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A DRAFT TEST PROTOCOL FOR DETECTING POSSIBLE BIOHAZARDS IN MARTIAN SAMPLES RETURNED TO EARTH

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October 2002

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PREFACE

This document provides the final version of a *Draft Test Protocol for Detecting Possible Biohazards in Martian Samples Returned to Earth*. This Draft Protocol was developed through an iterative process of discussion and review during the Mars Sample Handling Protocol Workshop Series, as well as afterwards. The table below is a chronological list of key workshops, reviews, and publications that led to the development of the Draft Protocol, and gives the terminology used in this document to refer to earlier versions. The final reports from the Workshops are cited in Appendix B, and contain full documentation and details of the sub-group discussions at each Workshop. The discussions from Workshops 1 through 3 led to a consensus that was reached during Workshop 4, resulting in the first complete protocol (denoted below as the "Completed Working Draft Protocol"). That document underwent review and revision by a special Oversight and Review Committee (see Appendix C), and a reading by the NASA Planetary Protection Advisory Committee. This "final" version of the Draft Protocol resulted from their critical reading and revisions, and supercedes all earlier versions. It is anticipated that this Draft Protocol will be subject to extensive further review and debate prior to development of any final protocol for use in receiving and testing samples from Mars.

Terminology Used	Date/Location	Report Citation or Annotation
Workshop 1 Final Report	March 2000, Bethesda, MD	*Race and Rummel, 2000*
Workshop 2 Final Report	October 2000, Bethesda, MD	*Race et al., 2001a*
Workshop 2a Final Report	November 2000, Rosslyn, VA	*Bruch et al., 2001*
Workshop 3 Final Report	March 2001, San Diego, CA	*Race et al., 2001b*
Penultimate Working Draft Protocol	May 2001	First compilation of the developing protocol from recommendations of Workshops 1, 2, 2a, and 3
SSB/COMPLEX Report: The Quarantine and Certification of Martian Samples	May 2001 Advance Copy	*SSB 2002*
Workshop 4 Final Report	June 2001, Arlington, VA	*Race et al., 2002*.
Completed Working Draft Protocol	June 2001	A consensus working draft resulting from the entire Workshop Series — published in WS 4 Final Report (see *Race et al., 2002,* Appendix A, page 71,); submitted to the ORC for comment and review.
Oversight & Review Committee (ORC) review process Oct-Nov 2001	12 November, 2001, ORC Meeting, Rockefeller University New York, NY	Review of the Completed Working Draft Protocol.
A Draft Test Protocol for Detecting Possible Biohazards in Martian Samples Returned to Earth	October 2002	*Rummel et al., 2002* (this document); the final version of the Draft Protocol incorporating comments and recommendations from the ORC.

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Introduction to the Draft Protocol

In anticipation of missions to Mars that will involve the return of samples, it is necessary to prepare for the safe receiving, handling, testing, distributing, and archiving of martian materials here on Earth. Previous groups and committees have studied selected aspects of sample return activities, but a specific protocol for handling and testing of returned samples from Mars must still be developed.

For upcoming Mars sample return missions, NASA is committed to following the recommendations developed by the Space Studies Board (SSB) of the National Research Council (NRC) in its report on sample handling and testing *[SSB 1997].* In particular, the NRC recommended that:

> *a) "samples returned from Mars by spacecraft should be contained and treated as potentially hazardous until proven otherwise," and b) "rigorous physical, chemical, and biological analyses [should] confirm that there is no indication of the presence of any exogenous biological entity."*

To develop and refine the requirements for sample hazard testing and the criteria for subsequent release of sample materials from precautionary containment, the NASA Planetary Protection Officer convened the Mars Sample Handling Protocol (MSHP) Workshop Series from March 2000 to June 2001. The overall objective of the Workshop Series was to produce a Draft Protocol by which returned martian sample materials could be assessed for biological hazards and examined for evidence of life (extant or extinct), while safeguarding the samples from possible terrestrial contamination. In addition to U.S. and international participants invited by NASA, significant participation and support by French scientists were provided in all aspects of the Workshops and protocol development through arrangement with the Centre National d'Études Spatiales (CNES).

The stated objective for the Workshop Series was:

> *"For returned Mars samples, develop a recommended list of comprehensive tests, and their sequential order, that will be performed to fulfill the NRC recommendations that 'rigorous analyses determine that the materials do not contain any biological hazards.'"*

Throughout the Workshop Series, these analyses were anticipated to comprise not only a series of tests to detect a possible living entity ('life detection'), but also tests to look for biological activity, even if a living entity were not detected ('biohazard testing').[1] Therefore the Workshop Series was designed to devise a protocol that could rigorously analyze returned martian sample materials to determine that those materials are free from biohazards and/or extraterrestrial life-forms, and are therefore safe to be released from containment in their native state for further scientific research. To accomplish this, Workshop Series participants focused on a variety of questions that had to be addressed about the protocol to meet the Series' objective (see Appendix A). This Draft Protocol is intended to incorporate the answers developed to those questions.

To keep the Workshop Series focused, a set of basic assumptions (see Appendix A) was given to the participants at each of the Workshops to guide and constrain their deliberations. Subsequent to the failure of the Mars Surveyor 1998 missions, these assumptions were subject to some modification during the re-planning process that NASA and its international partners undertook (i.e., the change of the return date from '2007' to 'in the next decade' in Assumption #2). However, none of the modifications affected the basic premises under which the Workshop participants undertook their task. These assumptions are consistent with the plans of NASA and its international partners as of the publication of this report

1. This two-pronged approach is consistent with the Space Studies Board's recommendations for returned martian samples *[SSB 1997, p. 27]*: "The initial evaluation of samples returned from Mars will focus on whether they pose any threat to the Earth's biosphere. The only potential threat posed by returned samples is the possibility of introducing a replicating biological entity of non-terrestrial origin into the biosphere. Therefore, the initial evaluation of potential hazards should focus on whether samples contain any evidence of organisms or biological activity."

(October 2002), and are expected to remain current despite the inevitable program delays and likelihood of future changes.

In addition to the development of this Draft Protocol through the NASA-led Workshop Series, the SSB was asked by NASA in early 1999 to develop recommendations for the quarantine and certification of martian samples—both as an input to the NASA Workshop Series, and as recommendations to NASA to be assessed in their own right. The SSB report *[SSB 2002]* was released in preliminary form in May 2001, just prior to Workshop 4. Thus participants of Workshop 4 had access to an Advance Copy of the SSB report during their review of the Penultimate Working Draft Protocol. Therefore, both the completed Working Draft Protocol (as published in the Workshop 4 final report *[Race et al, 2002]*) and this final version of the Draft Protocol reflect, to a great degree, an examination of the findings and recommendations of the Space Studies Board study.[2]

This document is the first complete presentation of the Draft Protocol for Mars sample handling that meets planetary protection needs, and represents a consensus that emerged from the work of sub-groups assembled during the five Workshops of the Series.[3] Over the course of the Workshops, participants converged on a conceptual approach to sample handling as well as on specific analytical requirements. Further discussions identified important issues remaining to be addressed, including research and development necessary for optimal protocol implementation. This Draft Protocol also incorporates the review comments of an Oversight and Review Committee (see Appendix C) that examined the Completed Working Draft subsequent to the end of the Workshop Series.

2. See Appendix B for a complete list of workshops and reports contributing to this Draft Protocol.
3. The final reports from the Workshops in the Series *[Race and Rummel, 2000; Race et al., 2001a, 2001b, and 2002; Bruch et al. 2001]* contain full documentation and details of the sub-group discussions that fed into this final version of the Draft Protocol.

Why a 'Draft Protocol'?

What is reported here is termed a 'Draft' Protocol because it is intended to be just that. While it is a responsibility of NASA's Planetary Protection Officer *[NASA 1999]* to prescribe "standards, procedures, and guidelines applicable to all NASA organizations, programs, and activities" to achieve the policy objectives of NASA's planetary protection program, including ensuring that Earth is "protected from the potential hazard posed by extraterrestrial matter carried by a spacecraft returning from another planet or other extraterrestrial sources," (in this case, Mars), it is neither practical nor useful for this Draft Protocol to be developed into a final form at this time. The final protocol that will guide the process of assessing the martian samples should owe much to new knowledge about Mars that will be gained in robotic exploration on Mars leading up to the sample return mission, as well as detailed information available only on the sample return mission itself. In addition, the final protocol should take into account the *specific* nature of the receiving facility that is developed for the initial processing and testing of the returned samples, as well as the requirements and abilities of the *specific* instrumentation and personnel selected to undertake the challenging task of testing the samples while protecting Earth from possible hazards, and preserving the scientific value of the sample return undertaking. It is anticipated that the final protocol will receive its final review at or about the time the first samples leave the martian surface.

Meanwhile, this Draft Protocol is intended to provide a proof-of-concept model of the final protocol, demonstrating one approach (and more importantly, a sufficient approach) to testing returned Mars samples for possible biohazards or biological activity of martian origin. This Draft Protocol has been developed to provide a sequential series of tests that can be applied to martian samples to provide data that can be used to make decisions about the release of unsterilized samples from containment—either wholly or partially—while allowing for an earlier release of samples subjected to a decontamination process ("sterilization") to ensure they are safe for analyses outside of containment.

Containment in the Sample Receiving Facility and Elsewhere

In order to preserve the scientific value of returned martian samples under safe conditions and avoid false indications of life within the samples, the capability is required for handling and processing Mars samples while preventing their contamination by terrestrial materials (i.e., cleanroom conditions, technical criteria TBD) and while maintaining strict biological containment. This requirement is a major challenge in the design of what will be described here as a Sample Receiving Facility (SRF).[4] To some degree, the cleanroom requirement is likely to constrain the working space inside an SRF even more than might normally be experienced in a "typical" Biosafety Level 4 (BSL-4) facility of similar size. An SRF will require combining technologies currently found in maximum containment microbiological laboratories (e.g., BSL-4, BSL-3)[5] with those used in cleanrooms to preserve the pristine nature of rare samples. Such an integrated facility is not currently available anywhere. Some of the challenges of providing such a facility may be alleviated through a design and development process that will include mock-ups of containment/cleanroom combinations whose efficacy can be tested thoroughly (see Figure 1 for some options). Some of the overall facility constraints may be lessened through the use of multiple containment facilities to accomplish different aspects of the protocol, especially where material (as opposed to biological) contamination constraints can be relaxed. It is anticipated that samples may be shipped among appropriate containment facilities wherever necessary under procedures developed in cooperation with the U.S. Centers for Disease Control and Prevention, the U.S. Department of Transportation, and appropriate international authorities. Nonetheless, it is envisaged that all samples initially

4. A variety of names have been used in reference to the place where returned samples will be handled and tested initially (e.g. Sample Receiving Facility (SRF), the Quarantine Facility, the Mars receiving laboratory, primary containment facility, quarantine facility, etc.). A recent NRC report *[SSB 2002]* has used "Quarantine Facility," but it is more useful in this report to use the generic SRF. The actual name and location(s) of the facility or facilities where the protocol will be executed is TBD. Use of these facilities beyond the receipt of martian samples may be anticipated.

5. "BSL" levels are a North American convention. European equivalents will be considered and described as necessary in implementation of the final protocol.

returned from Mars will be placed in a single SRF and held there through the preliminary examination phase (i.e., “Preliminary Evaluation,” as envisaged in Figure 2 on page 18), and for those subsequent steps compatible with SRF design and capacity.

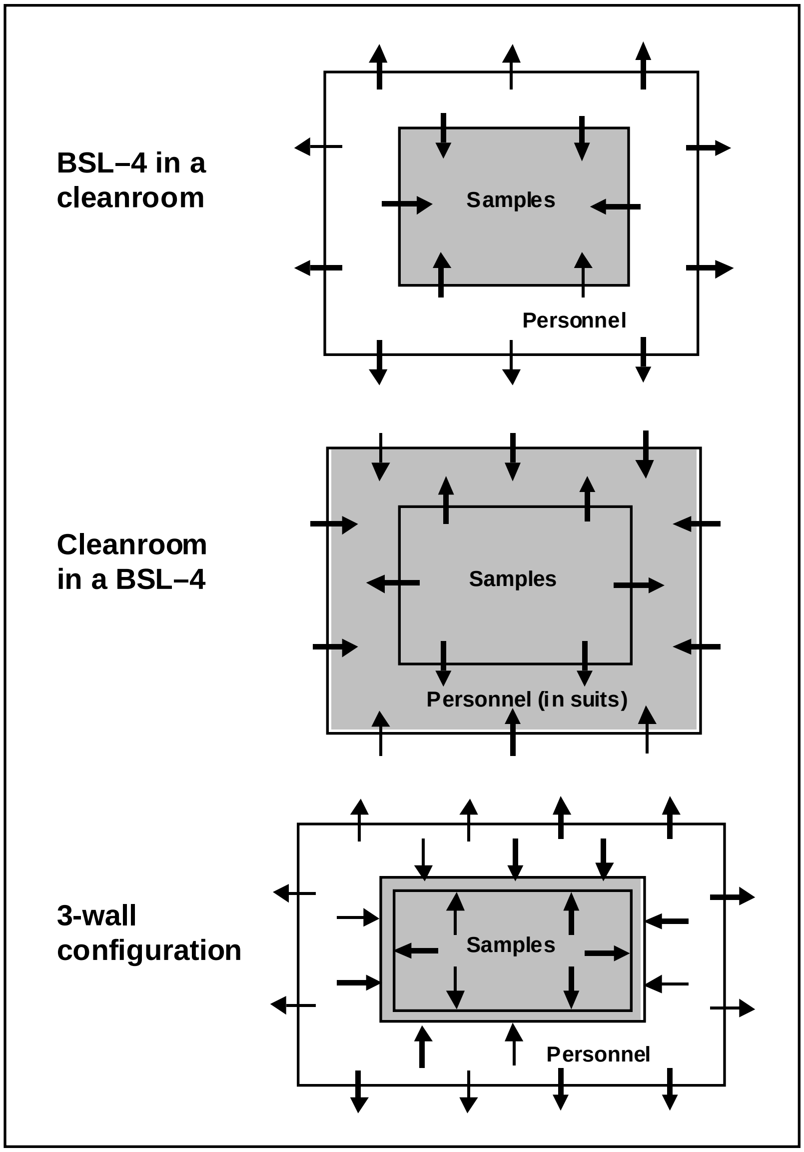

Figure 1. Top and Center: Simple options for the combination of a biological containment facility with a cleanroom. Arrows show gas flow (*via* leakage) caused by pressure differentials in the spaces shown. Gray areas are potentially contaminated by any organisms the Mars samples might contain. Bottom: A more complex arrangement with double walls separating workers from samples, and in which the gases from the workers and the samples both are exhausted through the space between the walls (and in the case of the gases from the personnel, to the outside atmosphere). *From SSB 2002.*

BSL-4 is required for work with dangerous and exotic agents that pose a high risk to the individual of aerosol-transmitted laboratory infection and life-threatening disease. The unknown nature of any possible biohazard in returned martian samples demands, at least initially, this most stringent containment presently afforded to the most hazardous biological entities known on Earth. In the biomedical community, biohazard testing is a pathway towards gradual "decontainment" of dangerous and/or exotic bioagents, when supported by experimental evidence. Decisions about the appropriate biosafety level for a particular bioagent can be made when sufficient data are obtained to support either the need for continued work at a high level of containment, or allowance to conduct work at a lower level.

Generally, lower biosafety levels are assigned to bioagents with less human virulence. If sufficient data are gathered to rule out concerns about human virulence and infection, a decision could later be made to allow subsequent work at a lower containment level during tests investigating possible environmental effects. A lower level of containment would potentially enhance sample access within the scientific community while still providing adequate biosafety conditions under existing biosafety guidelines and regulations.

In addition to satisfying both biosafety and cleanliness needs, the SRF will need to provide different types of laboratory environments for carrying out the various aspects of protocol testing. During the Workshop Series, the new term 'Planetary Protection Level' (PPL) was developed for the purpose of categorizing and describing the different combinations of containment and cleanliness conditions required within the SRF for different testing needs. Although details of various PPL designations will require further definition, it is possible to anticipate a number of laboratory conditions that may be required during the protocol testing. The four PPLs are described in the following text and in Table 1:

- PPL-α – for incoming samples and archived samples; maximum biocontainment and cleanliness; maintains samples in an inert gas environment and Mars-like conditions (TBD).[6]
- PPL-β – maintains maximum biocontainment and protection for workers and the environment; maximum cleanliness, but allows exposure to ambient terrestrial conditions.
- PPL-γ – maintains maximum biocontainment with moderate cleanliness and ambient terrestrial conditions (i.e., for animal testing scenarios).
- PPL-δ – maintains BSL-3-Ag containment conditions, with less emphasis on cleanliness, and ambient terrestrial conditions.[7]

PPL-type	Biocontainment	Cleanliness	'Ambient' Conditions	Used For:
PPL-α	Maximum (BSL-4)	Maximum	Mars-like (pristine); *Although at 1 atm w/inert gas environment.*	Incoming container and materials; some preliminary tests; sample bank/storage; some Life Detection
PPL-β	Maximum (BSL-4)	Maximum	Earth-like	Life Detection; some Physical/Chemical; TBD
PPL-γ	Maximum (BSL-4)	Moderate	Earth-like	Some Biohazard testing, some Physical/Chemical processing, and animal testing
PPL-δ	Strict BSL-3-Ag	Ambient	Earth-like	Some Biohazard testing; 'post-release' tests TBD

Table 1. Anticipated laboratory conditions and PPL categories. Note: Levels of cleanliness associated with each PPL are TBD and should be defined explicitly well in advance of sample return.

6. It is anticipated that only the primary SRF will be required to have PPL-α conditions. If other facilities beyond the SRF are used as part of the protocol testing, they will be certified for conducting particular tests or studies at the appropriate PPL conditions.
7. PPL-δ provides a level of containment for the samples that allows investigators to work in a laboratory situation providing protection to personnel through an engineered environment with HEPA filtered air entering and leaving the area, containment of water and/or waste to the laboratory, and protection through personnel protective equipment consistent with U.S. BSL-3 Agriculture and French P4 standards. It was recommended that the BSL-3-Ag facilities used should be designed to accommodate large instruments, rather than miniaturizing the instruments to fit into a pre-existing lab.

It is important to note that, regardless of cleanliness requirements or ambient conditions, all initial testing will be done under maximum biocontainment equivalent to United States BSL-4 *[CDC-NIH, 1993]*. In addition, Biohazard testing will not require the extreme cleanliness levels to be used for initial sample processing, or certain Physical/Chemical or Life Detection tests. The majority of Biohazard tests will be done in PPL-γ. If the results of the initial Life Detection and Biohazard tests are all negative, it may be appropriate to conduct some subsequent tests under less strict containment conditions. The first step in downgraded containment for untreated samples has been designated as PPL-δ, which is equivalent to BSL-3-Ag.[8]

"Sterilization" of Martian Samples

Recognizing that a species' adaptation to physiological stress may evolve through natural selection, it is expected that possible extant life on Mars could be able to survive extremely hostile conditions. Surface temperatures at the equator of Mars range from –100°C during the martian winter to 20°C during the martian summer. Mars is extremely dry; the partial vapor pressure of water on the surface is approximately 0.1 bar. The martian atmosphere is 95% CO_2 and provides no protection against exposure to 200-300 nanometer ultraviolet light, which may generate strong oxidants in the surface material. It is believed that organic compounds on the surface of Mars are subject to oxidation by this UV-induced photochemistry. Since this combination of conditions cannot be found on Earth, it is unlikely that a single terrestrial species will be found that can serve as a surrogate for a putative martian organism when evaluating methods for sterilizing martian samples. There are terrestrial environments, however, that are sufficiently similar to the martian environment to allow the isolation of species that exhibit extreme resistance to a subset of the conditions (e.g., desiccation, radiation, or

8. PPL-δ applies at the point in the protocol where samples do not require atmospheric isolation and may be moved to outside laboratories with suitable facilities for further testing. In general, level 3 biosafety laboratories (BSL-3) abide by different standards within the U.S. and Europe. For clarity, the U.S. standard for BSL-3-Ag will be used.

cold) to be encountered on Mars. As an item for further research, it is anticipated that an effort will be made to identify and characterize terrestrial species from environments as similar as possible to those on Mars, and that these species will be used to validate sterilization processes.

In the context of this Draft Protocol and the relevant NRC reports *[SSB 1997; SSB 2002]*, the term "sterilization" is used to connote the decontamination process that will be used to ensure that the samples are safe for analyses outside of containment. It is possible, though very unlikely, that martian organisms are not carbon based, and martian biology could conceivably be based on other elements (e.g., Si, N, P, O, H, S, Al, B). But overall, it should be noted that the chemical elements on Mars and the forces holding molecules together are the same as on Earth. If there were a life-form on Mars based on other than carbon-containing molecules, the energies holding such molecules together would not be much different than those for proteins and polynucleotides. Hence, bond breakage by heat or gamma radiation should be similar for Earth and Mars life-forms, and sterilization conditions for Earth microorganisms should eradicate microorganisms of similar size from Mars. There is no absolutely optimal approach to decontamination under these circumstances, but enough is known about the relationships among organism size, repair mechanisms, and survivability, that the maximum survivability of any martian organisms can be estimated with some confidence.

Whether we assume that life on Mars is based on the same building blocks as terrestrial life, or on other covalently bonded complex molecules, only two methods of sterilization are considered viable options at present—dry heat and gamma radiation, either alone or in combination. These methods will penetrate the sample and, therefore, provide the highest level of assurance that putative organisms will be destroyed. It is recognized that the application of heat, and in some cases gamma irradiation, will modify the geological properties of the sample. Within reason, every effort should be made to develop and implement a method of sterilization that protects the scientific integrity of the sample.

Many of the key parameters measured by geochemists are unaffected by sterilizing representative geological samples with gamma radiation *[Allen et al., 2000].* Gamma photons from ^{60}Co (1.17 – 1.33 MeV) in doses as high as 30 Mrads do not induce radioactivity in rock and mineral samples. Such doses also produce no measurable changes in isotopic compositions, elemental compositions, or crystallographic structures. The only detectable effects are changes in albedo, color, and thermoluminescence in selected minerals. Isotopic and elemental compositions will not be affected regardless of gamma dose. Sterilization at doses significantly above 30 Mrads may induce changes in crystallographic structure (*caveat:* research required) and dose-dependent changes in albedo, color and thermoluminescence may affect sample science. On balance, if samples returned from Mars require biological sterilization, exposure to gamma rays may provide a feasible option.

For the development of a final protocol for use with martian samples, a program of research should be initiated to determine the effects of varying degrees of treatment by heat and by gamma irradiation on organic compounds in rocky matrices, and also on microscopic morphological evidence of life. This research should be started well in advance of the return of the Mars samples, so that the decontamination process can be designed to allow data obtained from analyses of sterilized samples to be interpreted with minimal ambiguity and maximum utility for the scientific purposes intended. Research should also be conducted to determine the efficacy of various supercritical fluids and commonly used organic solvents in killing model microorganisms, allowing the possibility that solvent extracts might be safe to remove from containment without the damage to dissolved biomarker compounds that would be caused by heat or ionizing radiation. Whether decontamination is systematically achieved by any supercritical fluids used in making extracts is a matter that must be investigated further, prior to the removal of any such samples from the SRF. Also critical will be the atmospheric conditions (gas mix, humidity) under which irradiation conditions are qualified for use. Lethality of irradiation is enhanced by the presence of oxygen, whether from O_2, H_2O, or other sources.

The aim of a sterilizing process is to reduce the risk of significant adverse effects of samples distributed to the scientific community. The sterilization levels will be defined to be such that the likelihood of adverse effects, given exposure to humans, animals, and the environment, is less than 10^{-6}. A suggested process for sterilization consists of irradiation with gamma rays at temperatures up to approximately 105°C *[Bruch et al., 2001, page 5]*. This procedure has the advantage of being able to kill all known terrestrial organisms, while doing minimal damage to the non-biologic constituents of the Mars samples.

The survival rate of a large number of terrestrial organisms exposed to ^{60}Co gamma rays has been determined as a function of dosage, dose rate, and temperature. There are no terrestrial organisms known whose probability of survival is $>10^{-6}$ at a dose of 20 Mrads at room temperature. Nonetheless, populations of organisms may require higher doses to ensure that the probability of finding any survivor is $<10^{-6}$. The survival rate at a given total dose decreases with increasing temperature during irradiation. For example, the sensitivity of dry T1 bacteriophage to inactivation by X-rays increases, or the D_{37} decreases by approximately ten-fold between 60 and 105°C *[Pollard 1953]*.

Protocol "Sterilization" Conditions A large number of geochemical tests will be carried out in the SRF upon arrival of the samples. These tests will likely include X-ray tomography to determine loci of cracks and other separations where life-forms most likely would be, and total organic carbon (TOC), which permits a limit on the density of carbon-based organisms to be calculated.

Irrespective of the chemical basis of any life-form, a confidence level of sterilization can be provided with only two assumptions: 1) any reproducing life-form must be based on macromolecules (i.e., polymers) with interatomic covalent bonds (not crystal lattices), and 2) since all such bonds have similar strength, destroying these bonds destroys the life-form.

Evidence shows that (at or near room temperature) 55 Mrads of radiation will destroy almost all known bacteria, viruses, spores, and prions (i.e., the causative agent in Scrapie) by 1 million–fold. Using 100 Mrads would give a 10–fold safety margin. If worst-case estimates are used (10^6 –10^{12} organisms/gram of martian sample and a tiny target, such as a virus) sterilization would require 400 Mrads. Even after this higher dose, most geologic studies may still be accomplished. This amount of radiation could be safely reduced if the irradiation were carried out at elevated temperature (e.g., 105°C), and/or if the TOC (or equivalent for non-carbon-based organisms) is low enough to rule out large numbers of organisms being present in the sample.

