NASA Langley Research Center
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Game Changing Development Program

Synthetic Biology Project Plan

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This Synthetic Biology project plan will follow the NPR 7120.8 process for the research and development areas, and a tailored 7120.5 for the BioNutrients flight activities. The plan outlined in this document is valid for the life cycle of the project. A GCD Change Request (CR) is used to document changes from the plan. It is the responsibility of each of the signing parties to notify the others in the event that the plan needs to be revised.

Synthetic Biology - Signature Page

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1.0 Project Goals

Many of the current logistical strategies developed for crewed missions within low Earth orbit (LEO) are impractical for future, long-duration lunar and Mars exploration missions. To meet logistic requirements, current missions commonly rely on the four "R's" - resupply, repair by replacement, redundant systems, and retreat to Earth. This approach results in substantial mass and volume requirements that are highly proportional to mission duration. Additionally, there are no guarantees that all required items will be available by supply or resupply strategies. To enable greater mission self-sufficiency for long-duration missions to the moon and beyond, a new paradigm is required – one that enables mission planners to adopt a "make it , don't take it" approach by using advanced *In Situ* Resource Utilization (ISRU) and In-Space Manufacturing (ISM) techniques for on-demand production.

The Space Technology Mission Directorate (STMD) Game Changing Development (GCD) Synthetic Biology (Syn Bio) project is designed to demonstrate the value of emerging synthetic biology approaches to meet mission demands. It is the intent to leverage new techniques to design, build, and test biomanufacturing systems; capable of operating in demanding space environments, be able to withstand extended periods of storage before use, and perform safely and reliably.

In addition, the Syn Bio project is also focusing on methods to reduce the logistical requirements for biomanufacturing in space. In particular, the project is focusing on using *in situ* resources (i.e., CO_2) that can be converted to the organic substrates required for heterotrophic microbial growth. This is an important capability that will allow biomanufacturing systems to scale to viable production systems for mission products such as food components, pharmaceuticals, polymers, fuels, and a range of valuable chemicals. Such capabilities will also spur progress for purely chemical (non-biological) synthesis methods for *in situ* product generation.

Technology Gaps – While terrestrial microbial biomanufacturing processes are capable of making a vast array of commercial products, there are numerous technical challenges associated with implementing biomanufacturing during long-duration missions. The overarching needs include:

1. Maintaining viable microorganisms for numerous years to be ready for use when needed.

   This is made more complicated in that space-based, long-duration storage is likely to be at room-temperature, rather than the optimal -80°C typically used terrestrially. Therefore, improved microbial stasis / storage methods need to be evolved and tested in space and for long durations.

2. Low power, mass, and volume microbial reactors that perform well in reduced and / or microgravity conditions.

   Terrestrial reactors are commonly large, heavy metal reactors with a wide range of supporting hardware and require significant cleaning and sterilizing operations. In contrast, space-based bioreactors need to be developed to be small and light, require little maintenance and ensure sterility, desirable gas diffusion, effective fluids management, and efficient biomass and product harvesting.

3. Use of *in situ* resources to reduce required biomanufacturing consumables.

   Consumable usage can be substantial in terrestrial microbial biomanufacturing processes, particularly growth media supply and water requirements. Scaled space biomanufacturing will require the ability to efficiently and rapidly make readily-metabolized media from *in situ* resources such as CO_2 and water.

4. Developing methods to ensure products meet product safety and quality requirements.

   Products such as nutrients, food components, and pharmaceuticals require stringent quality standards for both safety and effectiveness. Common quality control processes used in terrestrial operations utilize extensive resources and methods that are not space-compatible. Low-overhead procedural and in-space monitoring systems will need to be developed to meet customized requirements.

The Syn Bio project includes two inter-related technology tasks to address the above technology gaps and develop and demonstrate valuable biomanufacturing capabilities that will serve future missions. They include:

1. BioNutrients

   This effort focuses on the on-demand production of fresh, high-value nutrients that supplement crew food supplies, thereby addressing the observed degradation of certain nutrients in stored foods and supplements during long-duration missions to the moon or Mars.

2. CO_2-Based Manufacturing

   This task is developing and testing a demonstration system that reduces media supply costs via a hybrid method of *in situ* physico-chemical (P / C) conversion of local CO_2 and water resources to small organic molecules that are subsequently used to feed microorganisms that are engineered to biomanufacture mission-relevant products.

These efforts directly address the STMD Strategic Thrusts:

ST1. Expand Utilization of Space: Enable servicing, assembly, manufacturing, and resource utilization.

ST5. Enable Humans to Live and Explore in Space and on Planetary Surfaces: Provide efficient / scalable infrastructure to support exploration at scale

ST6. Grow and Utilize the U.S. Industrial and Academic Base: Expand public-private partnerships for mutually beneficial technology developments and drive U.S. innovation & expand opportunities to achieve the NASA dream

The Syn Bio project also addresses the following NASA Strategic Plan (2018) objectives:

Strategic Objective 1.2: Understand Responses of Physical and Biological Systems to Spaceflight

Strategic Objective 3.1: Develop and Transfer Revolutionary Technologies to Enable Exploration Capabilities for NASA and the Nation

The Syn Bio project goals directly contribute to the technical capabilities and gaps identified in the GCD Technology Roadmaps in the following areas:

TA06 Human Health, Life Support and Habitation Systems

4. Habitation (Minimizing logistical burden for food and other supplies)

2. Long-Duration Health

TA07 Human Exploration Destination Systems

7.1.3 Consumables Production

Syn Bio Project Goals:

- Leverage emerging advances in synthetic biology and related areas to enable biomanufacturing for future long-duration missions to the moon and beyond.
- Produce valuable mission products on-demand, safely, and reliably during missions.
- Utilize *in situ* resources to enable the generation of mission consumables to increase mission sustainability.

2.0 Project Objectives

The Syn Bio project objectives are discussed below within each of the two development tasks.

BioNutrients Objectives

Future long-duration missions face significant challenges maintaining crew health. A critical area is supplying adequate nutrition, as certain vitamins and nutrients in supplied foods and supplements have been shown to degrade extensively during extended storage. It has been well established that a deficiency in only one required nutrient can lead to debilitating or fatal diseases such as scurvy (vitamin C deficiency). We are therefore creating a platform biomanufacturing technology that demonstrates *in situ* microbial production of targeted nutrients for long-duration missions such as sustainable lunar or Mars surface operations. The concept is similar to making familiar fermented foods (e.g. yogurt), but in this case with a focus on the production of a very specific quantity and quality of nutritive products with substantially less time and infrastructure than traditional plant-based production methods.

Our current on-orbit nutrient production process (BioNutrients-1 Flight Demonstration) utilizes a small “production pack” system that encloses a dried, edible, extended shelf-life growth substrate and one of two strains of a common food microorganism (*Saccharomyces cerevisiae* - a yeast) that have been genetically engineered to produce either beta-carotene or zeaxanthin. Upon crew-led hydration and mixing of the growth packet contents, the organism will activate and produce a desired amount of the nutrient. It is anticipated that current food/beverage hydration stations already employed in spacecraft will be suitable for packet hydration in eventual use scenarios. The amount of the nutrient produced is controlled by the eventual limitation of the growth media, theoretically leading to consistent levels of biomass and thereby the nutrient it contains.

In the eventual application, once growth is completed, the contents of the package will be heat-deactivated to kill the food microorganisms while still maintaining nutrient quality, and then be consumed by the crew. For the proposed flight experiments, the packet is not heat treated, but is instead frozen for return to Earth for analysis. The system employs a single-species inoculum with an otherwise sterile, single-use packet growth system, thereby ensuring a safe and simple food production environment.

On-going flight tests are planned to be conducted over a five-year period on the ISS. The intent is to store the production packs that contain the media and organism(s) over the course of five years, and to have the crew intermittently activate them to understand the shelf-life and performance. These tests also include ground controls to determine the specific effects of storage and growth in the space environment.

In addition to these on-orbit growth tests, a wide range of other microorganisms and treatments will also be stored on ISS during this 5-year period. These treatments will not be activated in space, but only tested upon return to Earth. This allows more specific information to be gathered that cannot be derived from on-orbit tests and allows the testing of many potential candidates without the costly overhead of crew time in orbit. These organisms include potential engineering host organisms, yogurt production species, probiotic organisms, and genetically modified organisms to help elucidate genetic features that enable sustained viability in the space environment.

As the flight experiment continues, the individual organisms that demonstrate enhanced survival in later years will be identified and characterized. This may provide either an actual “space-hardy” strain for future engineering and use, and / or specific genetic information that can be utilized to create custom strains in other species that would then also exhibit enhanced survivability.

An intrinsic need for the overall BioNutrients concept to be viable for mission use is the ability to make an array of required nutrients, rather than just one. Therefore, another objective of this project is to develop a means to produce multiple nutrients simultaneously. As part of this project, we will perform a use-case analysis to guide decisions as to how best to support multiple nutrient production. This can theoretically be done by having multiple single-nutrient production packs, or by having a single organism make multiple unrelated nutrients, or by using multiple organisms together in a single production pack that each make a single nutrient. This last option is not performed in terrestrial commercial systems and will require novel approaches to be derived and tested. Mission requirements such as duration, number of crew supported by the nutrients, and return on the investment of resources will be considered as part of the trade space.

The production pack developed for the BioNutrients-1 on orbit demonstration is a hard-shell system that is volume intensive during storage. While this system allows the necessary on-orbit testing for the BioNutrients-1 flight demonstration and readily met safety and operational requirements, actual use of the BioNutrients concept will require that future production packs use less mass and volume during storage, and eventual disposal. Therefore, we are developing a new reactor system that meets the growth and storage needs of the organism, while also occupying minimal volume. The preliminary design of the gen-1 pack is an FEP bag-based system that allows gas production during growth, sterility, optimal storage conditions, and the ability to consume the contents by the crew. It will be necessary to flight-test these concepts, hopefully with multiple opportunities to learn and iterate the design, to enable eventual flight use by the crew (no crew consumption is planned in this project plan).

Another objective of this effort is to develop an operational safety plan that demonstrates that the BioNutrients project concept can be safely conducted during missions. This plan will include a Hazard Analysis and Critical Control Point (HACCP) plan that creates an end-to-end food safety plan that guides every operation during the production process to ensure against unwanted contamination by-products. NASA food safety requirements are stringent and are almost exclusively employed during the ground-based preparation of foods for consumption on orbit.

Food safety testing for food items produced in space will also require the development of new methods and technologies to both control and monitor these processes. While preliminary methods of sanitizing fresh plant tissue have been developed, they rely on a sterilizing agent and an awkward disinfection process and are not approved for regular use. This system will not be applicable to the BioNutrients concept, and new and novel procedures will need to be developed and tested during spaceflight to enable implementation.

We therefore intend to conduct this demonstration of the overall platform technology which will inform and guide future R&TD efforts of BioNutrients.

Summary of BioNutrients Objectives:

1) BioNutrients: Develop and test a platform technology for the on-demand microbial manufacturing of multiple human nutrients to mitigate nutrient deficiencies in the current food system for long duration missions.

2) BioNutrients: Develop and flight test a production pack system that enables required nutrient production after long-duration storage of the microorganisms and media, requires minimal mass and volume, and complies with required food safety requirements.

3) BioNutrients: Develop and flight test procedures specified in the Hazard Analysis and Critical Control Point (HACCP) plan and in-space monitoring methods for assessing food safety for implementation during future missions.

CO_2-Based Manufacturing Objectives:

Future long-duration missions, particularly extended lunar and Mars surface missions, will require ISM using ISRU to greatly decrease launch costs, extend mission reach and increase self-sufficiency. While many ISRU / ISM proposals focus on the production of a single product, the overarching objective of this project is to extend ISRU and ISM

capability by developing a platform methodology for the rapid biomanufacturing of a wide range of valuable mission products using locally available resources.

As large-scale and long-term applications of synthetic biology for in-space microbial biomanufacturing will rely on a suitable media for microbial growth, a CO_2-based manufacturing platform could be a potential source of growth media carbon. CO_2 is readily available in spacecraft and habitats from human respiration and oxidation of wastes and is the principal gaseous component of the Martian atmosphere. As such, it is a ubiquitous feedstock for lunar and Mars surface missions and holds the potential to significantly reduce consumable logistics.

The overall approach taken in this project involves rapid physico-chemical conversion of CO_2 (and other needed constituents) to an organic substrate (microbial media) that can then be used in microbial biomanufacturing processes to make high-value mission products. This approach mimics the well-established Earth-based industrial biomass and petrochemical conversion processes that utilize CO_2-derived organic compounds (e.g. cellulosic biomass, oil, coal, natural gas) as the feedstock for producing thousands of different products. In contrast to terrestrial processes that are solely dependent on derivatives of biological photosynthesis for primary product generation, our approach relies on innovative physico-chemical (P/C) conversion technologies to more rapidly and efficiently convert CO_2 to selected organic biomanufacturing feedstock compounds. This feedstock will eventually serve as a critical component of a complete "*in situ* defined media" which is critical to enable a wide range of reliable biological ISM approaches, including the production of food and nutrients, bioplastics and polymers, fuels, biocements, and other compounds.

Candidate physico-chemical methods to convert CO_2 to feedstock compounds include electrochemical, photochemical, photoelectrochemical, thermal catalytic, and plasma technologies. These systems have the advantage of exhibiting rapid and predictable conversion of CO_2 to known products that can be used directly or as a molecular feedstock. For instance, electrochemical reduction of CO_2 to metabolizable compounds has already been well established using a wide variety of methods and catalysts. Some of the primary compounds derived from CO_2 include methane, acetate, formate, carbon monoxide, methanol, ethylene, oxalic acid, and propanol. Some of these chemicals are suitable precursors for biological processes, and numerous examples exist in the literature for microorganisms that metabolize a variety of these CO_2-derived compounds.

