Design, Testing, and Validation of the Search for Extra-Terrestrial Genomes Instrument

by

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B.S. Mechanical Engineering
California Institute of Technology, 2015

Submitted to the Department of Aeronautics and Astronautics in partial fulfillment of the requirements for the degree of Master of Science in Aeronautics and Astronautics at the MASSACHUSETTS INSTITUTE OF TECHNOLOGY

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Abstract

The development of any spaceflight instrument involves a systematic, iterative process of design, testing, and validation. This ensures that the system developed will meet the needs of stakeholders while minimizing costs and risks. Here, the needs for a life-detection instrument targeting nucleic acids are identified, the objectives for that instrument are determined, and system engineering analysis is used to demonstrate that a Search for Extra-Terrestrial Genomes instrument would fulfill those objectives and is feasible for a rover mission. Additionally, we show our design and build process for a testbed to rapidly prototype SETG components and subsystems, which has successfully automated nucleic acid extraction, sequencer loading, and parts of library preparation. We also experiment with thermal simulations and conduct a sequencing test at Martian conditions, using a custom built thermal vacuum chamber system. Finally, this thesis explores potential avenues for future development and identifies short term and long term engineering goals that would assist the SETG team in developing an instrument prototype.

Thesis Supervisor: Jeffrey A. Hoffman
Title: Professor of the Practice of Aeronautics and Astronautics
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A special thank you goes to my family and friends, who believed in me no matter my successes or setbacks. I couldn’t have written this without you.

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Chapter 1

Introduction

1.1 The Search for Extraterrestrial Life

1.1.1 Motivation

One of the oldest questions that faced early astronomers and scientists was whether we were alone in the universe. Finding life on other celestial bodies would fundamentally change the way we view our planet and its place in the cosmos. The way we view biology is tied to our single sample of Earth. As such, the search for extraterrestrial life is a natural continuation of humanity’s curious and scientific nature.

There are, however, substantial difficulties in finding life away from planet Earth. The challenge of even escaping Earth’s atmosphere has only been surmounted in the last century, and the great distances that separate our planet from other celestial bodies make traditional human exploration difficult. Several different approaches have been developed for life detection that address these problems. Many of these techniques use robotics and astronomy to identify measurable indications of biological activity, or biosignatures.
1.2 Biosignatures

Biosignatures are a fundamental aspect of astrobiology: the study of the origin, presence, and future of life in the cosmos. They can be broadly organized into two categories: the properties of life, and biomarkers. Life detection technologies have traditionally focused on one or the other.

1.2.1 Properties of Life

Searching for the properties of life requires being aware of what is essential for life to exist and searching for those aspects. For example, a fundamental feature of life is some form of metabolism, with all known organisms utilizing water in the process. Thus, searching for the presence of current or past water on other worlds is a valid way to search for evidence of extant life. Other methods have attempted to detect life by observing processes that lifeforms perform. The Viking lander conducted three experiments attempting to identify common life processes, including the metabolization of aqueous nutrients, carbon fixation, and the exchange of gases such as oxygen or methane [1]. Finally, the presence of life can lead to measurable indirect effects, such as atmospheric disequilibrium of gases or the seasonal variability in Earth's surface coverage by vegetation; these effects can be measured via an orbiter or even a ground based telescope [2].

These methods of identification are powerful and are a major part of current life detection missions, as well as NASA’s astrobiology strategy [3]. Mariner 9 was able to return images of riverbeds and evidence of water-based erosion [4], while the 2001 Mars Odyssey mapped the distribution of water on the surface of Mars [5]. The Mars Express orbiter used spectrometers to map water ice, methane, and ammonia starting in 2003. Pathfinder’s findings suggested a wetter Martian climate in the past, while Spirit and Opportunity found strong evidence to support the water hypothesis in the Columbia Hills and Meridiani Planum [6, 7, 8].
1.2.2 Biomarkers

Biomarkers on the other hand are physical objects, often molecules that indicate the presence of life. On Earth, for example, the physical remains of a lifeform would be a biomarker. On other worlds, biomarkers would likely be organic molecules of some form, such as proteins or complex carbohydrates.

The Viking lander carried one experiment to search for biomarkers: the gas chromatograph-mass spectrometer (GCMS). This instrument attempted to heat Martian regolith and identify organic chemicals. The Phoenix landers later also carried a mass spectrometer, in addition to a wet chemistry lab (WCL) that measured the presence of various ions in the Martian regolith. The Mars Science Laboratory (MSL) rover, Curiosity, is also searching for biomarkers, and has successively discovered several evolved gases from heated regolith that indicated the presence of organic compounds [9].

Biomarkers have the potential to be particularly useful; the presence of a complex organic molecule can provide strong evidence for extant life. Challenges remain, however, in their detection. Contamination can cast doubt on the detection of a biomarker, and basic organics such as methane are not completely conclusive evidence. The next stage of biomarkers should involve the search for an unambiguous biomarker that could be identified as either contamination or potential extant life. Nucleic acids, as the building blocks of life as we know it, are the natural next step in life detection biosignatures.

1.3 DNA Detection for the Discovery of Extent Life

1.3.1 Panspermia

There have been several theories throughout history that state that life on Earth owes its genesis to conditions both on and off this planet; this idea is known as panspermia. The hypothesis that life originated from the stars is an ancient one, traceable back to the Greek philosopher Anaxagoras; but in recent times mounting evidence has
made the theory more plausible [10]. Weak panspermia is the idea that some of the basic organic compounds that make up life were delivered to Earth via an outside mechanism. Actual meteorite samples and simulated comet samples contain amino acids, nucleobases and basic sugars [11, 12, 13, 14, 15]. Thus, there is evidence to suggest that the building blocks of life are not exclusive to Earth. These same building blocks were delivered to other planets including Mars.

In addition, several studies have been conducted on a microbe’s ability to survive inside a meteorite. Another form of panspermic theory, litho-panspermia, posits that microbes could be transferred from one celestial body to another via meteorites. Since Mars and Earth have exchanged nearly a billion tons of rock during their early lifespan, it is also possible that early microbes could have been exchanged between the two planets, surviving, thriving, and evolving in their respective early environments. In this case, Earth life and Mars life, if it exists, could have some common ancestor [16, 17, 18, 19, 20, 21, 22].

In summary, there is strong evidence that life could have emerged on early Mars, either through rock exchange with Earth or through delivery of organics via comets and meteorites, and in that case would either be related to life on Earth through a common ancestor, or might have used the same building blocks. As such, the search for life should include the search for life as we know it.

1.3.2 Informational Polymers

Informational polymers are one of the main macro-molecules that exist in all known life. Their existence allows organisms to reproduce and pass on their genetic information to descendants. Through natural selection, this allows populations to adapt to their environment. Primarily, the informational polymers found on Earth are ribonucleic acid and deoxyribonucleic acid, RNA and DNA. The central dogma of molecular biology is that information is transferred from nucleic acids to proteins, which then carry out the functions of a cell and thus an organism. Nucleic acids are fundamental to life’s processes, but unlike other macro-molecules like complex carbohydrates, they can carry data that is unique to the organism in question. The full genome of
an organism allows that organism to be placed within the broader picture of life as we know it, allowing for hierarchical classification. Whether delivered via a comet or transferred early in the solar system’s history, life on Mars would in all probability have evolved very differently. This provides the added benefit of identifying forward contamination. If organisms discovered on Mars do not branch too deeply from the tree of life, most likely those organisms were brought by a rover, lander, or human.