If martian organisms returned to Earth are similar to terrestrial organisms, a dose of 20 Mrads at 105°C should reduce their number to $<10^{-6}$ of their initial number (but not necessarily kill them all). It is not clear, however, that martian organisms should be similar to terrestrial organisms; it is possible that they could be much more resistant to gamma radiation. A good deal is known about the relationship between the size and the biochemistry of terrestrial organisms and their resistance to gamma radiation. For example, it has been shown that smaller organisms tend to survive higher radiation doses, but the strategies used by microorganisms to increase their resistance to radiation are not well understood. It might, therefore, be a useful exercise to explore hypothetical possibilities for the evolution of martian organisms adapted to the much higher radiation fluxes to which they would be subjected naturally, compared to terrestrial microbes. The radiation dose at various temperatures required to reduce the probability of the survival of even a *single* organism below 10^{-6} per sample could then be estimated and could become the basis of irradiation protocols for the sterilization of returned Mars samples. In particular, tests should be made against radiation-tolerant species like *Deinococcus radiodurans*, which possesses amazing radiation repair capabilities *[Daly 2000]*. In such tests, it will be important to consider the destruction of both the smallest and most hardy known Earth organisms, as well as the destruction of non-living surrogates (such as viruses and viroids) that can serve to provide effective sterilization doses for martian organisms that may be smaller—as small as conceivably possible (see *SSB 1999*). Such surrogates also can provide for the eventuality that, if Earth life and putative Mars life are somehow related, the sterilization conditions will provide effective protection against martian virus- or viroid-like entities that may be potentially hazardous.

Criteria For Release

As part of the charge to the recent NRC study of *The Quarantine and Certification of Martian Samples [SSB 2002]*, the Committee on Planetary and Lunar Exploration (COMPLEX) was asked to study "What are the criteria that must be satisfied before martian samples can be released from the facility?" The Committee's recommendations were weighed extensively in the derivation of the release criteria given here. For the most part, their recommendations are incorporated in spirit, if not in specific wording. Departures from the Committee's report were the subject of Workshop Series discussions, and were addressed in the review of the Oversight and Review Committee. The departures are most obvious where the NRC Committee made recommendations that were not fully consistent with their own assumptions. An example of this is given in a footnote to the NRC report *[SSB 2002, p. ES-5],* which states that, "The word 'life,' when used in the context of martian life, should always be understood to mean 'Life as we know it,' to allow for the possibility of life-forms distinctly outside our terrestrial experience." This is an important footnote, but it has been noted that not all of the Committee's release criteria (for example, 'no carbon equals no hazard') were consistent with this possibility. Additionally, COMPLEX's recommendations place a heavy emphasis on "sterilization" of Mars samples as a key to their release—yet the report states in a number of places that the effects of sterilizing doses of heat and/or gamma radiation on the geochemical and biological signals the samples may carry are not known. Overall, the release criteria listed below are slightly more stringent, as well as somewhat more comprehensive, than those recommended by COMPLEX.

Table 2 gives the basic overview of the questions that need to be answered prior to the release of unsterilized samples from the SRF. These questions will be asked of a representative sub-sample of the material returned from Mars.

Item	Question	Strategy
1	Is there anything that looks like a life-form?	Microscopy; beam synchrotron or other non-destructive high-resolution analytic probe, particularly one that would allow testing unsterilized (yet still contained) samples outside main facility.
2	Is there a chemical signature of life?	Mass spectrometer and/or other analytical measurement systems (to be used in containment) that would identify biomolecules, chiral asymmetry, special bonding, etc.
3	Is there any evidence of self-replication or replication in terrestrial living organism?	Attempts to grow in culture, in cell culture, or in defined living organisms.
4	Is there any adverse effect on workers or the surrounding environment?	Microcosm tests; medical surveillance of workers and monitoring and evaluation of living systems in proximity of receiving facility to ensure no release or exposure associated with operations of SRF.

Table 2. Sequence of questions and possible strategies for decisions about release of sample material from containment.

In any event, only evidence of measurable biohazards or active martian life-forms or their biomaterials should be regarded as relevant criteria for deciding whether to release any unsterilized samples (the specific release criteria are TBD). Depending on results of Life Detection and Biohazard tests, remaining portions of samples will either be released for allocation outright, or sterilized and then released for allocation. Hence, the following criteria are intended to govern the release of samples evaluated using this Draft Protocol:

Protocol Release Criteria

- No solid sample shall be released from containment in the Mars receiving laboratory until it or its parent sample undergoes preliminary examination, baseline description, cataloguing, and any necessary repackaging.
  - ➢ Samples to be used for Life Detection procedures or to be released from containment will be screened for radioactivity and potential chemical hazards.
  - ➢ Additionally, samples to be used for Biohazard testing will be screened for known toxicity to bacterial and eukaryotic cells.
- Samples containing any active martian form of life, *be it hazardous or not*, will be kept under appropriate level of containment, or be thoroughly sterilized before release.
- Samples providing indications of life-related molecules, including proteins, nucleic acids, or molecular chirality, will require more extensive testing, including additional Biohazard testing, prior to their release.
- Samples may be released if they are first subjected to a sterilizing process involving heat, radiation, or a combination of these agents, to ensure they are safe for analyses outside of containment. A sample that is 'safe' is stipulated to be free of any viable self-replicating entities or entities able to be amplified.
- Samples may be released if Biohazard testing does not yield evidence of live, extraterrestrial, self-replicating entities, or of harmful effects on terrestrial life-forms or environment under Earth-like conditions.
  - ➢ Biohazard testing will involve assays for: 1) replication in media with various organic and inorganic carbon sources, including enriched media (liquid/solid), and sparse media appropriate to photo- or chemo-autotrophs; 2) effect/growth on various cell cultures; 3) effect/growth on whole organisms (i.e., murine/specified rodent; plant); and, 4) effect on the ecosystem level.
  - ➢ Basic Biohazard testing will be required even in the absence of evidence of organic carbon in a sample returned from Mars.

Overview of the Draft Protocol

The Draft Protocol has one basic purpose—to ensure that a representative set of sub-samples undergoes sufficient testing to evaluate them against the release criteria. Samples must be characterized, categorized, and analyzed to ensure that they can be sorted according to a procedure providing 'statistical relevance' to any sub-sampling (whether homogenized or pre-sorted for 'biologically interesting features'), within a reasonable time using a minimal amount of sample. Early results in the Biohazard testing will need to be screened to ensure that potentially chronic effects are not overlooked. The tests themselves should be performed in an order that takes into account the relative harm posed by a potential biohazard (e.g., to humans, animals, environments) and takes into consideration a variety of routes of exposure and infection. Samples must be tested for biomolecules (known or suspected), for other organic compounds, and for non-carbon evidence of an active metabolism being present (e.g., alterations of sulfur, iron, or other compounds). Life Detection and Biohazard testing partially overlap, and both will depend on the processing of the samples and data from the Physical/Chemical processes to evaluate their results and how to interpret them.

The Draft Protocol has three main segments: Physical/Chemical (P/C) processing, Life Detection (LD) testing, and Biohazard (BH) testing. Figure 2 is a simplified overview of how these segments are related. In this protocol, P/C processing refers to all of the analytical testing and sample description that will be accomplished prior to materials being tested for signs of life, or in support of various forms of life and biohazard detection. LD testing is also mainly analytical and descriptive. LD testing seeks signs of life in either morphology, chemistry, or cultivation, as well as detecting a life-form in a manner that may be informed by hypotheses about what signs of life a martian biota might leave. BH testing seeks to challenge test sample materials against a variety of model systems to see if the sample contains any hazardous properties that can be shown to be the result of a self-replicating entity contained within the sample. BH testing should be as free as possible from assumptions about the putative nature of a martian life form.

OVERVIEW: DRAFT MARS SAMPLE RETURN PROTOCOL

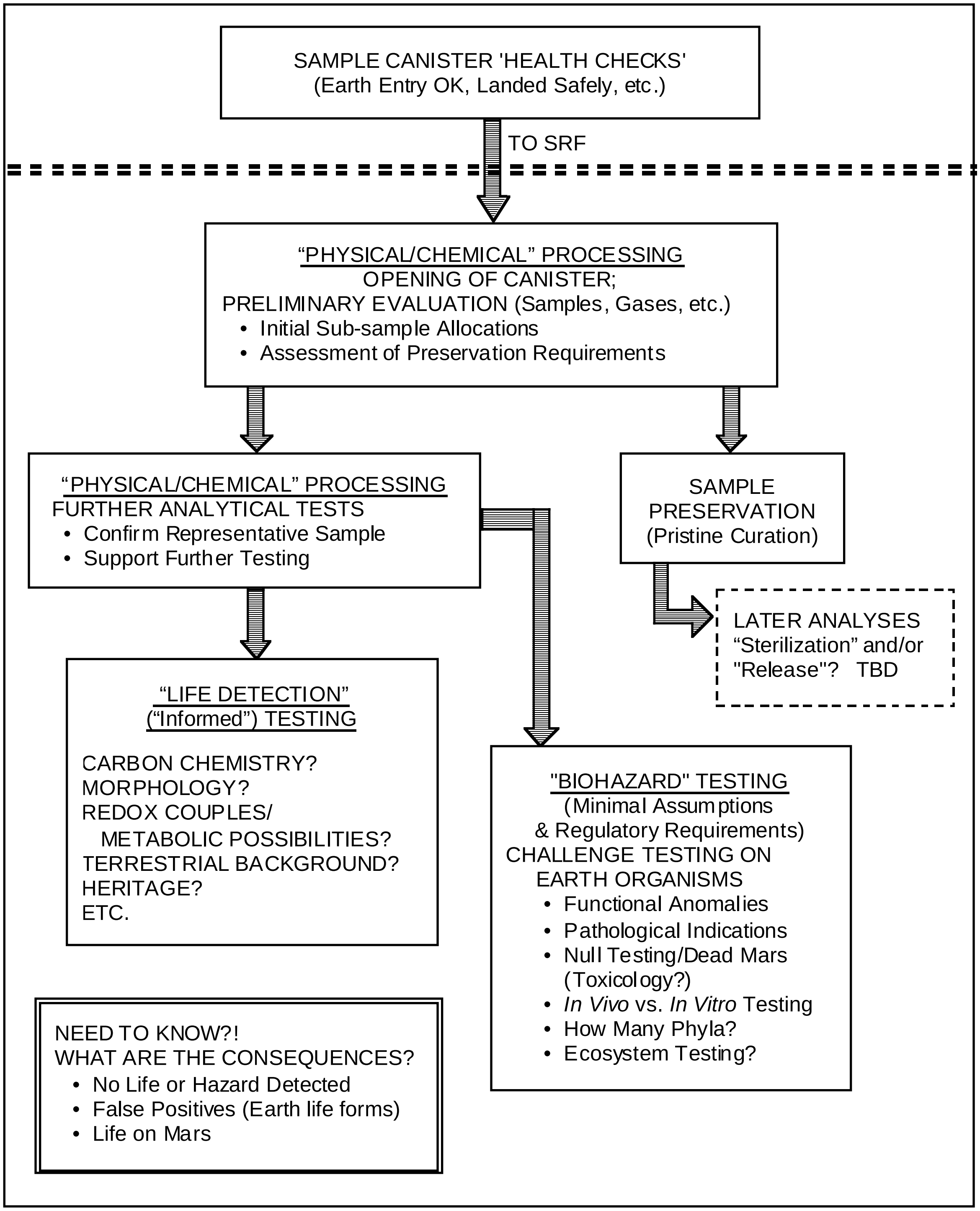

Figure 2. A simplified overview of the Draft Protocol showing the 3 main segments: Physical/Chemical processing, Life Detection, and Biohazard testing.

The overall process is as follows: the sample(s) will be removed from the Sample Return Canister (SRC) under maximum biocontainment in gloveboxes containing an inert gas atmosphere and housed within a combination cleanroom/biosafety lab. After initial documentation, samples will undergo preliminary characterization, splitting, and detailed examination using a variety of different methodologies. Ultimately, data from LD and BH testing will be used to determine whether to release materials from biocontainment. All sample materials not selected for further testing will be archived in sealed containers in an inert atmosphere glovebox within the lab for future scientific purposes. The Draft Protocol also addresses issues related to facilities, personnel management, monitoring, contingency planning, decision making, protocol review, implementation, and approval processes.

Physical/Chemical Processing

The overall objective for P/C processing is to specify information about the samples required to enable effective LD and BH testing, and curation. The focus is on sample characteristics that could be determinative in understanding the results of any *in vitro* and *in vivo* testing that may be required, as well as on information needed for sample preservation purposes. P/C processing includes actions affecting the returned samples between the time the SRC arrives in the SRF and the time sample aliquots are apportioned for LD and BH tests. P/C processing under this protocol should include only those actions required in support of planetary protection and future sample utilization. Figure 3 outlines the proposed P/C processing, which draws heavily from protocols proposed or used by others.[9]

9. This Draft Protocol is based on a framework developed at the first Workshop in this Series *[Race and Rummel, 2000, p.14-19]*, and on an earlier report by MSHARP *[Carr et al., 1999]*, which are, in turn, based on protocols developed at Johnson Space Center for handling and processing Apollo lunar samples, Antarctic meteorites, and cosmic dust. During the Workshop Series, modifications to the Draft Protocol were suggested by various sub-groups *[Race et al., 2001a, 2001b, 2002]*, and many of those have been included here resulting in several significant differences from the framework developed in Workshop #1. In general, the proposed Draft Protocol is consistent with the requirements and conditions set forth by the Space Studies Board *[SSB 1997]*, the MSHARP Committee *[Carr et al., 1999]*, an earlier workshop on sample quarantine protocols *[DeVincenzi et al., 1999]*, and CAPTEM *[Neal, 2000]*.

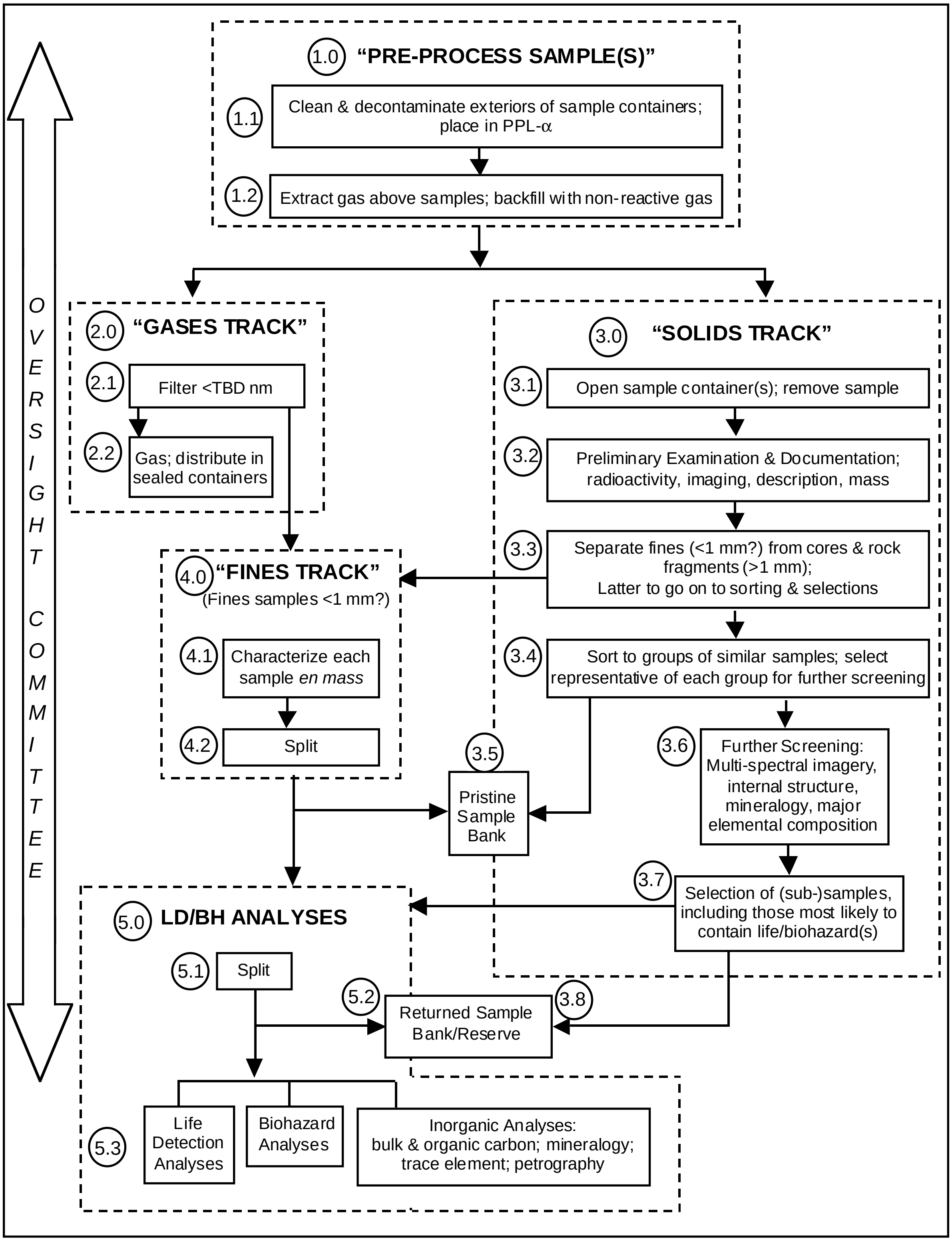

Figure 3. The Physical/Chemical processing will occur in four sequential stages leading into the Life Detection and Biohazard testing. The numeric annotations refer to numbered sections of text below, which elaborate on the proposed P/C steps.

Principles The selected steps and investigations in the P/C processing tracks are motivated by the following principles, as functions of the SRF: know what the returned samples are; preserve sample integrity; document everything; anticipate that different types of samples (e.g., gases, fines, rocks, and cores) require different treatment; recognize that all data obtained in the P/C processing must serve later scientific investigations; use the minimum sample possible; and provide real-time guidance and adjustment to the process. These principles, initially outlined by the report of the Mars Sample Handling and Requirements Panel (MSHARP) *[Carr et al., 1999],* have been endorsed by all the Mars Sample Handling Protocol Workshops *[Race and Rummel, 2000; Race et al., 2001a; Bruch et al., 2001; Race et al., 2001b; Race et al., 2002].*

The first two principles (know the sample; preserve sample integrity) are, to some extent, inconsistent because every characterization method or action on the returned samples will affect them in some regard. This inconsistency has been addressed in two ways. First, all characterization procedures in P/C processing are nominally non-contact and non-destructive—all the sample mass remains in the same physical and chemical state after each analysis. Second, most of the returned sample is subjected to only minimal investigations, while only a representative portion of the sample is subjected to more specific (and potentially sample-altering) analyses. The P/C processing and screening methods, except for weighing, involve sample interactions with electromagnetic radiation, principally near-visible wavelengths (near ultraviolet, visible, and near infrared). Several methods use X-rays to probe the samples, but it was recognized that X-rays can (at some dosages) affect biological/organic systems.

This Draft Protocol attempts a compromise between the desire to affect only a small proportion of the returned sample by planetary protection testing, and the need to assure safety by testing all portions of all samples. A range of strategies have been advocated to deal with the sample testing issue, from “characterize everything with all available non-destructive methods,” to “store most of the sample uncharacterized, and do only the minimum with the rest" (see discussions in: *Carr, et al., 1999, p. 37; Race and Rummel, 2000, p. 18; Race et al., 2001a, p. 35; and Race et al., 2001b, p. 34*). Here it is stipulated that it will be essential to examine all the returned material in at least a minimal fashion to: confirm spacecraft operations in sample transfer from Mars to the Sample Return Canister; correlate returned samples with documentation developed by the mission on Mars; and provide enough data to make informed choices about samples for LD/BH analyses. Examining all returned materials in at least a minimal fashion will help avoid a worst case scenario where an obviously biogenic sample could be stored unexamined and only discovered after nominal LD/BH tests were completed.

Documentation All treatments and actions with the returned samples need to be documented fully. Without a high level of documentation, it would be impossible to establish which samples are representative or particularly interesting, and to indicate what had been done to which sample during processing.

Different Samples It is clear that the different types of samples will require different processing techniques. Gases and bulk fines samples are expected to be inherently homogeneous to some level, and will require only minimal processing to derive characteristic and representative samples. However, solid materials are anticipated to be potentially heterogeneous and more extensive study and real-time decisions about their processing will be required.

Minimum Sample Mass The amount and size of returned Mars samples will be small, and it will be desirable to subject sample materials to a great range of biological, physical, and chemical tests. Thus, by necessity, each test on a returned sample must use the minimum mass consistent with achieving the scientific goal of the test.

Real-Time Adjustments – Oversight Committee Provisions must be made to adjust the P/C processes in response to changing technology and mission specifics, to monitor the processes in progress, and to adjust them in real-time to fit the actual returned samples *[Carr et al., 1999, pp. 7, 9]*. This Draft Protocol is being written more than 10 years before the nominal return of Mars samples to Earth. We do not know the spacecraft configuration, the types of martian samples that will be collected, their return configuration, and the exact nature of planetary protection measures. Similarly, we cannot anticipate all of the advances in instrumentation and analytical methods that are likely between now and the time of sample return.

It is likely that the returned samples will not be exactly as we imagine them now, and may include materials that are complex (e.g., breccias) or unusual (e.g., a possible stromatolite fossil). Treatment of these types of samples must be sample-specific, and cannot be defined in advance. Thus, there must be a mechanism such as an SRF oversight committee to adjust the final protocol to fit the actual samples.

Assumptions In preparing the P/C portion of the Draft Protocol, the mission profile and constraints outlined in the initial *Assumptions* of the Workshop Series *[see Appendix A]* were adopted. It is worth reiterating here a few of the key assumptions which hold particular relevance to physical chemical processing: the SRCs will be received at the SRF free of exterior contamination with Mars materials, intact, and with no breaches of containment (see page 96); the returned samples will include gas, fines material (bulk regolith), and solids; the total mass of all samples is expected to be ~ 500 to 1000 grams.

Overview of Physical/Chemical Processing Physical and chemical processing comprises the priority actions taken concerning the returned Mars samples between arrival of the SRC at the SRF, and initial examination for hazards and the LD/BH testing of fines and solids. These anticipated steps in P/C processing are shown schematically in Figure 3, which is based on portions of Figures 6-2 and 6-3 of *Carr et al. (1999),* Figure 2 on page 18 of *Race and Rummel (2000),* and the narrative of *Race et al. (2001a).* The numeric annotations in Figure 3 refer to similarly numbered sections of text below, which elaborate on the proposed P/C processing steps in narrative form.

P/C processing can be divided into three phases in roughly sequential order:

- Pre-processing, before preliminary examination of the samples;
- Preliminary examination and screening of gas, fines, and solids, to permit informed choices about samples for later detailed testing, banking, or curation; and,
- Sub-division of samples selected for Life Detection and Biohazard tests.

Following P/C processing, Life Detection and Biohazard testing will begin. Those processes may require information developed during preliminary examination and screening, and may also require subsequent and more detailed information of a physical or chemical nature; these additional analyses are not included here as they are contingent upon the results of the Life Detection and Biohazard testing.

The steps of preliminary examination and screening were judged to be different for three types of samples: gases, homogeneous particulate samples, and inherently inhomogeneous samples like rocks, rock cores, and regolith cores. Each of these sample types will follow a different track through preliminary examination and screening as described in the text below and shown on Figure 3 as the *'Gases Track,' 'Solids Track,' and 'Fines Track.'*

Pre-processing Samples

- *1.0 Pre-Processing Steps.* Pre-processing steps outlined here are those between arrival of the SRC at the SRF, and initial examination of gas, fines, and solids. Pre-processing steps refer to cleaning and decontaminating the exterior of any containers holding samples, as well as the initial steps in each of the gases-, fines-, and solids-tracks involving opening containers and removal of samples.

- *1.1 Clean and Decontaminate Exterior of SRC.* It is imperative that the exterior of any sample return containers or vessel(s) carry no terrestrial microbes, and are organically clean. (It is assumed that the exterior of the SRC is not contaminated with martian materials.)[10] If these states are not achieved, all subsequent analyses for life or biohazard are severely compromised. Actual methods of cleaning and decontamination are to be determined. An interesting new method to be considered is laser ablation of the SRC exterior.

Procedures for opening sample containers are mission specific as to number, types, and contents of containers. At a minimum, we assume that some solid materials with surrounding gas will be in the container(s). It is recommended that the gas be extracted for separate treatment, and that the solid samples be contained thereafter in an inert gas, such as dry nitrogen.

- *1.2 Extract Head Gas and Back-fill.* The returned solid samples will arrive on Earth with some gas surrounding them. Presumably, this “head gas” would consist originally of martian atmosphere. By the time of arrival on Earth, the gas might have been affected by chemical and physical reactions with the solids (rock and soil), by out-gassing from the solids (especially if the temperature rises above 25°C during return), and possibly by biological activity in the sample. This gas may contain information important to understanding the thermal, chemical, and biological histories of the solid returned samples. Therefore, extraction and analysis of the head gas is a high priority.

In this step of pre-processing, the head gas would be extracted from the SRC, and the SRC back-filled with a chemically unreactive gas to ambient “room” pressure. Exact procedures for extraction and back-filling will depend on the SRC design and construction, but might (for instance) include puncturing the SRC at an intentional thin point, extracting the head

10. It should be noted that planetary protection requirements will exist for a Mars Sample Return (MSR) Project to assure that the sample return container(s) is(are) intact and free of exterior contamination with Mars materials when delivered to the Sample Receiving Facility. Compliance with these requirements is the responsibility of the MSR Project Office and, therefore, not a function to be included in this protocol, which begins at the point of opening that clean and intact container.

gas to a pre-determined vacuum pressure, and refilling the SRC with dry clean N_2 gas. The extracted head gas would be processed as set forth below (see 2.0 – 2.2 *Gases Track*).