A key task of the pilot project was to conduct a comprehensive analysis to identify and characterize potential methods to enable an overall microbial production system using CO_2 as the primary carbon source, as represented in Figure 1.

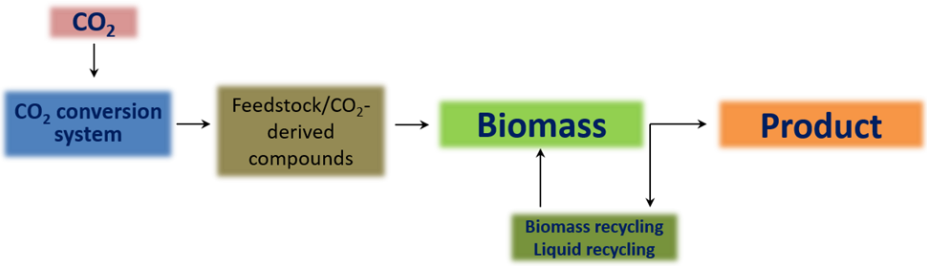

Figure 1. CO_2 – based manufacturing platform for bioproduct production.

To facilitate this process, there is a need to develop optimal CO_2 conversion systems to make effective microbial substrates. Globally, these systems are nascent, and further development is required to generate rich, high-energy carbon sources for microbial biomanufacturing systems. Therefore, an important objective of this task is to identify methods that can initially demonstrate this concept, while also supporting the overall development of systems

capable of making substrates such as sugars. This is approached in multiple ways, including NASA testing of commercial SOA reactors, directly supporting advanced technology development (our Stanford University collaborative agreement), and supporting the NASA CO_2 Conversion Centennial Challenge, which is requesting the abiotic production of sugars using CO_2 and hydrogen.

It is also critical that all the above-mentioned components and methods be integrated to allow concept demonstration. The initial activity needed is to conduct a preliminary integration of individual components which demonstrates the viability of the concept, followed by an increased fidelity prototype that is compatible for use in reduced gravity missions such as lunar and Mars surface missions. The results from these efforts will serve to guide use-case analyses, knowledge gap identification, and future research recommendations.

Summary of the CO_2-Based Manufacturing task objectives:

1) CO_2-Based Manufacturing: Perform an initial integration of the developed CO_2 conversion, bioreactor, engineered organism and purification systems to both demonstrate the validity of the concept, and to identify needs/challenges for prototype development.

2) CO_2-Based Manufacturing: Develop "space-ready" engineered microorganism(s) capable of making a targeted mission product (carbonic anhydrase), and test with an improved production and product harvesting system that is compatible with spacecraft environments during lunar and Mars missions.

3) CO_2-Based Manufacturing: Develop use case scenarios for this overall approach, identify needed future areas of research and development, and provide recommendations for forward work.

There are several **major deliverables** associated with meeting the objectives of both Syn Bio efforts:

For the BioNutrients effort, developing and demonstrating an overall biomanufacturing platform technology for space applications requires advancements and technology development in several areas. Of principal consideration is the development of microorganisms that can be effectively stored at ambient temperatures in space environments for durations of 5 years or more (necessary for a typical low energy round trip Mars mission), and reliably produce the nutritional compounds of interest in controlled amounts. Another important facet involves developing hardware to both store and grow the microbes, allow microbial inactivation and eventually crew consumption of the contents, all while being of very low mass and volume. Finally, methods must be simultaneously developed and tested that ensure that the BioNutrients approach can be safely conducted during missions and meet all operational and food safety requirements. This need will result in the development of a customized HACCP plan and any potentially ensuing technical monitoring system. All of these products will be required to enable a final flight demonstration that involves using highly evolved microorganisms in an efficient production pack to produce a verifiably safe process and product.

For the CO_2-Based Manufacturing effort, three major areas of research and technology development and testing are involved. First, as with the BioNutrients effort, there is a need to make microorganisms that will survive well in space, be safe and reliable, and generate a required mission product. In addition to these needs, the organisms must also be able to use substrates, such as formic acid and acetate, that can be currently be made via physicochemical conversion of CO_2. These products are not as effective as the readily metabolized substrates commonly used in commercial operations (sugars); therefore, these microbes will need to be able to effectively metabolize alternate substrates.

Second, the ability to make organic substrates from CO_2 must be identified and its products tested. Our previous efforts have led to the successful production of formic acid using a SOA commercial system. Because formic acid is not an energetic compound for biomanufacturing, we have collaborated with Dr. Matthew Kanan of Stanford University who has developed an electrochemical system for the production of acetate. Acetate is a significantly better substrate than formic acid and has shown to support moderate growth of our test microorganisms. A Cooperative Agreement between NASA and Stanford University has been established to support this effort.

Because future systems will likely require more effective substrates than either formic acid or acetate to enable small reactor footprints, we do not intend to focus on evolving these systems to a high TRL, but instead develop / use our currently available outputs as an initial demonstration of the overall process concept. As these CO_2 conversion technologies are matured globally, it is envisioned that future SOA capabilities will replace our current CO_2 conversion systems. To facilitate this eventuality, the team is participating in the NASA CO_2 Conversion Challenge, which is focused on the physicochemical conversion of CO_2 and hydrogen to sugars. These sugars would significantly enhance biomanufacturing processes by increasing food energy density and metabolic suitability, and result in lower mass, power, and volume biomanufacturing systems.

Third, suitable reactor systems will need to be developed that allow effective operation under the anticipated lunar / Mars surface mission conditions (reduced gravity and increased radiation). This includes fluid management, gas diffusion (oxygen supply and CO_2 removal), and product harvesting. Initial prototypes developed in our laboratory have been low volume and simple bags suitable for laboratory testing. We plan to develop and ground test advanced reactors that would be suitable for extended lunar / Mars surface missions. No flight testing is anticipated during the stated project period for this effort.

The major deliverables that support the BioNutrients and CO_2-Based Manufacturing objectives are summarized below in Table 1.

Table 1. Deliverables

Fiscal Year	Deliverables
FY19	(BN) Flight production and stasis packs prepared and delivered for flight. (BN) First returned sample results. (CO_2) Preliminary test results of integrated prototype components.
FY20	(BN) Year BN-1 1+ BN Flight Demonstration Results 2020 Annual Report. (CO_2) Improved Strain design report (CO_2) Preliminary Use Case review and report
FY21	(BN) Gen 1 Production Pack designed, fabricated and laboratory tested. (BN) HACCP Yeast protocol review and protocol (BN) Year 2+ BN Flight Demonstration Results 2021 Annual Report (CO_2) Lunar / Mars compatible demonstration unit tested (CO_2) Closeout Report
FY22	(BN) Gen-1 Production Pack flight test results (with improved microbial strains). (BN) Year 3+ BN Flight Demonstration Results 2022 Annual Report

FY23	(BN) Gen 2 production pack designed, fabricated and laboratory tested (BN) HACCP protocol review (BN) Final HACCP protocols for flight testing (BN) Year 4+ BN Flight Demonstration Results 2023 Annual Report
FY24	(BN) Engineered, space-ready host strains for future use (BN) Year 5 flight (Gen-2 production pack/HACCP protocols, advanced strains) demonstration results (BN) Close-out final report

(BN) = BioNutrients Project; (CO_2) = CO_2-Based Manufacturing Project

3.0 Technical Performance

The principal goals of both the BioNutrients and CO_2-Based Manufacturing Projects seek to advance the state of the art of in-space biomanufacturing. Both projects are geared to identify where advanced biological system development can address both general and specific mission needs for future exploration efforts. In both cases, the intent of the research and development is to both identify potential categories of mission materials that need to be made during a mission due to shelf-life issues or because of the potential savings of using *in situ* resources to offset launch / supply costs, as well as initiate production systems needed to synthesize these materials. In both tasks, it is sought to advance the TRL of both the individual components that comprise the system (such as bioreactors, CO_2 conversion systems, organism enhancement), as well as the overall capability of the integrated system.

Key Performance Parameters for the BioNutrients Task

For the BioNutrients project, the major goal is to develop and test a method that can produce a known amount of a targeted nutrient over a mission timespan of five years, without unwanted contamination and with minimal hardware/ consumable requirements. Also, as it is likely that more than one nutrient will need to be produced to meet the crew's nutritional needs, it will likely be beneficial to determine how best to make multiple targeted nutrients available in a given time window, either in a single production pack via advanced organism engineering (one organism makes multiple products), or using multiple organisms / multiple packs in innovative processing methods.

These goals lead to four key metrics that will drive technical system design and performance evaluation (see Table 2). First is the overarching goal of being able to produce nutrients during an extended period, as certain nutrients will need to be produced up to five years from launch. The approach of the BioNutrients system is to supply a known amount of media and maintain enough organisms to initiate growth, and then grow approximately the same amount of microbial biomass and associated nutrient during each pack activation operation.

KPP 1: ISS production pack viability lifespan: A production pack is considered to be "viable" if it demonstrates the ability to make targeted nutrients at >50% when compared to production packs that are activated shortly after arrival at the ISS. This KPP involves measuring the amount of the target nutrients that are produced each time the production pack is activated over the course of five years. SOA is taken as the mass of nutrient made from production packs at time one (initial ISS production run). This value will vary with respect to the nutrient being made, organism, amount of media and other factors, but in each case will serve as the reference point for later comparison.

The selected threshold value (TV) of three years for all samples to meet 50% of the initial ISS value, and a project goal (PG) of 5 years for all samples. The TV of 3 years is adopted as it represents a long enough duration to allow for prepositioning and the minimal timeframe needed for a roundtrip to Mars. The PG of 5 years indicates a desired shelf life that will meet the expected 5 yr. requirement for Mars exploration. This KPP is at the center of the BioNutrients project, with the remaining KPPs describing other key aspects of attaining this goal.

KPP 2: Microbial safety assessment: KPP 2 addresses the need for a high level of general safety and specific compliance with NASA food safety requirements. Test samples will be evaluated using the procedures listed in NASA-STD-3001 Vol. 2, Rev. A, which involve the standard microbial food safety techniques used to determine supplied food safety prior to launch. The SOA value listed (<100 CFU/100ml) is the acceptable value in the above document. However, our TV and PG goals are <50 and <1 CFU/100ml, respectively. These values are both lower than the accepted SOA and are selected as it is anticipated that future regulations governing consumable materials manufactured in space will be subject to more rigid standards. BN-3 test samples will be evaluated both with the anticipated in-space heat treatment for microbial kill, as well as without heat treatment for the specific purpose of looking for the presence of coliform, Staphylococcus and Salmonella bacteria as per the designated Petri-film techniques. This helps to determine if these organisms were present before heat kill. Samples will also be examined microscopically to assess presence of dead pathogens. Consistently attaining the PG of <1 CFU/100ml is highly conservative and would be a strong indicator of overall microbial safety.

KPP 3: Biomanufacturing platform supplies multiple nutrients: KPP 3 involves the eventual likelihood that multiple nutrients will be required to mitigate future nutrient deficiencies, rather than just one nutrient. SOA is taken as the current BioNutrients approach that is undergoing spaceflight testing on ISS, in which one compound is selected. The TV of 2 is selected as accomplishing the making of two compounds requires solving many of the underlying challenges of exceeding a single compound. A project goal of 4 nutrients is selected as representing substantial strides in the co-production of nutrients. It is currently unknown what nutrients need to be made for future mission scenarios, and to what levels. Therefore, this process is undertaken to develop a platform capability to address future needs that have not yet been defined.

Table 2. Key Performance Parameters – BioNutrients Task

Key Performance Parameters				
Performance Parameter	**Units**	**State of the Art**	**Threshold Value**	**Project Goal**
KPP 1: ISS production pack viability lifespan (1)	**years**	**N/A(2)**	**3**	**5**
KPP 2 : Microbial safety assessment(3)	**cfu/100ml**	<100	<50	<1
KPP 3: Biomanufacturing platform supplies multiple nutrients(4)	**number of nutrients**	1	2	4

Notes:

(1) A production pack is considered to be "viable" if it demonstrates the ability to make targeted nutrients at >50% when compared to initial ISS production packs. This KPP assesses effects of storage over time. Viable production packs that have been stored and tested on ISS for 3 years (Threshold Value) are assumed to be suitable for extended Lunar surface mission needs, and a 5 year lifespan (Project Goal) is needed for Mars surface missions as per the Human Research Program 5-year stored food shelf-life requirements.

(2) There is no SOA for this KPP because there are currently no means of making nutrients during spaceflight.

(3) Microbial safety is assessed using NASA food safety Petri-film testing procedures (NASA-STD-3001 Vol. 2, Rev. A) for the coliform, Staphylococcus and Salmonella tests expressed as Colony Formation Units (CFU)/100ml. Includes microscopic inspection for dead cells.

(4) Intent is to build a biomanufacturing platform that provides on-demand, simultaneous availability of multiple nutrients with minimal mass, power, and volume requirements.

Key Performance Parameters for the CO_2-Based Manufacturing Task

The principal drivers of the CO_2-Based Manufacturing effort involve developing the capability to microbially manufacture a wide range of required mission products, using *in situ* resources. Whereas the BioNutrients effort is currently supplying all the media required, this effort seeks to reduce mission costs and increase sustainability by supplying the bulk of the media with carbon-based substrates derived from local CO_2 and hydrogen. Therefore, a major premise of this work is that, within certain scenarios/use cases, this approach can result in reduced consumable costs and increased sustainability for biomanufacturing operations. The KPPs that support this effort are discussed below and are summarized in Table 3.