1.3.3 Search for Extraterrestrial Genomes (SETG)

If the search for extant life is important, if nucleic acids are the best biosignature available, and if we as a species are at the right time and technological level, then the development of a life detection instrument based on detecting the presence of nucleic acids is a key goal of astrobiology and space technology. The Search for Extraterrestrial Genomes (SETG) is an effort at MIT to develop such an instrument. The goal is to develop an instrument that would fly on a flagship mission to Mars (or, alternatively, to an ocean world) and process some form of sample, notionally regolith. In processing this sample, this instrument would attempt to sequence the remnants of ancient nucleic acids and characterize the results. By doing so, it would be possible to demonstrate a strong positive detection for life, sensing false positives via DNA sequence and preventing forward contamination, and placing extant life on the tree of life, allowing for classification and further study.

The development of such an instrument is a long endeavour, however. Several years have to be spent on the development of the core science and potential components before a core design is even developed. As a large, interdisciplinary project, the development of SETG requires significant systems engineering. In particular, systems engineering’s focus on interactions and interfaces is vital in the testing, verification, and validation phases of instrument design.
Chapter 2

Systems Engineering of SETG

First, this chapter explores the systems engineering process taken with the design of SETG. Following the flowdown from needs to objectives to requirements, we explore what shape the SETG system would take, culminating in rough values of volume, mass, and energy draw for a potential instrument.

2.1 Needs

As addressed in the previous chapter, future life detection missions will require strong in-situ evidence of past or present biology. That need can be addressed by the SETG instrument, utilizing nucleic acids as an unambiguous biomarker. Fundamentally, all other objectives are derived from this main goal.

2.2 Objectives

The primary goal of the SETG instrument is to extract and isolate informational polymers from environmental samples, which could be regolith, ice, or liquid [23]. A secondary potential goal, however, would be to provide in-situ sensing of forward contamination, which would help future life detection missions from engaging in false positives [23]. Any nucleic acids accidentally aboard the SETG instrument or other aspects of a rover or probe could be analyzed in addition to a target sample, allowing
for the characterization of contamination.

The SETG instrument is currently being developed under the NASA MatISSE program, which is focused on technology maturation. This means that while the overall objectives presented earlier are still valid, smaller objectives have to be established for the three year life cycle of maturation the project is focused on. Based off of an initial development schedule, our initial objectives involve reaching a technology readiness level (TRL) of 6 at the end of the three year funding cycle; TRL 4 should be reached after the first year and TRL 5 after the second. Technology readiness level is not a well defined metric, and can vary from instrument to instrument. The engineering challenge is to then define TRL values that are accurate representation of readiness while also being achievable within a limited timescale. To that end, TRL 4 was defined as a work flow with automated subsystems and manual handling between subsystems, TRL 5 an end-to-end automated subsystem, and TRL 6 a nominal Mars prototype that functions end-to-end automatically in a thermal vacuum chamber [23]. Sensitivity goals were also established for TRL 5 and TRL 6, with 10 part per billion (ppb) sensitivity at TRL 5 and 1 ppb sensitivity at TRL 6.

2.3 Requirements

Requirements are the next step of a systematic approach to instrument design; they describe the a system’s functions, attributes, and features. Establishing feasible, concise, descriptive requirements is key to preventing schedule issues, cost overruns, or technical failures.

For projects or systems still developing their technologies, it would make sense to establish requirements for specific development cycles rather than for the entire flight system; establishing requirements in order to allow an instrument to successfully apply for a flight proposal would be appropriate for relatively low TRL systems. The SETG instrument utilizes adapts components and protocols from diverse fields, so clear, well established requirements are important for communicating results across fields.
For SETG, the nominal mission was to aim for an instrument on a Martian rover. This places recommended requirements that the instrument should aim for a similar volume, mass, and energy draw of a typical Martian rover instrument. The instrument should also be sensitive enough to be able to successfully differentiate between Earth and potential Mars life in Martian regolith. These sensitivity and instrument requirements together drive the testing and validation that would occur Pre-TRL5 and Pre-TRL6; while some requirements are already on track to be fulfilled, others require some additional design iteration to approach. More general requirements involve surviving the trip to Mars and being able to communicate data back to Earth.

2.3.1 Technical Requirements

These general requirements flow down to more precise technical requirements. Creating an instrument that has a similar volume, mass, and energy draw to other Mars instruments means that rough values should be established for those parameters. Based off of the total payload mass of 80 kg for Curiosity, and discounting the large and central Sample Analysis at Mars instrument, each instrument is nominally estimated at 5 kg [24]. Those same instruments vary from 1 to 10 liters (L) of volume, leading us to set 2-3 L as a target volume. Energy draw is a difficult figure to bound, but Curiosity was able to generate 110 W of continuous power with roughly 1600 W hr of usable energy each day from the rechargeable batteries [25]. With that in mind, a stationary rover could theoretically provide 200 W hr to an instrument. Surviving the Martian transit trip means surviving the radiation environment of space, which was measured by MSL to be about 500 microGray per day [26]. Including operational time on Mars, 1 Gy radiation tolerance is a reasonable requirement. For sensitivity, the requirement to detect extent life in a regolith sample leads to the 1 ppb sensitivity requirement, established such that a theoretical SETG instrument could produce 1 Mb of data at a noise budget roughly equivalent to the one for reduced carbon detection on Curiosity’s SAM instrument [27]. These requirements are displayed below:
<table>
<thead>
<tr>
<th>Category</th>
<th>Target</th>
<th>Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass</td>
<td>~3 kg</td>
<td>6.0 kg*</td>
</tr>
<tr>
<td>Volume</td>
<td>2-3 L</td>
<td>4.3 L*</td>
</tr>
<tr>
<td>Energy</td>
<td>200 W hr</td>
<td>210 W hr*</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>1 ppb</td>
<td>100 ppb</td>
</tr>
<tr>
<td>Radiation Resistance</td>
<td>1-2 Gy</td>
<td>&gt;5 Gy</td>
</tr>
</tbody>
</table>

Table 2.1: SETG Requirements reported on Year 2 of the NASA report. Note that the mass, volume, energy draw, and sensitivity of the SETG instrument are currently higher than our target, while data return values are within budget.

### 2.4 Subsystems

In order to fulfill these technical requirements, preliminary designs for each individual subsystem and for the overall system must be established.

#### 2.4.1 Inlet

The inlet is, at the current stage, an under-explored aspect of the SETG instrument. While inlets on other systems such as the Phoenix lander can be used for inspiration, our workflow presents unique challenges. One of the challenges of the inlet is that it must be able to seal against the atmosphere to prevent the evaporation of reagents being introduced to the regolith. At the same time, the inlet has to open to accept regolith, without exposing liquids to the atmosphere. This necessitates some sort of airlock system, which has multiple points where sample can become clogged. In addition, the sample might have to be moved to other chambers where lysis and other steps can occur, either via a pump or pressure driven system, which is challenging if the sample is located in a reservoir that must unseal and then reseal. Additionally, the inlet is the first part of SETG that would interact with the regolith sample, and thus will depend heavily on the ability of a rover to introduce that sample to the SETG system. An assumption we are working under currently is that a ground, powderized sample can be introduced to the instrument via some sort of robotic arm, as is the case on MSL.