Three issues related to gases were identified for further consideration and possible research: 1) the effects of vacuum and non-martian gas on the chemical properties of the sample; 2) the effects of vacuum and non-martian gas on any live martian biota; and 3) the effects of extraction on gas isotope ratios.

For the first issue, experience with curation of the *Apollo* lunar samples has shown that few geochemical and other inorganic investigations are materially affected by holding and processing the samples in dry N_2 gas at 1 bar. Of course, the lunar samples originated at hard vacuum on the Moon. It is not clear what changes might be wrought on returned Mars samples (possibly containing clays or other hydrous materials) by first vacuum pumping, and then immersion in dry N_2 gas; further research is required in this area.

For the second issue, there is reason for the returned solid samples to be treated under an atmosphere as near to martian as possible, i.e., both to preserve key geochemical signatures *[Neal, 2000, p. 22492ff]*, and to maintain possible microorganisms in their native environment. It is unknown whether live martian organisms could be killed by removal of 0.006 bars of CO_2 and then immersion in 1 bar of N_2, and there may not be comparable terrestrial biota to test. Some samples eventually will be subjected to higher pressures merely because the biota of BH tests would not survive in martian atmosphere. On the other hand, there are serious problems in sample handling and geochemistry that would be caused by immersing the samples in a model martian atmosphere. Sample handling and LD/BH testing at reduced pressure (the near vacuum of 0.006 bars CO_2) present severe problems. Sample handling under vacuum was attempted during the *Apollo* program with lunar samples, and was found to be extremely difficult, expensive and contaminating (e.g., mercury or oil from vacuum pumps). Similarly, back-filling the sample container with a relatively reactive gas like CO_2 would change the isotopic nature of the sample. Terrestrial carbon and oxygen will exchange with the sample and compromise biological and geochemical inferences from these two stable isotope systems. This is an area of future research and discovery. One possible approach would be to backfill the SRC and perform sample handling and examination, where possible, under 1 bar of dry N_2 gas with 0.006 bars of CO_2 added. This might satisfy the constraints of easy sample handling, while being consistent with the desire to not kill live martian organisms, if any, and should be considered for the final protocol.

For the third issue, it is known that the elemental and isotopic ratios of a gas sample can be fractionated during transfer from one reservoir to another. With the head gas in contact with the abundant surface area of the returned samples, fractionation could become a serious potential problem.

Gases Track

- *2.0 Gases Track.* Gas withdrawn from the SRC, the "head gas," will be processed by filtering and subsequently split for Life Detection and Biohazard testing and would be available relatively rapidly for other investigations *[Race and Rummel, 2000, p. 17].*
- *2.1 Filter to <TBD Nanometers.* During or after removal of the head gas from the SRC, the gas should be filtered to remove particles *[Race and Rummel, 2000, p. 17]*. The purpose of filtering the head gas is to remove objects that could reasonably constitute viable organisms, or that might present biohazards. The size of objects passing the filter is to be determined. Sizes suggested by sub-groups in the Workshop Series have ranged from <0.5 µm *[Race et al., 2001a, p. 34]* to <0.02 µm *[Race et al., 2001b, p. 27],* both of which are realizable with current technology (currently, some methods are rated to remove particles larger than 0.003 µm). It is not clear if filtering could change the chemical or molecular composition of the head gas, for instance by preferential adsorption of heavy noble gases or by catalysis of reactions; this also requires additional research.
- *2.2 Distribute in Sealed Containers.* Filtered head gas should be released from the SRF and distributed in sealed containers. Unlike the returned solid samples (rock, regolith, etc.), a returned gas sample is only useful for investigation if it is contained. Typically, a gas sample like this would be placed in a glass bulb, which would then be sealed by melting the stem of the bulb. Containment at PPL-α or PPL-β levels is inherent in the

combination of filtration and this procedure. The filtered gas will be available for immediate allocation from the SRF without further processing or sterilization.[11]

Solids Track

- *3.0 Solids Track.* After removal and filtering of the SRC head gas, the remaining returned samples would be solids of various types, i.e., regolith samples, rocks, rock cores, soil cores, and fines. The specifics of this solid sample set are to be determined during mission design. These solid samples will be processed through two separate tracks, *Solids Track* (3.0) and *Fines Track* (4.0), for basic documentation, further preliminary testing, and selection for subsequent LD and BH tests.

  Some principles of this P/C process are worth restating here. The P/C process is a method to obtain the minimum data needed to characterize the samples adequately and to permit selection of suitable samples for LD/BH tests. The remaining samples will be preserved and made available for subsequent investigations and analyses. The samples will be changed as little as possible from their original state.

  The martian samples will only be touched by or come in contact with a limited set of materials under controlled temperature, pressure, humidity, and atmospheric conditions. Pristine lunar samples are touched only by stainless steel, aluminum, and Teflon™; these might also be suitable for returned Mars samples. Neal cites the considerations, from a geochemical perspective, for choices of materials for sample handling and suggests several types *[Neal, 2000].* Whether these materials are appropriate for returned martian samples should be determined through additional research with Mars simulants prior to sample return.

  The temperature of processing is TBD, and will depend in great part on technical mission constraints. The implicit assumption here has been that the temperature of processing will be between 0°C (273K) and ambient

11. To date, no decisions have been made about when and under what conditions sample materials will be eligible for release from containment at the SRF. Ultimately, it is likely that decisions about what is done with sample materials will be made after review by an appropriate international scientific oversight committee at the SRF in consultation with NASA's Planetary Protection Officer and other responsible officials.

(~298K), for which the protocols and experience with the *Apollo* samples are relevant. On the other hand, it will be important from geochemical and biological perspectives to maintain the returned sample at its ambient martian temperature, ~240K *[Carr et al., 1999; Neal, 2000].* This temperature may not be possible within mission constraints, and there appears to be no compelling reason to process at temperatures significantly below those experienced by the samples during their transit to Earth. It is not clear, at this point, what problems and attendant costs would be associated with sample curation and processing at sub-freezing temperatures.

It is suggested that an atmosphere of 1 bar of unreactive gas be used in processing, curation, and back-filling of the SRC. The steps outlined below assume that processing and curation will take place under 1 atmosphere of a pure unreactive gas (e.g., N_2). It is not known whether this gas would present problems for the LD and BH testing procedures. The composition and pressure of the atmosphere has implications for biological and geochemical testing, and is an area of concern (see sections 1.2, 5.0, and "Future Research"). It must be recognized that a requirement for processing at low pressure, like the atmosphere of the martian surface (0.006 atm), would have significant implications for the design and cost of a SRF.

- 3.*1 Open SRC and Remove Samples.* The SRC must be opened to retrieve and remove the solid samples. The procedures for opening the SRC and removing the samples are to be determined and will depend largely on the design of the SRC.

- *3.2 Preliminary Examination and Documentation.* As part of the P/C processing, *Preliminary Examination and Documentation* includes the minimal investigations deemed critical to an understanding of the nature of the returned sample, and to support initial biohazard investigations *[Race and Rummel, 2000, pp. 14, 17; Race et al., 2001a, p. 37].*

The first material-hazard investigation is a measurement of sample radioactivity. Some forms of ionizing radiation can penetrate the curation barriers between the returned sample and human processors. The purpose is not to measure abundances of indigenous radioisotopes (e.g., ^{238}U), nor cosmogenic radioactivities (e.g., ^{26}Al), but rather to determine whether radiation levels associated with the samples could pose a threat to workers at the SRF. Biohazard radioactivity can be measured on the bulk returned sample (safety level TBD), and need not be measured on individual samples unless the bulk presents a radiation biohazard. Only gamma radiation need be measured, as beta and alpha radiation will not penetrate the barriers between the returned samples and human processors. Based on prior experience with martian materials in meteorites, it is considered unlikely that returned martian samples will present a radiation safety hazard.

Imaging provides the first critical documentation of the returned sample *[Race and Rummel, 2000, p. 17].* Imaging at this stage serves multiple objectives: verification of mission success; correlation of specific samples with images of them taken on Mars and their sources; documentation of physical effects of transport to Earth (e.g., fracturing, disaggregation); preliminary identification of rock types; and measurement of sample volumes. It is anticipated that the returned samples would be imaged at a high spatial resolution (TBD, perhaps ~0.1 millimeter per pixel), over a range of perhaps seven to nine different wavelengths TBD, with at least three or four in the visible. These data will be critical to understanding the nature of the returned sample, and in processing and selection of samples for Life Detection and Biohazard tests.

The sample masses should be measured at this stage, and each time a sample is cleaned, split, or allocated. Measurement of mass is important as a mission requirement, for sample tracking and curation, and in allocating suitable samples for LD/BH testing. For instance, it is likely that a given mass of martian material would be returned to Earth as a mission requirements, and weighing at this stage will determine if that mission requirement has been fulfilled.

- *3.3 Separate Rock Fragments and Cores From Fines.* At this stage of processing, the solid samples would be separated into larger and smaller fragments. The larger samples would include drill cores, whole rocks, and rock fragments or rocklets (equivalent to the *Apollo* “coarse-fines”).[12] The

12. The terminology used to refer to small rocky materials has varied from workshop to workshop in this Series. The terms rock fragments, rocklets, and pebbles have been used to identify a general class of solid material that is distinct from fines, larger rocks, or rock cores. In addition to determining cut-off sizes at some later date, it will be necessary to use consistent terminology in all parts of the protocol.

smaller samples would include unconsolidated regolith, atmospheric dust, and dust generated by coring operations. This separation is necessary because the larger fragments cannot be treated as homogeneous powders, and must be examined individually for Life Detection and Biohazard analyses. It is possible that the regolith samples will include small rocks and rocklets, comparable to the case with the lunar regolith samples returned by the *Apollo* missions. As with *Apollo*, the small rocks and rocklets would be separated from the finer material, cataloged, and curated individually throughout subsequent processing and analyses. The cut-off size for rock fragments or rocklets remains to be determined. The standard cut-off size in the soil science community is greater than 2 millimeters. Sub-groups in the Workshop Series have suggested sizes ranging from greater than 1 millimeter to greater than 2 millimeters, and even "… greater than several millimeters …" for martian samples *[Race et al., 2001a, p. 34; Race and Rummel, 2000, p. 17].* Decisions about cut-off sizes for different classes of solid materials will be made when the sample is returned and first examined, based on a recommendation of the SRF Oversight Committee (see *Personnel Management Considerations* later in this document).

Given the dusty nature of the martian surface, and the likelihood of dust generated during coring, it is anticipated that the surfaces of cores and rock samples will be coated with fine-grained materials. After separation, preliminary examination, and documentation of the returned solid materials, it will be necessary to remove dust from surfaces of the cores, rocks, and rocklets *[Race et al., 2001b, p. 22].* These fine materials constitute distinct samples of martian material, and will require different processing and curation than the solids (i.e., the fines track). In addition, the fine materials on solids likely will hinder identification and processing of the latter by obscuring their surfaces. Selection of samples for Life Detection and Biohazard assays will require knowledge of the mineralogy, structure, and textures of the samples. The analytical probes available (primarily visual and near-infrared optics) will be unable to operate effectively on dust-covered samples.

The exact methods of fines removal are TBD. Suggested methods have included vacuuming the samples, blowing the dust off, a combination of vacuuming and blowing, and laser desorption. In all these cases, thought needs to be given to how the fines will be collected after removal. The fines collected from each solid sample would be identified individually, and treated as a separate fines sample within the *Fines Track,* as described in section 4.0 below.

- *3.4 Sort to Groups.* After removal of adhering fines, the solid samples should be sorted into groups of similar materials using visual clues and information from *Preliminary Examination* data *[Race and Rummel, 2000, p. 17; Race et al., 2001a].* This step assumes that the returned sample will contain several cores and/or multiple millimeter-sized rock fragments ("rocklets"). Criteria for sorting would include size, rock type (including color), grain size, texture, and other readily observable properties. This sorting is an important first step towards selecting representative samples for Life Detection and Biohazard tests *[Race et al., 2001a, p. 26]*.
- *3.5 Pristine Bank.* Samples and sub-samples that are not chosen at this point for *Further Screening* and/or for Life Detection and Biohazard tests will be stored in a *Pristine Sample Bank [Race and Rummel, 2000, p. 17]*. This "bank" will serve as a containment system designed to maintain the physical/ chemical, and biological integrity of samples while they await allocation for other analyses at a later date. According to recommendations by the Curation and Analysis Planning Team for Extraterrestrial Materials (CAPTEM), the "bank" should hold the samples under an inert atmosphere at temperatures below 240K *[Neal, 2000].* The pristine solid samples are those that have been affected by no procedures beyond those of preliminary examination, dust removal, and sorting. The pristine bank will serve the critical purpose of preserving a portion of the returned sample for analyses beyond and after the Life Detection and Biohazard assays associated with planetary protection. The pristine bank samples will become the principal resource for all subsequent chemical, geological, physical, and biological analyses on the returned samples.
- *3.6 Further Screening.* At this point, sub-samples of each rock type group sorted previously (see section 3.4 above) would be subjected to additional analyses in support of (and preliminary to) Life Detection and Biohazard tests *[Race and Rummel, 2000, p. 14; Race et al., 2001a, p. 37].* The exact analyses needed are to be determined in conjunction with the detailed

LD/BH tests (see *Future Research*, below). Whenever possible, selected analyses should emphasize non-destructive methods that are not likely to modify or destroy biological molecules or biohazards, and would not be anticipated to kill or weaken live martian organisms. Once the tests are defined, it will be possible to learn what characteristics of the returned samples might affect or interfere with particular tests, and what data are essential prior to the tests. With this information in hand, the *Further Screening* analyses can be tailored to meet the requirements of life and biohazard detection. Given these restrictions and uncertainties, the following screening methods have been suggested:

- ➢ Multi-spectral imagery of the samples in visible, near-infrared, and/or thermal infrared light can provide identification of the minerals (inorganic chemical compounds) and the presence and distributions of organic matter and water (molecular and bound) in the sample. Raman spectroscopy should be considered here, also, with the caveat that samples can experience significant heating during Raman analysis. For instance, 514.5 nanometer green light from an argon laser is absorbed significantly more than 1064 nanometer infrared light from a Nd:YAG laser. Heating can also be mitigated by distribution of laser power in space and time over the sample. The distributions of minerals on the samples' surfaces will be crucial clues to understanding their internal structures. X-ray diffraction analysis would also be valuable in defining the minerals in the samples (see *Race et al., 2001a*, p. 35ff*,* for more detail on these methods.)
- ➢ It is important to know the internal structures of the samples (especially the larger ones), because biogenic material could reasonably be concentrated in cracks and open spaces (analogous to terrestrial endolithic organisms). Building on the multi-spectral imagery, tomographic analyses could provide three-dimensional visualizations of the internal structures of the samples. Among tomographic methods, the most developed at present is X-ray tomography. To provide X-ray tomographic maps of density (i.e., continuum absorption of X-rays) now requires only a bench-top instrument. X-ray tomographic maps for individual elements like carbon require at present the X-ray intensity of a

synchrotron light source, and is considered impractical for this *Further Screening* step.

- ➢ Abundances and distributions of major elements and several minor elements will likely be important for sample selection in Life Detection and Biohazard analyses. It is also possible that abundances of certain elements could produce false positives or negatives on Life Detection and Biohazard tests. A likely method for elemental analysis is X-ray fluorescence, a mature technique used routinely in inorganic geochemistry and studies of human bone composition.
- ➢ It would be very useful at this stage to have bulk analyses for carbon as a guide to sample selection. However, a non-destructive test for bulk carbon that is sufficiently precise, and has low enough detection limits to be useful here, has not been identified; this requires future research.

- *3.7 Selection of Sub-samples.* Representative sub-samples will be selected for Life Detection and Biohazard tests based on data from the *Further Screening* tests (see section 3.6). The remaining unselected samples will be stored in the *Returned Sample Bank* (see section 3.8) for future research access. Additional research will be required to define representative sample and sub-sample criteria for all martian materials in light of a potential for extreme heterogeneity of rock and soil samples, and a concomitant likelihood that putative biohazards may be limited in terms of location. Selected samples will carry forward to the actual Life Detection and Biohazard investigations (see section 5.0).
- *3.8 Returned Sample Bank.* The *Returned Sample Bank,* distinct from the *Pristine Sample Bank* (see section 3.5), is for storage of samples that have experienced the analysis of *Further Screening,* but have not yet been allocated for Life Detection and Biohazard tests. These returned samples should be labeled and kept distinct from the pristine samples, as the former have had more chance for contamination than the latter.

<u>Fines Track</u>

- *4.0 Fines Track.* Fines samples are those with particle sizes smaller than some limit TBD; the size limit suggested in the MSHP Workshop Series was 1 or 2 millimeters *[Race and Rummel, 2000; Race et al., 2001a,* *2001b]*. In either case, it is anticipated that fines samples will contain so many grains, mixed homogeneously, that it will be readily possible to take representative splits for Life Detection and Biohazard tests. Fines samples may include materials from a variety of sources: material collected as such, like dust from a wind-deposited dune; regolith that has had coarser material removed (see section 3.3); dust filtered out of the SRC headspace gas (see section 2.1); or particulates removed from surfaces of rocks or cores (see section 3.3).

- *4.1 Characterization.* Characterization of fines samples would be limited to imagery of each bulk fines sample (possibly including multi-spectral imagery) and weighing of each bulk sample *[Race et al., 2001a, p. 35].* There is no need to image or otherwise characterize each individual particle within a bulk fines sample. Only these minimal analyses are needed to document each fine sample at this stage in order to select samples or representative sub-samples for Life Detection and Biohazard assays. Each fines sample may be subdivided into fragments larger and smaller than 1 millimeter *[Race and Rummel, 2000],* but the desirability of this further splitting is an area requiring additional research.

- *4.2 Split for LD/BH Tests and Banking.* At this point in P/C processing, fines samples would be selected for Life Detection and Biohazard tests, and split into representative aliquots. Some aliquots would be carried forward to Life Detection and Biohazard tests (see section 5.3), and some would be reserved in the *'Pristine Sample Bank'* (see section 3.5). Since additional chemical analyses will be included as part of the LD/BH testing, no separate elemental analyses will be conducted on fines at this point in the P/C processing.

  The methods for splitting the fines samples are TBD. Methods used in typical terrestrial applications (e.g., riffle splitter, or coning-and-quartering),[13] may not be appropriate or practical here *[Race et al., 2001a, p. 14].* First, these methods will involve considerable contact between and among the sample, tools, and surfaces, and may be deemed too contaminating.

13. A riffle splitter is a mechanical separation device that is able to split an unconsolidated soil sample into two equal parts that have the same grain size distribution (and presumably composition) as the parent sample. Coning-and-quartering is another commonly-used separation method (as described in *Maxwell 1968*).

Second, both methods have the potential for considerable loss of sample through embedding in metal surfaces or electrostatic adhesion to metal and plastic surfaces. The electrostatic adhesion problem will be exacerbated in the dry atmosphere of the PPL-α spaces, as has been found with curation of lunar samples. In fact, neither method is now used for splitting lunar fines samples. This clearly is another area of required research.

In this Draft Protocol, it is assumed that a sub-sample of fines is representative, based on confirmation of an adequate splitting method. However, it is suggested initially *[Race et al., 2001, p. 14]* that each sample of fines be split into multiple sub-samples, each of which should be analyzed for bulk composition and mineralogy (as under *Further Screening*, see section 3.6) to determine whether splits are homogeneous. Further consideration of this issue is needed.

Preparation for Life Detection and Biohazard Testing

- *5.0 Samples for Life Detection and Biohazard Testing.* At this point, samples have been selected for LD/BH tests as well as other P/C analyses.
- *5.1 Split into Representative Sub-samples for LD/BH.* The samples selected for LD/BH tests will be split into representative sub-samples at this point. This splitting is necessary to ensure that analyses are performed on similar materials, and so that the results of one test may be reasonably correlated with the results of another. Splits chosen for immediate analysis will proceed to various LD/BH tests (see section 5.3 below). Some splits will be held in reserve as part of the *Return Sample Bank* as described in section 5.2. below.
- *5.2 Reserve.* Some splits from section 5.1 will be held in reserve for LD/BH tests, in anticipation of future needs. Should a test fail or require repetition, this reserve material would be available. These reserve splits could reasonably be kept in the *'Return Sample Bank,'* but labeled accordingly.
- *5.3 Parallelism of Tasks.* It is beyond the scope of the P/C procedure to describe the actual operation of LD/BH analyses and supporting inorganic analyses. However, they are included on Figure 3 for completeness. It is anticipated that these three types of tests will be run in parallel, with the

results of each influencing the interpretation and course of the other tests *[Carr et al., 1999, p. 9]*.

<u>*Future P/C Research and Development Needs*</u> In the discussions of P/C processing of the returned martian samples, several areas were identified where data were not available or could readily be obtained without additional research. Each research suggestion discussed below is keyed to the particular numbered text section above, where it is called out:

- Exactly what analyses and data do the LD/BH analyses require from the P/C processing? (see sections 3.2, 3.6, and 4.1). The P/C process here reflects informed judgment about which analyses would be most useful in LD/BH studies, but it will be very important to know what information about sample characteristics, or about the particular P/C processing, will be useful when assessing LD/BH results (for example, to determine possible causes of false positives or negatives; to document abundances of specific elements of interest (e.g., arsenic) or minerals (e.g., saponite clay); or to characterize surface reactivity and constituents (e.g., super-oxidants, etc.).
- In implementing the final protocol, there must be close collaboration between biohazard, toxicology, and pathology disciplines on the one hand, and chemistry, biochemistry, geochemistry, physics, and geophysics, on the other, to coordinate a truly integrated testing outcome, pursuant to augmenting which physical sciences data should be ruled in or ruled out in ultimate interpretations of sub-sample biohazard and/or toxicity testing.
- Trial-testing initiatives should be developed before the protocol is fully implemented in a sample return mission. These trials should be refinements that take into account the prospective chemical and physical properties of martian soil and rock(s) (and/or use martian surrogates where applicable), as well as evaluate biohazard containment facility needs.
- Is there added value in separating each fines sample into grain size separates *[Race and Rummel, 2000, p. 17]*? What additional contamination might be introduced by this procedure? (see section 4.2)

- How can one remove terrestrial contaminants (including organics) from the exterior of the SRC before it enters PPL-α space? Laser ablation surfacing was suggested and should be studied (see section 1.1).
- How can one effectively remove and collect dust and other fines from the surfaces of rocks and rock cores? (see section 3.3) Three suggestions were vacuuming, blowing with compressed gas, and laser desorption.
- What effects do X-rays have on biological structures and molecules? Several analytical methods involve interaction of X-rays with the samples (e.g., XRD, XRF, XR tomography), and it is not known whether these X-ray doses interacting with Mars samples would affect LD/BH analyses (see section 3.6).
- How can one analyze a bulk sample for trace or ultra-trace quantities of carbon, non-destructively and without anticipated deleterious effects on biological molecules or viable organisms? (see section 3.6)
- Is the chemical composition of the head gas affected by filtration to remove small particles? (see section 2.1)
- What chemical and physical effects would removal of head gas and replacement with dry nitrogen have on the returned martian samples? (see section 1.2)
- What chemical effects would removal of head gas from the returned sample canister have on the gas itself? (see section 1.2)
- What effects would removal of head gas and replacement with dry nitrogen have on live martian and any contaminating terrestrial organisms in the returned martian samples? Would these effects be mitigated if samples were curated under dry nitrogen with 0.006 bars of CO_2 gas? (see section 1.2)
- What effects would gas with terrestrial carbon and oxygen isotope ratios have on live martian organism in the returned martian sample? Would live martian organisms ingest the terrestrial carbon and oxygen, and become isotopically indistinguishable from terrestrial organisms? (see section 1.2)
- How can one produce representative splits of martian dust and fines materials without unacceptable contamination or loss of sample? (see section 4.2)

- How can one confirm that splits of dust or fines material are representative before Life Detection and Biohazard analyses, or is such confirmation necessary? (see section 4.2)
- What are the overall requirements and statistical test methods necessary to ensure that a representative sub-sample of rock and soil material is available for further LD and BH testing?
- Using artificially constructed Mars simulants, determine whether materials and conditions recommended by CAPTEM *[Neal, 2000]* are appropriate for handling martian samples. (see sections 3.0 and 4.0)
- Petrographic thin sections are enormously valuable in characterizing the minerals, structures, textures and history of a rock. Can petrographic thin sections be produced in a manner consistent with the principles of minimal sample use and minimal contamination of the section material and the remaining sample? (see section 5.3)

Areas of Concern Several areas of serious or general concern have been raised during discussions of physical and chemical processing. These issues, listed below, are significant enough to affect mission design, and SRC and SRF design.

- The validity and significance of Life Detection and Biohazard procedures in the SRF are strongly dependent on sample collection procedures on Mars, and thus on spacecraft and mission design. How can the Life Detection and Biohazard teams influence the designs of sample return spacecraft and sample collection procedures?
- What if the return sample container is breached or its seal is compromised? What contingency plans are possible to achieve PPL-α containment and biosafety? (see *Assumptions*, Appendix A)
- Is measurement of sample mass important as a preliminary characterization step? Should it be deferred until the “Further Screening” step? (see sections 3.2 and 3.6)
- How is the head gas to be removed from the SRC without contamination? Is backfill with non-reactive gas justifiable in terms of possible effects on martian biology? Would it be adequate or preferable to backfill with 6 mbar of terrestrial CO_2 and the remainder a non-reactive gas? (see section 1.2)

- What should be done if a unique critical sample is smaller than the nominal requirements for LD/BH analyses? (see section 3.4)
- What should be done if the requirements for LD/BH testing evolve to consume an inordinate quantity of returned sample, to preclude other biological, organic, and inorganic tests that further NASA's other goals? (see section 5.0)
- Study the effects of sterilization measures that could have significant adverse effects on biochemical analyses outside of PPL containment *[Race and Rummel, 2000]*.