KPP 4: Biomass harvest efficiency:

A critically important design parameter of the overall system is the minimization of energy and mass losses within each step of the process. For example, very high rates of efficiency are desired for CO_2 conversion, substrate conversion to biomass/products, high harvesting efficiency and high product purification rates. Losses in each process will be additive and result in significant overall process inefficiencies. Therefore, all these factors are strong design and performance drivers.

KPP 4 focuses on the level of biomass that can be harvested from the bioreactor after the growth process. While large-scale terrestrial systems have the ability to use multiple rinses and heavy, energy intensive processes such as centrifugal separators to achieve ~97% harvest efficiency (used as the SOA value), this process will be much more challenging in space-based operations. The potential use of membranes in our designs, can also lead to microbial adherence and decreased harvesting levels. Likewise, small scale systems may experience an increased proportion of losses in tubing, reactor corners, membranes, etc. as opposed to large scale systems.

Considering that each process of the overall system will have an associated efficiency, and therefore each inefficiency is additive, it is critical to optimize each trophic level of the process. The harvesting procedure will therefore become a major design driver of the bioreactor system, which will include materials of construction, the design of corners and folds, fluid transport lines, and rinsing operations. It will likewise drive the design of the harvesting system itself, including the selection of the separation phenomenon such as filtration, centrifugation, etc.

85% is selected as the TV for this KPP, as this represents that the vast majority of the microbial biomass can be reliably separated and processed for product purification. This level of performance is deemed satisfactory for initial operations. The PG value of 97% matches current terrestrial SOA performance and serves as a reasonable expectation for the maximum amount of recovery that can be obtained in this system.

KPP 5: % of media components sourced from ISRU: This KPP indicates the desired level of reduced consumables that will be enabled through all aspects of the space-based manufacturing approach. This includes savings that will be realized both through *in situ* media generation, as well as advanced recycling techniques not currently implemented in terrestrial SOA biomanufacturing systems. Recycling of the spent biomass has the potential to substantially decrease needed media supplies. Additional savings from multiple uses of the water in reactors can also realize savings in water treatment costs. For KPP 5 This represents the percent of the overall media components that are sourced from CO_2 and hydrogen (both of which could be obtained through ISRU), microbial biomass recycling, and potentially crew wastes that result in a viable media for use in the developed bioreactor system growing the organism engineered to make the target product (nominally carbonic anhydrase). To be considered a viable growth media, it must meet the minimal criteria of providing at least 5% carbon substrate utilization efficiency (i.e., 5 gm biomass is derived from 100 gm carbon substrate). The BioNutrients production pack approach is selected as the space-based SOA technology. In this approach, all media components are launched. Therefore, the % media components sourced from ISRU equals 0%.

Based on the use of acetate as a carbon substrate for both systems, a TV of 50% reduction of supplied (launched) consumables is anticipated, with a maximum reduction of 80% (PG). These values will vary depending on the media composition and biomanufacturing use case scenarios. Multiple scenarios (varying media types/product types and amounts) will be investigated to identify the breakeven points for the use of this approach, and to identify plausible use cases for multiple products

It should be noted that as the CO_2 conversion field advances with time, the substrates that can be made from CO_2 will become of higher metabolic quality, and the conversion efficiency of this process will increase. Therefore, this KPP will directly benefit from activities such as the current NASA CO_2 Conversion Challenge, which is requesting teams to make sugars from CO_2. Additionally, advances are expected from planned continued collaborations with Dr. Matthew Kanan at Stanford University, who is currently developing SOA methods that lead the field in acetate production.

Table 3. Key Performance Parameters – CO_2-Based Manufacturing Task

Key Performance Parameters				
Performance Parameter	**Units**	**State of the Art**	**Threshold Value**	**Project Goal**
KPP 4: Biomass harvest efficiency [(1)]	**percentage**	**-** [(2)]	**85**	**97**
KPP 5: KPP 5: % of media components sourced from CO_2, H_2, and recycled mission wastes[(3)]	**percentage**	**0** [(4)]	**50%**	**80%**

Notes:

(1) This represents the amount of biomass that can be processed in relation to the total amount produced within the system (total amount produced will be assessed as amount from nominal operation plus amount recovered following enhanced manual recovery and inspection of system). Biomass loss due to biofilm formation or inaccessible due to geometry and flow design of the reactor can significantly reduce performance of the overall system. Maximizing biomass recovery greatly improves overall system efficiency, and drives organism selection and efficient reactor design/operation. Our current product is carbonic anhydrase, a non-secreted product for which biomass is an acceptable proxy. Note: Biomass containing carbonic anhydrase can potentially be utilized in a liquid amine system without further purification.

(2) There are no space-based biomanufacturing systems to serve as a SOA. Harvest efficiencies in commercial terrestrial systems can vary based on specific organisms and procedures used. Commercial bioreactor harvesting efficiencies are often quoted as ~97% and we will use this as a comparative value.

(3) This represents the percent of the overall media components that are sourced from CO_2 and hydrogen (both of which could be obtained through ISRU), microbial biomass recycling, and potentially crew wastes that result in a viable media for use in the developed bioreactor system growing the organism engineered to make the target product (nominally carbonic anhydrase). To be considered a viable growth media, it must meet the minimal criteria of providing at least 5% carbon substrate utilization efficiency (i.e., 5 gm biomass is derived from 100 gm carbon substrate).

(4) The BioNutrients production pack approach is selected as the space-based SOA technology. In this approach, all media components are launched. Therefore the % media components sourced from ISRU equals 0%.

4.0 Resource Requirements

The following documents the resources required to implement the project plan, including total budget and resources by Center (see Table 4 for the budget breakdown). The scope of the budget includes resources required for both the BioNutrients and CO_2-Based Manufacturing tasks. The budget spans the entire projected length of the BioNutrients flight demonstration, which is five years starting with launch in FY19. The resources for the CO_2-Based Manufacturing task are requested for FYs 19-21 only. The remaining funds in FYs 22-24 are solely for BioNutrients efforts.

Experience has indicated that the science team for the BioNutrients-1 flight experiment requires approximately half-time effort for the continuing analyses of returned samples. This may reduce as the timeline progresses if some of the samples show they are no longer viable, as they will no longer need to be analyzed.

For FYs 22-24, there is a consistent (flat) profile as it is anticipated that while the initial CO_2-Based Manufacturing efforts have ended, there are increased activities regarding organism engineering, the development and testing of a flight safety protocol, and a second generation flight reactor development and flight tests for BioNutrients BN-2 and BN-3 missions, as well as supporting the ongoing BioNutrients-1 flight sample analyses. The schedule of these activities should allow a balanced budget to be sufficient, with no anticipated significant variances year-to-year.

Table 4. Project Resource Allocations[1]

Budget	FY 20	FY 21	FY 22	FY 23	FY 24	Total
Full Cost ($K)						
FTE						

Labor ($K)						
Procurement ($K)						

[1]Outyear budgets may be modified based on outcome of approved appropriations.

5.0 Technical Approach

The technical approach to achieve the two task areas of the Syn Bio Project are discussed separately below:

BioNutrients Technical Approach:

The overall scope of the BioNutrients efforts is comprised of several sub-tasks as outlined below.

Aim1: Conduct ISS Flight Test of BioNutrients-1

The work contained in this Aim involves the development and testing of a platform technology that demonstrates *in situ* microbial production of targeted nutrients to address food and supplement storage challenges in long duration missions. The process utilizes individual production packs that enclose a dried, edible, extended shelf-life growth substrate and the common food microorganisms, *Saccharomyces cerevisiae* and *Saccharomyces boulardi* - both yeasts, that have been genetically engineered to produce zeaxanthin and beta-carotene respectively. Upon crew-conducted hydration and mixing of the growth packet contents, the organism activates and rapidly produces a desired amount of biomass and the target nutrient. The food/beverage hydration stations already employed in spacecraft will be used to supply the water for packet hydration. The amount of the nutrient is controlled by the eventual limitation of the growth media, leading to consistent levels of biomass and thereby the nutrient it contains. In future implementation, once growth is completed the contents of the package will be heat-deactivated to kill the food microorganisms while maintaining nutrient quality, and then consumed by the crew. The system would employ a single-species inoculum with a sterile, single-use packet growth system, thereby ensuring a safe and simple food production environment.

The overall strategy consists of identifying a safe food organism that will be genetically engineered to produce a mission-relevant nutrient. The organisms must also be able to be reliably stored for very long durations (multiple years), so the dormant form of the organism (spores) is likely the optimal storage format. An edible media must be used as this will be consumed by the crew along with the grown organisms, similar to how milk transformed by bacteria becomes yogurt. These components will be contained in a custom-developed storage packet, which will also serve as the growth vessel. The packet is a single-use system that would nominally be disposed of after use, similar to how space food / beverage bags are discarded. It contains features to manage the microbially-produced CO_2, while keeping the water and microorganisms safely contained in the growth packet.

The system is planned to be tested over a 5-year period on the ISS and ground controls. This involves having the prepared growth packets containing dried edible media and yeast spores contained in an outer storage bag that protects it from water and oxygen exposure. Sufficient numbers of these bags will be brought to ISS to allow a test to be conducted by the crew intermittently over a 5-year period. It is anticipated to conduct 6 hydration tests (4 replicates/test) over the five years. This will allow evaluation of overall performance in a timeframe that matches desired food shelf-life duration for future Mars surface missions.

A test will consist of a crew member removing 4 storage bags and removing the inner growth packet from the outer storage bag. Each growth packet will be hydrated using 33 ml of sterile water, the contents well mixed, and then placed within a temperature controlled incubator at 30^{o}C for 48 hours. The growth packets will then be removed and placed in a -80^{o}C freezer and maintained at that temperature until returned to the ground-based laboratory for evaluation.

In addition to these treatments, other samples will be tested during this 5-year test. They will include similar storage/ growth packet systems that are not hydrated in space, but rather just stored and returned to Earth to allow more detailed observations when activated in the laboratory. Similarly, various wild-type (non-engineered) and engineered food microbes (including probiotic organisms) will be stored in sealed vials and intermittently returned to Earth for evaluation (a total of 14 stasis pack return events are planned).

Ground evaluation of growth packets activated in space will undergo tests including; total yeast biomass present, zeaxanthin production, microbial contamination detection, and multiple “omics” analyses. Returned un-activated production packs and stasis pack microbes will undergo activation in the laboratory and then subjected to similar tests as noted above, as well as growth rate evaluation and stasis efficacy.

Payload Concept of Operations

- Experiment Schedule

The following points are criteria that influence the BioNutrients-1 experiment schedule:

- The science team requires sample returns for different sample types distributed over a 5-year period in order to obtain time-dependent data.
- For highest-quality comparison results, there is a need to execute the on-orbit experiment (ISS Production Packs) as closely as possible to returned controls (Earth Production Packs).
- An incubator (Space Automated Bioproduct Lab, SABL or comparable) must be available for ISS Production Pack experiment execution.
- Cold Stowage volume must be available for stowage and return of ISS Production Packs at the required temperature range (between -70°C and -100°C). 8 packs fit into ½ Minus Eighty Laboratory Freezer for ISS (MELFI) box.

Figure 2 summarizes the current BioNutrients-1 Experiment Schedule. Due to unknown future flight scheduling and the various dependencies listed above, the experiment schedule is notional and may need to be re-negotiated over-time. An updated figure with as run schedule and refined future flight scheduling will be provided with each yearly report.

As depicted in Figure 2, it is desirable to return Earth Production Pack Kits to the PI’s lab for experiment execution and then execute the corresponding ISS Production Pack experiments on-orbit such that Earth and ISS Pack execution occurs at approximately the same time. As a result, ISS Production Pack experiments will occur after SpX unberth. The desired timeframe for incubation is U+7 to 9d (±4d), which allows for adequate time for the PD to deliver the recently-descended Earth Production Packs to the PI’s lab.