To address these challenges, several design options have been proposed, and several
members of the SETG group have looked at potential designs, including a rotational inlet and an inlet that consisted of a sealing screw. For the budgets, a simple rotational acrylic airlock operated by a servomotor, inspired by the Phoenix lander's inlet, was envisioned, with rough values of 70 cubic centimeters (cc) and 150 grams for volume and mass. Energy draw was estimated as what was required to turn the servomotor and seal the inlet.

2.4.2 Sample Preparation

The sample preparation portion of the instrument is one of the more developed systems, due in part to our laboratory's partnership with Claremont BioSolutions (CBIO). CBIO actively develops and sells a system called PureLyse that performs mechanical lysis of cells through the spinning of micron-scale beads in a motor [23]. To gauge the performance of this subsystem, we use \textit{B. subtilis ATCC 6633} spores as a model for a tough-to-lyse extant organism. Based off of a 4 megabase genome, with one genome per spore, 1 ppb sensitivity translates to 10000 spores in a 50 mg sample. In order to produce 1 Mb of data with single molecule sequencing, we require 2 picograms of DNA, which would require us to extract >5% of the DNA from \(10^4\) spores [27]. This assumes a 0.06 percent yield through library preparation and sequencing, which we are still working on achieving.

This system has been a major focus of technology development in our laboratory with the goal of attaining >5% DNA yield from a variety of Martian regoliths doped with \textit{B. subtilis} spores. In addition, we are working with CBIO to develop solutions that would integrate all the pipetting steps in one automated cartridge. The PureLyse cartridge that was prototyped for us was based on the SimplePrep instrument, which was used to meet our TRL4 milestone. The prototype cartridge used a motor to drive a switching valve and evolved gas pumps to push fluid into a reservoir that would contain the OmniLyse motor (Figure 2-1). This cartridge was a one-time use device that would run the standard PureLyse protocol. Over the last two years, however, new protocols have been developed in our laboratory in an effort to create a "universal protocol" that can address the challenges of extracting DNA from regolith
that may contain salts, acids, or other disadvantageous chemical compounds [28]. The next cartridge design is in progress and should hopefully implement some of these new protocols. The volume and mass budgets were calculated based on the CAD diagrams for the prototype cartridge. Power and energy budgets were calculated to account for running the PureLyse motor and actuating the valves and pumps.

Figure 2-1: Prototype Sample Preparation Cartridge

2.4.3 Library Preparation

Library preparation is a key step that prepares DNA for sequencing. While there are many variances in library preparation that are dependent on the technology being used, traditionally it involves the fragmentation of DNA to the proper length, end repair of the strands to remove overhangs, ligation of adapters added to the ends of
the strands, and amplification of regions of the molecules to increase the amount of DNA available for sensing and sequencing. In using sequencing for space applications, several changes and considerations are introduced, however. First, amplifying nucleic acids may amplify contaminant DNA as well and might introduce bias. Library preparation is also technology specific, and can vary based on the sequencer being implemented in a final system.

Library preparation for the MinION sequencer involves first taking DNA from the extraction subsystem and cleaning and purifying the sample. Then, since many double stranded DNA fragments will have single stranded overhangs after being sheared, these fragments have their tails enzymatically removed. A single nucleotide base, adenine, is added to both ends of the DNA strand. After further cleaning, motor proteins are added to the end of each fragment; these motor proteins ratchet the strand through the pores of the sequencer base-by-base. The sample is then ready for loading into the MinION (Figure 2-2).

Most library preparation protocols, and in particular the ones that are being used with the MinION sequencer, involve the transfer and heating of the fluid sample and reagents. As such, for the volume, mass, power, and energy budgets, a similar system to the sample preparation cartridge was envisioned and used for a rough approximation. As our library preparation protocols have changed, however, these budgets might need to be revised. New protocols are developed to be more efficient or meet different needs, and the MinION's protocols have undergone several changes through our time working with it. For example, the current library preparation protocol involves successive cleaning steps that could necessitate a change in design, depending on the results of the testing discussed later in the thesis.

2.4.4 Sequencing

A key components of the current SETG instrument is the MinION nanopore sequencer. The system functions by utilizing a polymer membrane perforated by protein nanopores. A current is passed through each nanopore that changes as a nucleic acid passes through. That disruption in current can be used to characterize the nucleic
acid, thus allowing for the sensing and identification of the molecule and the sequence of bases that it is comprised of. While there are other single molecule sequencers, the MinION is attractive due to its low mass, volume, and power requirements, as well as its space heritage from being run on the International Space Station [27]. The MinION is structurally composed of the sequencer itself as well as a FlowCell. The FlowCell contains the polymer membrane and fluidic channels, while the sequencer contains the electronic components to complement the FlowCell. The MinION is small both in mass and volume, estimated at 87 grams and 80 cc, with only a 1 Watt power requirement. A standard sequencing run, however, can take time, and results in a large energy draw: a 48 hour run would use 48 Watt-hours. Additionally, the sequencer is designed to function at room temperature, and will control the temperature of the internal sequencing reactions based off of that assumption.
2.4.5 Data Processing

Data processing from the MinION involves recording the ionic currents from the system, and relies on the MinKNOW software package, which runs and saves the sequencing data from the MinION. These saved files are then run through a basecaller, a program that interprets the signals and assigns bases to a read signal based on its probability. Traditionally, MinKNOW is run on a computer connected to the MinION, while basecalling might be done either on the cloud or on a powerful local machine. Typical runs from the MinION can produce data volumes on the order of hundreds of gigabytes after basecalling; however, shorter runs can be performed to produce smaller datasets.

Initial designs for the SETG instrument envision a flexible programmable gate array (FPGA) that would process read signals, which would then be stored for later engineering analysis. The instrument would likely store raw signals on a limited basis, in addition to processed bioinformatics data such as histograms and quality scores. Storage is necessary, due to the modest data return rate from Mars of 1 Gb/day [23].

The budgets for the data processing system were based off of a nominal system on a circuit board, like the Xilinx Kintex-7 [23]. While significant volume and mass margin was provided for the computing system to grow, the instrument should hopefully not exceed these values. The power draw of the data processing subsystem is likely to be substantial, and that is reflected in the power budget.

2.4.6 Waste Management

Waste management is included in the original budgets as a necessary aspect of the instrument. Reagents cannot just be vented to the Martian surface, due to contamination control concerns. Storage of those reagents is also important, since many of them could lose their efficacy upon freezing or even potentially damage tubing and structures. Despite the importance of this process, the waste subsystem was not a primary focus so far in development. Many other aspects of the SETG instrument, such as the Sample Preparation subsystem, already have some amount of space defined
for waste reagents. The final design of the subsystem could be simple and involve an unheated chamber that would seal and allow internal reagents to freeze, or it could include a more complicated method involving some sort of absorptive material like a foam. Additionally, the design of this component is intimately related to the number of runs the instrument has planned; if very few runs are planned other subsystems such as Sample and Library Prep could have their own waste sections. If many runs are expected, a more dedicated waste subsystem would be required.

For the budget, a small, simple chamber for reagent storage was envisioned and planned for in terms of mass and volume. This system would likely draw very little power or energy, especially if it used vacuum to drive fluid to its chamber.