Life Detection Testing

Introduction The proposed Life Detection (LD) analyses are intended to detect specific evidence whether life of any kind exists in the sample, or rule out the presence of such evidence of life.[14] These analyses will use a broad definition of and criteria for life, and an approach for detecting life, not intended to be limited by the specific features of life as we know it on Earth. This approach will begin with, and rely on, 'signatures' of various types that encompass all known terrestrial life, and that might encompass non-terrestrial life. These signatures structures, structural and biosynthetic chemistry, isotopic patterns, and geochemical features that help define the underlying principles of life (see *Biosignatures,* page 45). The LD tests will take advantage of, but will not be constrained by, knowledge of the structural and metabolic intricacies of terrestrial life. In particular, the recent recognition of our limited ability to cultivate terrestrial microbial life[15] emphasizes the importance of relying on methods beyond *in vitro* cultivation for detecting extraterrestrial life. Life is likely to be catalytic and carbon-based. The most parsimonious scenarios for the existence of extraterrestrial life posit the presence of a prebiotic mix similar to that which existed on the early Earth. The similarity of Mars to Earth in this regard is anticipated under current models of solar system

14. The final reports from each Workshop contain detailed documentation of the discussions which occurred at those Workshops *[Race and Rummel, 2000; Race et al., 2001a, 2001b, and 2002].*

15. At the time of this writing, only about 1% of known microbes can be readily cultured.

formation. Evolutionary paths different from those that occurred on Earth may have led to the generation of slightly different building blocks and polymers. The LD methods should be potentially capable of recognizing the products of these variant paths, and be capable of recognizing the various known forms of life on Earth.

An overall strategy for LD is illustrated in Figure 4, showing the expected flow of materials into the various testing queues to be established for the protocol. This strategy, originally developed in the first Workshop of the Series *[Race and Rummel, 2000],* was refined and elaborated upon in the subsequent Workshops *[Race et al., 2001a; 2001b; and 2002].*

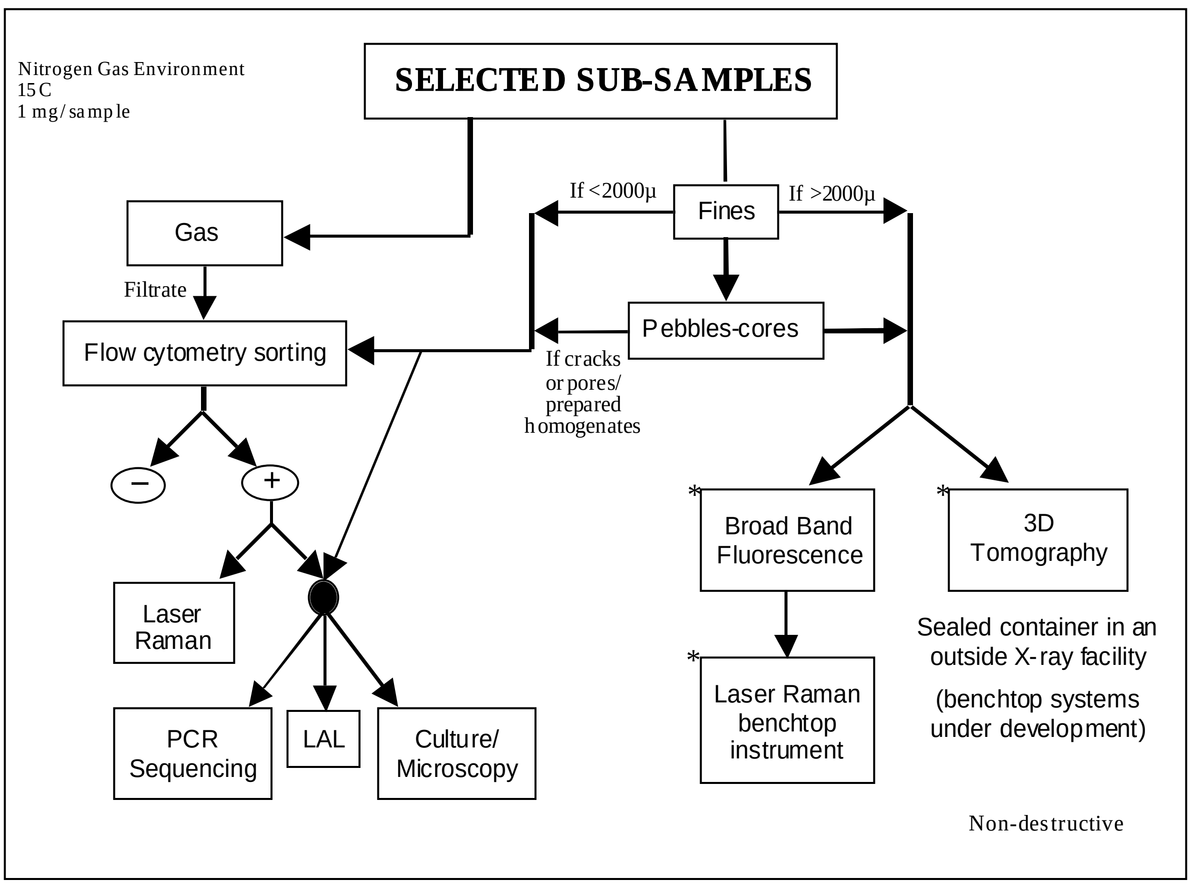

Figure 4. Life Detection Process Flowchart.

Table 3 lists what could be considered ‘universal’ properties of life. Many of these properties are directly measurable, although some of them, such as replication or evolution, can, in all likelihood, only be inferred. Evidence for only a subset of these properties in an extraterrestrial specimen might constitute a sign of life (e.g., evidence for a self-sustaining catalytic system). However, it is the presence and combination of all of these properties that define life as we know it.

- Life is catalytic
  - + There should be significant deviations from what chemical kinetics predicts
  - + Life modifies its environment
  - + Life consumes energy
  - + Life creates waste products
  - + Life is exothermic
  - + Life uses thermodynamic disequilibria to build and maintain other thermodynamic disequilibria (in open systems or within a “wall”)
- Life is genetic
  - + There will be some system for storing and propagating information
  - + There will be molecular distributions with significant capacity for complexity
- Life replicates and evolves
  - + There will be evidence for replication of structures and complexity
  - + There may be evidence (structural & chemical) of evolution of form& function

Table 3: Universal properties of life, as we know it.

LD Principles General principles to follow in searching for life or biosignatures (i.e., signs of life) are shown in Table 4 on the next page. These principles guide the search from the selection of samples to be tested through the application of analytical methods, as shown above in Figure 4. Analytical methods can be divided into those that facilitate a wide survey of a representative portion of different sample types, and those that facilitate a more focussed, but high-resolution, examination of areas of interest. Survey methods are less destructive of samples, and include microscopy, broad band fluorescence, surface scanning and chemistry, tomography, and isotope release experiments. These methods seek structural and basic chemical signatures, and local inhomogeneities. Higher resolution methods are generally more destructive, and include mass spectroscopic methods, combustion, isotope analysis, and electron microprobe procedures for elemental mapping. These methods seek to characterize inhomogeneities and more complex structures, and are discussed below in further detail (see *Sample and Time Requirements*, page 53).[16]

- Begin with a broad survey of a portion of different sample types for more general features suggestive of life, then turn to a higher resolution examination of sites with suggestive features for a more complete characterization
- Emphasize structural signatures of life and other inhomogeneities that can be easily detected as a first order task
- Emphasize less destructive methods in the early stages of investigation, since they can guide the use of more definitive but destructive methods
- Start with samples least likely to contain life (e.g., surface fines); if negative, use these as blanks and controls for spiking experiments
- Recognition of life will require the coincidence of multiple independent signatures
- Inactive or “past” life will be treated as potentially active life
- Generalize a carbon-centered methodology to other chemical species
- Use an iterative approach for the Life Detection protocol
- Invest significant time in the design of controls and blanks, as early in protocol development as possible.

Table 4: General principles guiding the search for life.

One factor that may complicate the Life Detection efforts is the difficulty in detecting or interpreting many of these signatures if the life-forms are inactive, or have been for long periods of time (e.g., hibernation or quiescence), or have become fossilized. One of the large challenges in Life Detection is a more complete understanding of the stability of various biosignatures over time and their dependence on continued metabolic activity. Attempts to induce activity and replication are also posited as a means of amplifying potentially detectable

16. An estimate of the amount of sample required for the survey/less-destructive methods is 200 milligrams, and 3 grams total for all tests (see page 53).

biosignatures. Some indicators, either structural and/or chemical, which may indicate “past” or inactive life should be treated as potential indicators of active life.

One potentially useful strategy for detecting active life-forms is based on replicate measurements over time. Repeated analyses for any of the biosignatures described above may reveal changes in the sample due to metabolic activity. The search for significant changes in these signatures offers an important potential source of information, and does not require a thorough understanding of the signature. The probability of life based on a chemical species other than carbon is low, but cannot be eliminated. With this in mind, carbon centered methodologies and approaches which dominate our present thinking need to be generalized to other chemical species whenever possible. An iterative general approach is recommended for the Life Detection tests, with results obtained by one method or analysis being used to specify and direct any subsequent use of such methods or analyses.

There are three possible outcomes of the Life Detection procedures:

1. *Failure to detect any of the biosignatures described above, and absence of any carbon or complex carbon in representative samples.* This result would lead to proposals for downgrading of the containment level for controlled distribution.
2. *Clear and overwhelming evidence of living organisms that appear to be of non-terrestrial origin (for example, evidence of motile structures with no DNA or RNA present).* This finding could result in the continued containment of all unsterilized samples for an indefinite period of time—until the living organisms are better understood. Biological experimentation and biohazard assessment would be given highest priority. It must be emphasized that the most likely source of life detected in the martian specimens is expected to be terrestrial contamination (introduced just prior to, or following the spaceflight portion of the mission).
3. *The third and most likely scenario lies between these extremes, where clear evidence of life or its absence is not forthcoming.* An example would be a

situation in which complex carbon-containing compounds are detected in the sample, but without other evidence of life or biosignatures.

Extraction of Representative Sample It is anticipated that sample material will differ widely in size and composition. For discussion purposes, a representative aliquot of approximately 1 gram would be subjected to extraction for further destructive tests. This initial extract will be made using ultra-clean water. Mechanical disruption may be necessary, but should be kept to a minimum so as not to damage cellular structures or potentially viable cells. A fraction of this aqueous slurry should be designated for organic solvent extraction. Obviously, future planning on the extraction of a representative sample will be dependent on mission capabilities and sampling equipment employed.

Biosignatures The signatures and signs of life that are the principal targets of LD testing may be defined through different prisms, perspectives, and methods. Broadly-defined signatures offer the greatest opportunities for detecting life that is unfamiliar to us in its detail; however, broad signatures also carry the greatest chance for misleading or false-positive findings. In general, the greater the number of independently-defined signatures that are detected, and the greater the spatial co-localization of these signatures, the stronger the evidence for life. As a simple example, self-sustaining catalytic processes should create a localized overabundance of a discrete set of related compounds. Useful biosignatures may exist in a variety of types:

- *Morphological.* As we know them, all forms of life are defined by a boundary (e.g., a wall) that delineates them from the surrounding environment. This “spatial-physical incongruity” often contains patterns, complexity and recognizable features (e.g., size, shape, structure, morphological indicators of replication or specialized features such as attachment and motility structures, septae, etc.).
- *Structural Chemistry.* Life can be defined by basic chemical features, such as organic or complex carbon, or by higher-order features, such as polymers, membranes, and attachment and motility structures. Methods

need to be improved for characterization of complex polymers and criteria developed for interpreting the patterns associated with complex carbon. We are even less well-informed about the possible structural complexity that can be incorporated into silica and silica-carbon polymers.

- *Metabolism and Bioenergetics.* The waste products that are released and the energy expended by all forms of life as we know them can be detected with physical and chemical methods. Some products are created through specific enzyme catalyzed reactions, such as the reduction of nitrogen that can occur from inorganic reactions. Other products are predicted to result from reactions in the absence of protein-enzymes, such as those involved in energy and CO_2 reduction. More work is needed to assess the range of metabolic mechanisms and products that occur on Earth, as well as theoretical studies of those that might occur in the absence of carbon.
- *Biosynthetic Mechanisms.* All life has mechanisms to synthesize structural, metabolic and replicative macromolecules. Carbon-based life on Earth uses protein-enzymes and, to a limited extent, ribozymes (catalytic RNA). The synthesis of macromolecules involves a sequence of reactions that depends on the availability of basic organic components, such as amino acids for protein synthesis. Such synthetic mechanisms should provide detectable biosignatures, if they are present. In taking a broader view, we must consider the possibility of biosynthetic mechanisms and pathways catalyzed by inorganic metals and minerals in non-protein matrices, or that are dependent on physical gradients (temperature, pH, Eh, magnetism), catalytic mineral surfaces, or various energy sources (UV and other forms of radiation and light). Such mechanisms may exist, but their detection may be as a consequence of first detecting other signatures of life.
- *Isotopic Signatures.* All forms of life with which we are familiar fractionate various elements; thus, fractionation patterns can be indicative of life. Organisms that express different metabolic capabilities display distinctive patterns in the fractionation of carbon, nitrogen and sulfur. This might be particularly important in assessing the possible origins of organic compounds and various volatiles such as methane, carbon dioxide, and carbon monoxide, if detected on Mars. While one cannot assume that extraterrestrial life will fractionate elements in the same manner as terrestrial life, it is reasonable to assume that local patterns of fractionation

within or at sites of life-forms in the sample will vary from those measured in the surrounding sample environment. Some isotopes, such as those for oxygen (detected in carbon dioxide and phosphate), can be indicators of environmental temperature. There is promising new technology for measuring carbon isotope fractionation patterns in single organic molecules and fractionation patterns in transition metals. The latter may be very important in identifying a biological source for various minerals such as magnetite.

- *Geochemical Signatures.* This family of signatures includes findings such as magnetite, and other minerals out of equilibrium with their normal distribution in the environment. Redfield-like ratios[17] of key elements (e.g., C, H, N, O, P, and S) are found in the pigments of terrestrial life, such as those known to be associated with photosynthesis, and other inorganic chemical anomalies (e.g., based on iron, sulfur, etc.). When specific biologically important elements are limited in the environment, there will be higher concentrations associated with life-forms or colonies of life-forms. Usually, the limiting element in the environment will limit the extent of growth and productivity of organisms (known as Liebig's Law of the Minimum). Some key elements that are limited in terrestrial environments include iron and molybdenum (essential for nitrogen cycle reactions), and tungsten (essential for specific enzymes in hyperthermophilic archaea).

Analytical Methods Because deep and surface mineral particles are common micro-environments for microbial life on Earth, the chemical analysis of Mars samples at a micrometer scale can yield information about the presence of active or fossil life on Mars. Raman, IR, and fluorescence micro-spectroscopy are valuable tools to perform non-destructive analysis of mineral matrices and surface compounds.

- *Microscopy.* As part of the preliminary examination of returned samples, light microscopy of fines as well as surfaces of pebbles or rock should be used to look for obvious signs of cellular structure and mineral deposits associated with microbial life.

17. The ‘Redfield Ratio’ describes the ratio of carbon to nitrogen to phosphorous (C:N:P) found in marine organisms.

- *Analysis of Gases in Head Space.* One potentially important analysis for Life Detection would be to compare a pristine atmospheric sample from Mars to the gas occupying the head space above collected soil and rock samples. If a pristine sample is available, the comparison may yield differences that could be due to chemical interaction of the gas with samples, or that may be signs of metabolic activity within the specimens.[18]
- *Laser Desorption Mass Spectroscopy and Laser Raman.* Laser desorption mass spectroscopy (LD/MS) is a rapid, non-destructive method for detecting low levels of organic matter in geological specimens. It has been successfully used to analyze PAHs in meteorites and interplanetary dust particles. Minimal sample preparation is required, and small particles as well as fresh fracture surfaces of larger specimens can be analyzed. In LD/MS, a 10-40 micron diameter spot is positioned on the specimen, organic species are thermally desorbed from the outer few microns of the specimen, they are photo-ionized and directed into a time-of-flight mass spectrometer. Continuing developments offer the prospect of high selectivity in detection of specific classes of organic compounds, (e.g., amino acids). Additionally, recent studies suggest that for organic compound detection UV-Raman spectroscopy (especially deep UV Raman, ~224 nanometers) may be 5-7 orders of magnitude more sensitive than longer-wavelength Raman spectroscopy, and can use a smaller focused light source that is less sensitive to rough surfaces. At UV wavelengths, the mineral fluorescence disappears and the signal, even when small, has little or no noise attached from that source. Automated scanning technology will be critical for application of these techniques to the maximum amount of sample. These techniques are limited to surface analysis.
- *3D Tomography.* Given the present state of the art, 3D tomography would require transport of a specimen outside of maximum containment facilities to a synchotron; however, the specimen can remain in a sealed container, under the equivalent of PPL-α containment conditions. The availability of an appropriately qualified synchrotron facility capable of applying this method to detect specific elements within a sample would be of great interest in the

18. Although not a requirement of the protocol *per se*, the desirability of this analysis suggests the importance of collecting separate gas-only samples from the sample collection sites on Mars.

preliminary examination of rock samples that might have heterogeneous interior structures.

- *Carbon Analysis.* High priority should be given to quantitative analysis of carbon, especially organic carbon. Techniques having the greatest sensitivity should be applied, including progressive heating/oxidation, coupled to GC/MS. It is anticipated that multiple samples and sites with suspicious findings from survey methods will be analyzed to detect and characterize localized organic or inorganic carbon.
- *Flow Cytometry.* An aliquot of the aqueous slurry will be subjected to flow cytometry. Flow cytometry will be used to analyze single particles in the range of 2 to 100 microns in diameter, at rates of tens to hundreds of thousands of particles per second. Based on initial, non-destructive characterization of laser light scatter and auto-fluorescence, particles will be re-analyzed, with or without staining with fluorochromes specific for DNA, proteins or functional viability assays. During subsequent analysis, at least four pre-selected sub-populations can be sorted from each sample for further analysis by other techniques. Positive fractions can be sorted and directed toward further chemical and biochemical testing.

Cultivation Elaborate forward-contamination controls will be used on the mission, but it is still possible that viable terrestrial microbes may be detected in returned Mars samples (either from contamination on the original spacecraft, the sample container that made a round-trip, or through sample handling contamination). To rule out possible terrestrial microbial contamination, an aliquot of the sample should be subjected to the standard microbiological examinations currently used for planetary protection, as well as other routine methods for detecting and identifying terrestrial organisms.

In addition to the procedures used to identify any terrestrial contamination, culture attempts should be made that represent Mars-like conditions. Culture conditions that would be compatible with martian micro-environments are not well-understood and the likelihood of success is small (only about 1% of Earth organisms can readily be cultured), yet attempts should be made to create such conditions and propagate life-forms. The composition of gases in the martian atmosphere, including plausible ancient atmospheres, should be replicated, especially with CO_2 as a carbon source. Given the current extremely dry conditions on Mars, the degree of sample hydration should be varied. The range may fluctuate from partially hydrated specimens to totally aqueous conditions. Energy sources should include light for any possible photosynthetic organisms and pairs of electron donors and acceptors for chemosynthetic organisms. Mineralogical information from samples should be integrated into the decisions in media formulations. Likewise, any organic compounds detected in the samples should be considered as carbon sources for possible microbial growth. Cultures will be monitored by simple microscopy as well as through multiple sequential analyses by GC/MS, LC/MS, micro-calorimetry, nucleic acid amplification, and other methods.

Distinguishing Earth-based from Mars-based Life If viable cells are found in the samples, and especially in cultures taken from samples, it will be important to address the possibility (even likelihood) of terrestrial microbial contamination. Detected cells will be subjected to phenotypic and genotypic analyses, with sequence searches against databases containing large numbers of known terrestrial organisms to quickly identify contaminants (though it is important to remember that only a small percentage of Earth microbes are currently known). Because of the harsh conditions on Mars and the relatively small amount of sample to be returned, the most likely source for familiar complex polymers such as nucleic acids is from terrestrial contamination. Amplification techniques such as the polymerase chain reaction (with broad range primers directed against targets such as rDNA, and with random oligomers) and subsequent sequencing methods offer a sensitive and rapid means for detecting and characterizing DNA and RNA (as a marker for terrestrial contamination), and should be applied to the outbound spacecraft and container surfaces before and after return, as well as to the samples themselves. Other assays, such as the *Limulus* Amoebocyte Lysate

(LAL) assay, may assist in detecting extremely small amounts of terrestrial contamination, but are less specific.

It must also be kept in mind that detection of terrestrial contamination in a specimen does not exclude the possibility that the same specimen also contains martian life. The presence of terrestrial contamination could compromise the detection of potential martian life in a number of ways—e.g., if martian life is closely related to Earth life, or if the “noise” of terrestrial contamination drowns out the “signal” of Mars life; this is a key reason for requirements to be imposed on the sample collection mission that will restrict the transfer of terrestrial contamination to the sample and/or sample container.

Considerations Concerning Controls and Blanks

- Prior to departure, the spacecraft and specimen containers should be examined, and samples should be archived; witness plates[19] should be employed.
- Strong consideration should be given to the return of a sample of martian atmosphere in a separate, but identical container. If collected and stored under increased pressure, extra aliquots of atmosphere could be used for replication of martian conditions in other experiments after specimen return.
- Early determination of negative findings for life in low-likelihood martian samples may allow these samples to be used as negative controls.
- Because negative results are expected in many of the Life Detection procedures, determinations of assay sensitivity using known specimens of terrestrial life would aid in the interpretation of these negative results.
- Methods should be validated and evaluated using a wide variety of terrestrial life-forms.
- Simulants of martian samples and conditions should be refined for protocol development prior to sample return. Particular attention should be given to the probability of highly-oxidizing sample surfaces.

19. ‘Witness plates’ are controls for forward contamination, used to monitor the bioload on a spacecraft before launch.

- Exposure of the sample surface to PPL-α conditions will inevitably lead to deposition of particulate matter from the surrounding enclosure. The features of this process should be characterized prior to specimen return.
- Questions that yield answers for which a statistical assessment of confidence can be performed should be identified. Principles to be applied in order to generate statistically robust findings should be determined.

Life As We Don't Know It The possibilities of dealing with "life as we don't know it" need to be considered seriously, including: a composition devoid of organic carbon; the unconventional reliance on "non-biological" elements such as Si, Fe, and Al; structures less than 100 nanometers in diameter; and a composition based on organic monomers. Of course, it is difficult to evaluate the probability of encountering forms of life with these features.

Discussions of the possibility of non-carbon based life have had a rich history, especially in the realm of science fiction.[20] Life based on organic monomers has recently been proposed as a model for the 'metabolism-first' scenario for the origin of life.[21] According to this model, a set of self-sustained chemical reactions might be considered 'living' if metabolism is considered to be more important than replication as a fundamental basis of life. Some of these unlikely scenarios might require alternative laboratory conditions for proper study (e.g., use of inert gases).

Existing theories of the origin of life on Earth suggest that life will arise as a consequence of chemical and physical principles anywhere prebiotic carbon compounds accumulate in suitable environments (e.g., water, temperature, etc.) in sufficient amounts for sufficient time. Although the precise process for life's

20. H.G. Wells, writing in the Pall Mall Gazette in 1894, scolded scientists for thinking of only carbon-based life: "It is narrow materialism that would restrict sentient existence to one series of chemical compounds – and the conception of living creatures with bodies made up of the heavier metallic elements and living in an atmosphere of gaseous sulfur is no means so incredible as it may, at first sight, appear."

21. Wächtershäuser, G., *Science* **289**:1307-1308 (2000).

origins on Earth is not known, it is perceived to have been a progression in complexity beginning from an original prebiotic mixture, at some stage involving RNA catalysis, and probably at later stages catalysis by peptides and proteins, ultimately culminating with the first simple organisms that had a metabolism, the ability to replicate, and the capability of preserving useful information during the replication process. The most likely scenario we can conceive of for the independent development of life on Mars is by a similar process, which if stochastic, may have deviated from our own terrestrial process and resulted in different fundamental amino acids or nucleotides used, types of lipids, chirality, etc. The primary indicator of past or present life of this type would be the finding of unusual macromolecular assemblages (e.g., peptides or oligonucleotides with nonstandard amino acids, nonstandard bases, nonstandard linkages). If deviation occurred only later in the process, we might find Earth-like complex structures such as recognizable ribosomal RNAs.

It also should be noted that if there is, or has been, life on Mars, it might be related to life on Earth by descent. If an evolved living organism reached Earth from Mars, or less likely, reached Mars from Earth, the two life forms should be closely similar in their biochemistry. They should, for example, use DNA as a genetic molecule and might have the same genetic code. If two life forms originate and evolve independently, however, there is no *a priori* reason to expect them to be similar.