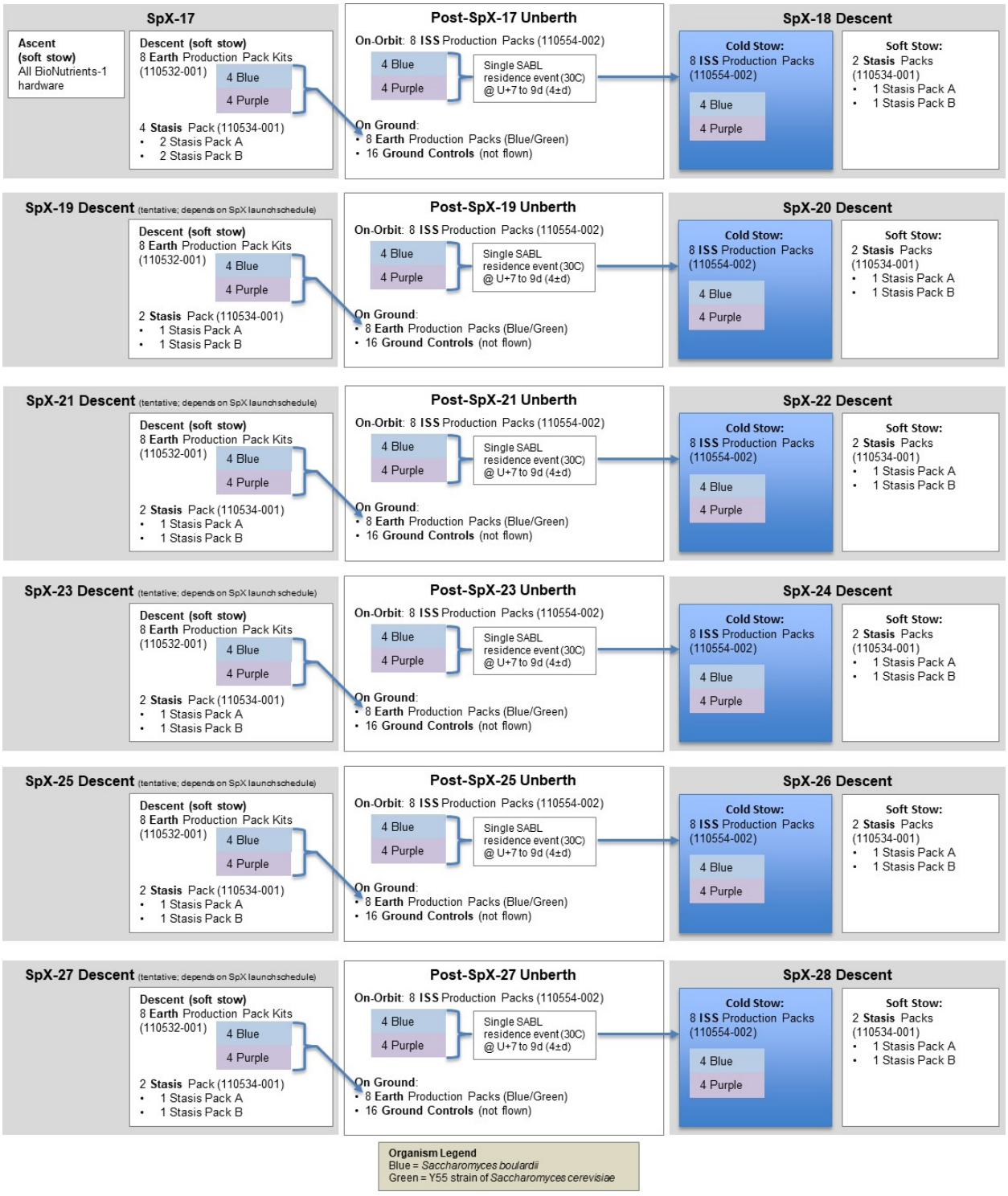

Figure 2. Initial BioNutrients-1 flight experiment operations scheduling

During the assembly of the hardware to be launched, ground control hardware for the Production Packs and Stasis Packs are simultaneously assembled. Ground control hardware remains in the PI's lab for ground control experiment execution that is "near-synchronous" with the on-orbit experiment (see the middle column of Figure 2).

- Launch

All the BioNutrients-1 Payload hardware is launched simultaneously in ambient soft stowage. Launching all hardware simultaneously ensures that the "start time" for exposure to the ISS environment is identical for all samples.

- On-Orbit Experiment with ISS Production Packs

a. Hydration and Initial Shaking

After arriving on the ISS, the BioNutrients-1 hardware is stowed at a to-be-determined location per direction from the ISS stowage team. At periodic intervals, ISS Production Packs are retrieved by crew for hydration.

On the day of hydration, the crew retrieves BioNutrients-1 ISS Production Pack Kits of the appropriate quantity and sample type. Additionally, crew will retrieve one BioNutrients-1 Support Kit and a 60mL syringe from the Wet Lab Pantry.

The crew opens the ISS Production Pack Kit. After the ISS Production Packs are removed from the kit bag, the crew trashes the kit bag with the oxygen scavenger and desiccant inside.

The ISS Production Pack Assembly (Figure 3) includes a tube component for providing strain relief to connectors during launch and storage. Prior to hydration, the crew disconnects the tube from the ISS Production Pack and trashes the tube.

Figure 3. ISS Production Pack Upon Removal from Kit Bag

The crew connects the Potable Water Dispenser (PWD) adapter to the PWD and then the 1L bag to the PWD adapter, then dispenses 1L of potable, ambient temperature, non-iodinated water into the bag. The crew draws 33mL from the 1L bag into the 60mL syringe, then attaches the syringe to the luer connector on the filter of the ISS Production Pack. The crew depresses the syringe plunger to push the water through the filter and into the Production Pack. (The filter ensures that injected water is sterile.) After water transfer, the crew disconnects and trashes the filter.

Figure 4. Capped ISS Production Pack (SABL and MELFI Configuration)

After hydration, the crew uses the tethered cap to cap the ISS Production Pack port, which is a one-way check valve (Figure 4). The crew then manually shakes the ISS Production Pack for one minute. The crew repeats the hydration and shaking process for the remaining packs in the experiment event (for a total of 8 packs), using the same bag of water and syringe. The crew places the packs inside the incubator. Note that the *Saccharomyces boulardii* (light blue) and the Y55 strain of *Saccharomyces cerevisiae* (purple) packs (yeast) are incubated concurrently, because they both require the same incubation temperature (30°C).

b. On-Orbit Incubation and Second Shaking

The SABL Incubator facility is planned to be used for BioNutrients-1 ISS Production Pack incubation. SABL is operated by BioServe and is ground commanded for the target setpoint temperature in advance of Production Pack insertion.

Immediately after hydration and shaking, the ISS Production Packs are inserted into the incubator. Each of the ISS Production Packs are attached by Velcro to a removable tray, provided by BioServe. In order to prevent product

gases (most notably, carbon dioxide and ethanol) from accumulating inside SABL, gas exchange with the cabin is required. Therefore, a BioServe-provided fan is installed and a SABL door gap is established, to encourage air exchange between the SABL volume and the cabin. This is important for the fermentation (yeast) by-product of ethanol, to minimize impact on ISS water system catalysts, and for carbon dioxide, to minimize crew exposure to a carbon dioxide "bubble" when retrieving packs from SABL.

The crew reports the time of insertion and removal of Production Packs so that an accurate incubation duration is recorded for use in earth activation ("flown control") and ground control samples. BioServe provides to the payload developer (PD) real-time on-orbit incubation temperature data to allow any deviations to be accommodated for in the ground incubations.

After 6 hours of incubation, the crew returns to the incubator, removes the ISS Production Packs, and manually shakes each pack for one additional minute to ensure adequate breakup of the organism pellet and homogenous mixing of the contents of the pack. After the second shaking, crew then places the packs back into the SABL to complete the remainder of their 48-hour incubation.

Photography of the packs is needed to assess sample color and to gain an understanding of where the fluid migrates to in a microgravity environment. This requires photography through both the base and the side of the packs immediately before and after both the 6-hour and the 42-hour incubation periods. This will likely be achieved by photographing strategically-oriented packs while inside SABL, before door closing events and after door opening events.

c. Freezing

After 48hrs of incubation, the crew removes the ISS Production Packs from the incubator and reports the time of removal. Once the packs are removed, the crew inserts each pack into a Bitran bag and the packs are then stowed in cold stowage (i.e. MELFI) at a temperature between -70°C and -100°C. The samples remain in cold stowage until they return to the PI's laboratory.

d. Earth Production Pack Kits and Stasis Packs Return

Earth Production Pack Kits and Stasis Packs are returned in soft stowage at intervals that enable the PI to obtain time-dependent data, per the return schedule indicated in Figure 2. Samples are picked up by the PD at the early destow location and delivered to the PI's lab.

e. Near-Synchronous Ground Control

Samples for a Near-synchronous ground control (NSGC) will be prepared at the same time as the flight sample preparation, using identical procedures. A full-scale (sample and hardware quantities same as flight), mission-length NSGC is performed in the PI's lab at ARC. Environmental temperature data is retrieved by the PD via the HOSC data interface to simulate flight environments in the NSGC. Each time the crew hydrates, shakes, incubates, and freezes a set of samples onboard, a corresponding set of ground control samples is processed. The actual on-orbit operations timeline and as-run crew procedures are followed for the near-synchronous ground control. Hydration, incubation, and freezing of the NSGC samples shall occur within a reasonable timeframe (as determined by the PI) following on-orbit execution. On-orbit incubation durations and environmental conditions are mirrored for the NSGC samples. Freezing and transportation modes and timelines also match those used for the flight samples.

f. Sample Analyses

The returned hydrated and frozen production packs will be evaluated with respect to overall biomass growth, nutrient production, and genetic analyses to determine if changes have occurred during growth in space. The un-hydrated production packs and stasis packs undergo hydration and growth in the PI's laboratory and undergo similar analyses. Together, and with time, these data will indicate the performance of the individual treatments and indicate the potential for the overall BioNutrients concept to contribute to in-space nutrient production. Returned samples will also be employed (see Aim 2 for more detail) to identify specific organisms that survived for long durations in space environments and assess their genetic alterations that may contribute to their success.

Aim 2: Development of Space-Ready Host Strains

As part of the five-year BioNutrients-1 flight test initiated in 2019, a range of industrially relevant microbial production hosts were selected for long-duration storage (stasis packs) aboard the ISS. Expected outcomes of this stasis pack study include: a) an identification of individual microbial hosts that survived long-term at ambient temperature aboard the ISS; and b) identification of the unique genes or genetic changes in the survivors that can be mapped to traits that can be engineered into other wild-type hosts to assist in long-duration survival at ambient temperature and increased radiation environments.

Included in this testing are strains with deliberate UV-generated random mutations and yeast "knockout libraries" that will be analyzed to help identify new or unique sets of genes beyond those currently known that can aid in space-based storage. In addition, literature studies will also be conducted to identify potentially beneficial genes. These varied sources of information will guide engineering efforts to develop space-ready production hosts for further ground and flight tests.

As part of the final flight test campaign (tentatively in 2024), the selected enhanced survivors from the stasis packs will be engineered to produce nutritive compounds of interest. The engineered strains will be ground tested for both product formation as well as survivability following desiccation. Flight testing of the organism will be conducted as part of the final flight test in conjunction with the production pack demonstration and evaluation (see Aim 3). Though long-term storage testing aboard ISS is not currently planned, the enhanced production strains will be ground tested long-term at temperatures that mimic those experienced onboard the ISS to preliminarily assess stasis performance.

Aim 3: Development of Operational Production Pack and Flight Testing

The original concepts for BioNutrients production pack development and testing involved using a plastic bag similar to the food bags currently used by astronauts. Early prototypes included a membrane sealed to the side of the bag to allow CO_2 gas diffusion out of the bag. The concept of operations involved containing the required media and organisms in the bag under otherwise sterile conditions. This bag would then have been contained in an outer bag with a vacuum created to minimize volume and exclude oxygen and water vapor to ensure a long shelf-life.

Despite initial successful testing, the initial flight tests employed a hard-shell design that ensured that all safety requirements could be met within the strenuous flight development schedule. This design approach required using more mass and volume than is likely feasible for extensive future use in missions. Therefore, it is intended to develop a substantially reduced mass and volume production pack that meets all safety requirements, and that also provides the capabilities to allow usage by the crew in future missions.

These features include: easy hydration/activation of contents preferably straight from the Potable Water Dispenser, functional delivery system for pack content consumption by crew (Note: crew will not consume contents during this project), a high degree of gas exchange capability, and functionality in both micro and reduced gravity conditions. The bag will also be fabricated with materials that will be compatible with a final, post-growth microbial kill, which is likely to be a thermal processing step.

The development is anticipated to occur with two iterations/tests. The first effort will develop a Gen-1 Production Pack concept, or possibly multiple concepts, for initial flight testing in microgravity aboard the ISS. These tests are intended to assess the performance of the design(s) with respect to gas diffusion and overall growth effectiveness. The fluid management within the bag will be a principal design driver for both gas diffusion and post-growth delivery of contents. Anticipated features include an integrated de-bubbler to provide a dense fluid for drinking, and the capability to easily dispense the contents.

After crew testing of the Gen-1 production pack, the lessons learned will be used to create an improved version – the Gen-2 production pack. This version will be improved by using Gen-1 test results, and by incorporating the features required to allow multiple compounds to be produced with the pack (see Aim 4 below). The Gen-2 version will be used for the final flight tests, which are intended to include the enhanced organisms, producing multiple compounds, and also demonstrate the identified food safety requirements and operations (see Aim 5 below).

The intent of the second flight experiment is to result in the deliverable of a flight-tested production pack design that would be ready for commercial manufacture and ultimate use by the crew (if deemed needed). This production pack will include many of the features to produce a wide range of potential compounds and could yield use by further researchers for future investigations.

Aim 4: Develop Multiple Compound Production Capability

It is anticipated that several nutritional products will need to be made simultaneously for the crew's use. Multiple vitamins have been identified as being problematic in supplying the crew's nutritional needs and may need to be generated together during the mission. Therefore, it is the intent to develop the capability of being able to ensure the system can reliably provide the simultaneous supply of multiple compounds.

Biosynthesis of products such as nutrients into microorganisms like yeast and bacteria require either engineering the production organisms' exogenous metabolic pathways to make the product of interest or enhancing their innate capacity. Either of these methods have their own associated metabolic cost. Adding more than one product into the same organism may not be metabolically efficient, and novel ways to produce more than two compounds at a time need to be developed and tested.

To ensure a capability to provide multiple nutrients, multiple pathways for producing various products of interest within the same production pack, or in multiple multi-user or longer duration packs will be designed and evaluated against Lunar and Mars use-case scenarios. Options will be down-selected to methods that show the highest potential for success in a lab-based production system for further development.

The major potential methods that will be developed and refined to accomplish this goal include:

a) Insert multiple pathways in one organism. This approach allows simplicity of growth and harvesting, but as mentioned above may be technically difficult to perform.

b) Employ two engineered strains with substrate specificity. This requires two or more strains that have been engineered to produce a separate nutrient, and that can grow using a substrate that the other organism cannot use. In this way, starting levels of surviving inoculum do not affect final growth and product levels, thereby assuring consistent production levels regardless of initial conditions.

c) Identical host organisms making a product each independently and operating within the same bag. We currently have been testing *Saccharomyces cerevisiae* Y55 to make zeaxanthin an ocular protective carotenoid as part of BN-1. We can similarly engineer into the same parent strain Y55 the ability to produce a secondary product such as vitamin C and mix the two zeaxanthin and vitamin producing strains together.

d) Provide separate growing areas within one production pack. It is possible to develop a custom production pack with multiple chambers that allows separate storage and growth of multiple strains, and a final combining of fluids for consumption. This approach would be preferable to a "single compound-single pack approach" as it would likely substantially reduce total pack mass, volume, and operations.

e) Provide packs optimized for a given single nutrient and designed to either supply enough nutrient for multiple crew members per run, or to supply the given nutrient for a longer period of time, thus decreasing the impact on resources of having multiple packs to supply the required assortment of nutrients.