2.4.7 Power Conditioning and Thermal Control

The current SETG instrument is being designed to function off of a 24V bus, such as the one on the Mars Science Laboratory. The power draw of certain systems, such as the MinION, is well defined, while some components, such as the data processing subsystem, have a range of potential values. The pump actuation required for fluid movement could use evolved gas generators that would require very low currents, and the valves required for flow direction could take advantage of vacuum actuation to minimize power. Continued testing and iteration is required to gain a more substantial handle on the power requirements of many subsystems.

Heating could potentially be a major component of those requirements. From an estimated Martian temperature of \(-55^\circ C\), the system would require a large amount of power to bring all its components up to operating specification; heating just the sequencer and sample prep cartridge to operating temperature is estimated to require 4 Watt-hours, based on a typical survival heater Watt density. Maintaining the temperature band required for sequencing is another question, involving the interplay of power draw from components and heat losses through the vessel. To that end, we are developing a thermal model to help manage the system’s heating and cooling.

The volume and mass of heating elements was not considered a large factor, with insulation and resistive heaters both being low mass temperature control options. The
power and energy draw, however, is large, as previously discussed. Over time, however, as our thermal model improves, we expect us to fall well under budget for power and energy, potentially at the expense of mass growth for passive heating/cooling elements.

2.4.8 Structure

The structural form of the SETG instrument was ill defined at the initial design stage. We opted for a strong, wear-resistant, easily machinable material for the outer structure: aluminum, commonly used on many other space instruments [29]. Aluminum’s conductive properties can be balanced with insulation internally to assist in thermal control. The structural mass and volume budget were estimated based on the total volume of the other components of the system; the volume of each other subsystem was calculated with wiring and air considerations in place, so a cube that would be allow for all the components to be housed inside was used as a first estimate. The mass of the structure was then established from the volume of the box and the density of aluminum. Structure is a subsystem, however, that can be redesigned and likely will be as the other scientific subsystems of the SETG instrument grow or shrink in volume and mass. As such, it was provided plenty of margin during design.

2.5 Mass, Volume, and Energy Budgets

These design considerations lead to the creation of budgets for mass, volume, and energy for the instrument. The nominal system at the time of the creation of these budgets was one that would process four samples with two sequencers for redundancy. The initial budgets are presented in the table below (Table 1):

A current best estimate for mass, volume, and energy draw was established for each subsystem. From there, we allocated extra margin to each subsystem based off of our expectations for how the system might grow. Then, after summing all of the subsystems together, a total system margin was added to create our specification, providing our budgets with a lower and upper bound.
<table>
<thead>
<tr>
<th></th>
<th>Best Estimate</th>
<th>Allocation</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (cc)</td>
<td>2500</td>
<td>3200</td>
<td>4000</td>
</tr>
<tr>
<td>Mass (g)</td>
<td>2860</td>
<td>3680</td>
<td>4930</td>
</tr>
<tr>
<td>Energy (W-hr)</td>
<td>130</td>
<td>170</td>
<td>200</td>
</tr>
</tbody>
</table>

Table 2.2: Current Budgets for SETG System

2.6 Discussion

The initial budgets for volume and mass fall within the expectation for a rover based Mars instrument. The energy draw of the system was high, at 130 Watt-hours. Much of this power can be attributed to sequencing and computation, however, and typically power estimates tend to decrease over the course of a system’s design due to better understanding of the thermal requirements [30]. Developing these budgets involved diving into the individual subsystems of the SETG instrument and how they functioned. Continued work has been done to describe the details of these subsystems more accurately, but the budgets are still valid ways to look at the instrument from a requirements perspective. Additionally, they help direct the testing effort in the lab to attain more accurate values for these budgets as well as prototypes for key subsystems. The TRL5 and TRL6 efforts are directly informed by the initial design steps taken during this phase of development.
Chapter 3

TRL5 Development

3.1 SETG Automation

The data presented in the preliminary SETG budgets are informative for making future decisions on system design. In order to move forward with technology development, however, it is necessary to verify whether these budgets are reasonable. In addition, it is necessary to be able to automate the SETG process; manual handling is common to many protocols, and existing liquid handling systems are not spaceflight-ready. These two fundamental ideas drove the creation of the Automated SETG Testbed, a system designed to automate the SETG process from sample to sequence. Testing and verifying the subsystems of the SETG instrument will help us evaluate if our original budgets were reasonable or if some components need to be redesigned. In addition, in developing the system for repeatability, we can show the sensitivity of sequencing results to subsystem inputs, which will provide information for the development of more effective protocols in terms of power, energy, and timing.

3.1.1 Overview

The SETG testbed functionally has three main subsystems: extraction, library preparation, and loading/sequencing. The extraction subsystem receives, as an input, an environmental sample and produces, as an output, eluate of concentrated DNA. That
output is the input to the library preparation subsystem, which produces ready-to-sequence DNA. The loading/sequencing subsystem then takes the ready-to-sequence DNA and produces sequencing data (Figure 3-1). Other previously discussed subsystems, such as power, thermal, structure, and waste management, serve to support these three main functional subsystems. This outlook does not necessarily reflect the specific hardware layout of the system, but is an organized way to test and evaluate important subsets of the overall testbed (Figure A-1).

![Automated SETG Testbed Block Diagram](image)

Figure 3-1: Automated SETG Testbed Block Diagram. Power and Waste Management are shown as additional subsystems. Structure and Data Processing are not, and are less relevant at this stage of testing.

### 3.1.2 Current Best Solution

To the best of our knowledge, a full sample-to-sequence automated system does not exist. Thus, in order to evaluate the performance of our testbed, we need to compare the performance of each of the three main subsystems to the current solutions.

**Extraction**

The current solution we are exploring is extraction of DNA from spores via the PureLyse system, described in the previous chapter. This method, a mechanical lysis method rather than a chemical or enzymatic lysis method, is considered to be our current best extraction solution. Our team has published several results from this
system in terms of percent yield, i.e. mass of DNA extracted divided by percent of mass of DNA added in [28]. The current protocol is a custom designed SETG protocol, meant to be agnostic to the choice of Martian regolith.

Library Preparation

Oxford Nanopore has several library preparation protocols designed specifically for the MinION flow cell, all designed to be done by hand. These protocols have different purposes, based on what device we would be using, how long we expect our strands to be, whether we can use polymerase chain reaction (PCR), and so on. With benchtop experimentation, we found that a modified version of Oxford's 1D Genomic DNA Ligation would be ideal. The protocol is called the One Pot Ligation Protocol, and was designed by Josh Quick of the University of Birmingham; we found it attractive due to its low number of bead cleanups and that it delivered high yields with a minimal number of steps [31]. We have quantified yield efficiency on the bench very well, though the only way to test if the library was successfully adapted for the MinION is to attempt to sequence it. The current best solution is still a human researcher or robotic arm moving reagents via a pipette.

Sequencing

The current solution for sequencing involves loading the MinION sequencer by pipette, before connecting it to a computer and running the MinKNOW program for anywhere from 1 to 48 hours. Successful loading of the MinION is determined by whether any leakage occurred from the Flow Cell and whether the MinION sequences the DNA we expect to find. While leakage is a catastrophic form of failure, and a large amount of data could be lost by inducing leaks during loading, the amount of data produced by the MinION also depends on other factors, such as flow cell quality. Regardless, if the MinION can sequence the nucleic acids that have been loaded, that is a good sign that library preparation was at least partly successful. A full success from sequencing would involve the MinION reporting a large number of reads within 24 hours, within an order of magnitude of the number of reads reported for a manual library.
3.2 Components and Methods

It is clear that an automated SETG system would improve upon our current solution and would move us towards TRL6 and subsequently a flight prototype. The following sections expand upon how that automated testbed was designed and created, first starting with the protocols requirements set by SETG and selecting hardware and software to meet those needs.