Sample and Time Requirements It is estimated that approximately 3 grams of sample will be required to conduct the proposed preliminary Life Detection tests on returned martian sample materials.[22] As methods mature and new approaches become available, these sample requirements may change. Estimates of the time needed for Life Detection are difficult to make. Survey methods can be completed within weeks-to-months, in some cases. However,

22. Estimates for sample amounts are based on what is necessary to conduct the tests outlined in the Draft Protocol; however, actual amounts may depend on definitions of "representative samples" made at the time samples are returned.

any positive or suspicious findings may impose additional time requirements, depending on the strength of the findings and the follow-up methods required for further assessment. For example, enrichment culture experiments as part of the Life Detection protocol may extend for many months, even though they are not considered a strong methodology for detecting martian life.[23]

Future LD Research and Development Needs

- Miniaturization of many chemical/physical analyses
- Sample registry, for re-interrogating precisely defined sites within the sample
- Micro-calorimetry
- Database development
- Software for "multiple sequential analysis" search logic
- Effect of Mars atmosphere versus inert atmosphere on proposed methods
- Cleaning/cleanroom technologies
- Validation of controls
- 3-dimensional nano-scale structural mapping of specimens
- Characterization of complex compounds based on Si, Al, Fe
- More complete inventory of life on Earth, using molecular methods

Biohazard Testing

Introduction The Biohazard testing process is intended to determine if samples from Mars pose any threat to terrestrial organisms or ecosystems, regardless of whether the samples are found to contain life-forms or non-replicative hazards. In this Draft Protocol, it is recognized that potential hazards could take one or more of a multitude of forms (e.g., toxic, mutagenic, life-cycle altering, hazardous through genetic recombination, disruptive to ecosystems, capable of biasing phenotypes, or even behavior). Thus, the spectrum of tests selected is deliberately diverse.

23. Attempts to culture potential microorganisms from Mars samples will be done recognizing that, even on Earth, the vast majority of terrestrial organisms cannot be cultured under known conditions. Bearing this in mind, the length of various culture experiments may be allowed to extend into months even though the likelihood of positive outcomes is extremely low.

Both conventional whole-organism animal and plant *in vivo* testing are planned, in addition to *in vitro* cellular assays and molecular biology tests (see Figure 5).

In light of the robust nature of emerging molecular, cellular, and conventional testing procedures, specific methods will be selected later in accordance with state-of-the-art practices and refinements at the time the final protocol is implemented *[Race et al., 2002].* Selections should take into account evolving test methods (e.g., toxicogenomics) that are anticipated to replace many current conventional practices over the coming years. These newer procedures may ultimately become refined state-of-the-art approaches. In such instances, advances in testing methodologies that presently await standardization and validation should allow modifications and refinements to Biohazard testing adopted for the final protocol applied to samples from Mars.

The proposed tests and procedures for Biohazard testing reflect the current state of knowledge and practice. It is anticipated that this Draft Protocol will evolve both in content and implementation as a result of new or improved methodologies or expanded states of knowledge prior to sample return, and in response to real-time information about sample materials learned during implementation of the various processes at the SRF. A sketch of the pathway of experiments for Biohazard testing is given in Figure 5 and further details of those pathways are in Table 5. The approach outlined in Table 5 was developed early in the MSHP Workshop Series *[Race et al., 2001a]*, and refined at subsequent Workshops in the Series *[Race et al., 2001b and 2002]*. Throughout the Workshop Series, the development of a general approach for Biohazard testing, rather than a specific list of tests, was considered the most useful and responsible approach for deliberations at this time. *[Race and Rummel, 2000; Race et al., 2001a, 2001b, 2002],*

The data from Biohazard testing will be used in combination with those from Life Detection and Physical/Chemical testing to determine what level of containment, if any, will be required for the further study of the samples. In practical terms,

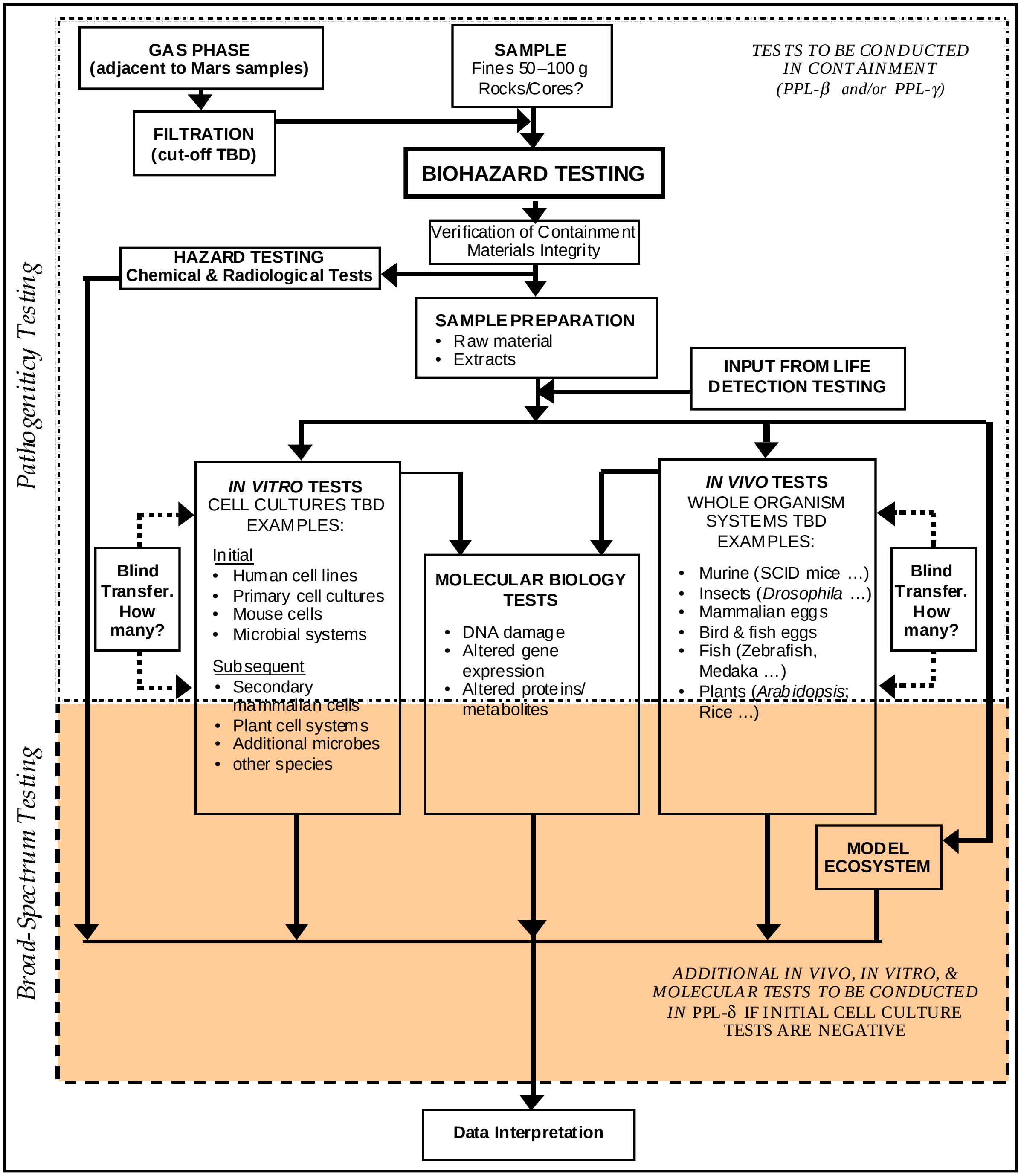

Figure 5. Proposed Flow Chart for Biohazard testing. The clear region contains tests (chiefly for pathogenicity) that should be done in strict containment (PPL-α/β/γ), while the shaded region represents similar tests for broader-spectrum biohazards done in less strict, but still secure, containment (PPL-δ).

Test Type	Procedures/Questions	Sample Usage and Time Required
Verification that any potential organisms do not attack biocontainment materials (e.g., Silastic™, rubber, etc.).	Do samples affect test coupons of containment materials at various humidity levels and temperatures?	Sample expended: 1 gram Time: 1 - 3 months?
Input from Life Detection Procedures (discussed separately): • If life detected, this would radically change/focus the approach to Biohazard testing by providing focus in terms of conditions for replication, agents that can kill the organism(s), etc. • If no life is detected, still run subsequent tests for toxicity and biohazard.	• Carbon? • Carbon-carbon bonds? • Complex carbon compounds (indicative of metabolic processes)? • Skeletal remains or fossilized remnants? • Indication of live organisms (organelles, membranes, structures on microscopic evaluation)? • Life-like structures? • Living agent (replicates in environment, with co-agent/host, in terrestrial cells)? • Mutual/commensal/parasitic relationship? • Kills cells or organisms? • Kills complex multicellular organisms? • Kills everything?	Sample expended: TBD Time: TBD
Multi-species infectivity, pathogenicity, toxicity testing. • Look at broad host ranges (assuming that any pathogens would not be too host-specific) with well-known and standardized model systems. • Use small organisms in small volumes, allowing for maximum sample conservation. • Initial work all done at BSL-4 biological containment level.	Sample preparation (rough cut): • Crush larger clumps/rocks but do not pulverize particulates. • Filter? • Mix into sterile water. • Chelate heavy metals? • pH buffer? • Use serum for some samples? Heavily irradiate sterilized control samples w/ ^{60}Co. Introduce appropriate amount of sample (10 -100 milligrams for statistical relevance) to culture of unicellular organism and cell lines. Inoculate whole organisms (animals as human models) with primary (not passaged) material. Monitor: • Cell proliferation, • Cell morphology, • Deferential analyses of biochemicals and gene expression • Comparative genomics (any inserted genes in host?) • Reporter assays (?) • etc.	Sample expended: Three trials plus sterilized control per organism, assuming 100 mg per sample = 1.6 grams. Time: ~ 6 months to allow for passage times.
Negative results with multi-species tests may lead to downgrading to PPL-δ.	The following tests/criteria are proposed: • First passage from infectivity analysis (+ or –), but second and subsequent passages all neg. • DNA damage assays (mutagenesis: Ames- test, strand break analysis). • Environmental damage. • Whole plant inoculations. • Diversity of growth conditions extant on Earth (extremophiles, etc.) and other media. Monitor: cell viability, expression of toxic response genes. Negative results on these tests may allow a decision to downgrade to a lower containment level or release.	Sample expended: ~10 - 20 grams (very rough estimate). Time: ~6 months to allow for passage times. Note: There was consensus on the 'first round' (infectivity), but it was also clear that the containment-level determination issues need considerably more analysis and study.
		Total = 15–25 grams

Table 5. An outline of a possible pathway of experiments for Biohazard testing.

Biohazard testing should allow a determination—with a high degree of confidence and a clear understanding of the conditions of release—of whether the samples contain any biohazard and whether to distribute sub-samples. A determination about releasing a sample from containment will be made with careful consideration of applicable regulatory requirements and will provide a reasonable assurance that the samples will not put humans or other terrestrial organisms at risk.

Biohazard Defined In general terms, hazards of concern to biological systems may be caused by materials or entities of biological origin, and by those materials or entities replicating or being amplified[24] toxic and by a biological system. Such hazards are capable of producing an adverse effect on or significant alteration of a biological system at the level of individual organisms or ecosystems.[25] In the special case of hazards from returned martian samples, a distinction can be made between replicating and non-replicating hazards. For the purpose of this Draft Protocol, a *biohazard* is defined as a hazard that can either replicate or be amplified by a biological system. In practical terms, replication is a key distinction between a biohazard (i.e., replicating and potentially contagious) and a simple toxin or hazard (e.g., a non-replicating substance that can be diluted down below an initial toxic concentration). Only replicating entities, or entities that are able to be amplified by a biological system, pose a potential widespread threat. While other hazardous materials are of concern, the quantities returned from Mars will be extremely limited, and they thus represent a potential hazard of real significance only to scientists and others who may be exposed to them.

24. In this context, biohazards are not limited to 'living' entities—and may include biohazards such as viruses that are not living or self-replicating *per se*.

25. In the context of potentially biohazardous extraterrestrial entities, "adverse effects" includes any significant alteration on a biological system, and is not limited to adverse effects that are immediately or acutely toxic.

If the distinction between a biohazard and a non-biological hazard is made, the level of containment and procedure for distribution of the samples can be appropriately defined. The existence of either biohazards, which are self-replicating or able to be amplified by another biological system, or toxic hazards would require further study and characterization of the nature of the hazard (e.g., strong chemical oxidizer, radioactive, replicating life-form, etc.) so that appropriate subsequent containment and/or handling procedures can be determined and stipulated to avoid potential biological impacts during future research.

Assumptions About Containment Containment at the SRF will be designed to provide a range of environmental conditions for the martian samples, while maintaining them at appropriate biocontainment levels. It is important to understand the various containment types at the SRF and the anticipated containment needs during Biohazard testing. Life Detection and Physical/Chemical tests will seek to characterize the sample materials and determine if evidence for “life” can be found under conditions that are both Mars-like and Earth-like. In contrast, Biohazard tests are designed to determine the effect of martian samples on terrestrial life-forms under Earth-like conditions. Thus, containment requirements for execution of the Biohazard testing will not require the same stringent clean room conditions associated with the preliminary P/C tests, certain Life Detection studies, and ‘banking’ or curation. The appropriate initial containment level for the Biohazard testing is thus anticipated to be PPL-γ, which translates to the maximum BSL-4 biocontainment, but with less demanding cleanliness restrictions than PPL-α.

The unknown nature of any possible biohazard in returned martian samples demands, at least initially, the most stringent containment presently afforded to the most hazardous biological entities known on Earth. If sufficient data are gathered to rule out concerns about human virulence and infection, a decision could be made later to allow subsequent work at a lower containment level during tests investigating possible environmental effects. The Biohazard testing process is designed to allow for gradual decontainment or adjustment to less stringent containment levels if justified upon review of accumulated data about the sample materials during implementation of the Draft Protocol. If the initial Life Detection and Biohazard tests are all negative, it would be appropriate to conduct subsequent tests under less strict containment conditions once sample materials have been shown to be non-biohazardous. In particular, additional geophysical testing can be done at a reduced level of containment, as well as using selected biological tests associated with the biohazard analysis. A lower level of containment would potentially enhance sample access within the scientific community, while still providing adequate biosafety conditions under existing biosafety guidelines and regulations.

Biohazard testing will be conducted within containment at the primary receiving facility or at other secure containment facilities. Since neither all the necessary scientific expertise, nor all of the high-end scientific instrumentation required, are located at a single facility, there may be a need to allow samples to be distributed for study/curation at facilities other than the initial receiving laboratory. The rationale for the use of multiple containment facilities and the ability to test unsterilized sample materials outside the primary containment facility depend on the availability of an adequate means for containing and transporting the samples, for sterilizing or cleaning the outside of the sample container, and for returning the remaining samples to the primary containment facility after non-invasive or non-destructive analyses (e.g., synchrotron analyses). Mobile containers certified at the appropriate PP level (as distinct from traditional BSL transportation requirements) should be developed and used for transport of samples between facilities.

Considering that Biohazard testing should yield results within a “reasonable time” (e.g., most testing completed within approximately 6 to 9 months), the majority of tests should be started synchronously and conducted in parallel. Nonetheless, the need to conduct preliminary sample examinations and to work on Life Detection require that Biohazard researchers proceed with some tests before others. Common sense and gradual decontainment strategies require tests identifying deleterious effects on containment equipment before those identifying biohazards to people, and the latter before identifying biohazards to the environment.

After the equipment-compatibility tests, the types of assays to be accomplished are prioritized by their likelihood of identifying potential pathogenicity and identifying any restrictions on the distribution of samples to other laboratories for further testing. If a possible human pathogen were detected, the strictest of handling protocols would remain in place. If, in complementary fashion, a pathogen specific to another host were detected, less stringent handling methods might be possible. If the only hazard identified were a non-replicating toxic agent (e.g., a toxic chemical), containment could be less restrictive, and would be definable on the basis of dose-response characteristics and the nature of the toxicity.

Model Systems for Biohazard Testing Prior to conducting Biohazard tests, decisions will be needed to identify the exact model systems that will be used for the specific assays. Working criteria for choosing the models are as follows:

- The models should be relevant to a probable hazard scenario, deliberately avoiding models that would only be sensitive to an improbable danger (i.e., very unlikely event, very artificial route, extreme doses, rare species confined to remote niches, etc.) as such models would be of little relevance to initial Biohazard testing with Mars samples. The emphasis will thus be placed on modeling of biological systems likely to be in contact with samples (e.g., workers, their microbial flora, their pets, insects, life-forms common to the surrounding of sites of future experimentation with the samples), *via* probable routes of exposure (e.g., aerosol, etc.), at probable (low) doses.
- Subsequent models should be relevant to systems of ecological and/or economic interest.
- Models should be sensitive, meaningful and, if possible, clear to interpret. Equivocal answers can needlessly prolong the time required to reach a

decision on sample release, and will likely cause samples to be consumed unnecessarily.

- Models should be robust. Samples are likely to contain complex minerals, oxidative agents and other elements that should not interfere with its function.
- Models should be well documented. Observations and analyses should identify known behavior of the biological system in the model. Preferably, its genome should be fully sequenced, and extrapolation to other species/situations should have been evaluated.
- Models should provide answers in a reasonably short time.
- Models should be compatible with handling within the SRF, under containment. For instance:
  - ➢ *Cellular and 'small' models.* Should the model organisms or cells for Biohazard testing be chosen or developed as of this writing, these would include:
    - ♦ wild type, mutant and recombinant yeast bearing special sensitivity to hazardous materials (e.g., radiation mutants; green and blue fluorescent protein [GFP and BFP] recombinants to test for recombinogenicity; etc.);
    - ♦ human cell lines that are as sensitive to pathogens as standard cell lines used for Biohazard testing (e.g., a human equivalent to vero E6 cells, as sensitive as BHK-cells to mutagens, etc.);
    - ♦ bacteria and other microbes associated with people (e.g., *E. coli, Staphyloccocus, Bacteroides*, *Chlamydomonas*, etc.);
    - ♦ bacteria found in niches likely to be similar to martian underground ecosystems (e.g., cold and possibly oxidizing, low-oxygen and with high radiation levels, etc.);
    - ♦ relevant algal/planktonic unicellular organisms;
    - ♦ mammalian (e.g., mouse) egg before re-implantation;
    - ♦ fish eggs (e.g., Zebrafish, Medaka, etc.);
    - ♦ models for testing effects on development (e.g., *Neurospora crassa);*
    - ♦ cells and seeds from *Arabidopsis* and rice;

    - complete *C. elegans*; and,
    - complete *Drosophila melanogaster* (likely a flightless variant).
- *Larger organism models.* For tests in which whole organisms are required, model organisms would include:
    - *Arabidopsis* and rice at different stages of development;
    - zebrafish and medaka;
    - bird eggs; and,
    - a variety of types of mice (e.g., germ-free, humanized, wild type, mutant, recombinant, immunosuppressed, knockout), whether reimplanted, newborn, or pregnant.
- *Ecosystem-level models.* For tests of multi-species systems, stable, replicable, laboratory-scale ecosystem models need to be developed and tested. Microbial mats may form a promising basis for such a model.

<u>*Verification of Containment Materials Integrity*</u> As a first order of business, a set of preliminary tests is required for materials used in containment equipment. It is important to verify that sample materials or potential organisms growing from them do not attack rubber, Silastic™, and other bio-containment materials. For example, ten 10-milligram samples would be taken for each seal/containment material (e.g., latex, Silastic™, Plexiglas™, cyanoacrylate, epoxy, etc.). ‘Coupons’ (i.e., small, regular samples) of each material would be incubated with martian sample material at a few different humidity levels, bounding those actually to be used for sample curation, and including liquid water. Test vessels for these experiments (i.e., primary containment) should be extremely non-reactive, such as refractory metals (e.g., titanium). For this example, if ten materials are tested, a total of one gram (or less) of martian sample would be expended.

At regular intervals (over weeks to months), the sample coupons should be monitored for degradation using optical methods, mechanical tests, and chemical analyses. ‘Failure’ criteria would be defined in terms of parameters that would compromise containment, such as outright consumption, pitting/erosion, pinhole formation, substantial changes in bulk chemical or mechanical properties, etc. The results would be used to provide a high level of confidence that the samples could be kept in storage vessels made of the tested materials without risk of inadvertent release.

Pathogenicity Testing These Biohazard tests, which have a specific focus on determining adverse effects on humans, will be done in PPL-γ (containment: BSL-4; environment: normal terrestrial). Toxic effects on cultured cells and microorganisms should be anticipated due to the chemical (mineral) composition of the Mars samples. Appropriate controls (terrestrial or meteoritic) must be run and interpreted. It is assumed that toxic effects, if any, should diminish rapidly in sub-culturing ('passaging') experiments, since a replicating agent or one able to be amplified would not be involved in a toxic response *per se*.

Since fines can be considered 'homogeneous' and can be sub-sampled as a single category in a statistically relevant way, Biohazard testing should begin with fines. Whether and when other materials should undergo the full array of Biohazard testing will be based on the results of initial P/C screening and processing.

Tests will involve exposing model organisms to the martian sample material. Specific cell and tissue systems should be used for Biohazard testing, as noted above in the "model" discussion and below in the discussion of each test. It is envisaged that a large amount of the cell culture work will be accomplished robotically using existing or new technologies.

The following specific initial exposure tests *[Race et al., 2001a]* should be included, based on the knowledge available should it be carried out today:

- Human cell lines and primary cell cultures, with particular emphasis on epithelial cells (e.g., skin, lung, gut). All cells will be observed for abnormal growth (e.g., cytopathic effect, morphological changes, genetic response to stress, integration into host genome, co-growth [mycoplasma-like], and mutation rates). Cells can be checked for transformation (growth on soft agar). Both supernatant and homogenized cell pellets should be passaged, typically twice each week for 3 months. Other replicate cultures must be observed for 1-2 weeks to look for delayed effects. Cell cultures (and concentrated medium) should be examined, as well, by electron microscopy to search for microorganisms that may have replicated without causing abnormal changes in the cells being cultured.

- Mouse cells should also be tested in similar fashion, with “culture-adapted” material being injected into mice; three mouse systems should be employed (i.e., wild-type, SCID, and SCID-Hu).
- Microbial systems to be tested should include *Chlamydomonas* (stress response), *S. aureus*, yeast, and *E. coli*. In addition, microorganisms that grow in high salinity should also be considered.

Subsequent Pathogenicity Testing and Possible Decontainment Subsequent testing should be designed to accommodate a variety of test systems and representative organisms from different biological domains and ecologically and economically important phyla. If the initial Biohazard tests (above) and Life Detection tests are all negative, it may be appropriate to conduct these subsequent tests under less strict containment conditions (e.g., PPL-δ). In particular, additional P/C testing, as well as some additional Biohazard tests, can be conducted at a reduced level of containment using the following models:

- Secondary mammalian cell culture systems.
- Plant cell systems (*Arabidopsis*) and whole-plant growth experiments.
- Additional microbes (e.g., nanobacteria, cyanobacteria, thermophiles, anaerobes, gram-positive bacteria) and microbial systems (e.g., various temperature ranges, pH ranges, salinity).
- Other species, such as *Drosophila melanogaster* (e.g., wingless mutants), worms (*C. elegans*), and amphibian and bird eggs. Horizontal and vertical transmission studies should be done. All animal species

should be observed for behavior change, toxic and teratogenic effects, and pathological changes.

Additional experiments can employ a variety of techniques to test for biologically active compounds, micro-arrays (for proteins), etc.

<u>*Broader-Spectrum Biohazard Tests*</u> Beyond strict pathogenicity testing, the Biohazard tests that should be completed include:

- *Direct culture.* This is also part of the Life Detection testing process; any cultured organism which cannot be clearly identified as terrestrial will be subjected to further Biohazard studies.
- *Exposure of cellular and 'small' models.* Unicellular organisms, or very small animals can be used with a limited amount of sample, i.e., ~10-1000 micrograms per test. These tests would be based on exposing the organisms to the sample and using some form of signal readout, such as gene expression.
- *Molecular and biological tests (altered levels of proteins and metabolites).* Rapid progress is being made in developing chip-based, as well as other, methods that allow one to measure the level of particular proteins or metabolites in a biological sample. Within the next five years, driven by the demand of genomics research and drug development, these techniques are likely to become broadly available. It is difficult to make specific recommendations at this time before standardized procedures are established. It is expected, however, that the comparative measurement of proteins and metabolites associated with the biological response to infection or toxic exposure will become part of the biohazard assessment procedure.
- *Genetic testing.*
  - ➢ *Mutagenesis Assays.* Another possible approach is mutagenesis assays that look at genetic changes over several rapid reproductive cycles. Typically, bacteria are used (e.g., the Ames test for mutagenicity uses *E. coli*). The consensus is that these tests will be problematic in that mutagenesis results tend to be oversensitive and controls would be

difficult to realize. A related assay type is teratogenicity, but these require breeding animals, and, thus, can require more time (for some species) than other assay types.

- ➢ *DNA Damage.* Assessment of DNA damage should include the measurement of mutation frequency, recombination frequency, and the occurrence of DNA strand breaks. Standardized methods are available to carry out each of these measurements, for example, genetic reversion assays for DNA mutation, transposon rearrangement assays for recombination, and terminal transferase assays for strand breaks. Such approaches, focusing on general measures of DNA damage, are likely to be more fruitful than highly specific measurements of DNA damage, such as comparative sequencing or the measurement of a particular type of DNA damage.
- ➢ *Altered Gene Expression.* Techniques are available for measuring the relative expression level of almost any gene under various conditions. For purposes of biohazard assessment, however, it would be preferable to narrow the focus to genes that are expressed at a significantly altered level in response to infection or toxic exposure. Testing for altered gene expression due to toxic exposure is being refined as “toxicogenomics,” and is anticipated to reach a sophisticated level of standardization by the time the selection of methods is made for the final protocol.