These techniques will be investigated to determine feasibility and will be down-selected to the most promising approach(es). The remaining approach(es) will be developed and tested, possibly in combination with required production pack design accommodations. It is intended to incorporate this capability in the second flight test of the Gen-2 production pack (see Aim 3), and this will serve as the major deliverable for this sub-task.

Aim 5: Develop and Test Flight Food Safety Protocol

The *in situ* production of high microbial load products require new food safety procedures and operations to be developed, tested, and approved for spaceflight. Terrestrial safety processes are effective largely due to standardized production and commercial distribution control, with many of the commonly employed safety practices being

untenable in space exploration systems. To address the safety of in-space production of nutrients, three task areas have been identified:

a) Develop a HACCP plan for microbial nutrient production and crew consumption in space. Following guidelines set forth by the US Food and Drug Administration (FDA) and NASA, a HACCP plan will be developed that identifies and controls potential hazards during all phases of the *in situ* storage and production processes. Example areas of focus include: media formulation, contamination, and storage; culture purity; packet materials, assembly, and operations; packet hydration and incubation in space; and final product testing. NASA food regulations will serve as testing standards. Procedures will be developed for two versions of the product, one with live cultures, and the other a Pasteurized version (to satisfy the existing total aerobic plate count standard). In the development of the HACCP plan, personnel from NASA's Human Research Program (HRP) Food Systems Program will be consulted to ensure that NASA regulations are addressed and adhered to.

   In addition to monitoring microbial loads throughout the BioNutrients production, storage and activation process, the HACCP plan will also include monitoring of pH and potential biological toxins. In addition, initial shelf-life studies to monitor the conditions of the packet and its contents will be conducted. The HACCP plan developed here will be tested as outlined in c).

b) Identify techniques for *in situ* detection of microbial contaminants. To determine if the growth process has resulted in a safe product during actual operations, it will be necessary to verify safety in situ. Nucleic acid-based detection methods such as the Specific High-Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) system, digital-droplet PCR and qPCR are techniques to detect pathogens and have highly sensitive detection levels. The individual components are also amenable to lyophilization for long-duration storage.

   Systems currently available on ISS, such as the WetLab-2 facilities, utilize stable lyophilized reagents in a commercial qPCR machine. Similarly, as part of the Genes in Space experiment, a miniPCR system is currently being used onboard the ISS and sequencing on board ISS has been carried out using MinION™. These flight-validated systems provide confidence that lyophilized reagents are stable in space and can be used for amplification of nucleic acids (although the shelf life may not support the longer missions needed for Mars exploration). In addition to nucleic acid-based methods, novel electrical biosensing techniques will be investigated to monitor microbial contamination. These electrical methods can reliably demonstrate microbial growth and utilize a small footprint and will be compared to the nucleic acid-based systems if commercially available. In addition, we will investigate other techniques that could be further developed. Successful methods will then be integrated into the proposed HACCP plan test in section c).

c) Development and flight evaluation of comprehensive *in situ* safety plan. Integrating the HACCP plan and the novel detection systems discussed above, a comprehensive safety protocol will be developed and tested against standard NASA and FDA safety methods. FDA approved techniques in the Bacteriological Analytical Manual (BAM) contain protocols for quantifying different microbes. Commercially available systems will be used where appropriate to detect food pathogens, such as the Colilert kit which provides very sensitive detection of coliforms. As we are intentionally producing live cultures, we will determine if only the intended Generally Regarded as Safe (GRAS) cultures remain in the packet. Heat processing of product and resulting effects on the microbial composition will be conducted. Comprehensive ground testing will first be conducted and evaluated, with eventual flight testing on ISS during the Gen-2 production pack tests (see Aim 3 above).

Combining the above methods, it is anticipated that the significant progress will be made towards the approval, and eventual implementation, of the BioNutrients concept. Additionally, this safety plan will help inform many other future mission food production efforts, including microbial, plant, and animal foods. The project is communicating regularly with the JSC Advanced Food Technology group and the microbial monitoring efforts supported by NASA at multiple centers in order to leverage advances from other teams in this arena and to ensure our approach is in alignment with potential future customers and stakeholders in this project. The team is also planning to take commercial food safety training and invest in external reviews of our HACCP plan and test results with industrial, academic and NASA food safety experts.

CO_2-Based Manufacturing Technical Approach

This task leverages technologies emerging from two rapidly advancing fields: 1) Carbon capture and reuse researchers are developing new ways to convert CO_2 to useful organics and other products and; 2) Biopharmaceutical producers are improving techniques for employing cells as chemical production plants, engineering them to synthesize complex organics from simpler substrates. Rather than competing with these specialized commercial entities, project cost-effectiveness will be maximized by a technical approach that leverages emerging commercial advances while focusing on NASA-specific R&D challenges and goals, such as ease of operation, safety, low overall system ESM (Equivalent System Mass), and reduced gravity operation.

Aim 1: Prototype Component Assembly

Building on previous work within our laboratory, we will conduct a preliminary integration of the multiple components required for an end-to-end demonstration of CO_2-based manufacturing is planned for FY19. This includes the integration of the overall system components and initial system performance testing. The intent is to provide a system that demonstrates the capabilities needed to implement an overall strategy, but that does not yet incorporate advanced features that address size and mass reductions, crew operation on Lunar and Mars surface missions, and general in-space operational challenges.

At this point in the overall development cycle, it is intended to employ system modularity to allow the ability of interchanging components as different or improved systems become available. For example, it would be beneficial to allow improved CO_2 conversion systems to be swapped in/out as technology capability improves. This prototype unit is not designated to be a flight system or be compatible with mission deployment – this is an initial ground test system and laboratory development unit.

Overall tasks include:

- Complete the CO_2–to-formic acid conversion system development and testing.
- Continue the development and testing of an advanced acetate production reactor concept in collaboration with Dr. Matthew Kanan of Stanford University. This is conducted through a continuing cooperative agreement. This capability is to be conducted separately, and the actual reactor will not be emplaced in the NASA tested system. Rather, the product generated by Dr. Kanan's systems will be either used or adequately simulated (to address quantity limitations) in system testing.
- Complete integration of validated system components. Install and test all required sensors and controls, and data collection systems for performance evaluation.
- Develop and test the preliminary engineered microorganism capable of producing the targeted compound carbonic anhydrase.
- Develop and test performance of a space-relevant bioreactor concept with the engineered organism to produce the targeted product.
- Demonstrate the ability to purify and potentially use the targeted product from the engineered microorganism.

Together, these tasks will enable conducting Aim 2, which involves initial performance testing of the integration of the individual components (see Aim 2 below).

Aim 2: Integration and Testing

The tasks associated with this Aim involve the integrated testing of the individual system components. This includes the use of the NASA-led formic acid electrolysis system, products formed from the Stanford-led acetate electrolysis unit, the space-relevant bioreactor, the engineered microorganism, and product separation. This effort is not intended to be a refined, single unit that automatically conducts these processes. It is instead a first attempt at using the developed hardware and resultant component products in a stepwise process to demonstrate overall capability. It will also reveal areas for future improvement, and guide component integration into a single, functioning mission-relevant (not flight ready) prototype. The tasks for this initial integration and testing include:

- Demonstrate complete CO_2-based manufacturing prototype system. Emulate Martian CO_2 and H_2 inputs using representative/simulated CO_2 and hydrogen sources.
- Demonstrate methods to generate a complete "*In-situ* Media". This involves amending organic CO_2-derived organic molecules with other required components and determining potential in-space sourcing of these components.
- If feasible, demonstrate the potential use(s) for the produced mission product. i.e., demonstrate that the carbonic anhydrase product is functional for CO_2 capture enhancement.
- Conduct an overall analysis to provide recommendations for future work, including system enhancements, scaling, mission integration scenarios, and other potential mission products.

The tasks outlined above will provide a comprehensive knowledge base and demonstration of capability that will guide FY 20/21 tasks (as discussed in Aims 3 and 4 below).

Aim 3: Space-Ready Production Strain

The initial demonstration of the CO_2-based manufacturing system as discussed in Aim 2 will be conducted with a traditional microbial engineering host (*E. coli* K-12). This organism is used because it has a wide array of available engineering tools which simplify the initial demonstration. It is not necessarily well suited for use in space operations, as its ability for long duration and ambient temperature storage is not well established. Also, even though this *E. coli* strain is not pathogenic, it may still raise safety concerns.

Therefore, this task will focus on the development of an alternate "space-ready" microorganism that is well adapted for the unusual substrates that will be available, product generation effectiveness, and its capacity for long-duration storage and activation.

Information derived from the on-orbit BioNutrients-1 tests will be utilized to guide the development of this organism. This includes the potential of using information gained from the selected engineering hosts contained in the stasis packs for initial engineering, desiccation engineering and procedures, and pellet encapsulation process effectiveness assessments. Additionally, any initial genetic information derived from "omics" analyses of durable and intentionally mutated strains may be exploited to increase space readiness.

The major elements of this task include:

- Using literature and information derived from the BioNutrients-1 flight experiment, as well as initial testing in intended substrates (acetate and formic acid), identify and down-select potential engineering hosts for use in the CO_2-Based Manufacturing system.
- Using metabolic modeling techniques, design and implement the metabolic pathways for production of the intended demonstration product (carbonic anhydrase) in down-selected hosts.
- Test the engineered microbes in relevant conditions, and begin storage testing as soon as possible in the development cycle to provide preliminary storage capacity estimates. Sufficient samples will be stored for the potential continued analysis after the completion of this project segment (follow-on R&TD).
- Provide a final microorganism for testing in the space-compatible prototype developed in Aim 4 (see below).

These efforts will provide an organism that should provide stable performance for testing in the prototype. This organism is not intended to exhibit the highest levels of product synthesis that can be realized. In general, it is our intent to demonstrate capability, and not to try to maximize product titer within biomass. Maximizing product titer is a cost and time intensive process that is not warranted until it is established that this particular organism will be used with a mission scenario. At that point, it would be prudent to acquire the services of biotechnology firms that specialize in this capability, as they have the dedicated facilities for rapid, high-throughput development and advanced metabolic modeling techniques at their disposal.

Aim 4: Mission-Compatible Prototype

This work focuses on building upon the work completed in Aims 1 and 2 and developing a prototype that represents a single working unit that is also compatible with the reduced gravity conditions of both lunar and Mars surface missions. It will leverage the previous work associated with developing individual systems components, and initial integration results. The organism developed within Aim 3 will eventually be utilized for final tests. Prior to its suitability for testing, initial versions of the microbe will be employed for preliminary testing as appropriate.

It is anticipated that having reduced gravity will be beneficial as compared to operation in microgravity conditions. The presence of gravity will facilitate separation of the three phases within the bioreactor. Gases will be able to gravity-separate and will likely provide mechanisms for improved gas diffusion and availability to the microbes. Solid biomass may also be more easily separated with gravity. Fluid management can also be enhanced in gravity environments.

In contrast, reduced gravity can still pose design challenges with respect to bubble formation and separation and create increased capillary influences that must be accounted for in the overall system design. Therefore, it is intended to seek consultation with microgravity fluid dynamics expert Mark Weislogel at Portland State University to assist in these design issues.

A major design effort regards the alteration of the bioreactor system to be better suited for mission utilization. Because it is intended for this system to be a platform technology that allows a variety of products to be produced, it must also allow a variety of different microbes to be used. As compared to the BioNutrients-1 effort, which employed a fermentative (no O_2 required) yeast, many production hosts will actually require the input of O_2 as a final electron acceptor. When organisms grow on lower-energy substrates such as acetate, aerobic respiration is preferred or even required. Supplying oxygen requires a bioreactor with better mass transfer efficiency than a BioNutrients-style container that simply allows gases to escape. Semipermeable membranes and mixing strategies will therefore be investigated and incorporated into the prototype.

Terrestrial SOA bioreactors that produce high value compounds such as pharmaceuticals are designed are not as constrained regarding ESM as are space systems. Selection of lighter materials, smaller sensors, and simpler control strategies can quickly yield ESM improvements. Additionally, trade-offs must be assessed. Higher cell growth rates allow for smaller reactor volumes, but require the costs of additional mixing equipment, process control systems, and / or consumables. Gravity-independent operation requires gas/liquid management. Operation in space also requires reliability and simplicity. This set of NASA-specific considerations helps define the design space for a mission-compatible prototype.

A soft-sided bioreactor, i.e., a bag, can be expected to offer lower ESM than a rigid vessel, but may be perceived as less safe due to the possibility of puncture or rupture if pressurized. Disposable bioreactor bags are commonly used on Earth; design ideas and regulatory certifications from these COTS products will be leveraged. A bag incorporating semipermeable membrane materials can contain liquid and maintain sterility while permitting gas exchange. Additional mechanical mixing, shaking, or forced aeration features may or may not pay for themselves on an ESM basis. The bags must also allow for a high degree of biomass harvesting and overall substrate conversion to biomass/products (see KPPs 6 and 7) to enable acceptable efficiencies. Fabrication and testing bags with such features will answer these questions and lead to an optimized prototype.

In addition to bioreactor enhancements, improved product purification methods will be required. This process can often be extremely specific to the organism and product characteristics. For example, cells may need to be disrupted and solvent extracted to obtain high product yields. Alternatively, an organism may be engineered to secrete the target product, potentially improving separation ease and economy. Therefore, while a specific method will be developed for carbonic anhydrase separation, focus will be given to the potential to enable multiple methods of product purification.