3.2.1 Work-flow

The section that follows is a heavily abridged version of the current protocols for extraction, library preparation, and sequencing, in order to understand the motivation for the testbed hardware. These protocols may not reflect what is currently being done on the bench, but rather the procedures being developed for automation.

**Extraction**

1. The input sample (regolith or water) is desalting and suspended in buffer, along with competitive binders.

2. The slurry is pushed back and forth through an OmniLyse device as it runs, lysing any spores in the slurry.

3. Buffer is pushed through the OmniLyse device to wash the system.

4. Elution buffer is delivered to the OmniLyse and heated.

5. The elution buffer is pushed through the device to the next subsystem.

**Library Preparation**

The library preparation subsystem can then be subdivided into three sub-subsystems.

1. First Bead Cleanup

   (a) The input eluate is introduced to a reservoir of Ampure XP solution.
(b) A magnet is moved such that it holds the beads in place on the inside of the reservoir.

(c) Elution buffer is removed and the chamber is washed with ethanol twice.

(d) The magnet is moved away. Elution buffer is added to the bead solution and heated.

(e) The magnet is moved back. The beads are pulled back to the wall.

(f) The water is pushed along as eluate.

2. End Prep and Ligation

(a) End preparation buffer and enzymes are added to the eluate, to prepare the ends of the nucleic acid strands.

(b) Ligation master mix, ligation enhancer, and nucleic acid adapters are added to the solution and allowed to incubate.

3. Second Bead Cleanup

(a) The solution is introduced to another reservoir of Ampure XP solution.

(b) The steps of the first bead cleanup are repeated with different buffers, to clean the library after the previous End Prep and Ligation step.

(c) The elution buffer is pushed to the next subsystem.

Sequencing

1. The priming buffer is loaded into the MinION.

2. The elution buffer, mixed with other buffers to become the library, is loaded into the MinION

3. The MinION is activated and sequenced.
3.2.2 Hardware

Testing the automation of the subsystems was the most important factor in the testbed’s design. While the final SETG instrument may be a custom designed, integrated device, that would not be conducive to modifying protocols and shifting design requirements. Since the automated testbed is meant to inform the final design, we were interested in selecting components that were modular and could be easily controlled via software, while still being able to drive small amounts of fluid precisely. LabSmith is a company that produces reservoirs, tubing, valves, pumps, sensors, and other fluid handling components for micro-fluidic applications, all made with inert components and designed to minimize dead volumes. LabSmith components a large majority of the current testbed. Additionally, a custom-built solenoid bay combined with a pump is used to generate pressure to move fluids from one container to another. The control computer powers LabSmith components, while two power supplies provide power to the non-LabSmith components. Unique components were also used for each individual subsystem.

Extraction

The Omnilyse device is a major part of the extraction subsystem, and is integrated via adapters to the 1/16" outer diameter (OD) tubing used throughout the subsystem. A PhD Ultra syringe pump is currently used for the lysis portion of the testbed; since it actuates a BD 3 ml syringe, it simulates the traditional PureLyse protocol more accurately than a pump with its own reservoir. An 8-way flow selection valve is also used centrally in the subsystem.

Library Preparation

In addition to previously discussed components, two magnetic assemblies were created by attaching neodymium permanent magnets to the shafts of two brushed small motors. The motors can be spun by a motor controller to move the magnets away from and up against the wall of a reservoir, in order to actuate paramagnetic
beads used in each protocol. Bio-Chem Fluidics one way valves prevent backflow, while resistive heaters raise the temperature of reagents for reactions.

**Sequencing**

The Oxford Nanopore sequencer is naturally implemented in this subsystem. Additionally, we built a custom-designed fluidic interface, designed by Kendall Saboda, that connects LabSmith tubing to the MinION Flow Cell’s ports for automatic loading. Additional components include a bubble trap, a Sensirion flow meter, and Bio-Chem one way valves.

### 3.2.3 Software

The testbed is designed to be controlled via a computer running MathWorks Simulink, communicating with components through serial ports or a National Instruments data acquisition board (DAQ). Simulink provides for unified control for many different components from different vendors, and allows for the precise timing and repeatability required for automated SETG protocols. In addition, the block-based programming approach lends itself to code modularity alongside component modularity, and allows for open-loop or closed-loop control further along development.

### 3.3 Design

To design the testbed and plan for its construction, Dr. Jacopo Tani created several flowcharts to establish the automated versions of the SETG protocols. These flowcharts describe the processes that a nominal instrument would take from sample to sequence, and were living documents that morphed as our design shifted through construction. The current version of the flowchart is split into phases, where each phase is a step in the instrument process. Phases 1 and 2 are sample preparation phases, while Phases 3 through 7 are library preparation phases (Figure 3-2).
Physical Layout

The physical layout of the automated testbed is not required to follow its functional layout. The phases of the system provide a rough framework for the layout, but the testbed footprint can be reduced if need be. Additionally, large components such as power supplies have to be located such that they can be accessed by several different components. The layouts of each subsystem as of writing the thesis are described below.

3.3.1 Extraction

Extraction on the lab bench consists of the OmniLyse device, tubes of reagents, and actuation via syringe. As such, the automated extraction subsystem primarily consists of the OmniLyse device, reservoirs of reagents, and actuation via the PHD Ultra Syringe Pump (Figure 3-3). The automated extraction protocol is designed to emulate the bench protocol, discussed in the previous section, with several key
differences. The Bio-Chem Fluidics 8-way valve is used such that the syringe pump can interface with multiple reservoirs. 3-way valves allow for pushing used reagents to waste. Desalting is not implemented via a centrifugal method, but rather through pushing fluid through a 0.2um filter; this is effectively desalting since cells are typically bigger than 0.2um and free DNA will be bound to sediment, while salt ions would pass through. While this is effective and can be implemented into our testbed, it is worth noting that this is not a true desalting approach, since salt ions are far smaller than the filter cutoff size. The input to this subsystem is the environmental sample of spores, while the output is an eluate of extracted DNA.

Figure 3-3: Extraction Subsystem. The subsystem has been spread over a large area to facilitate easy access and modification.

3.3.2 Library Preparation

The Library Preparation subsystem has the largest number of components, since the library preparation protocol contains a larger number of reagents and steps than
extraction or sequencing. Primarily, the library preparation subsystem consists of more than 20 reservoirs, with the pressure generator moving reagents and sample from one reservoir to the next (Figure 3-4). The small liquid volumes used in library preparation necessitate thinner tubing and greater control over dead volumes. The heating elements are used to heat several reagents for reactions. 3-way valves are used to divert fluid to waste while the magnet assemblies (made up of a motor and a physical magnet) can hold or release the ferromagnetic Ampure XP bead solution, discussed in previous chapters. The input to the subsystem is the extracted DNA eluate from the extraction subsystem. The output is cleaned and prepped DNA to be loaded into the MinION Flow Cell.
3.3.3 Sequencing

The sequencing subsystem is mainly comprised of two parts: the loading apparatus and the MinION sequencer. The loading apparatus consists of the previously discussed custom-made acrylic interface with connectors that mate with tubing from the rest of the automated testbed (Figure 3-5). This interface has four ports that correspond to the four ports of the MinION Flow Cell: the Loading Port, the Spot On Port, and the two Waste Ports. A precise 80 ul LabSmith automated syringe pump is used to deliver the sample into the MinION, since loading involves delivering small volumes of fluid with precise timing, without disturbing or damaging the flow cell's nanopore membrane. The sequencer itself is a standard MinION sequencer, with holes drilled in the lid to allow the loading interface to access the flow cell. Additionally, a 3D printed clip compresses the interface against the flow cell. The air bubble trap prevents bubbles from forming and interfering with MinION loading, while the flow meter monitors the fluid volumes inserted into the flow cell. The completed library is the input to this system, and the output is sequence data.