- *Whole organisms.* This approach includes ingestion/inhalation/injection of samples by living organisms with subsequent monitoring of physiologic functions, behavior, gene expression, inflammatory cascade (e.g., cytokine levels), etc. Hosts can include animals, plants, and modified organisms (such as SCID mice, xenograft systems, etc.). Another key aspect of this approach is the ability to evaluate the infectivity of the potential organisms to other organisms via passage, and in subsequent generations. The benefits of this approach to whole organism testing include: direct measurement of physiologic effects; ability to handle multi-organ interactions in toxicity; inherent inclusion of complex host characteristics (tough to execute with cell-based and other assays); and, the possibility of detecting infectivity (if hosts are appropriate for replication).

  Nonetheless, some significant drawbacks exist, including: the difficulty in seeing long-term effects; it would be impossible to cover all possible organisms (many terrestrial pathogens are very host-specific); large samples may be required; tests may be confounded by the presence of inorganic materials; and, results may depend on the mode of introduction of sample to test organisms (terrestrial pathogens have specific routes of infection). A major drawback of this approach is that it requires more sample, i.e., ~100-5000 micrograms per test. Approaches/organisms include:

- ➢ Exposure by direct contact and/or aerosol—*Arabidopsis* and rice at different stages of development;
- ➢ Exposure to the sample by routes to be determined (e.g., water solution, etc.)—Zebrafish and Medaka;
- ➢ Injection with powdered sample—bird eggs (notably embryonated chicken eggs); and,
- ➢ Exposure of a variety of types of mice (such as: germ free, humanized, wild-type, mutant, recombinant, newborn, pregnant, immunosuppressed, reimplanted), to the sample as an aerosol, by intraperitoneal injection, or *per os*. There may also be genetic designer knockout mice exposures included, which could alleviate some of the above mentioned drawbacks.

The selection of particular species for whole-organism Mars sample testing should be based upon (i) state-of-the-art methodology and practices at the time of the mission and (ii) expert opinion about the suitability and applicability of employing certain species over other disqualified candidates. NASA will keep abreast of research developments in whole organism testing, as well as cultivate and maintain strong liaison relationships with national and international scientific experts to assure that appropriate state-of-the-art methods and practices are ultimately employed and followed.

- *Ecosystems.* Multi-organism population testing is important because potential biohazard effects may only manifest within the complex interactions present in ecosystems. The development of microarrays for analyzing RNA from soil and water will allow both bacterial community structure and function to be followed in microcosms. Although the development of reproducible test microcosms will require further research and development, such assays could be sensitive, fast (on the order of a week), and include environmental genomics monitoring capabilities. Microcosm tests could allow monitoring for ‘global’ characteristics (e.g., system metabolism, biochemical profile of solid/liquid/gas phases, etc.), as well as for specific parameters associated with subtle or complex changes in community structure and function. Additional research will be required to develop these comprehensive and effective tests.

Sample Size Two different approaches were used to estimate the amount of sample required for analysis. The first was based on a pre-sorting of the sample that assumed that ‘relevant’ biologically interesting sub-samples would be used. Under this assumption, the amount of sample to be used is dictated by:

- the relevance of the dose being modeled,
- the amount with which the model biological system can be physically dosed,
- the sample preparation procedure,
- the number of tests to be conducted, and
- the total time Biohazard testing should take.

With this approach, the crudely estimated sample consumption for Biohazard testing was ten grams.

The second approach did not assume a particular sorting of ‘relevant’ samples, but instead used simple statistical methods. Using Earth soil as a crude reference, a conservative calculation suggested that 15–25 grams of sample should suffice. These two estimates were quite close despite the very different approaches used to arrive at them.

Ruling out biohazards in one sample will not allow for extrapolation to other samples. It will remain a case-by-case task, at least for a considerable period. This applies even when sub-sampling returned materials. One consideration is whether samples should be 'homogenized' prior to Biohazard testing. Such a homogenization is inadvisable because of the loss of information it represents. For example, sedimentary rocks (which may be in the minority) are more likely to harbor signs of life than igneous rocks. In addition, since surface conditions may be toxic to organisms, homogenization with deeper sample components may not be advisable.

In general, small sample sizes will be required to conserve the returned specimens, so biological assays that require small quantities are highly desirable. Examples include cell-based assays (requiring as little as 100 microliters of total fluid volume, making milligram samples potentially adequate) or the use of small organisms, such as *Arabidopsis* and *C. elegans.*

It was noted that the amount of material needed for destructive testing (consumed) in biohazard assessments must be determined in consultation with biostatisticians. Regardless of what starting assumptions are made, the statistics of sampling will apply, and confidence in 'hazard exclusion' statements can only be made in the form of "no hazard exists at a concentration greater than *X* per gram."

Time Needed The time to conduct Biohazard testing was estimated to be twice the time to conduct the slowest test. It was estimated that most of the results would be acquired within 90 days, but that 4 to 6 months would be a good estimate for the completion of the bulk of the testing on the initial samples, including opportunities to conduct tests on subsequent generations of whole organisms involved in the testing. As an example, it was estimated that all Biohazard testing necessary to downgrade the samples from BSL-4 to BSL-3-Ag would take approximately 6 months, while another 6 months would be required to downgrade the sample to a lower level of containment or release, as appropriate.

Comments on Controls Control samples clearly are needed for all of the above experiments. Methods for generating control samples (e.g., dealing with oxidants, iron, etc.—these contaminants could greatly confound bioassays and not be modified by some sterilization methods such as high-level irradiation) must be developed.

Irradiated samples, while somewhat modified, apparently are suitable for much of the geologic investigations of interest, and along with simulants can be used as controls. Interestingly, "clean" in terms of geology can mean knowing that certain elements such as lead are present in concentrations in the parts-per-trillion range. The important point here is that typical biological containment systems are not designed with such cleanliness (e.g., molecular/atomic) in mind. A practical impact of this is that containment/handling equipment and materials should be characterized in terms of trace concentrations of elements that may be irrelevant biologically, but damaging to geological and other scientific analyses.

One additional point is that there is a need for pre-launch controls to help rule out terrestrial contamination. Swab samples, etc., from the assembly and launch phases and test facility should be taken periodically for two years before mission launch. This will be a vital piece of the process to establish positive and negative controls. Negative controls can also be generated at the time of analysis by treating samples with DNAses, proteases, etc., to subtract out any terrestrial or Mars biomarkers, so that effects of Mars soil on subsequent assays can be evaluated.

Future BH Research and Development Needs Further efforts need to be undertaken to perfect many steps in the final protocol, including:

- A sub-sampling procedure needs to be developed and validated so as to provide statistical relevance and innate conservatism. This is essential to ensure that the Biohazard testing is capable of determining the safety of the samples. Without an effective representative sub-sampling strategy, testing of the entire sample may be necessary, and untested samples may need to be kept in containment indefinitely.

- Specific models for use in Biohazard tests have to be chosen or developed. Each one of them should be validated with terrestrial mimics of martian soil (possibly with meteoritic minerals from Mars) used "as-is," or spiked with known agents to provide a positive control in Biohazard testing.
- Relevant, robust, and reproducible methods of sample preparation and sample delivery must be developed to ensure the Draft Protocol can be accomplished effectively.
- The selection of optimal cell and culture systems for use in biohazard and toxicology assays will be critical. Prior to protocol implementation, research is needed to select optimum cell and/or molecular assays for BH testing.
- All assay refinements should take into account biohazard containment issues in their design and implementation. Moreover, it is likely that NASA will need to coordinate these refinements, and any attendant research developments, with the toxicology and infectious disease programs at the National Institutes of Health (NIH), the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), and the Centers for Disease Control and Prevention (anticipating forthcoming funding increases to integrate extensive research into infectious diseases and bioterrorism issues). NASA also must stay abreast of developments in toxicogenomics at the NIH and in industry, a new field anticipated to replace conventional toxicology methods over the next five years.

Facility Requirements

The size and scope of the facility required to complete the elements of this Draft Protocol will depend on whether all protocol functions and activities (e.g., sample receiving and processing, physiochemical characterization, Life Detection studies, and Biohazard testing) will be conducted at a single SRF or if some elements will be distributed to secondary labs beyond the SRF. In either case, based on experience following receipt of lunar samples, the primary SRF should be designed to be expandable and allow great flexibility in switching functions as needed. In particular, the SRF should be able to support investigator-driven research, both to accomplish science objectives that should be addressed prior to release of unsterilized samples, and to accommodate initial work following the possible discovery of extraterrestrial life, if necessary. The primary SRF should be designed to allow continuous and long-term operation in addition to accomplishing its primary goal of receiving the Mars samples and implementing the final protocol. There also should be a backup PPL-α facility to contain a subset of the initial samples for banking purposes.

The various elements of the Draft Protocol and appropriate levels of containment for completing them are depicted in Figure 6. From a planetary protection perspective, these functions can be performed at any facility that meets the containment requirements, but as of this writing, no facilities exist which meet PPL-α or PPL-β requirements, and only a handful worldwide meet PPL-γ. Similarly, no specific test or instrument is precluded from use during the completion of the protocol if that test or measurement can be accomplished or placed in containment.

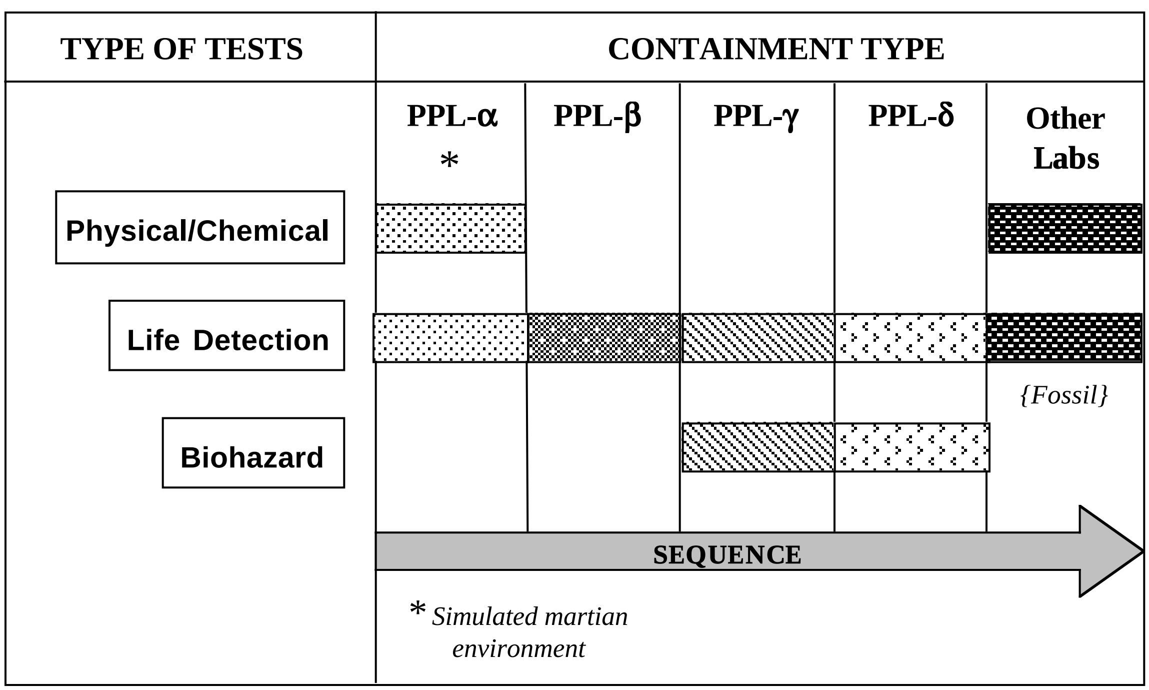

Figure 6. Sequential containment requirements by test category.

Regardless of how the final protocol functions are distributed, all ancillary facilities must meet the same containment guidelines and standard operating procedures (for items such as personnel monitoring, security assessment, chain of custody tracking for samples, etc.). There are advantages of utilizing a single facility, at which the samples are received and all functions up to PPL-γ are performed before some materials are transferred to PPL-δ facilities to complete the testing. These advantages include a streamlined management and advisory structure, decreased sample volume for testing, fewer personnel to monitor for potential exposure, consolidation of appropriate experts at a single site, and diminished transportation and logistics concerns. Significantly, this approach assures that the samples are in the fewest number of facilities practicable, should special actions be necessary if they are found to contain life or a biohazard. Likewise, there are disadvantages to building a single large facility instead of a smaller one to be used in combination with other, existing facilities. Potential disadvantages include increased cost and complexity, a possible decreased breadth of instrumentation that can be accommodated, potential delays in recruitment of personnel or complications for personnel visiting from international partners, and the lack of a second containment laboratory for the corroboration of test results.

In the final analysis, the facilities required to implement this Draft Protocol, or its successors, should be the minimum set needed to accomplish the required planetary protection and science requirements for Mars sample handling in containment. A variety of facility strategies can be pursued, depending on the availability of personnel and resources among the partners pursuing a Mars sample return mission. Further studies of this issue are required, since several of those strategies can provide for protocol completion as well as the optimal availability of the samples for scientific studies at the earliest possible time consistent with Earth safety.

Future Research and Development Needs Additional facility-related tasks that should be addressed in further work include:

- Completely define the PPL containment guidelines and any qualifying or disqualifying site-related criteria;
- Continue to work with the appropriate agencies and groups[26] to explore containment issues, options, and requirements regarding the refinements that will be necessary over the coming years to design or retrofit the appropriate and applicable biohazard containment facility;
- Develop a self-contained structure that could be placed inside of a BSL-4 laboratory, and, as a composite, meet PPL-α containment requirements (this structure should be able to use robotics to handle the specimens);
- Develop a comprehensive list of equipment, and the required facility accommodations, for all proposed tests in the Draft Protocol;
- Develop systems needed for some Life Detection testing under simulated martian environmental conditions, while maintaining PPL-α/β containment; and,
- Develop cooperative agreements with appropriate BSL-3 and BSL-4 laboratories that can provide experience to NASA personnel prior to the receipt of Mars samples, or that may act as PPL-δ laboratories thereafter.

Environmental and Health Monitoring and Safety

Procedures for monitoring the health and safety of the personnel of the SRF and the environment in and around the SRF (as well as at secondary sites if used) must be developed and implemented as part of the final protocol. These will require a consideration of monitoring over time and an assessment of how long to continue monitoring, beginning prior to the arrival of Mars samples and continuing during work on the samples at the SRF and at secondary sites, and for some time thereafter.

26. Appropriate agencies such as: NIH, USAMRIID, and CDC in the U.S. and Institut National de la Santé et de la Recherche Médicale (INSERM) in France.

Assumptions

- The actual risks associated with the Mars samples are unknown.
- The greatest potential risk is biological. Additionally, the potential existence of "life as we don't know it," although considered remote, must be acknowledged and addressed in testing.
- The potential primary exposures will be limited to a small group of trained professionals in the SRF until more information about the nature of the specimens is available.
- A high level of security for the SRF and the samples will be maintained as part of the PPL designation.

Recommended Principles for Development of a Monitoring Program for SRF

Whenever possible, the monitoring plan should use existing regulations and standards. Since international teams will be working on the Mars samples, the regulatory standards from participating countries should be reviewed and considered when developing the final monitoring plan. When considering existing regulatory standards, the strictest standards, as appropriate for the anticipated hazards, should apply. Exemptions from existing regulations may be necessary. For example, differences in the protection of medical information between the participating countries may be in conflict. The first principle for personnel monitoring and safety must be to provide optimal protection from anticipated hazards for the individuals working with Mars samples. Because of the unique nature of the potential hazards, additional controls beyond those routinely used for hazard monitoring may be required. The monitoring plan should be designed to maintain a balance between the estimated risks to individuals, the environment, and the general population, and the personal and practical impositions of the monitoring program. The monitoring plan should allow for cross-correlation of the data from the Life Detection and Biohazard testing with the data from the monitoring of the SRF personnel and environment, and allow for subsequent modification of either set of tests.

Potential Hazards Five categories of potential hazards to personnel were considered: physical hazards, potential chemical hazards from non-biological toxins, biological hazards, psychological hazards, and loss of containment itself. The physical hazards include predominantly radiation from the Mars samples (which is expected to be negligible) and hazards associated with equipment within the SRF. The potential chemical hazards are predominantly from non-biological toxins. Any biological hazards will clearly be the most difficult to monitor. Psychological hazards may arise for personnel working under PPL conditions, although the psychological risk perception will be far greater for the general public than for committed risk-taking workers, if generally less immediate. Finally, ensuring that there is no loss of containment is a significant part of the monitoring program.

Recommendations for Monitoring

- *Physical Hazard Monitoring (Radiation and Equipment).* Radiation is a standard hazard with well-established protocols for protection, handling, and monitoring. To confirm the expectation that the Mars samples will not present a radioactivity hazard, a radioactivity measurement should be one of the initial measurements conducted during the Physical/Chemical assessments (though technically it is part of the Biohazard testing). The measurement should be at a level appropriate to assess a biohazard risk, and need not assess the absolute level of radioactivity present. Standard radiation safety protocols should be in place prior to the arrival of the Mars samples, but if the radioactivity level does not represent a biohazard, monitoring for radioactivity can be discontinued (unless required for equipment used in the SRF). If a biohazardous level of radioactivity is detected in the Mars samples, the radioactivity monitoring program would be continued. Other risks from equipment or facilities can be addressed by the use of standard procedures, training, and maintenance.
- *Chemical Hazard Monitoring.* A chemical hazard from the Mars samples would be most likely caused by non-biological, non-replicating toxins, if present. The presence of toxins will be assessed early in Physical/Chemical testing. If an unusual substance or chemical is

identified, specific monitoring methods for that substance can be designed. The substance could also be used as a marker for Mars sample breach of containment monitoring in the SRF and the environment.

- *Monitoring of Containment.* Standard methods for monitoring of containment can be adapted for use in implementing the PPLs, and can be used to define a breach of containment or potential personnel exposure. If a breach occurs within the SRF it can be corrected by standard procedures, and personnel exposures can be assessed. If a breach occurs to the environment outside the SRF, a standard procedure should be developed to assess possible consequences to the environment and/or to humans. Procedures for handling a breach of the SRF due to different causes (e.g., leak, disaster, security breach, etc.) should be considered in the development of the plans for handling a breach.
- *Monitoring of the Environment.*
  - ➢ *Before Mars Sample Arrival.* An assessment of the environment around the SRF should be made prior to the arrival of the Mars samples. Environmental monitoring should be implemented in compliance with the applicable and appropriate regulatory requirements, and in consultation with relevant U.S. and international agencies. The environmental assessment should survey the pre-existing conditions, and include an assessment of the water, air, flora, and fauna. This survey will likely be accomplished as part of the Environmental Impact Statement (or Environmental Assessment) required by the U.S. National Environmental Policy Act and that will be done prior to building the SRF. During the survey, sentinel species (including microbes, insects, plants, and animals) can be identified for use as baseline organisms for monitoring of environmental changes. Consideration should be given to including some of the same organisms, or closely related organisms, in Biohazard testing. In case changes in the environment around the SRF are noted after arrival of the Mars samples, the Biohazard testing results could assist in determining if the changes are related to the Mars samples. Environmental monitoring may also include surveillance of humans in the nearby population, if the facility's location warrants it. If so, NASA will use attendant, sensitive risk communication practices in implementation of all public health surveillance initiatives.

- ➢ *During Mars Sample Handling at the SRF.* Once the Mars samples are in the SRF, environmental monitoring can focus on the identified sentinel species and any novel components of the Mars samples, if identified. It also will be useful to track and record basic weather conditions in the area of the SRF as part of baseline data. In the event of a breach to the outside or any unusual occurrences or observations around the SRF, these data could prove useful in demonstrating either positive or negative correlation with actual or alleged impacts from SRF operations. Also, if routine monitoring reveals changes in the environment, procedures could be undertaken to assess whether an undetected breach has occurred. SRF personnel would assist with investigating the cause of the environmental change to establish whether it is related to the SRF and Mars samples. In the event of a breach, procedures should be followed to re-establish containment and clean up any detected contamination.
- ➢ *After Completion of Life Detection/Biohazard Testing.* The required level of continued environmental monitoring should be reassessed based on the outcome of the Mars sample testing protocols. Consideration should be given to the requirements for maintaining security and containment within the SRF to assure the proper transition to the long-term curation of the Mars samples.

- *Monitoring of the SRF Personnel.*

  - ➢ *Before Mars Sample Arrival.* A process of certification for people who will work in the SRF should be developed that will include security clearances, medical examinations and tests, and a thorough program of education about procedures to be employed in health monitoring as well as on the risks and requirements for employees. Clear inclusion and exclusion criteria for employees, based on the requirements of the certification process, should be developed prior to hiring of personnel.

    Baseline medical evaluations of personnel should use the existing medical evaluation standards appropriate at the time the evaluations are performed. Since the SRF will be functional for a period of time prior to the arrival of the Mars samples, monitoring before the arrival of the Mars samples should include several evaluations over time (a period of two years has been proposed). Recommended baseline evaluations include a medical history, physical examination, tests on the person (e.g., chest X-ray), and tests on samples from the person (e.g., blood and urine). All testing should be as non-invasive as possible, and maintain a balance between estimated risks from the Mars samples and the risks associated with the tests. Test specimens should also be archived for future comparison, if needed, and may include serum, lymphocytes, semen and/or hair. In addition, neuropsychological evaluations using standard testing techniques with well-established interpretation methods should be administered. Symptom data should be obtained using standardized instruments available at the time of the SRF commissioning.[27]

- *During Mars Sample Handling at the SRF.* A schedule for regular evaluations of personnel should be established, using the same evaluation methods adopted for the baseline data collection. Procedures for standard medical management of personnel illnesses should be available either on-site or with adequate transportation to a medical facility, as needed. Intervention should be correlated with exposure, or an identified risk of exposure, to the Mars samples. If an exposure occurs and the exposed individual has or develops symptoms, the person should be transferred to a medical facility with BSL-4 containment capabilities until proper assessment of the individual is accomplished. If an exposure occurs and the individual does not have or develop symptoms, procedures for quarantine of the individual should be developed with specific guidelines as to the length of quarantine required if the person remains asymptomatic. If an individual becomes symptomatic and there is no evidence of an exposure, the individual should be treated as appropriate for the symptoms, and monitoring should continue as prescribed by the Draft Protocol.

27. The exact survey instrument has not been identified, but it would be possible to use currently existing surveys, similar to the Millennium Cohort Study (U.S.) or the GAZEL Cohort survey (France), sponsored by the U.S. Department of Defense and INSERM, respectively. Current information about these two surveys, may be found online at: <http://www.gazel.inserm.fr> and <http://www.millenniumcohort.org>.

- ➢ *After Completion of Life Detection/Biohazard Testing.* The question of how long to continue monitoring of SRF personnel has to be addressed. Certainly, the duration of monitoring will be influenced heavily by the outcomes of the Life Detection and Biohazard testing. Several factors may need to be considered in this decision, such as the protection of the workers versus the protection of the general population. Clearly articulate decisions will be needed on whether to have lifetime surveillance for the personnel, or to have a mandatory period followed by optional reporting (if the risk is determined to be low). Monitoring could become optional if the samples are deemed safe by the Life Detection and Biohazard testing. The need for surveillance of relatives or people living close to the personnel should be considered. A distinction should be made between monitoring for risk management and the continued collection of data for a research study. The interpretation of personnel evaluations may require the use of a control group or population-based estimations of frequencies of different events. If so, sources for this information should be specified. Finally, the issue should be addressed on how to ensure provision of adequate health insurance or services to support any required long-term monitoring and care for the SRF personnel.

- *Monitoring at Secondary Sites.* The level of monitoring to be used at secondary sites receiving and working on portions of the Mars samples should be based on the results of the Life Detection and Biohazard testing. If the Mars samples are still potentially hazardous, or their biohazard status is unknown, several points should be considered in developing a protocol for monitoring at secondary sites. First, secondary sites should be identified prior to the arrival of the Mars samples, to allow for pre-certification of personnel and baseline data gathering. Second, all distributions of sample materials should be tracked, and procedures for monitoring of containment at the secondary sites should be developed. Third, consider monitoring personnel at secondary sites using the same protocols used at the SRF. The number of additional personnel exclusively located at secondary sites is expected to be small.

If the Mars samples are deemed safe, either through "sterilization" or by Biohazard test results, the methods should be used for tracking all sample distributions and all individuals in contact with the samples. In such a circumstance, only event reporting is needed.

Database Issues A central database with data analysis capabilities and procedures should be used for environmental data (baseline, monitoring), personnel data (baseline, during operations, follow-up), secondary site data, and sample tracking data. Procedures for regular data analysis and reporting should be developed. Access to, and confidentiality of, the data should be defined and assured. Data analysis should distinguish between surveillance and research, with consideration given to the requirements for ethical review and approval for any research protocols.

Future Research and Development Needs

- Criteria for inclusion/exclusion of personnel to work at the SRF or at secondary sites.
- The time frame of personnel monitoring, i.e., "lifetime" versus limited period (according to hazards).
- If long-term monitoring is implemented, which parameters to monitor on a long-term basis?
- Need for informed consent for testing and possible long-term monitoring.
- Level of baseline testing and monitoring for secondary site workers as compared to workers at the SRF.
- Protection of individuals from life-insurance or health-insurance discrimination.
- Procedures for database management and data analysis, with consideration of confidentiality and security issues.
- Should monitoring be restricted to relevant public health measures, as opposed to extending the Draft Protocol to allow for epidemiological research?
- Level of medical facilities needed at the SRF.

