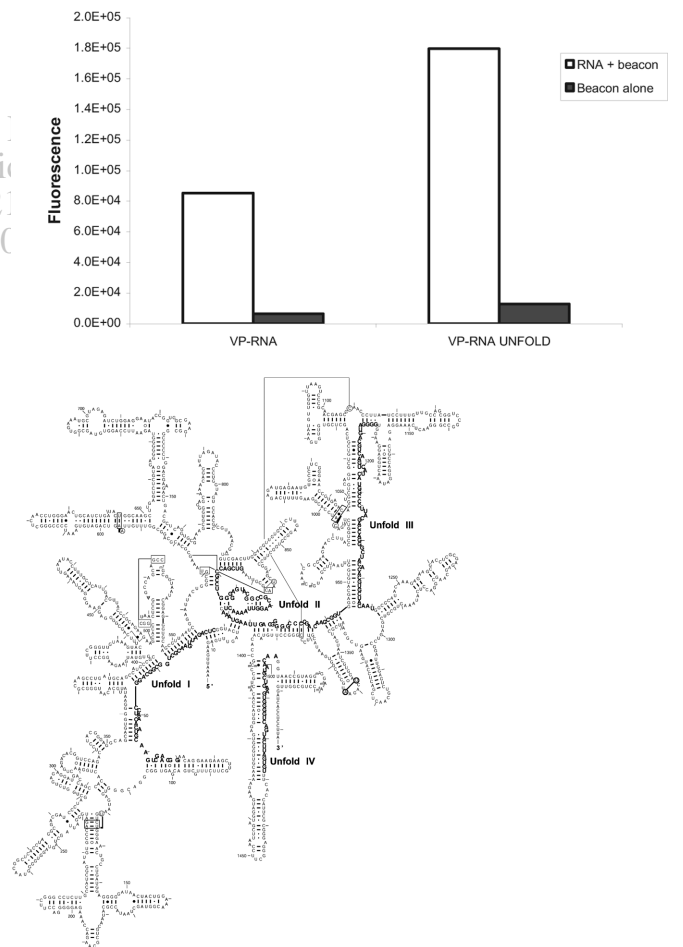


Fig. 4. A (Upper): Hybridization signals are enhanced by prehybridization with unfold oligonucleotides. Hybridization signals obtained with 40 pmol target *V. proteolyticus* total RNA and 20 pmol of the molecular beacon V3VPR. B (Lower): Location of helper oligonucleotides Unfold I-IV on a schematic secondary structure diagram of the 16S rRNA molecule.

ing approach that can greatly simplify sample handling procedures. The assay format explored herein bypasses complications such as extensive sample purification, labeling, wash conditions, background signals, etc., that are encountered with methods such as PCR or array hybridization. Moreover, it is clear that one could paralyze a molecular beacon assay and thereby test for many organism groups in a single experiment.

Different approaches to circumvent 16S rRNA target inaccessibility in solution assays can be integrated into the molecular beacon assay. We observed enhanced hybridization signals when magnesium buffer was used to fragment the RNA sample, although target fragmentation with magnesium needs to be optimized for the desired fragment size with respect to both temperature and time of incubation. It is instructive to mention that optimization of such RNA fragmentation should be done by means of an assay and not simply by observation of fragments on a gel. Long unfold oligonucleotides, on the other hand, provide a non-destructive, general tool to force target unfolding and can be designed to target multiple bacteria. The implementation of a system that utilizes surface attached molecular beacons might also be possible. Such a development would allow creation of molecular beacon arrays.

ACKNOWLEDGMENTS

This research was supported in part by grants from the National Space Biomedical Research Institute (NASA Cooperative Agreement NCC 9-58), NASA's Office for Human Systems Research and Technology (NNJ0-4HF43G), and the Institute of Space Systems Operations to G.E.F and R.C.W. The authors wish to thank Drs. Clarence Sams and Brian Crucian for the information they provided on space-flight immune system changes, as well as NASA/Mir astronauts, and the Office of Technology Transfer for assistance with microbial studies on the Mir space station.