3.3.4 Additional Subsystems

In order to serve these three main subsystems, other subsystems were designed as well, described below.

Data Processing

The MinION sequencer is operated via a connection to a computer running MinKNOW. For the testbed implementation, a separate computer is used for the MinION so that the testbed computer can focus on automation. Data acquisition and base calling are all handled by that local computer. For the testbed, we used the UDOO X86, a single board, quad core, x86 architecture computer. This board is notable both for its use of Intel architecture, making it MinKNOW compatible, as well as its small footprint; at 102 square cm, it is a viable computer for a future flight prototype.
Figure 3-5: Sequencing subsystem, focused on the acrylic loading interface.

**Power Conditioning and Thermal Control**

The automated testbed is not operating off of the same constraints as the final instrument; there is no singular 24 V bus powering the entire system. All components, however, are powered with less than 12 V from one of two power supplies. Using power supplies set at different levels accurately simulates multiple power rails on a bus, while allowing for rapid changes in voltages during subsystem testing.

**Structure**

There is no structural housing to the current automated testbed. The system is developed on the lab bench and is allowed to take a larger surface area than the final instrument would require. This allows for modularity and ease of assembly to the testbed; however, as the system is tested and optimized, the volume of the testbed can be reduced. These results will allow the final device to occupy a much smaller
footprint.

Waste Management

The waste generated by a run of the testbed is designed to be stored in separate waste reservoirs. Additionally, a container that has been emptied of its contents can be used to store waste as well. This policy works well with the cleaning protocol established for the testbed. No physical component, outside of the BD 3 ml syringe and OmniLyse device, is disposable; every component has to be cleaned between test runs. Thus, after a test run, waste containers are manually emptied and the full system is flushed with sporicide. After emptying the reservoirs again, the system is flushed with pure water and emptied twice. The sporicide eliminates organics inside the system and the water flushes out the sporicide. This allows for simple waste management while eliminating contaminants from run to run.

3.4 Testing and Results

3.4.1 Extraction

The sample preparation step has been the primary focus of the initial stages of testing. Due to ongoing work done by our laboratory and our partners Claremont BioSolutions, we have an extensive data set for extractions in a bench-top environment [28]. Thus, we can compare the yield efficiency of automated extraction with manual extraction. The majority of testing was conducted with an aliquot of roughly $10^8$ *B. subtilis* spores in water.

Initial testing built up to extraction slowly. First, long fragments of DNA, extracted from *E. coli*, sheared to 6 kilobases and suspended in pure water, were pushed through the OmniLyse device while it was running, using no additional buffers. This was to test where we were liable to lose sample in the system, as well as compare values to manual lysis. Afterwards, the same was done with *B. subtilis* spores. Finally, full extraction was done with *B. subtilis* spores.
Initial lysis testing resulted in low yields, which led to new design iterations that included reducing tubing length and removing components that were potential sources of sample loss, such as the flow meter. The testing protocol was also modified to allow for larger elution volumes and heated elutions. Minor changes in layout were also made after testing, such as tilting the OmniLyse device to ensure that it fills before lysing. The modularity of the system made these changes easier to implement after realizing that the protocol had to be modified.

While these tests resulted in better yields, the greatest jump in functionality was in implementing a pre-treatment step in extraction. I theorized that with the extensive tubing length of our system, it was possible that DNA from the lysed spores was binding to the inside of our tubing, leading to significant sample loss. As such, I introduced a pre-treatment step where I flush the extraction system with sodium pyrophosphate.

Sodium pyrophosphate (NaPP) is known to inhibit binding of nucleic acids to surfaces; the molecules occupy the same charge sites that DNA would, reducing the number of sites that DNA could bind to while traveling through tubes. By flushing the system with NaPP prior to extraction, we would allow many of the binding sites inside the tubing of the system to be bound to NaPP molecules, allowing more DNA to pass through to the final elution. After implementing this step, I saw our yields increase. With spores in water, I was able to attain yields on par with manual extractions (Figure 3-6). Initial tests with basalt were also promising, potentially leading to developments that could allow the automated system to surpass manual handling. Unfortunately, the introduction of regolith began to damage one of the valves in the 8-way BioChem valve, rendering that port inoperable. Automated regolith testing has been paused until a better fluid selection system has been designed or purchased. We plan for future systems to avoid moving regolith as much as possible. Additionally, an in-situ SETG instrument would process a limited number of samples, minimizing the potential for valve damage. For now, future integration testing will continue to use water, as this does not damage the flow selection valves in the testbed.

While the system has produced promising results and demonstrated the ability
Figure 3-6: Extraction Yields with Water, before and after using NaPP as a pre-wash. Basalt data is included to compare with water. Water extractions and basalt extractions with NaPP match manual extraction yield numbers.

to automated extractions, providing a path to the next version of SETG, there is additional work to be done. Desalting has not been implemented into the system, since neither water nor basalt regolith need desalting for extraction. Additionally, manual research suggests that heating the OmniLyse device during elution is better than delivering a heated elution buffer; that has yet to be implemented, as well. Looking ahead, the current testbed could have future issues with clogging, since it cannot dynamically account for clogs in the system. While manual handling allows for simple dynamic pressure and speed adjustment based on visual and tactile data, the testbed would have to evolve and implement several more sensors and a more sophisticated feedback control loop to achieve the same result. For now, however, the subsystem has successfully demonstrated spore extraction from water and basalt, with sufficient yields to meet system requirements.
3.4.2 Library Preparation

As of the writing of this thesis, all of the components have been selected and tested individually for the library preparation subsystem. Additionally, the entire subsystem has been assembled. Full testing is ongoing; while a successful end-to-end library preparation has not been achieved, the testing to date has already provided valuable information and data for future designs.

The first phase of testing was conducted on the first sub-subsystem: the first Ampure XP bead cleanup. Testing this step involved taking a raw DNA sample, purifying it via a bead cleanup, and measuring the percentage yield. On the lab bench, we expect at least 70 percent yield through this process. Initially, testing was done in a quasi-automated fashion. In this context, that meant using the assembled Ampure sub-subsystem, but delivering pressure via a manually operated syringe and moving a magnet by hand. This allowed us to control pressure and timing while observing the testing results dynamically. Quasi-automated testing revealed a concern that would continue to cause challenges for this subsystem: fluid handling. Fluid handling for library preparation involved pressure delivery, where pressurizing a reservoir would move fluid from it to another reservoir. This has proven to be less precise than a syringe pump, since fluid ends up retained in the edges of the reservoirs. Additionally, fluid delivered to another reservoir would end up bubbling and splattering as pressure pushed air in after reagent is expended. Both of these problems lead to sample and reagent loss, which is particularly damaging to this subsystem due to the small volumes in library preparation. Many library prep reagents are on the order of 10s of microliters, with several being less than 10 ul.