Summary Monitoring methods for personnel and the environment should be developed with consideration given to international regulatory, cultural, and ethical issues. The radiation and chemical risks are considered to be of low probability and can be assessed early in the chemical testing procedures to reduce the monitoring burden. Procedures must be developed for database management and data analysis, with assurances of confidentiality and security of the data. Procedures for monitoring personnel should include procedures for education and certification.

Personnel Management Considerations in Protocol Implementation

The staffing of the Sample Receiving Facility(-ies) can be accomplished in a number of ways. For example, scientists can be recruited to fill permanent positions at the SRF, or could be selected through a competitive grants program for work at the SRF, or some combination of the two approaches. Considering the variety of tasks that must be accomplish during design, construction, and operation of the facilities, as well as during implementation of the final protocol, it will be advisable to use a variety of different personnel selection processes. Personnel should be hired progressively during the development of the project and the facility(-ies). The functions and responsibilities of the Director's position will be substantially aided by appropriate committees and advisory groups. In the event that more than one facility is used, the required methods and procedures outlined in the Draft Protocol should be applied beyond the SRF to any facility or site planned to handle martian samples during the implementation of the final protocol. Because researchers and the public worldwide will have an interest in returned martian materials, the international character of the program should be respected throughout the entire process. Figure 7 on the next page presents a high level schedule and overview of the process from now until the samples are returned to Earth. One concept of the functions, staffing requirements, and organization for a Mars Sample Receiving Facility, is further elaborated in Figures 8, 9, and 10. These figures outline staffing needs and proposed organizations at 10-, 5- and 3-years before the arrival of actual samples at the SRF.

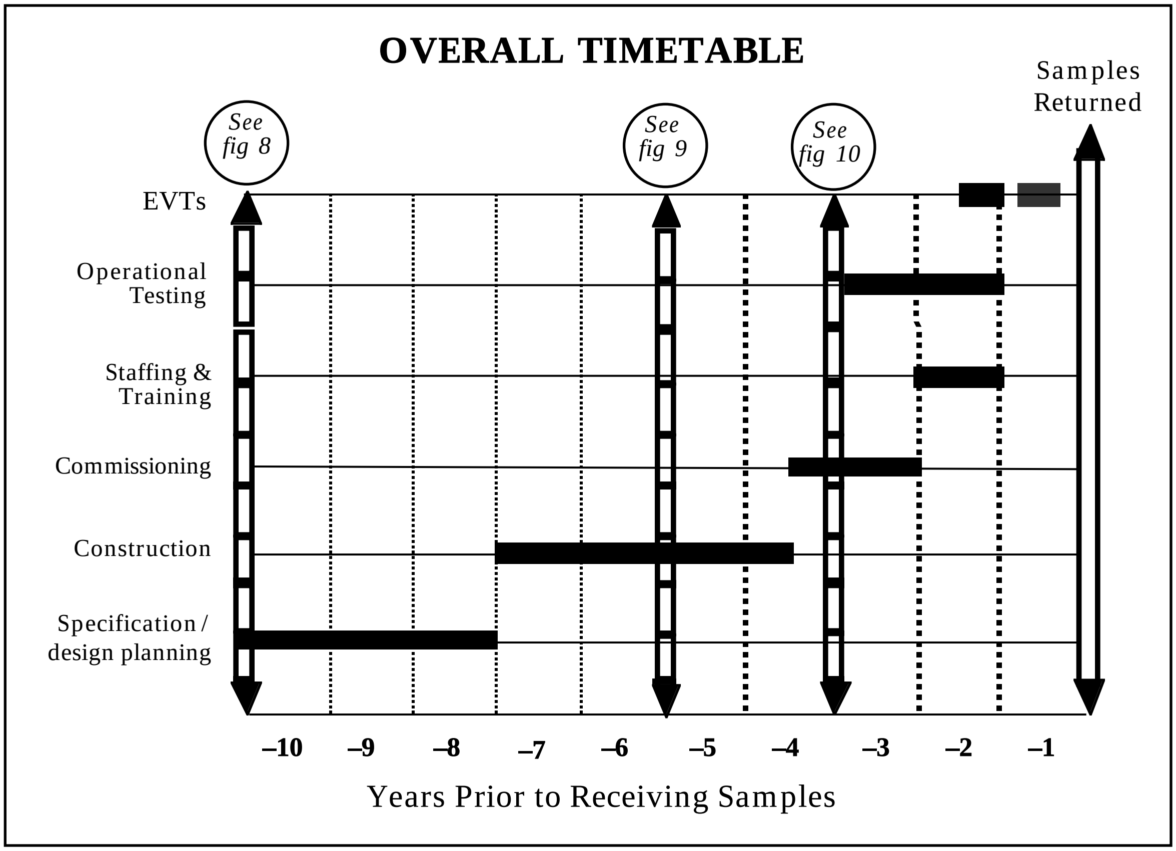

Figure 7. Example overall timetable of the required activities to design, build, and operate the SRF. The double-headed arrows indicate timing of the staff organization described in the subsequent figures (EVT = Experiment Verification Test).

These proposed management, staffing, and organizational frameworks amount to a working hypothesis for the design of the building and operation of the SRF, based on the following assumptions:

- The protocol must be fully and successfully tested before the actual handling of the martian samples. The exact makeup and sequence of the Experiment Verification Tests (EVTs) are TBD.
- It is estimated that a complete EVT will last approximately 6 months and at least one complete EVT must be demonstrated successfully before actual handling of the returned samples. Thus, the first EVT must begin no later than 18 months before the returned samples arrive at the SRF in order to

allow enough time to adjust and repeat the EVT, if necessary (at least 9-10 months before experiments begin on actual returned samples).

- These EVTs are consistent with the recommendation of the SSB (1997) and earlier Workshops in this Series that the SRF be operational two years before the arrival of the actual Mars samples. These EVTs are part of the normal operational testing.
- Based on experiences at other BSL-4 laboratories in the United States and France, no less than one-year is required to staff and properly train the technical and scientific personnel.
- Commissioning of the SRF, which can be performed in parallel with the staffing and training, will require at least 18 months.
- In order to accommodate the staffing, training and commissioning requirements of the SRF, construction of the facility must be finished 3 years before the actual operations. From past experiences, in France and the United States, construction of the facility itself will also require 3 years.
- It is estimated that about 3 years will be needed to develop design specifications and plans for the SRF, and obtain necessary authorizations to build the facility. To accommodate all the activities necessary to design, build and operate an SRF, the entire process must begin fully ten years in advance of sample return.

To illustrate one approach to staffing and organization that meets facility and protocol requirements, the text below provides specific details related to the recommended staffing and organizational plans. It is emphasized that these scenarios are not fixed requirements of this Draft Protocol, but are intended to provide a conceptual structure on which to base future organizational and staffing plans.

10 Years in Advance As soon as the decision is made to build and/or update a Mars SRF, ~10 years before the actual operations, four positions should be staffed in order to prepare specifications for future activities and a substantive review of the design of the facility (see Figure 8). The key positions to be filled 10 years prior to sample return are the Project Manager/Director, a Director for Administration, a Project Scientist/Director for Science, and an Environment, Health, and Safety Officer. The Director, who is responsible for the overall sample handling project implementation, will have the assistance of an SRF Oversight Committee. This Committee will monitor progress and assure compliance of the project with the final protocol and with whatever science requirements are to be implemented in the Facility. In this example, it is anticipated that the initial Director will have a background in scientific facility engineering, and that transition to a Director with a science background will occur after construction of the facility is assured. The

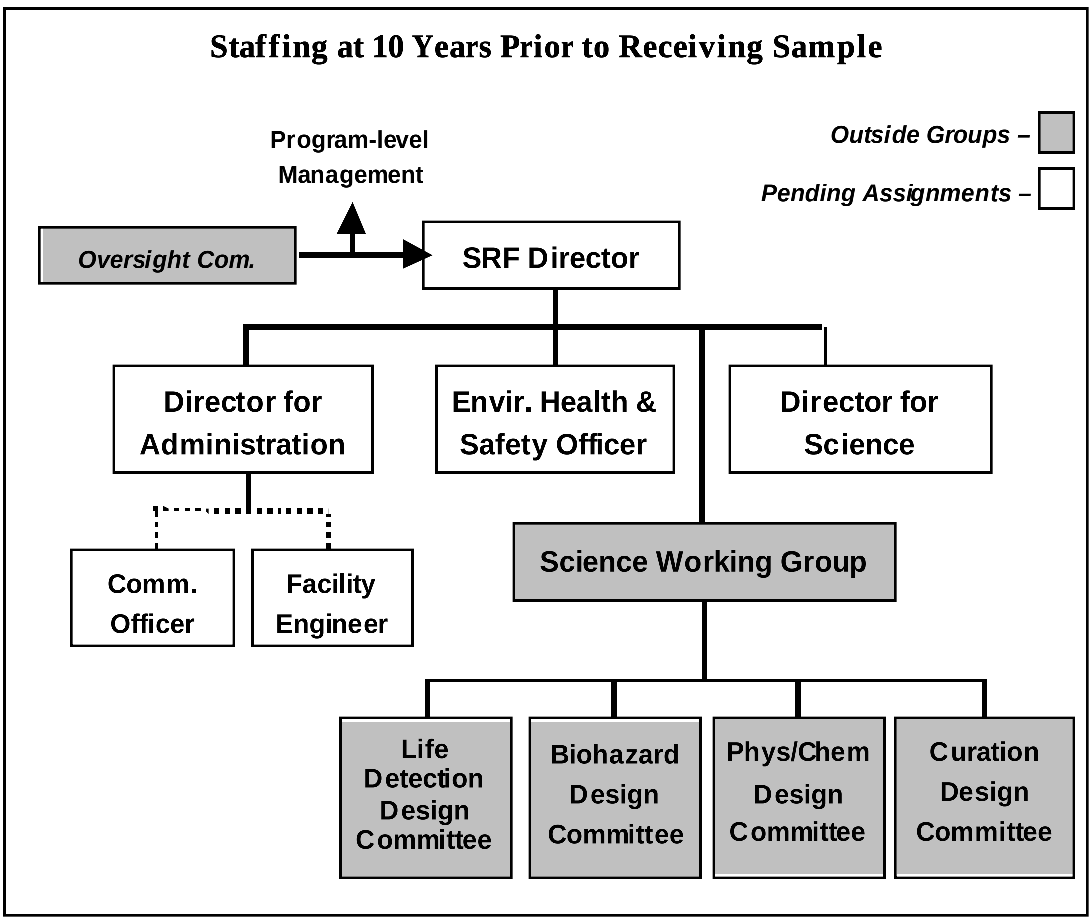

Figure 8. Top-level staffing requirements and structure of the SRF at 10 years prior to arrival of the returned sample(s). Permanent positions are in plain boxes; committees are in grey boxes. Not all positions are full-time.

Director will be assisted by the Environment, Health, and Safety Officer to ensure that the actual design requirements related to these critical topics are implemented properly. A Director for Administration will focus on budget and staffing issues, and the development of the staffing plan to cover the life of the project. Additional engineering support (e.g., the Facility Engineer) would be added as necessary.

The Project Scientist/Director for Science will coordinate the work of scientific committees and working groups that will develop science specifications and support the design process for their respective disciplines or areas. Also at this point in the project, a Communications Officer should be available, at least on a part-time basis, to ensure attention to risk communications and outreach—keeping the community informed and identifying and answering questions regarding the SRF. All communications, plans, and activities at the SRF should be consistent with those outlined in any comprehensive communication plan developed for the mission and the Mars exploration program as a whole (see the section titled "Maintaining and Updating the Protocol," below).

From the beginning of the process, three different kinds of committees should be installed to help the Directors and Scientific Discipline Heads in overseeing their changing responsibilities:

- The Science Working Group (SWG) will be charged with helping to guide the overall project during the construction phase, to provide recommendations and expertise in assuring its compliance with sample scientific requirements and the final protocol. The members of the SWG will be chosen from an *ad hoc* set of scientists representing the required disciplines and expertise. Later, they will be replaced by the Investigators Working Group, comprised of selected Principal Investigators from an open competition seeking proposals for sample analysis activities within the Facility.
- Scientific design committees will be specialized in four disciplines, Life Detection, Biohazard testing, Physical/Chemical, and Curation, with

members designated by the agencies participating in the mission. These committees will prepare the design and review and oversee the project to ensure the facility can operate consistent with the operational aspects of the planned protocol. As soon as the Scientific Discipline Heads are hired, these committees will become Discipline Advisory Panels to assist them.

- Finally, the SRF Oversight Committee will be composed of 12 to 15 members selected by the Program leadership, perhaps with some cross-membership from the NASA Planetary Protection Advisory Committee and the French Planetary Protection Committee. These committees will be in charge of reviewing the overall process and the proposed measures to comply with the requirements of the final protocol. The Science Oversight Committee will report to Program Management and the Planetary Protection Officer, above the level of the Project Manager/Facility Director. However, it is expected that they will interact directly with that Manager on a regular basis.

Membership on the various committees will be staggered to ensure an appropriate turnover without losing the "project memory." Agencies involved with the SRF should set up jointly an international search committee for recruitment of the Directors, various functional managers, the Facility Engineer, and the Scientific Discipline Heads**.**

5 Years in Advance At roughly midway through the construction of the facility, the Scientific Discipline Heads should be hired for each required scientific discipline (see Figure 9 on the next page). These managers will ensure that construction is completed properly to accommodate the specific needs of their disciplines. With the help of experts working as part of the scientific working group and discipline advisory panels, they will complete the general and specific operating procedures to handle the martian samples and the training program for staff to be hired. At this point, a Facility Administrative/Staff Manager will also be hired to assist in the hiring of the technical staff and prepare for future administrative and personnel needs of the facility.

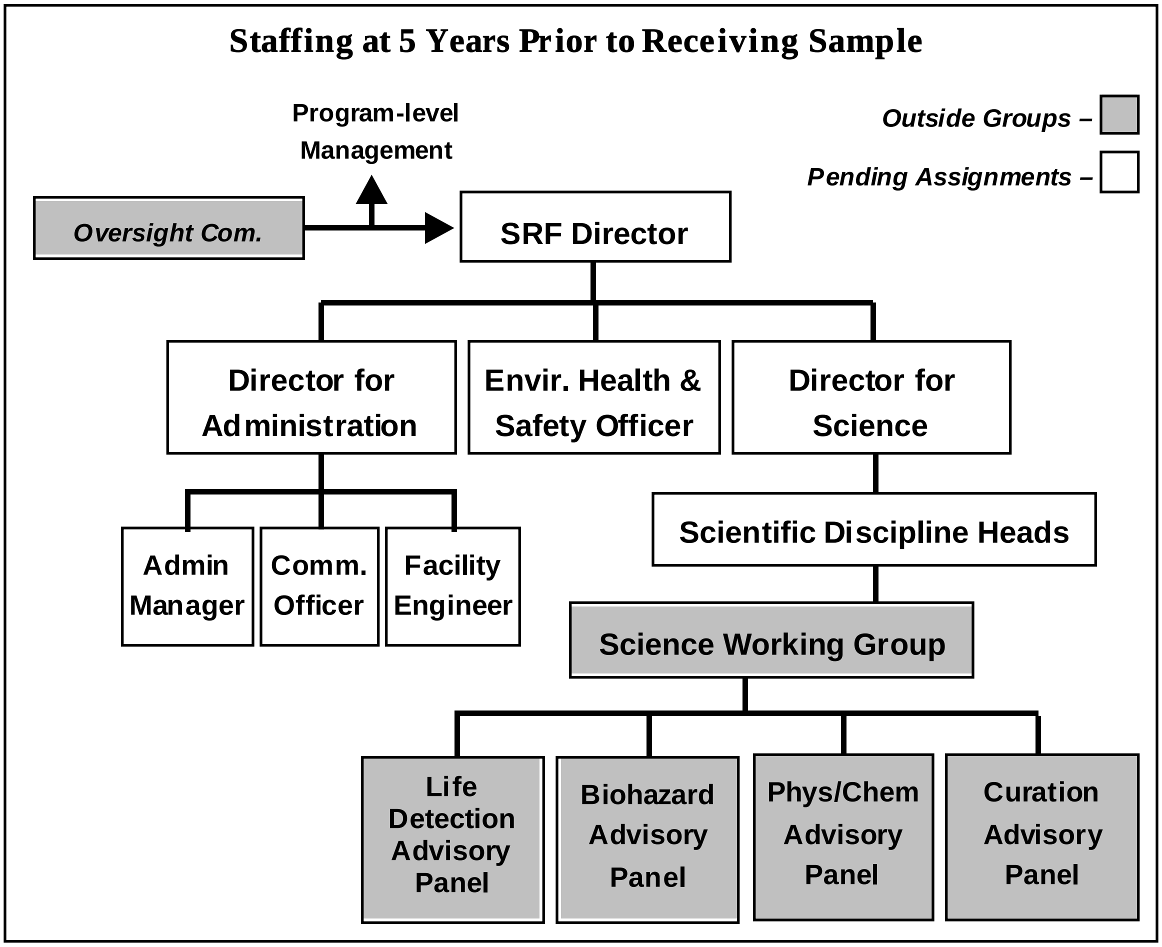

Figure 9. Top-level staffing requirements and structure of the SRF at 5 years prior to arrival of the returned sample(s). Permanent positions are in plain boxes; committees are in grey boxes.

<u>*3 Years in Advance*</u> In order to have a fully operational facility two years before samples are returned, the final staffing and training of various operational positions must begin three years prior to actual operations (see Figure 10). At this time, the required supporting groups, such as an Institutional Bio-Safety Committee (IBSC) and an Institutional Animal Care and Use Committee (IACUC), will be formed, and staff necessary to support facility operations, administrative functions, communications, and safety program implementation will be added, Also at this time, it is anticipated that the *ad hoc* Science Working Group (which until this time would have dealt with both science issues and issues of planetary

protection protocol compliance), will be supplanted by an Investigators Working Group selected through an open solicitation that would provide for scientific investigations to be accomplished within the facility. The relationship of these selected science investigations to the accomplishment of the protocol objectives may be close or distant, depending on the strategy undertaken to implement the protocol in its final form.

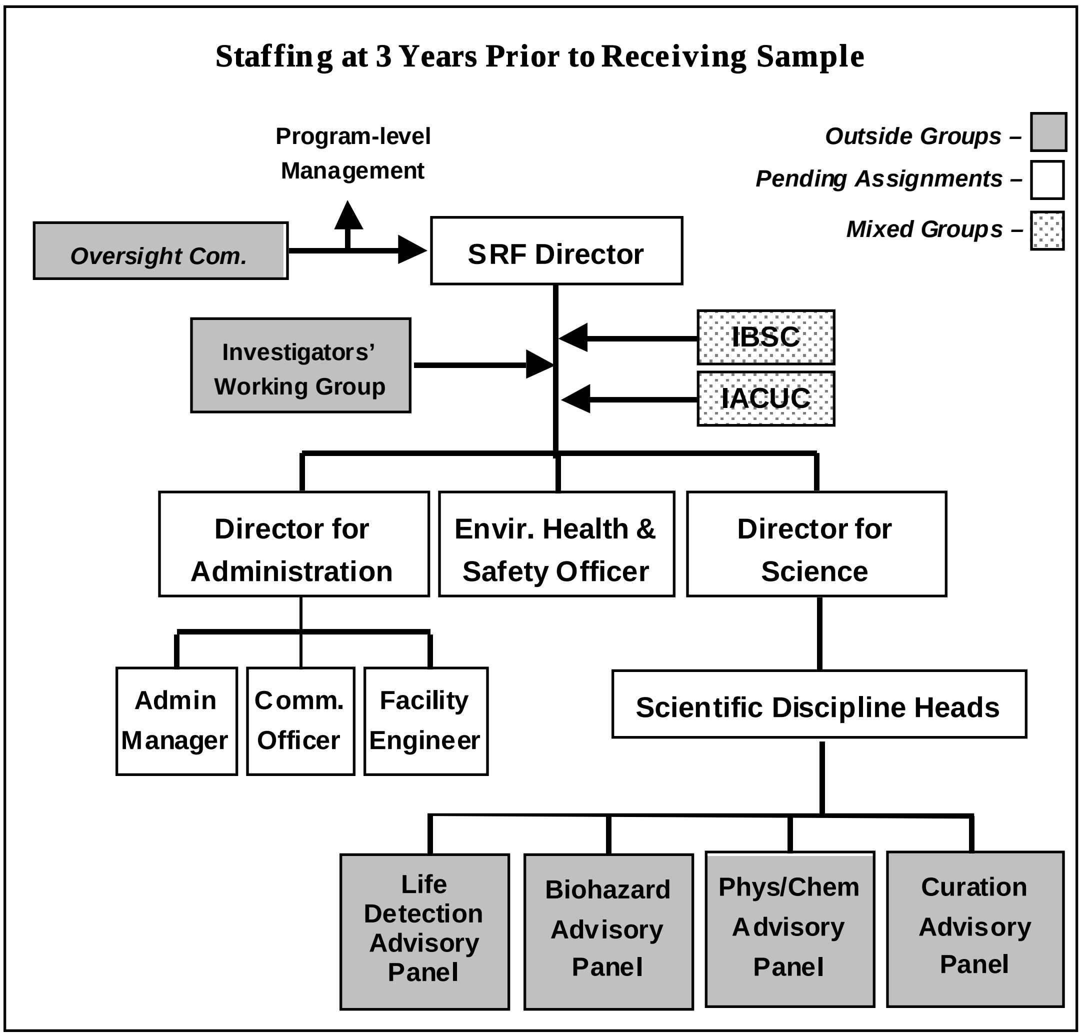

Figure 10. Staffing requirements and structure of the SRF at 3 years prior to arrival of the returned sample(s); permanent positions are in plain boxes; committees are in grey boxes; stippled boxes indicate an Institutional Bio-Safety Committee (IBSC) and an Institutional Animal Care and Use Committee (IACUC).

Future Considerations Three major issues will require further consideration in the overall staffing of the SRF.

1. Currently, no one has experience in simultaneous operations or activities in combined BSL-4 and cleanroom conditions as will be needed for PPL-α through PPL-δ. The advice of experts from the pharmaceutical or micro-process industries would be helpful.
2. Details on the optimal staffing mix at the SRF must be considered further. It is not clear what mix of government employees, semi-permanent staff employees, outside contractors, and guest scientists will be needed to staff the facility and implement the final protocol. In planning for facility staffing and operations, international access and participation should be considered throughout the process.
3. In order to comply with planetary protection constraints and protocol requirements, a sustained and adequate budget will be needed throughout the design, construction, and implementation phases of this project.

Contingency Planning for Different Protocol Outcomes

Developing contingency plans for different outcomes of the final protocol will require anticipating how the scientific community might interpret test results and react under a variety of possible scenarios following the return of martian samples. In addition to considering how to interpret possible scientific results, it will be important to plan how to respond in the face of possible breaches in containment. Recommended response to various likely scenarios are discussed below:

Organic Carbon It is likely that carbon will be found in sample materials. The sensitivity of current and future methods will be very high, so that at least some level of contaminants should be detected, and perhaps carbon compounds from Mars, as well. The existing base of knowledge on meteorites and other material collected from space will be useful in providing baseline information to help guide these investigations. Since the Viking results focused on volatile organics, further attention to the question is appropriate. *In situ* measurements of non-volatile organics on missions prior to the sample return mission would be useful to gauge predictions of anticipated sample organic content.

<u>*Extant Life or Biomarkers Positive*</u> If extant life or evidence of biomarkers are detected in the samples, all work on the samples will continue to be done in strict containment until more definitive data can be gathered (see *Release Criteria* and *Biohazard Testing* sections, above.) Maximum effort should be made to determine if any of the positive results are originating from Earth life or Mars life. Information management will become an issue, both for scientific communication and in shaping the debate among scientists. It will be important to plan for how and when initial information, with its attendant uncertainties, should be disseminated to the public.

<u>*Non-Earth Life Confirmed*</u> In keeping with the SSB recommendations *[SSB 1997]*, and the stated release criteria, sample materials will be released from containment only if they are shown to contain no extraterrestrial life-forms, or they are sterilized prior to release. If non-terrestrial life is confirmed, a previously constituted SRF Oversight Committee will need to review the protocol, the steps taken in support of the protocol, and ongoing provisions for containment. If a portion of a sample is confirmed as positive for non-terrestrial life, subsequent testing and analyses on all sample materials will continue in containment. This means that all physical, chemical, and geological characterization, as well as Life Detection and Biohazard tests requiring non-sterilized material should continue to be done in strict containment, either in the SRF or in any other test facilities that may be used. Experimentation on methods to sterilize samples containing the newly-discovered life should begin in conjunction with investigations of appropriate biological culture conditions. Once appropriate biological sterilization techniques can be validated, detailed plans for distribution of samples can be developed or revised in order to meet the established or revised scientific objectives. Management issues will include administrative and technical procedures for scientific study and curation, as well as informing the public.

Although it is premature to develop specific recommendations at this time, it is possible to identify issues that will need further discussion in advance of sample return. The concerns fall into three broad categories: Science and Testing; Facility and Technological; and Policy and Administrative.

Science and Testing Confirmation of a preliminary discovery of martian life should require a careful reconsideration of results from many parts of the final protocol, ranging from a review of preparation, through scanning and testing methods, to verification of biocontainment materials and sterilization techniques, and a reassessment of conditions for banking, storage, transportation and curation. If evidence of any martian life is found, there should be a plan to aggressively expand the studies with the expectation that there will be multiple, additional life forms, given that evidence that life can be supported on Mars. In addition, it will be important to understand the culture and environmental conditions that are required to maintain and perhaps to grow the new life-form to obtain more material for study in the lab, and what precautions are needed in the process. Also, it will be important to review the final protocol to recommend modifications in physical, geological, and chemical tests of sample materials, adding or deleting tests as needed.