REFERENCES

- Borchers A, Keen C, Gershwin M. Microgravity and immune responsiveness. Implications for space travel. *Nutrition* 2002; 18:889.
- Broude NE. Stem-loop oligonucleotides: a robust tool for molecular biology and biotechnology. *Trends Biotechnol* 2002; 20: 249-56.
- Bugbee B, Salisbury FB. Wheat production in the controlled environments of space. *Utah Sci* 1985; 46:145-51.
- Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; 162:156-9.
- Crucian BE, Cabbage ML, Sams CF. Altered cytokine production by specific human peripheral blood cell subsets immediately following space flight. *J Interferon Cytokine Res* 2000; 20:547-56.
- Fuchs BM, Glockner FO, Wulf J, Amann R. Unlabeled helper oligonucleotides increase the in situ accessibility to 16S rRNA of fluorescently labeled oligonucleotide probes. *Appl Environ Microbiol* 2000; 66:3603-7.
- Hall LB, Bruch CW. Procedures necessary for the prevention of planetary contamination. *Life Sci Space Res* 1965; 3:48-62.
- Juergensmeyer MA, Juergensmeyer EA, Guikema JA. Long-term exposure to spaceflight conditions affects bacterial response to antibiotics. *Microgravity Sci Technol* 1999; 12:41-7.
- Kacena MA, Merrell GA, Manfredi B, et al. Bacterial growth in space flight: logistic growth curve parameters for *Escherichia coli* and *Bacillus subtilis*. *Appl Microbiol Biotechnol* 1999; 51: 229-234.
- Konstantinova IV. Immune resistance of man in space flights. *Acta Astronaut* 1991; 23:123-7.
- MacElroy RD, Wang D. Waste recycling issues in bioregenerative life support. *Adv Space Res* 1989; 9:75-84.
- MacElroy RD, Kliss M, Straight C. Life support systems for Mars transit. *Adv Space Res* 1992; 12:159-66.
- Manie S, Konstantinova I, Breittmayer JP, et al. Effects of long duration spaceflight on human T lymphocyte and monocyte activity. *Aviat Space Environ Med* 1991; 62:1153-8.
- Muniesa-Perez M, Jofre J, Blanch AR. Identification of *Vibrio proteolyticus* with a differential medium and a specific probe. *Appl Environ Microbiol* 1996; 62:2673-5.
- Nefedov YG, Shilov VM, Konstantinova IV, Zaluguyev SN. Microbiological and immunological aspects of extended manned space flights. *Life Sci Space Res* 1971; 9:11-6.
- O'Meara D, Yun Z, Sonnerborg A, Lundeberg J. Cooperative oligonucleotides mediating direct capture of hepatitis C virus RNA from serum. *J Clin Microbiol* 1998; 36:2454-9.
- Pierson DL. Microbiology. In: Nicogossian AE, Huntoon CL, Pool SL, eds. *Space physiology and medicine*. Philadelphia: Lea and Febiger; 1993:157-66.
- Race MS, MacGregor DG. Integrating public perspectives in sample return planning. *Adv Space Res* 2000; 26:1901-9.
- Rummel JD. Implementing planetary protection requirements for sample return missions. *Adv Space Res* 2000; 26:1893-9.
- Sonnenfeld G, Mandel AD, Konstantinova IV, et al. Spaceflight alters immune cell function and distribution. *J Appl Physiol* 1992; 73:191S-5S.
- Sonnenfeld G. Space flight, microgravity, stress, and immune responses. *Adv Space Res* 1999; 23:1945-53.
- Taylor GR, Neale LS, Dardano JR. Immunological analyses of U.S. Space Shuttle crewmembers. *Aviat Space Environ Med* 1986; 57:213-7.
- Tyagi S, Kramer FR. Molecular beacons: probes that fluoresce upon hybridization. *Nat Biotechnol* 1996; 14:303-8.
- Vila A, Viril-Farley J, Tapprich WE. Pseudoknot in the central domain of small subunit ribosomal RNA is essential for translation. *Proc Natl Acad Sci USA* 1994; 91:11148-52.
- Zaluguev SN, Viktorov AN, Gorshkov VP, et al. Prevention of staphylococcal infections in manned space flight. *Kosm Biol Aviakosm Med* 1981; 15:27-9.