A summary of specific tasks for this Aim include:

- Develop the overall set of design requirements for the space-compatible prototype system. This includes the required CO_2-derived substrate characteristics and the additional needed media components, the bioreactor operational needs, and product harvesting and purification.

- Using these requirements as a baseline, develop a reduced-gravity bioreactor system that is compatible with the lunar and Mars surface missions. It will be designed to optimize substrate to biomass conversion and harvesting effectiveness. This involves selecting materials of construction, gas/fluid/solids management, and interfacing with media and organism input methods.

- Develop the required biomass harvesting and product purification methods required to efficiently recover targeted products. This includes appropriate interfaces with the production pack, as well as the development of novel methodologies.

- Using the developed prototype, conduct tests with the "space-ready" engineered microbe (developed in Aim 3) that produces the targeted product carbonic anhydrase, and that is compliant with the requirements and desirements of the bioreactor production system.

- Develop a technology assessment and a forward plan for maturing this technology for future surface missions, including potential products/use cases, estimated ESM, and identify the remaining R&D challenges that require further investment.

It must be noted that this effort will not focus on the integration of a CO_2 conversion system to make the microbial substrates within the prototype. Our research to date has indicated that the NASA-led formic acid electrolysis unit will not provide a suitable substrate within the context of this prototype demonstration. Instead we plan to continue support for the development of the Stanford-led acetate electrolysis unit in order to investigate scale-up and improved consumable logistics. The actual chemical products and / or simulants from this system will be used in a manner that simulates the physical inclusion of the system. This is partially because of the cost and difficulty of developing a unit for integration with the remaining prototype components. Another factor is that the NASA CO_2 Conversion Centennial Challenge may result in even better substrates for eventual testing.

This work will culminate in a close-out report that will document the results of the entire project and include recommendations for future directions in CO_2-Based Manufacturing systems development for space applications.

Technology Readiness Assessments

Assessments of the five major technology deliverables for the Syn Bio have been conducted as part of an internal team review. These will be updated and assessed annually as part of the GCD Technology Assessment Periodic Review (TAPR). The results are discussed below and are summarized in Table 5.

- **Space qualified bioreactor development**: Pre-packaged, hydratable, disposable, flexible bioreactor capable of safely growing engineered organisms to produce targeted nutrients. The ongoing BioNutrients flight experiment intends to demonstrate that the overall concept is implementable in space. It utilizes a hard-shell production pack that is not the ultimate design desirement and will be replaced by packs with improved mass and volume reductions. From this on-orbit experience, an entry TRL = 3 has been determined. It is anticipated that the production and testing of the Gen-1 and Gen-2 packs will bring the TRL level to 5, and perhaps higher depending on performance.

- **Long duration ambient storage of microorganisms:** Ambient storage system that involves three components, a microorganism that is both naturally and synthetically engineered for long duration storage at ambient temperatures, optimization of the preparation of the organism for stasis, and packaging optimization. Over the course of the five-year effort, it is expected that microorganisms will be identified/ developed that are both excellent engineering hosts and well adapted for use in long duration missions. This information will be published, and the organisms made available to other researchers to allow high-value capture and dissemination of this experiment. The microorganism's entry TRL is assessed at approximately 3, with a final TRL of 5, and possibly higher.

- **Methods for ensuring quality and safety of biomanufactured nutrients:** HACCP Plan for safe preparation of biomanufactured nutrients, post growth processing, safety assurance, and product quality verification. As there is no current system that is appropriate for ensuring food safety for microbial foods, the entry TRL is assessed at 2. After development and testing on-orbit, the resultant processes should elevate to an overall level of TRL = 5. Some individual components may vary from this estimate.

- **Development of ISRU based growth media:** Abiotic production of CO_2 derived organic carbon substrate required for the growth of the microorganisms that manufacture the products of interest, produced using in situ and mission waste materials. A prototype design for CO_2-based physicochemical conversion of CO_2 to acetate has been tested at a bench scale and individual subsystems such as growth of E. coli on the acetate-based media has been performed. Building a higher fidelity prototype and improvements to the physico/chemical conversion system, modeling, and follow-on testing to optimize the media composition should allow system design tradeoffs to be better understood (moving to TRL3 / 4). The Use Case scenario being worked by team and discussed externally by stakeholders may allow this to move to TRL4 / 5.

- **Space relevant biomanufacturing system development** (Integrated CO_2-Based Manufacturing Prototype)**:** Integrated prototype biomanufacturing system that couples physico-chemical conversion of CO_2 to a microbial growth supporting bioreactor and product purification system. There has been demonstrated progress in developing the individual components of the overall prototype as a terrestrial system but work towards meeting likely operational space relevant requirements has not been incorporated yet, therefore, the entry TRL is assessed to be a value of 2 or less. Significant uncertainty still exists of the integration performance and further development is required. After the completion of that work, it is anticipated that the overall TRL will become a composite value of 4, with potential component-level variances.

- **Space qualified organisms for biomanufacturing:** Microorganisms demonstrated to have sustained viability in preliminary spaceflight tests, readily engineered, and optimized for growth on ISRU derived media. We have a microorganism that is engineered to express a product of interest and this organism has been shown to survive utilizing the ISRU derived media. Models predict multiple organisms can be supported in our system (TRL 3). Questions remain as to the long duration shelf life, radiation hardening, and optimization of these organisms needed to meet the full requirements of the CO_2 based manufacturing platform. More testing and analysis are needed and results from the BioNutrients project will be used to reach a higher TRL value for the space qualified organism element allowing this to move to TRL 4 or even 5, results dependent.

Table 5. Technology Readiness Assessments

Technology Readiness Assessments			
Technical Capability Elements	**TRL**		**TRL Verification**
	Entry	**Exit**	
Space qualified bioreactor development	3	5 (1)	BioNutrients Fight tests will demonstrate on-orbit technical and safety performance from current hardware and result in the design of flight verifiable unit. Milestone ID = C9-BN

Long duration ambient storage of microorganisms	3	5	5 year flight/ground tests will demonstrate growth performance, product formation, survivability and stability of test organisms, and result in enhanced hosts for future use. Milestone ID = K19 -BN
Methods for ensuring quality and safety of biomanufactured nutrients	2	5	On-orbit test of Gen-2 production pack with the developed food safety measures (e.g. HACCP plan) to ensure product quality. Milestone ID = K17-BN
Development of ISRU based growth media	3	4	Further development of CO_2 conversion unit from collaborator, Lunar / Mars compatible CO_2-Based Manufacturing prototype tests complete. Milestone ID = C6-CO_2
Space relevant biomanufacturing system development	2[(2)]	4	Integrated prototype laboratory testing complete. Milestone ID = C4-CO_2
Space qualified organisms for biomanufacturing	3	5	Production strain testing complete and data from BioNutrients long duration storage studies. Milestone ID = K5-CO_2

(1) Flight demonstration will indicate concept feasibility / performance but does not render it flight-ready for actual use.
(2) Bag-based reactor is newly developed concept for this technology. Expect successful use to raise it to TRL 3-4 via integrated laboratory testing.

6.0 Management Approach

The STMD GCD Space Synthetic Biology (Syn Bio) Project activities within this plan will be managed by ARC. The organization for this project is represented in Figure 5 and Table 6. ARC management is overseen by the ARC STMD point of contact. The Project Manager (also the Principal Investigator) is based at ARC and will oversee the Flight Payload Managers, Resources Manager and the project Science Leads.

Communication and reporting will flow up the STMD GCD organizational structure. The Syn Bio Project Manager (PjM) will report to both the ARC center point of contact and to the STMD GCD Program Manager/Program Element Leads on a regular basis. The Syn Bio PjM will track progress of the tasks monthly and provide monthly reports as requested, including progress, schedule and technology variances from the plan and include an evaluation of the project risk plan. The Syn Bio PjM will also support GCD program reviews to evaluate project performance and provide information as necessary to potential customers.

Interactions with other NASA centers will occur primarily through flight activities. The project expects to continue interactions with the JSC Payload Integration Manger (PIM) for BioNutrients flight operations. Additionally, appropriate personnel from supporting flight centers (e.g., KSC, Wallops) will be engaged. General consultation is expected to continue with other centers throughout the entirety of this project. In particular, the JSC Human Research Program (HRP) will be frequently consulted regarding nutrient selection and food safety guidelines. *In situ* Resource Utilization (ISRU) and In-Space Manufacturing (ISM) personnel will also be consulted regarding potential project interfaces.

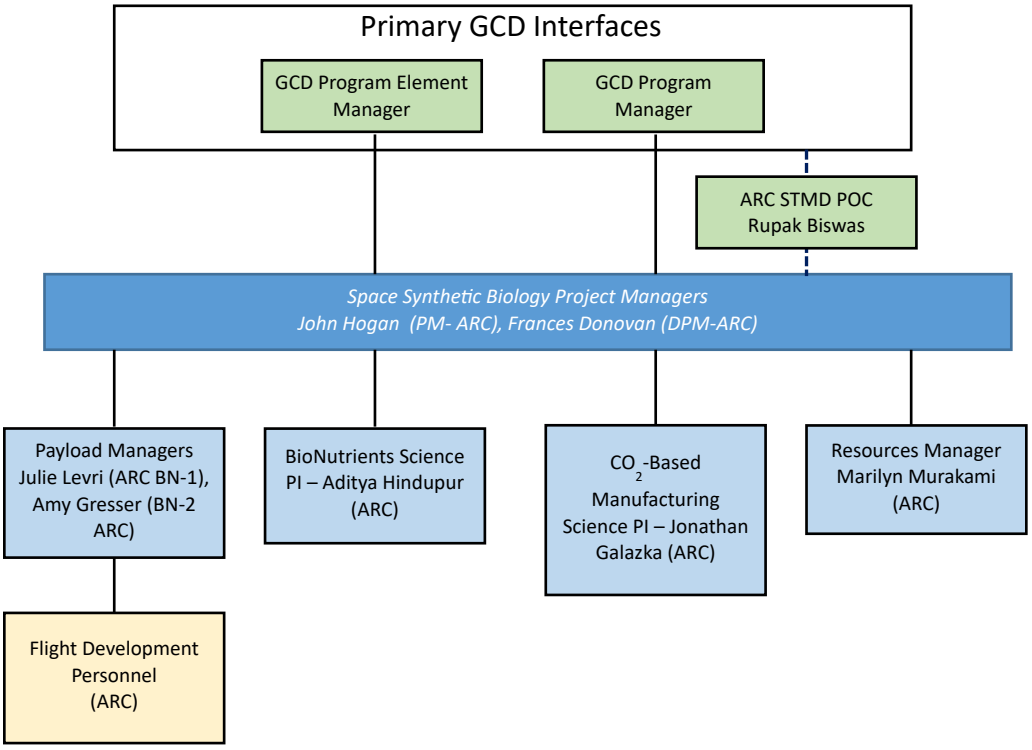

Figure 5. Organizational Structure

Table 6. Roles and Responsibilities

Roles and Responsibilities		
Role/Title	Name	Responsibility
Program Manager	Drew Hope	Project plan approval authority.
Program Chief Engineer	Amanda Moore Cutright	Program engineering technical authority.
Program Element Manager	Kevin Kempton	Primary program contact with the project.
Program Chief Safety Officer	Duane Pettit	Program Safety Technical Authority for safety requirements and waivers.
Project Manager	John Hogan (PM) Frances Donovan (deputy PM) Julie Levri (Payload Manager BN-1) Amy Gresser (Payload Manager BN-2)	PM and DPM manages project technical objectives and direction. Payload Managers manage flight demonstration preparation and operations.

Project Resource Manager	Marilyn Murakami	Manages project labor and procurement resources.

There is no significantly unique infrastructure or institutional requirements that are critical to the successful execution of the project during its life cycle. The primary infrastructure required for this work is contained within Codes SCB and SCF within ARC. Code SCB (Bioengineering Branch) contains the laboratories and flight payload development areas needed for the vast majority of the proposed efforts. These laboratories house the required assets regarding the necessary molecular biology activities, as well as hardware development. Code SCF (Flight implementation Branch) facilities are utilized while preparing hardware for flight. ARC fabrication facilities and external vendors are also utilized for certain portions of the hardware development efforts. Management from codes SCB and SCF interact and cooperate regularly to maintain the required facility resources and maintenance.

There are no leveraged resources from funding organizations outside of GCD.

The flight work encompassed in this plan manages technical data using processes levied by the NASA ARC Space Biosciences Division (SC). Table 7 lists the procedures and work instructions that address technical data management from change control through documentation practices, record keeping and data retrieval, all of which ensure that NASA quality standards for flight projects are satisfied. All technical data is recorded for retrieval as needed from the ARC SC Engineering Resource Center (ERC).

Table 7. Ames Procedures and Work Instructions

Document Number	Document Title
AI-04172	Document Change Preparation and Processing
AI-04173	Document and Change Release Distribution
AI-04170	Document Preparation and Review
AI-04171	Document and Product Identification
AI-04246	Review and Approval Matrix
AI-00007	Test Procedure Requirements
AI-06711	Build Instruction Requirements
AI-04229	Preparation, Registration and Revision of Forms

7.0 Work Breakdown Structure

Figure 6 depicts the Syn Bio Project Work Breakdown Structure (WBS), and Table 8 provides the WBS dictionary.