Our initial Ampure cleanup tests failed due to a variety of factors, such as imprecise heating and inability to control the paramagnetic fluid. Kendall Saboda designed a separate circuit board to control heating, while I briefly experimented with electromagnets for holding Ampure solution in place. The heating circuit board was able to control reservoir temperature to within several degrees, so we considered that a success. The electromagnets were less successful; in order to exert sufficient magnetic
force, we would be forced to use high mass and high power components. Instead, we opted for a single brushed motor, holding a permanent magnet on a prong. By actuating this motor, the system can move a magnet towards or away from a reservoir and thus simulate the bench protocol simply and accurately. After these modifications, we found that Ampure XP yields peaked at a consistent 41 percent. This peak yield is still less than the manual protocol’s yield, and current research is focused on why this is the case.

![Automated Ampure Tests vs Manual](image)

Figure 3-7: Automated Ampure XP yields compared to the lower bound of manual tests. In addition, yields are shown for failures due to paramagnetic bead control as well as failures due to imprecise heating. Standard deviation is shown via error bars.

While Ampure XP yield is lower than expected, it should be sufficient for a basic library preparation. The subsystem consists of two bead cleanup steps, so we would expect a 16 percent yield, assuming no other losses, from the subsystem. As such, we assembled the full library preparation system in order to attempt a full preparation. This library preparation failed, as in that the final elution did not contain DNA. Faced with this quandary, we resolved to conduct tests systematically: test the output of
each individual sub-subsystem one by one, to identify where we were losing our DNA library. As of the writing of this thesis, the final sub-subsystem is identified as the point where library is being lost, most likely due to the small volumes of fluid being moved in that process.

Despite the lack of a successful library preparation, the challenges that the system has been facing have led to lessons for future iterations. For example, the fluid handling system for SETG will have to be robust to large differences in volume; some reagents in library preparation are 200 ul in volume, while others are 2 ul. The system will also be required to precisely control heating for small volumes; the ligation enzyme needs to be heated to 65° C, but overshooting that temperature by even several degrees can start to lead to denaturing of DNA, depending on the presence of AT and GC pairs; a higher AT pair concentration lowers the melting point of a double-stranded length of DNA [32]. These challenges, however, could lead to new technology solutions. Since the problems with the second subsystem has been isolated to the last sub-subsystem, a successful library preparation is imminent, which will lead to the final step of subsystem integration, and then development of a TRL6 system.

3.4.3 Sequencing

The sequencing subsystem can essentially be split into two parts: preparing the MinION sequencer to sequence and sequencing itself. The second part of the subsystem is already automated; assuming the MinION is prepared properly and a sample is delivered, the MinION will function as an off the shelf (OTS) product from Oxford Nanopore. The testing and results from the sequencing subsystem are primarily from the first part of the subsystem, preparing the MinION.

Initial loading testing involved injecting pure water via the custom MinION interface into used flow cells to check for leaks in the component-flow cell interface. These tests failed, with partial leakage occurring at two of the four ports. Leakage was also consistent, indicating that the interface-flow cell connection was to blame. Since leakage was only partial and only occurred in some of the ports, I decided to
redesign rather than overhaul the interface component, designing slightly longer and wider fittings. These ports were designed to seal better with the flow cell ports.

Continued loading tests showed that the new interface component was more effective, but would still allow a small amount of leakage. This amount varied with pressure; if the interface was held tightly against the MinION, I found that the interface would seal against the flow cell. As such, I replaced the clip holding the interface with three double wrapped elastic bands, which exerted far more downward pressure on the interface; the subsystem no longer leaked. The rest of the subsystem was simple to assemble and test, since it was comprised of primarily LabSmith components.

Our next test was to load a premade library, prepared manually on the bench, and sequence it. Kendall Saboda prepared a 72 ul library with 772.5 ng of sheared Lambda phage DNA, which was loaded into the input reservoir of the loading subsystem; this input reservoir would be the same as the output reservoir of the library preparation subsystem. Upon starting and running the loading test, the subsystem successfully primed and loaded the flow cell without any large-scale problems; a small bubble formed in the flow cell membrane, obscuring 12.5 percent of pores, a frequent concern even in manual handling. Despite the bubble, the test produced a wealth of data, producing over 900,000,000 events in twenty hours. The test was only run for 24 hours rather than a full 48 hours, but we did not expect to produce many more events in the second day, judging by previous sequencing run experiences.

This test was highly promising; the loading subsystem showed that with a good library it could successfully prepare and load a MinION. We do not expect major revisions to the loading subsystem at this stage, compared to extraction or library preparation. Steps will have to be taken to translate the subsystem to a flight compatible system, discussed below.

3.4.4 Additional Subsystems

The supporting subsystems were also evaluated for results that could be applicable to future SETG instrument designs.
Data Processing

The testing computer performed well with the DAQ, and was able to precisely control all of our testbed components. A point of concern was that different components were communicated with in different ways (USB, Serial, or directly through the DAQ). This was an inefficient solution and was primarily a function of using off the shelf hardware versus a custom solution. The MinION computer performed well, and while it was not asked to basecall at the same time that it was acquiring data, its success here is informative, since we expect to be using a similar computing solution in TRL6.

Power Condition and Thermal Control

Our benchtop power supplies were sufficient for our power needs. We initially provided an adjustable power supply, in order to account for different components being tested at various voltages. As the testbed design solidifies, we can identify which voltages would be required to operate all of our components. At this current stage, we know that we would require a 12 V and 6 V power rail for the system to operate ideally.

Structure

The structure of the testbed was somewhat functional during TRL5 testing. Leaky connections were a frequent concern throughout the testbed, however. A custom designed device would likely avoid many of these concerns; the trade-off for a modular system is introducing more points of failure in fluidic connections. Moving forward, an integrated device should solve this problem, while being more flight compatible.

Waste Management

Waste management was successful throughout the testing. At no point did a waste reservoir leak or overflow; additionally, storing the waste separately allowed us to access it later in case we needed to track down sample loss.

The cleaning protocol was also effective in removing DNA and spores from the system, while not damaging any component. It was, however, insufficient for clearing
regolith from the system; basalt particles would be trapped in reservoirs during extraction and had to be manually removed via delicate task wipes. Moving forward, it would be ideal to design a cleaning protocol that would be agnostic to the presence of regolith, or avoid moving regolith altogether.

3.5 Future Work

Despite the fact that an end-to-end test had not been conducted at this time, the design and testing process of the testbed has provided information that will influence the development of SETG, including the advantages and disadvantages of the testbed itself, challenges that future work will have to address, and the path that the SETG team will have to take to meet those challenges.

3.5.1 Advantages

The previously discussed advantages of automation still hold true during testing. Automation allowed for consistent, repeatable experimental technique, with the number of factors that could cause variation in results is reduced. In addition, modifying a protocol to introduce changes is simple. Additionally, after starting a run, no other human interaction is required in the middle, allowing researchers to work elsewhere. This has allowed for us to be able to conduct tests and assemble other portions of the testbed in parallel.