Facility and Technological Concerns Questions about the adequacy of the SRF to maintain the new life form must also be addressed, including the possible need to add equipment, change operations, review emergency plans, or upgrade the facilities because of what has been found. Concerns about security should also be reconsidered, especially in view of the potential disruptive activities of any terrorists or 'radical' groups that may be opposed to sample return. The advisability of allowing distribution of untested sample material outside the SRF may need to be reconsidered, as well.

Policy and Administrative Concerns If martian life is detected, both short-and long-term policy issues will arise. The short-term listing of concerns relates to procedures regarding access to and distribution of sample materials, as well as to the publication and review of research findings. The chain of custody of sample materials will be important in the assessment of data quality, as well as in addressing the legal requirements of who is allowed to "touch" the sample (or verifying who has handled the sample appropriately or inappropriately). It will be critical to incorporate chain-of-custody considerations into the final protocol well in advance of sample return.

As part of sample return planning, it will be important to develop an organized communication plan which will lay a strong foundation in public understanding and acceptance prior to the mission, and allow for an open dialogue with all sectors of the public. Such a plan should include consideration of the diverse questions, concerns, and issues likely to be raised, including those related to the mission and spacecraft operations, the sample return and Biohazard testing, the administrative and legal matters associated with the effort, and to the potential implications of discovering extraterrestrial life. Plans should be developed well in advance in order to avoid a frenzied, reactive mode of communications between government officials, the scientific community, the mass media, and the public. Any plan that is developed should avoid a NASA-centric focus by including linkages with other government agencies, international partners, and external organizations, as appropriate. It will also be advisable to anticipate the kinds of questions the public might ask, and to disclose information early and often to address their concerns, whether scientific or non-scientific.

In the long term, the discovery of extraterrestrial life, whether extant or extinct, *in situ* or within returned sample materials, will also have implications beyond science and the SRF *per se*. Such a discovery would likely trigger a review of sample return missions, and plans for both robotic and human missions. Legal questions could arise about ownership of the data, or of the entity itself, potentially compounded by differences in laws between the United States and the countries of international partners. In any event, ethical, legal and social issues should be considered seriously. Expertise in these areas should be reflected in the membership on appropriate oversight committee(s).

Contradictory/Inconsistent Results Given the number of techniques, spanning several scientific disciplines, it is very likely that contradictory or inconsistent results will be found. Differences in the sensitivity of methods will exist and confidence in the reliability and level of experimental controls will differ among procedures. It is important to stress the need for replication of experiments and duplication of results among multiple sites to add confidence to the results assessed. In addition, it will be important to follow a strict scientific procedure for interpreting data and making decisions about sample materials. There is a need to involve multidisciplinary experts and groups in the overall decision making process as well as in devising procedures for drawing conclusions, certifying results, and deciding whether samples are safe enough to be released to lower containment levels.

Application of Release Criteria According to the COMPLEX report on '*The Quarantine and Certification of Martian Samples' [SSB 2002]:*

> *"If the samples are shown to be altogether barren of organic matter, to contain no detectable organic carbon compounds and no other evidence of past or present biological activity, untreated aliquots of the samples should be released for study beyond the confines of the Quarantine Facility."*

The stated goal of the MSHP Workshop Series was to design a protocol to test returned sample(s) for biohazards and the presence of martian life, to ensure that a sample is safe to be released without sterilization, for further study. The release criteria listed in this Draft Protocol are consistent with the cited NRC recommendation, but this Draft Protocol imposes the additional requirement to complete Biohazard testing on all samples, taking into account the possibility of non-carbon-based life. As such, this Draft Protocol is more conservative than the most recent NRC recommendation *[SSB 2002]*, but justifiably so in terms of what is known and not known about life elsewhere.

Conversely, arguments have been advanced suggesting that a sterilization step be added to the protocol for "good measure," for the release of any materials, even if the samples are devoid of organic compounds and do not demonstrate any biohazard. After an evaluation of the arguments advanced regarding this concept, both pro and con, this additional step is not required by this Draft Protocol. Central to an understanding of the arguments is the question of risk, i.e., Can *any* protocol be guaranteed to be absolutely risk-free? If not, what is an acceptable level of risk (for example, one that approximates the risk from the natural influx of martian materials into Earth's biosphere)? And, is there any treatment method that can eliminate all risks from the returned samples, while preserving them for the detailed scientific study envisaged by the scientific community? Clearly, the issue of sterilization will require serious additional attention and research well in advance of sample return. Likewise, the safety of releasing materials that have passed both Life Detection and Biohazard testing should be carefully challenged through a rigorous quality assurance program applied to the completion of the Draft Protocol.

Breach of Containment Anticipating a containment breach and planning for such an event is an essential element of facility management. The responses to a breach will depend on where it occurs and what happens. Conceivably, it could occur in an area with a high population density or in a remote location. The breach could be a result of an accident or a crime—as a result of activity either outside or within containment. Required steps on how to handle breaches (based on long term experience and emergency plans for handling pathogenic biological material under BSL-3 and BSL-4 containment), are known. Additional information for responding to breaches and containment problems has been gained through decades of experience in handling lunar and other extraterrestrial materials.

Clearly, an emergency plan will be needed well in advance to develop recommended responses to various breach scenarios. The first steps will involve investigation of the degree of compromise, considering both biosafety and sample integrity. Full documentation of any breach event will be required as well as identifying the degree of sample compromise, what organizations or personnel should be involved in all phases of a response, and how notifications and communications should be handled. The plan should focus on all aspects of mitigation, cleanup, and recovery from perspectives of both biosafety and sample integrity (e.g., decontamination of the area, sample recovery, re-packaging and labeling as compromised, or destruction if required, etc.).

Maintaining and Updating the Protocol

The recent report from the NRC *[SSB 2002]* recommended:

> *"A continuing committee of senior biologists and geochemists that includes appropriate international representation should be formed and charged with reviewing every step of the planning, construction, and employment of the Mars Quarantine Facility. The committee should be formed during the earliest stages of planning for a Mars sample return mission. Members of the committee should also participate in the design of the spacecraft and those portions of the mission profile where biological contamination is a threat."*

This Draft Protocol refers to the necessary committees, including the SRF Oversight Committee, and the NASA Planetary Protection Advisory Committee (PPAC). The protocol implementation and update process will require establishment of these expert oversight and review committees, re-evaluations of proposed plans at key points in time before sample return, and open communication with scientists, international partners, and the public regarding risks, benefits, and plans. The scope of the task is summarized in Figure 11. A narrative explanation of recommendations and activities in the process follows.

Final Scientific and Policy Reviews Reviews of the Draft Protocol should provide for the highest degree of scientific scrutiny and evaluation.[28] The evaluation should be conducted jointly by scientific organizations from both the United States and

28. This Protocol was jointly derived by NASA and CNES, reflecting their intention to jointly accomplish the sample return mission. A final protocol should reflect reviews by all of the eventual mission partners.

France (and other countries, as appropriate) to avoid prolonged negotiations and resolutions that may arise when such reviews are conducted separately. This review should probably occur at the level of the National Research Council in the United States, and its equivalent scientific organization in France, whichever is most appropriate (among the French institutions discussed were Centre National de la Recherche Scientifique (CNRS), or representatives of various Etablissements Publics à Caractère Scientifique et Technique (EPST), including – but not exclusively – CNRS or Académie des Sciences). Final decisions about which institutions should be involved in scientific reviews are TBD, but should include NASA's Planetary Protection Advisory Committee, and the French multi-Ministry-sponsored Planetary Protection Committee.

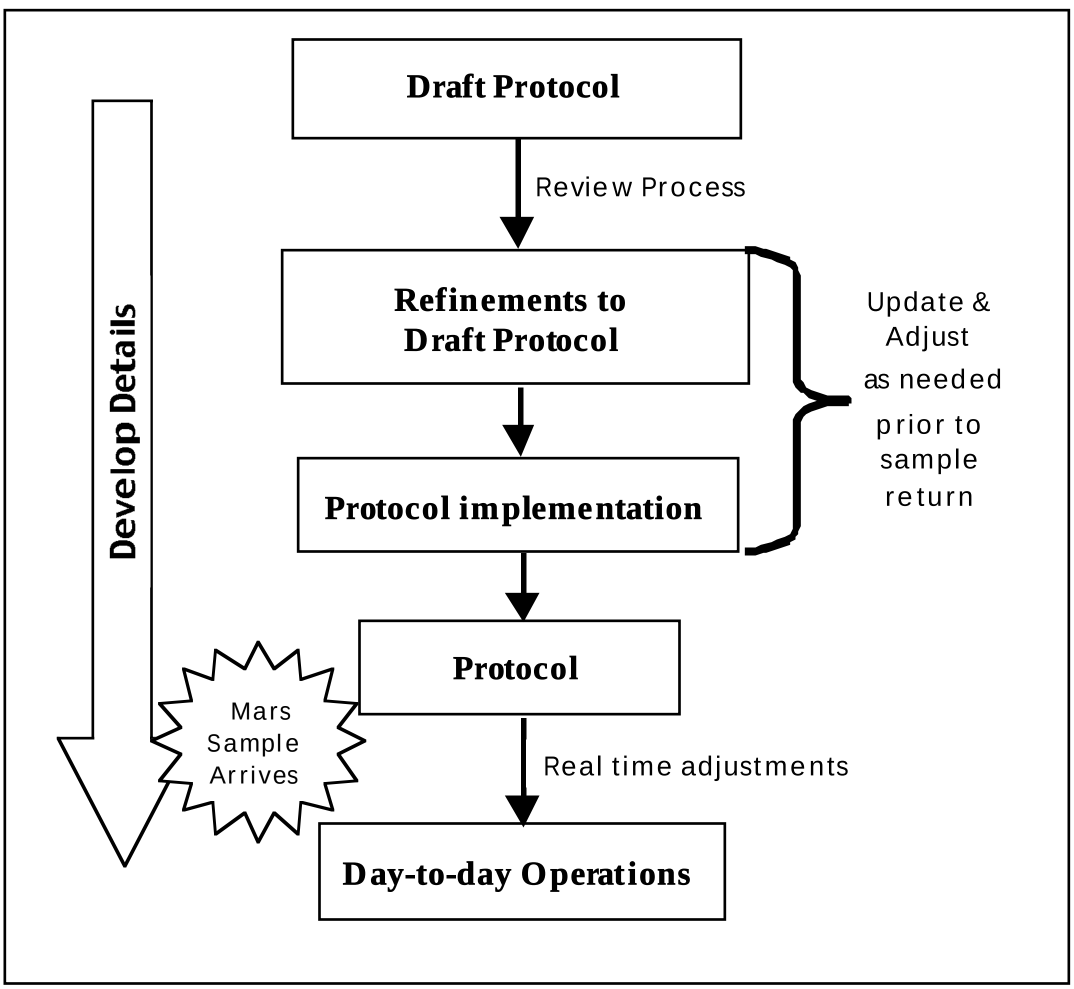

Figure 11. Protocol update and implementation process.

Clarity of Meaning and Terminology Clarity of meaning is essential to the implementation of any process especially when the process involves international agreements. Therefore, absolute consistency should be used in the language for any documents and charters associated with the eventual final protocol. When the actual definition of a word or phrase is in dispute, reference should be made to those definitions or meanings that are standard and accepted when interpreted at the international level. Clarity in terminology will be especially important when describing levels of containment to avoid confusion caused by mixing United States and French definitions of BSL and P4 containment. PPL containment definitions should be jointly derived to avoid these mixed meanings.

Ethical and Public Reviews Evaluations of the proposal should be conducted both internal and external to NASA and Centre National d'Etudes Spatiale (CNES) and the space research communities in the nations participating in the mission. An ethical review should be conducted at least at the level of the Agencies participating and these reviews made public early in the process (in France, the national bioethics committee, Comité Consultatif National d'Ethique pour les Sciences de la Vie et de la Santé, CCNE, is the appropriate organization). The final protocol should be announced broadly to the scientific community with a request for comments and input from scientific societies and other interested organizations. Broad acceptance at both lay public and scientific levels is essential to the overall success of this research effort.

Future Modifications to the Protocol When a final protocol has been adopted and approved by a consensus of appropriate scientific organizations, few changes should be made to its content. Changes should be made as scientific information, methodology, and/or technology improve between the time of the approval and the actual physical implementation of the final protocol within the SRF laboratories. Changes in methodologies or technologies to be used in implementing the final protocol may be considered if a proposed change would meet the following criteria:

- Increases the sensitivity or selectivity of the test,
- Reduces the length of time necessary for a test without a reduction in sensitivity or selectivity,
- Reduces the complexity of the sample handling process,
- Increases the overall safety of the process,
- Reduces the chances of contamination to the sample or the environment,
- Reduces the cost of the process, or
- Represents a new technology or method that has the broad, general acceptance of the scientific community.

Changes to the final protocol should receive appropriate expert review at the same level as the initial document.

Advisory Committees and Expert Panels Changes in scientific methodology and instrumentation are inevitable due to the long development time envisaged for this mission. This necessitates long term, consistent, input and advice from the external scientific communities of the partners engaged in the mission. To facilitate this process, a standing Planetary Protection Advisory Committee (PPAC) is being appointed in the United States to provide input to the NASA Office of Space Science and the NASA Planetary Protection Officer, and that a similar standing committee (Planetary Protection Committee, PPC) is being appointed in France. Both of these committees should provide for the participation of representatives of governmental regulatory agencies to make use of their particular expertise as well as to enhance communications among those various agencies, NASA, and CNES.

Standing joint working committees or specialized expert panels should be appointed (perhaps in cooperation with the SRF's Science Working Group) with appropriate expertise to provide support and advice to the United States PPAC and the French PPC in each of three specific areas: technical processes, scientific procedures, and safety/biosafety issues. To provide the most effective level of support, these groups should be comprised of members with expertise in a particular area of concern and organized into individual panels. No expert should be a member of more than one panel. The overall membership of the committees and expert panels should be selected to meet the specific needs of the agencies, and should represent the scientific goals of the agencies and the external science communities. Their work should aim at providing the respective agencies with information essential to the success and safety of the Mars sample return missions. These panels and committees may function jointly or independently depending on the specific need.

The PPAC and French PPC should receive the annual reports of the three panels, which will also provide annual written reviews to the NASA Planetary Protection Officer and, in France, to the appropriate Minister to whom the committee reports. These reviews should include relevant operational issues and concerns and provide risk assessments of the technical processes, scientific procedures, and safety/biosafety plans and processes. These reviews should be made available to scientific and professional organizations with interests in the mission activities.

Communications Unusual or unprecedented scientific activities are often subject to extreme scrutiny at both the scientific and political levels. Therefore, a communication plan must be developed as early as possible to ensure timely, and accurate dissemination of information to the public about the sample return mission, and to address concerns and perceptions about associated risks. The communication plan should be pro-active and designed in a manner that allows the public and stakeholders to participate in an open, honest dialogue about all phases of the mission with NASA, policy makers, and international partners. Risk management and planetary protection information should be balanced with education/outreach from the scientific perspective about the anticipated benefits and uncertainties associated with Mars exploration and sample return. The communication plan should also address how the public and scientific community will be informed of results and findings during Life Detection and Biohazard testing, including the potential discovery of extraterrestrial life. Because of the intense interest likely during initial sample receipt, containment, and testing, procedures and criteria should be developed in advance for determining when and how observations or data may be designated as "results suitable for formal announcement." Details about the release of SRF information, the management of the communication plan, and its relationship to the overall communications effort of the international Mars exploration program should be decided well in advance of the implementation of this protocol.

Flow Charts and Timelines In order to assure the rational use both of the facilities and sample materials, development of appropriate flow charts and time lines will be needed to coordinate the complex series of interrelated procedures. Safety issues must be prominent at all significant decision points in the process (e.g., release from containment, and downgrading to lower level of containment). It is essential to identify the critical points for these decisions in advance so that all participants understand their timing, and to ensure that such decisions are not negotiated in haste. Flow diagrams are intended to coordinate complex testing and inclusion of all required elements, especially those concerning biosafety and biohazards leading to the sharing of sample material with the external scientific community. In addition to containing timelines, procedures and processes, flow charts should also include key decision points for changing the status of the sample to a less restrictive PPL and proceeding in a particular direction along branches of the decision tree. Each such chart should incorporate a risk tree and assessment process.

Workshops/Reviews The need to change schedules and procedures may be anticipated during the time between now and sample return. To provide assurance that rules exist between the involved international partners and the scientific communities, two workshop/reviews should be scheduled prior to sample return to Earth in order to reaffirm details about process, methodology, safety, and release criteria. The first review should be conducted at the conclusion of the facilities design phase to determine if the physical structure meets the scientific and safety standards as defined within the specifications. In addition, the first workshop should review the existing procedures that will be conducted within the facility(ies) to confirm the specific flow chart outlining the approved sequence of tests and analyses. A second similar workshop/review should occur after the samples have been collected on Mars, but in advance of their actual return to Earth for evaluation. Details about who should coordinate these workshop/reviews and modify schedules or procedures are TBD.

Preparations and Processes for Decision Making about Release of Samples It will be important to make advanced preparations for organized data interpretation and decision making. These preparations will be especially critical in the event that a distinctly martian life-form is found within the returned samples. While it is impossible to develop details of the protocol at this time, it will be crucial to have considered how decisions will be made, by whom, and based on what principles if an extraterrestrial life-form is discovered. A specific committee should be established at least a year ahead of sample return to develop contingency protocols and processes that will be in place if and when martian life is found and verified. It is likely that protocol test results will not lead to unanimous decisions in all instances. It will thus be important to have a review and approval infrastructure for handling decisions about whether to release sample materials from containment, or reduce containment to a lower level upon completion of the final protocol tests. Addressing the overall decision making process in a formal manner will be critical for drawing conclusions, certifying results, and deciding whether samples are releasable or not. Any decision to release samples should involve selected multidisciplinary experts and groups, such as an Interagency Committee on Back Contamination (ICBC) similar to the one used during the *Apollo* program. The U.S. PPAC and French PPC should be involved in reporting to relevant bodies in their respective countries. Details on the structure(s) associated with decision making are TBD.

The organizational structures, management plans, charters and reporting lines for many of the proposed committees and groups will need to be developed in the coming years. Many questions cannot be resolved until additional details on facility design, operational logistics, mission architecture or anticipated schedules are made available. Future work should use this Draft Protocol to support the development of these items.

APPENDIX A:
MSHP WORKSHOP SERIES BASIC ASSUMPTIONS

The Mars Sample Handling Protocol (MSHP) Workshop Series was designed to touch on a variety of questions in pursuit of the stated objective, such as: "What types/categories of tests (e.g., biohazard; life detection) should be performed upon the samples? What criteria must be satisfied to demonstrate that the samples do not present a biohazard? What constitutes a representative sample to be tested? What is the minimum allocation of sample material required for analyses exclusive to the Protocol, and what Physical/Chemical analyses are required to complement biochemical or biological screening of sample material? Which analyses must be done within containment and which can be accomplished using sterilized material outside of containment? What facility capabilities are required to complete the Protocol? What is the minimum amount of time required to complete a hazard determination Protocol? By what process should the Protocol be modified to accommodate new technologies that may be brought to practice in the coming years (i.e., from the time that a sample receiving facility would be operational through the subsequent return of the first martian samples?)

To keep the Workshops focused, a set of basic assumptions were provided to guide and constrain deliberations; these assumptions were:

1. Regardless of which mission architecture is eventually selected, samples will be returned from martian sites which were selected based on findings and data from the Mars Surveyor program missions.
2. Samples will be returned *sometime in the next decade*.
3. Samples will not be sterilized prior to return to Earth.
4. The exterior of the Sample Return Canister will be free from contamination by Mars materials.
5. When the Sample Return Canister (SRC) is returned to Earth, it will be opened only in a Sample Receiving Facility (SRF) where samples will

undergo rigorous testing under containment and quarantine prior to any controlled distribution (‘release’) for scientific study.

6. The amount of sample to be returned in a SRC is anticipated to be 500-1000 grams.
7. The sample will likely be a mixture of types including rock cores, pebbles, soil, and atmospheric gases.
8. The amount of sample used to determine if biohazards are present must be the minimum amount necessary.
9. Samples must be handled and processed in such a way as to prevent terrestrial (chemical or biological) contamination.
10. Strict containment of unsterilized samples will be maintained until quarantine testing for biohazards and Life Detection is accomplished. Sub-samples of selected materials may be allowed outside containment only if they are sterilized first.
11. The SRF will have the capability to accomplish effective sterilization of sub-samples as needed.
12. The SRF will be operational two years before samples are returned to Earth.
13. The primary objective of the SRF and protocols is to determine Whether the returned samples constitute a threat to the Earth’s biosphere and populations (not science study *per se*) and to contain them until this determination is made.

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APPENDIX E: GLOSSARY OF TERMS AND ACRONYMS

ALH	Alan Hills (Antarctica)
BFP	Blue Fluorescent Protein
BHK cells	A cloned cell line widely used as a viral host, in studies of oncogenic transformation and of cell physiology.
BSL	Biosafety Level
CAPTEM	Curation and Analysis Planning Team for Extraterrestrial Materials (NASA)
CCNE	Comité Consultatif National d'Ethique pour les Sciences de la Vie et de la Santé (French)
CDC	Centers for Disease Control and Prevention (U.S.)
‘cleanliness’	Free from biological or chemical contamination
CNES	Centre National d’Etudes Spatiale (French)
CNRS	Centre National de la Recherche Scientifique (French)
COMPLEX	Committee on Planetary and Lunar Exploration (U.S.)
‘coupons’	Small, regular samples of solid laboratory materials such as plastic
CP	Conference Proceedings (NASA)
D_{37}	The average radiation dose required to inactivate a live or infectious particle
DNA	Deoxyribonucleic Acid
Eh	Oxidation Potential
EPST	Etablissements Publics à Caractère Scientifique (French)
EVT	Experiment Verification Test
GC/MS	Gas Chromatograph/Mass Spectrometer
GFP	Green Fluorescent Protein
HEPA	High Efficiency Particulate Air (filter)
HHS	Department of Health and Human Services (U.S.)

IACUC	Institutional Animal Care and Use Committee
IBSC	Institutional Bio-Safety Committee
i.c.	Intracranially
ICBC	Interagency Committee on Back Contamination
INSERM	Institut National de la Santé et de la Recherche Médicale (French)
i.p.	Intraperitoneally
IR	Infrared
Knockout mouse	A mouse that is genetically engineered (both alleles of a critically targeted gene are replaced by an inactive allele using homologous recombination) to produce a particular designer alteration whereby a specifically targeted gene becomes inactivated (or "knocked-out")
LAL	*Limulus* Amebocyte Lysate
LC/MS	Liquid Chromatograph/Mass Spectrometer
LD/BH	Life Detection/Biohazard (Testing)
LD/MS	Laser Desorption Mass Spectroscopy
MeV	Mega Electron Volts
Mrads	Megarads
MS	Mass Spectroscopy
MSHARP	Mars Sample Handling and Requirements Panel (NASA)
MSHP	Mars Sample Handling Protocol
MSR	Mars Sample Return
NAS	National Academy of Science (U.S.)
NASA	National Aeronautics and Space Administration (U.S.)
Nd:YAG	Neodymium-doped:Yttrium Aluminum Garnet (Laser)
NIH	National Institutes of Health (U.S.)
NPD	NASA Policy Directive
NRC	National Research Council (U.S.)

Nude mouse	A mouse that lacks a thymus and, therefore, cannot generate mature T lymphocytes to mount most types of immune responses
PAH	Polycyclic Aromatic Hydrocarbon
‘passaging’	A sub-culturing technique
P/C	Physical and Chemical (Testing)
PCR	Polymerase Chain Reaction
per os	Oral administration (e.g., that a drug is to be swallowed)
pH	Measure of hydrogen ion concentration (acidity)
PP	Planetary Protection
PPAC	Planetary Protection Advisory Committee (NASA)
PPC	Planetary Protection Committee (French)
PPL	Planetary Protection Level
rDNA	Ribosomal DNA
‘readout’	A measure of potential biohazard effect
‘riffle splitter’	A mechanical separation device used for geological samples
RNA	Ribonucleic Acid
‘rocklets’	Millimeter-sized rock fragments
SCID	Severely Compromised Immunodeficient
SCID-Hu	Severely Compromised Immunodeficient (human)
‘simulant’	Analogue
SP	Special Publication (NASA)
SRC	Sample Return Canister
SRF	Sample Receiving Facility
SSB	Space Studies Board (U.S.)
TBC	To Be Confirmed
TBD	To Be Determined
TEM	Transmission Electron Microscopy
TM	Technical Memorandum (NASA)
TOC	Total Organic Carbon

USAMRIID	U.S. Army Medical Research Institute of Infectious Diseases
USDA	U.S. Department of Agriculture
UV	Ultraviolet
WHO	World Health Organization
'witness plates'	Controls for forward contamination; used to monitor for bioload on spacecraft
XRD	X-ray Diffraction
XRF	X-ray Fluorescence

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14. ABSTRACT

This document presents the first complete draft of a protocol for detecting possible biohazards in Mars samples returned to Earth; it is the final product of the Mars Sample Handling Protocol Workshop Series, convened in 2000-2001 by NASA's Planetary Protection Officer. The goal of the five-workshop Series was to develop a comprehensive protocol by which returned martian sample materials could be assessed for the presence of any biological hazard(s) while safeguarding the purity of the samples from possible terrestrial contamination. The reference numbers for the proceedings from the five individual Workshops (1, 2, 2a, 3, and 4) are: NASA/CP-2000-20964, NASA/CP-2001-210923, NASA/CP-2001-210924, NASA/CP-2001-211388, NASA/CP-2002-211841.

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