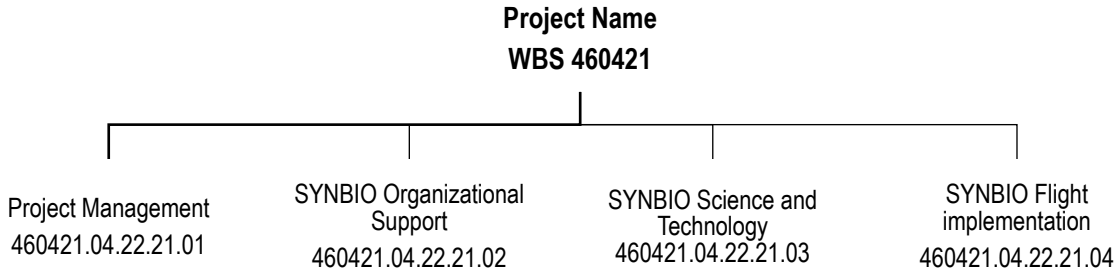

Figure 6. WBS Structure

Table 7. WBS Dictionary

WBS Dictionary		
WBS Element	Title	Description
1.0	Project Management	Includes project management, and task leads, budget, schedule.
2.0	Organizational Support	Includes Resource Manager and associated personnel
3.0	Science and Technology	Includes Science Leads, and Science Team personnel
4.0	Flight Implementation	Includes Flight Payload Managers, flight implementation personnel (ARC/JSC)

8.0 Schedule and Milestones

The project milestones and key and controlled milestone lists are presented in Figure 7 and Table 9, respectively.

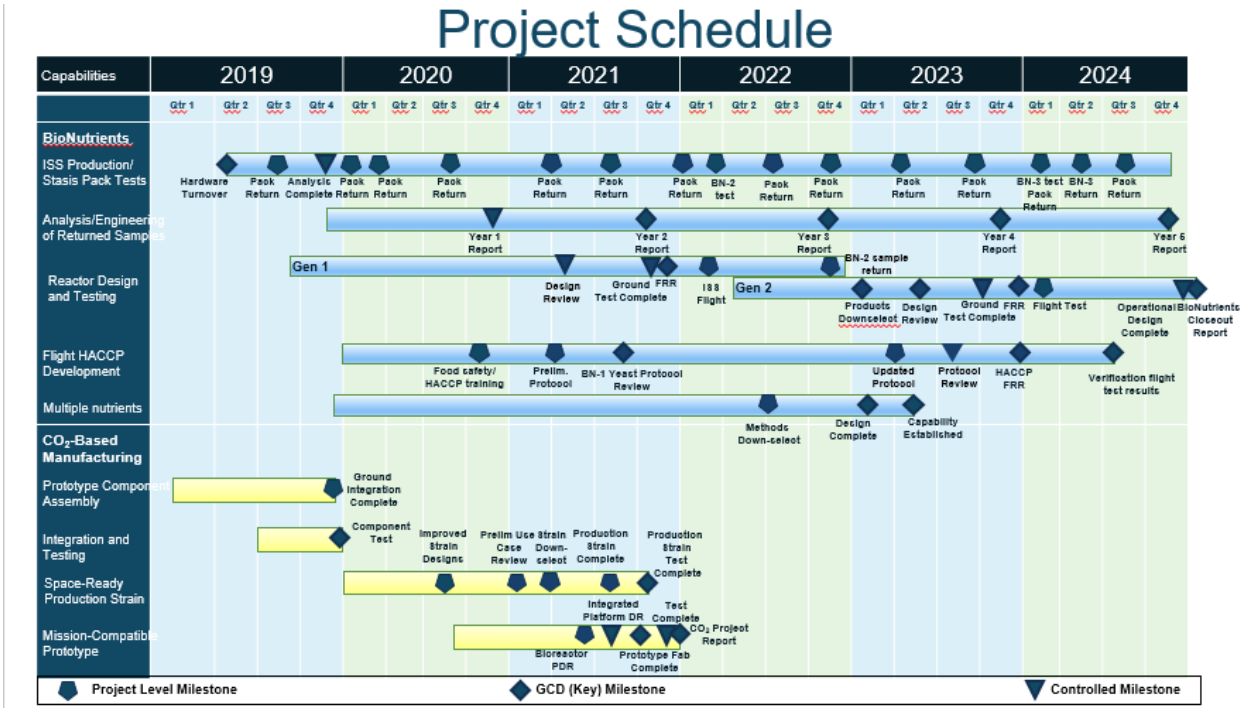

Figure 7. Project Schedule for BioNutrients and CO2-Based Manufacturing Tasks

Table 8. Key and Controlled Milestone List

Project Key and Controlled Milestone List				
ID	**Title**	**Description**	**Deliverable**	**Due Date**
K1-BN	Flight Hardware Turnover	BioNutrients-1 nominal load hardware turnover for SpX-17 ISS	Hardware	01/30/2019
C1-BN	Analysis of 1st Sample Return	Growth Analyses of BioNutrients first samples returned from ISS (Earth Production /Stasis Packs)	Test results	08/04/2019
K2-CO_2	Component Test	Demo of CO_2-Based manufacturing integrated prototype and in-space implementation analysis	Hardware	09/15/2019
C2-BN	BioNutrients Flight Demonstration Results 2020 Annual Report	Results of flight/ground BioNutrients testing	Report	07/20/2020
C3-BN	Design Review	Generation 1 Flight Pack Design Review	Review	01/19/2021

C4-CO_2	Space Compatible System Design Review	Design review/GCD TAPR held for overall CO_2-Based Manufacturing prototype	Review	04/15/2021
K3-BN	Yeast Food Safety Protocol Review	NASA review of flight HACCP and other safety protocols for BN-2	Review	05/15/2021
K4-CO_2	Space Compatible platform Fab.	Fabrication of the lunar / Mars compatible platform complete	Hardware	07/01/2021
K5-CO_2	Production Strain Ground Tested	Space-ready production strain testing completed	Test Results	07/07/2021
K6-BN	BioNutrients Flight Demonstration Results 2021 Annual Report	Results of flight/ground BioNutrients testing	Report	07/20/2021
C5-BN	Gen-1 Pack GroundTest (EVT)	Bionutrient Gen-1 ground tests completed	Test Results	07/28/2021
K7-BN	FRR (targets SPX-24 Nov Launch)	BioNutrients flight readiness review for Gen-2 pack	Review	09/01/2021
C6-CO_2	Space Compatible Prototype Tested	Lunar / Mars compatible CO_2-Based Manufacturing prototype tests complete	Hardware/ Test Results	09/15/2021
K8- CO_2	Closeout Report	CO_2-Based Manufacturing closeout report	Report	09/30/2021
K9-BN	BioNutrients Flight Demonstration Results 2022 Annual Report	Results of flight/ground BioNutrients testing for year 3 (Reported at GCD TAPR)	Report	07/20/2022
K10-BN	Products/strains Down-Select	Products and strains selected for further engineering and flight tests	Down-select	9/15/2023
K11-BN	Multiple Nutrient System Design	Design of system able to produce multiple nutrients completed	Design	10/15/2023
K12-BN	Multiple Compound Nutrient Production Established	Ability to make 2 or more compounds nutrients demonstrated	Report	02/10/2023
K13-BN	Gen-2 Pack Design Review	Design review of Gen-2 pack complete	Review	03/01/2023
C7-BN	Food Safety Protocols (HACCP) Review	Food safety protocols reviewed by NASA and external experts	Report	05/01/2023
C8-BN	Gen-2 Pack Ground Tests	Ground testing of BioNutrients Gen-2 packs complete	Test Results	08/15/2023

K14-BN	HACCP FRR	Flight readiness review for food safety flight test/procedures	Review	09/30/2023
K15-BN	Gen-2 FRR	Flight readiness review for Gen-2	Review	09/30/2023
K16-BN	BioNutrients Flight Demonstration Results 2023 Annual Report	Results of flight/ground BioNutrients testing for year 4 (Reported at GCD TAPR)	Report	07/20/2023
K17-BN	HACCP verification test results	Results of HACCP implementation with flight samples	Report	04/15/2024
K18-BN	BioNutrients Flight Demonstration Results 2024 Annual Report	Results of flight/ground BioNutrients testing for year 5	Report	07/20/2024
C9-BN	Flight Compatible Pack Design	Design of flight operational BioNutrients production pack complete	Design/ Report	08/30/2024
K19-BN	BioNutrients Closeout Report	BioNutrients closeout report	Report	09/30/2024

Legend: C = Controlled Milestone; K = Key Milestone; BN = BioNutrients Project; CO_2 = CO_2–Based Manufacturing Project

9.0 Strategy for Technology Transition

In general, the primary stakeholders for these technologies are housed within HEOMD. In particular, Advanced Exploration Systems projects are potential customers for a range of possible products. The Life Support Systems (LSS) project has expressed strong interest in the product (carbonic anhydrase) we are manufacturing for the CO_2-Based Manufacturing task. This is valuable to them as it may be a required component for successful operation of a liquid amine-based CO_2 removal system currently under development. In this vein, the LSS project is currently funding our laboratory to develop a customized carbonic anhydrase enzyme suitable for use in their system. The goal of this effort is the specific properties of the enzyme, not the capability of producing during space missions.

The AES Logistics Reduction (LR) project, and in general the goals of the ISRU program, will also be potential customers of possible products. For example, LR goals include reducing mission consumables and repurposing wastes. The Syn Bio Project has the potential to be able to make several mission products using *in situ* resources.

The Human Research Program (HRP) is also a potential customer for eventual products developed in the BioNutrients project. HRP personnel are kept aware of our activities and are consulted for their system requirements.

At the time of writing this document, there are no commitments from potential customers regarding needed hardware/ system delivery.

Appendix A: Additional Plan Paragraphs

A-1 Risk Management Plan

The Project will manage risks per GCD Risk Plan guidance in GCDP -01-RPM. In addition, the flight payloads development effort for BN-1,2, and 3 will follow the IAW NPR 8000.4, tailored to meet the needs of the project.

As is standard for flight payloads, the BioNutrients effort further defines the project "risk" as any circumstance or situation that poses a threat to project control cost, project control schedule, or major mission objectives, and for which an acceptable resolution is deemed unlikely without a focused management effort. Project risks are managed in compliance SCF Work Instruction 6487 – Space Biosciences Division (SCF) Risk Management Plan, which is consistent with NPR 8000.4 and Ames Procedural Requirement (APR) APR 8000.4. The risk management process is tailored for BioNutrients as described below.

Step 1 – Team members report any new risks at team meetings. In addition, the Risk Management Board also brainstorms risks at every monthly (approximately) scheduled risk meeting.

Step 2 – Based upon the nature of the risk, the Risk Manager (RM) assigns a Risk Owner (RO).

Step 3 – The RM works with the RO to identify the appropriate risk statement and draft a consequence and likelihood score based upon the criteria shown in the below tables, which hare taken from SCF Work Instruction 6487.

Step 4 – The Risk Management Board is convened. The Board is composed of the project team, including representation as needed by the project manager, science, engineering, safety, quality assurance, operations, and budgeting. The board assesses documented project risks and decides on a plan of action to ensure risks are minimized. The board assesses the consequence and likelihood rankings, prioritizes the set of risks, and assigns each risk a rating of i) Accept – risk is accepted and not mitigated, ii) Mitigate – action steps are taken to lessen the probability or impact of the risk, iii) Watch – the risk is regularly monitored, to determine if different action should be taken, and iv) Research and some investigation is conducted in order to determine proper action. By default, all risks with a likelihood ranking of "low" and a consequence ranking of "low" are accepted.

Step 5 – The RM works with the RO to create a mitigation plan and checks in with RO regularly to track progress on that plan.

Step 6 – The RM updates the BioNutrients-2 Risk List on the team SharePoint site.

Step 7 – The RM keeps the PM informed of risks evolution and provides updates at team meetings. Risks are also presented to and reviewed by Space Biosciences Division management as needed. For reporting purposes, presented risks are exhibited in a 5x5 risk matrix.

Level	*Probability*	*Probability (Safety)*	*... or – the current process...*
5	Very Likely 80 – 100%	Likely to occur immediately ($X > 10^{-1}$)	Cannot prevent this event, no alternate approaches or processes are available.
4	Likely 60 – 80%	Probably will occur in time ($10^{-1} > X > 10^{-2}$)	Cannot prevent this event, but a different approach or process might.
3	Possible 40 – 60%	May occur in time ($10^{-2} > X > 10^{-3}$)	May prevent this event, but additional actions will be required
2	Unlikely 20 – 40%	Unlikely to occur ($10^{-3} > X > 10^{-6}$)	Is usually sufficient to prevent this type of event.
1	Highly Unlikely 0-20%	Improbable to occur ($10^{-6} > X$)	Is sufficient to prevent this event.

Level	*1*	*2*	*3*	*4*	*5*
Mission Success Operational Performance (Technical)	• Minimal or No Impact to mission objectives • Nominal execution of mission • Minor reduction in performance • Minor or no impact to design or operating margins	• Failure to meet any single mission objective • Operating in degraded state • Moderate reduction in performance • Can handle with design or operatin gmargins • Damage to non-critical system, element, ground facility, function or emergency system	• Significant impact to mission objective • Operational worakarounds avilable • Significant reduction in performance • Significant loss of design or operating margin • Loss of any non-critical system, element, ground facility or function • Loss of emergency system	• Loss of multiple mission objectives • Major increase in flight operations timelines or complexity • Major degradation in performance • Loss of all design or operating margin • Damage to critical system, element, ground facility, or function	• Loss of entire mission/scienc e • No alternatives exist • Loss of payload or any critical system, element, major ground facility or function
Schedule	Minimal or No Impact	Additional Activities Required. Able to Meet Need Dates	Level 2 Milestone Slip of ≤ 1 Month.	Level 2 Milestone Slip of >1 Month, or Program Critical Path Impacted	Cannot Achieve Major Program Milestone
Cost	Minimal impact (0 to 2.5% increase)	Moderate impact (2.5% to 5% increase)	Significant impact (5% to 7.5% increase)	Major impact (7.5% to 10% increase)	Major impact (>10% increase)
Safety	No Injury	Minor Injury, minor illness	Significant or longterm injury, illness incapacitation or impairment	Permanet injury, impairment or incapacitation	Loff of life, disabling injury

A-2 Security Plan

The Project will provide protection for sensitive classified documents, materials, information, or by-products commensurate with the assigned classification level, which prevents unauthorized persons from gaining access during use, dissemination, storage, movement, or transmission.

All systems used for communication and for technical data management are approved for use with NASA ARC projects, as required by NPD 1440.6, NASA Records Management and Ames Procedural Requirements document APR 1440.1, Records Management Program Requirements.

A-3 Safety Plan

The Project shall comply with Occupational Safety and Health Administration (OSHA) and NASA SMA Requirements. For test activities that involve NASA personnel or property at locations not governed by the Agency and which can potentially lead to injury of NASA personnel or property damage, the project shall provide information as a supplemental appendix to the STMD Mishap Preparedness and Contingency Plan (MPCP) STMD MPCP which details the specifics of such an activity. The MPCP supplemental appendix shall be submitted to the Program Element Manager and GCD Chief Safety Officer for processing and approval well in advance of the scheduled test date.

The project complies with Occupational Safety and Health Administration (OSHA) and NASA safety and mission assurance requirements, as detailed in the following Ames Procedural Requirements APR 8705.1, System Safety and Mission Assurance and APR 8715.1 Ames Health and Safety Manual.

A-4 National Environmental Policy Act (NEPA) Compliance

The Project shall comply with the National Environmental Policy Act (NEPA), Executive Order 12114, and the requirements of NPR 8580.1. Additional guidance on compliance with these documents can be found in the GCD Project Manager Handbook.

The Project complies with the NEPA, Executive Order 12114, and NPR 8580.1, as required by Ames Procedural Documents APD 8500.1, Ames Environmental Policy and APR 8500.1, Ames Environmental Procedural Requirements.

A-5 Planetary Protection

As a technology development project, the Planetary Protection Provisions for Robotic Extraterrestrial Missions requirement, NPR 8020.12D, is not directly applicable to execution. For technology developments which are anticipated to matriculate to a flight mission, the approach for planetary protection and impact assessment will be coordinated with the appropriate mission directorates and NASA officials at both the center and HQ level for the flight program.

A-6 Systems Engineering

General Information The Synthetic Biology Project encompasses flight payload development for the BioNutrients system and non-flight/terrestrial platform development for the CO_2-based manufacturing system. The systems engineering approaches for these two endeavors differ accordingly. As a flight project, the BioNutrients approach is guided by NASA Ames Flight Systems Implementation Branch (SCF) and is consistent with NPR 7123.1B and the NASA Systems Engineering Handbook. The CO_2-based manufacturing effort will result in building and testing of a terrestrial lab prototype system and will be managed with a more agile systems engineering approach. Each approach is presented in more detail below.

The BioNutrients approach is tailored to the level of project complexity and is based upon NPR 7123.1B. The project technical team will plan, schedule, and participate in the reviews necessary to assure project workflow, processes, and technical product performance requirements are clear, well defined, and documented. Specific review gates will provide periodic assessment of the project's technical and programmatic status at key points in the life cycle. Configuration management and archival of all documents will be managed via the SCF Engineering Release Center (ERC).

1. The BioNutrients Review gates: All project reviews have specific entrance and exit criteria established prior to the review. Review completion documentation is maintained under configuration management in the controlled document archives of the ERC. Typically, gate reviews follow NASA 7120.5 and are tailored for the scope of the payload. Considering COVID-19 and the associated reduction in workforce availability, BioNutrients has received agreement from the Ames Chief Engineer's Office to streamline some review gates as indicated below.

Review	Description and Significance
System Requirements Review (SRR)	The SRR examines the functional and performance requirements defined for the system and preliminary project plan and ensures that the requirements and selected concept will satisfy the mission. Successful completion of the SRR baselines project requirements and leads to a formal decision by the cognizant authorities to proceed with preparations for project implementation. For BN-2, the Science and Demonstration Requirements Document (SDRD) and Concept of Operations Documents will be distributed to SCF, SCB, SC and ACE for comment by email by a specified deadline. SRR will be an internal review to assess the documents, consider comments, agree upon edits, and baseline. No slides will be developed. The baselined SDRD and ConOps as well as a summary sheet of disposition of received comments will be provided back to SCF, SCB, SC and ACE.

Review	Description and Significance
Design Review (DR)	Typically, both PDR and CDR are conducted for a flight project but, due to the relative simplicity of BN hardware, only one of these two reviews will be conducted. Since this is a milestone carried on the Project Plan, this will be a standard review per NPR 7120.5 appropriately tailored for the scope of the payload. The DR determines that the technical effort is on-track to complete system development and meet performance requirements within the identified cost and schedule constraints.
Experiment Verification Test (EVT) Test Readiness Review (TRR)	The EVT TRR certifies the all the following are in place to conduct a flight-like EVT: • Flight hardware is fully assembled, and flight materials are available in quantities needed • Flight procedures are complete and available • Facilities and ground support equipment are available • Staffing and facility planning are complete
Completion of EVT – Ground test complete	Successful completion of the EVT indicates successful ground-based, execution of the full duration of the planned experiment in a scenario that is as flight-like as possible.
System Acceptance Review (SAR)	The SAR verifies the completeness of specific end products in relation to their expected maturity level and assesses compliance with stakeholder expectations. The SAR examines the system, its end products and documentation, and test data and analyses that support verification. It also ensures that the system has sufficient technical maturity to authorize its shipment to the designated operational facility or launch site. Successful completion of the SAR, results in system acceptance by the customer, authorization to ship the hardware to the launch site or operational facility, and authorization to install software and hardware for operational use. For BN-2 and BN-3, the Requirements verification matrix (RVM) will only include science requirements and any qualification/acceptance requirements that are not already addressed by the ISS or Safety requirements. This is a low-risk approach, as adequate closure of ISS requirements is tracked in VERITAS and adequate closure of Safety requirements is determined by the PSRP. The RVM, along with any updated versions of the SDRD and ConOps, will be distributed to SCF, SCB, SC and ACE for comment by email by a specified deadline. SAR will be an internal review to assess the documents, consider comments, agree upon edits, and revise, as necessary. No slides will be developed. Baselined RVM and a summary sheet of disposition of received comments will be provided back to SCF, SCB, SC and ACE.
Pre-Ship Review (PSR)	At the PSR, all flight hardware, flight materials, relevant GSE and procedures needed for pre-flight are accounted for, reviewed for proper configuration, and approved for shipment.

Review	Description and Significance
Flight Readiness Review (FRR)	The FRR examines tests, demonstrations, analyses, and audits that determine the system's readiness for a safe and successful launch and for subsequent flight operations. It also ensures that all flight and ground hardware, software, personnel, and procedures are operationally ready. As a result of successful FRR completion, technical and procedural maturity exists for system launch and flight authorization and, in some cases, initiation of system operations.
Flight Sample Delivery to PI	Completion of sample delivery to the PI indicates turnover of responsibility for samples from the payload developer (PD) to the PI for initiation of sample analysis.
Project Closure	Project Closure occurs upon completion of the following activities: • PI has reported on key parameters from flight samples • Mission data has been documented and recorded • Final versions of all procedures, build instructions, and other PD-related hardware development documentation have been recorded in the ERC • Hardware has been appropriately inventoried and secured, either in MCS bonded stores or in the BioNutrients-2 PD hardware laboratory • Lessons learned have been appropriately documented

2. Requirements Management: The following table describes the BioNutrients requirements-related documentation and the responsibility for managing document content:

Document	Description of document content management
Science and Demonstration Requirements Document	The Payload Manager manages the content of the SDRD. All requirements are managed through a change control process that includes change impact assessment of all candidate modifications from the perspective of the PI and the PD. Accepted changes are incorporated into official revisions of the document, which are reviewed and approved prior to being recorded in the ERC.
ICD to SSP 57000	The BioNutrients-2 Interface Control Document Engineer (ICDE) manages the content of the ICD to SSP 57000. All requirements are managed through a change control process that includes change impact assessment of all candidate modifications from the perspective of the PI and the PD. Accepted changes are incorporated into official revisions of the document, which are reviewed and approved prior to being recorded in the ISS PIRN database.
System Requirements Document and Verification Matrix	The Payload Manager manages the content of the System Requirements Document and Verification Matrix. All requirements are managed through a change control process that includes change impact assessment of all candidate modifications from the perspective of the PI and the PD. Accepted changes are incorporated into official revisions of the document, which are reviewed and approved prior to being recorded in the ERC.

3. The BioNutrients experiment has the following key stakeholders:

- PI – The Principal Investigator of the BioNutrients Project, Dr. John Hogan at NASA Ames Research Center
- STMD – The Space Technology Mission Directorate Office at NASA HQ – Game Changing Division - Advanced ECLSS and ISRU
- HRP – The Human Research Program Office at NASA Johnson Space Center
- ISS – The ISS Payload Program at NASA Johnson Space Center
- Code S – The ISS Utilization Office in the Science Directorate at NASA Ames Research Center
- Code SC – The Space Biosciences Division at NASA Ames Research Center
- Code SCF – The Flight Systems Implementation Branch at NASA Ames Research Center
- Code SCB – The Bioengineering Branch at NASA Ames Research Center
- ARC OCE – The Office of the Chief Engineer at NASA Ames Research Center

4. Stakeholder communication will be maintained through regular updates and reporting, and stakeholder participation in reviews. Note that GCD project milestones include the review or reports generated from the reviews highlighted in blue above. Presentations and publications to the larger scientific community, as well as ad hoc meetings with stakeholders within NASA, will also be used to obtain feedback and guidance throughout this project.

The CO_2-based manufacturing platform will take an agile systems engineering approach. The team will focus on having smaller, frequent, and iterative technical exchanges with stakeholders, experts, and collaborators, and have fewer formal technical reviews. Requirements definition will be done through iterative testing and discussion with our collaborators and potential technology adopters within NASA, as part of ongoing research and development. Documentation will be done via reports to GCD and publication in scientific literature and conference presentations. All data, documents, and drawings will have configuration management by date and be archived in the Synthetic Biology Bioshock server. Specific artifacts and reviews are listed in Table 9 - Key and Controlled Milestones and will be uploaded and under configuration management on the GCD SharePoint site. If required by the Program Office, a more detailed approach to systems engineering will be documented in a separate Systems Engineering Management Plan.

The CO_2-based manufacturing platform has the following key stakeholders:

- PI – The Principal Investigator of the BioNutrients Project, Dr. John Hogan at NASA Ames Research Center
- STMD – The Space Technology Mission Directorate Office at NASA HQ – Game Changing Division - Advanced ECLSS and ISRU
- HRP – The Human Research Program Office at NASA Johnson Space Center
- Code S – The ISS Utilization Office in the Science Directorate at NASA Ames Research Center
- Code SC – The Space Biosciences Division at NASA Ames Research Center
- Code SCB – The Bioengineering Branch at NASA Ames Research Center
- ARC OCE – The Office of the Chief Engineer at NASA Ames Research Center

Stakeholder communication will be maintained through regular updates and reporting for GCD, listed as major milestones in this project plan in table 9, Key and Controlled Milestone List.

A-7 Commitments from External Organizations

No commitments from external organizations exist at this time.

Appendix B: Acronym List

ARC	Ames Research Center
CE	Chief Engineer
CFU	Colony Forming Unit
CO_2	Carbon Dioxide
CONOPS	Concept of Operations
COTS	Commercial-Off-The-Shelf
CTP	Common Technical Practices
CR	Change Request
CSO	Chief Safety Officer
ERC	Engineering Release Center
ESM	Equivalent System Mass
EVT	Experiment Validation Test
FDA	Food and Drug Administration
FTE	Full-Time Equivalent
FRR	Flight Readiness Review
FY	Fiscal Year
GCD	Game Changing Development
HACCP	Hazard Analysis and Critical Control Point
HRP	Human Research Program
ISM	In-Space Manufacturing
ISRU	*In situ* Resource Utilization
ISS	International Space Station
JSC	Johnson Space Center
KPP	Key Performance Parameter
LEO	Low Earth Orbit
LR	Logistics Reduction
LSS	Life Support Systems
MATS	Material Acquisitions and Tracking System

MCS	Materials Control System
MELFI	Minus Eighty-Degree Laboratory Freezer for ISS
MPCP	Mishap Preparedness and Contingency Plan
NASA	National Aeronautics and Space Administration
NEPA	National Environmental Policy Act
NPD	NASA Policy Directive
NPR	NASA Procedural Requirement
NSGC	Near Synchronous Ground Control
OSHA	Occupational Safety and Health Administration
P/C	Physico-chemical
PCD	Project Content Document
PD	Payload Developer
PG	Project Goal
PI	Principal Investigator
PIM	Payload Integration Manager
POC	Point of Contact
PEM	Program Element Manager
PjM	Project Manager
PM	Program Manager
PSR	Pre-Ship Review
PWD	Potable Water Dispenser
QA	Quality Assurance
R&TD	Research and Technology Development
SABL	Space Automated Bioproduct Laboratory
SAR	System Acceptance Review
SCF	Flight Systems Implementation Branch
SDRD	Science and Demonstration Requirements Document
SE	Systems Engineering
SHERLOCK	Specific High-Sensitivity Enzymatic Reporter UnLOCKing

SOA	State Of Art
Syn Bio	Space Synthetic Biology
STMD	Space Technology Mission Directorate
TAPR	Technical Assessment Periodic Review
T-REx	Technical Risk Executive
TRL	Technology Readiness Level
TRR	Test Readiness Review
TV	Threshold Value
UV	Ultraviolet
WBS	Work Breakdown Structure