In addition, the automated testbed has provided a platform on which novel solutions could be rapidly prototyped. These solutions, such as motors to control Ampure magnets or resistive heaters to heat reagents, will be valuable in the design of the TRL6 instrument. Other solutions, such as electromagnets, were rapidly tested and discarded as an idea without major expense.
3.5.2 Limitations

At the same time, there are limitations to the testbed. One limitation is the extended amount of time required for automated testing. While a full automated sample to sequence run will take roughly the same amount of time as a hand run, the automated solution will require cleaning steps. With the current ’1 run of sporicide, 2 runs of water’ protocol, an automated run will take 4 times as long as a manual test, not including sequencing time. During the test itself, researchers will be free to do other work, but this does slow down the number of tests that can be conducted in a certain time frame, such as a day.

Currently, the system also has a large footprint on the bench. This allows for modularity and ease of access to every component; however, when the design of the instrument is finalized, extraneous components and tubes can be removed. Eventually, the system will have to be reduced in size to fit inside the volumes described in the system engineering budgets.

3.5.3 Challenges

In building and operating the testbed, several major testbed-wide challenges have become apparent and would need to be resolved moving forward. First, benchtop protocols for sample extraction and library preparation assume a human in the loop. This human can make dynamic decisions based off mid-run data. For example, a lab technician can note that insufficient mixing is occurring during a pipetting step, and increase pipetting speed accordingly. Automatic control would have to involve either universal protocols to account for eventualities a priori, or constant measurements that could sense for something like insufficient mixing. The former involves an intimate knowledge of all failure modes of the system and is dependent on the existence of some protocol to account for those modes; the latter involves multiple data streams being processed constantly at every step of the sample to sequence process, with decision trees being programmed for each eventuality. Both paths increase development time.
Secondly, long tubing allows for the loss of liquids and DNA. On a lab bench, pipettes offer an easy solution for transferring liquids from one reservoir to the other with minimal fluid loss, with humans able to adjust timing and position to preserve droplets. The automated system relies on PEEK tubing. A tube in between two reservoirs would likely be filled with air at the beginning of a test that would need to be purged first. Then, at the end of a fluid delivery step, an additional purge step would be required to push the last small volume of liquid out of the tube. Small errors in purging steps can multiply over the phases of the process, leading to fluid being left behind in tubing, reducing the efficiency of the testbed.

3.6 Continued Development

The next major steps for continued development of the automated testbed are the completion of library preparation and subsystem integration. Completing library preparation will involve identifying where fluid loss is occurring, improving control over temperature during heating, and increasing our ability to mix fluids and re-suspend beads. While these are complicated issues, addressing them will lead to a completed library preparation subsystem, and successes here will provide valuable information for designing a TRL6 system.

Integration is our next major step after that: connecting extraction with library preparation and library preparation with sequencing. Since library preparation is still being developed, integration has not been started. The design of the subsystems and the modularity of the system is meant to make this process as simple as possible when started. Each subsystem is designed to produce the output needed for the next subsystem’s input. At the same time, this could also produce new issues: the fluid movement issues presented in library preparation could lead to issues with loading, since library splattered on the walls of the reservoir could not be accessed by the loading pump. These issues could either be addressed through testing and small redesigns, as was the case with the individual subsystems, or through entirely new subsystem designs, closer to TRL6 designs. A potential future avenue for development
is to combine integration with pre-TRL6 work, resulting in an integrated, end-to-end system that is closer to TRL6 than the current design.

3.6.1 Preparing for TRL6

Readying the system for TRL6 also means assuring that individual subsystems can address the challenges of an analog environment, while assuring we can hit our previously discussed accuracy requirements. Sequencing has only recently been done in micro-gravity on the International Space Station, and has yet to be tested under reduced pressures or extreme temperatures [33]. Developing that capability concurrently with the testbed will lead us in parallel to a system that is not only automated end-to-end, but built to withstand the Martian environment; this directly motivates the next chapter of work.
Chapter 4

Towards an Integrated SETG

4.1 Lessons Learned from TRL4 and TRL5

The objective of the SETG-MatISSE project in its current iteration, as described in Chapter 2, is to reach technology readiness level 6. More specifically, this entails a "...System/subsystem model or prototyping demonstration in a relevant end-to-end environment (ground or space)." [27]

Through the work done to achieve TRL4, and the testing that has been done with the TRL5 testbed, several key areas were identified where continued research could provide valuable data going into TRL6 development. One such area is in better understanding and bounding the temperature maintenance requirements of the SETG instrument. The initial system budgets identified thermal control as a major component of our total energy consumption, the third highest after the sequencer and processing unit. The SETG system has components and reagents that need to be kept at certain temperatures over months in storage. During run-time, those same reagents and components need to be held at different temperatures on the order of hours to enable enzymatic reactions during sample preparation, library preparation, and sequencing; for example, the MinION has to maintain a temperature near $32 \pm 2^\circ$ C, with a temperature $10^\circ$ C higher leading to total loss of the MinION Flow Cell. A key system driver, then, is to verify our thermal control system, validate the SETG thermal requirements, and potentially reduce thermal control system energy
usage. An informative test would be to successfully sequence DNA at Mars-like conditions. While this would not be an end-to-end test involving extraction and library preparation, it would be a rapid way of assessing our ability to design an instrument housing and maintain its internal thermal conditions.

4.2 Preliminary Steps

4.2.1 Thermal Vacuum

The thermal vacuum chamber is a vital component of SETG technological maturation (Figure 4-1). The chamber is a custom-built Abbess Cube thermal vacuum chamber. The chamber is capable of controlling the temperature of an internal thermal plate via heating elements and a cryo-line fed with liquid nitrogen, in addition to its internal pressure via a pump and a purge gas system connected to either a tank of N2 or CO2. Using a Watlow EZ-Zone temperature controller and a SuperBee CVM 201 Convection pressure controller, the chamber can reach temperatures as low as $-179^\circ$ C and pressures as low as 30 Pa. Thus, the chamber can provide a reasonable first-order approximation of Martian in-situ pressure, temperature, and gas composition conditions. Electrical connections are available at several ports in the side of the thermal vacuum, and provide access to power, sensor, and data wires connected to external supplies, boards, and computers.

The chamber was developed during the TRL4 phase of the project to assist in developing space-capable hardware. The system has already been used to cold test conductive bridging random access memory (CBRAM) chips, subjecting them to Martian and lower temperatures [34]. In addition, the thermal vacuum has been integral in helping to develop the Pre-TRL6 pressure vessel, discussed below.
4.2.2 Testing and Validating

Pre-TRL6 Pressure Vessels

In order to test our ability to sequence under Mars-like conditions, as well as rapidly iterate on TRL6 designs, we assembled and designed several pressure vessels that would enclose the MinION sequencer and a processor inside the thermal vacuum. Connections through the vessel would provide power to the system while the chamber was taken to average Mars conditions. The original pressure vessel (pressure
vessel v0.5) was a cylindrical structure made from acrylic with Teflon end caps, designed primarily to rapidly test sequencing and measure the resultant thermal loads (Figure 4-2). The initial design, however, was too isolated from the thermal plate; at low pressures, conduction is the dominant form of cooling over convection, and the system’s only contact with the thermal plate was through the Teflon end caps, which have low coefficients of heat transfer. During our initial tests within the chamber, a software update for the MinKNOW software package was released and pushed. The updating process introduced unexpected delays in initiating sequencing; ss such, the system overheated before sequencing could be started, damaging the MinION flow cell.
The next pressure vessel iteration (pressure vessel v1) was constructed by Kendall Saboda. Taking into account a potential volumetric design for the SETG instrument, the next vessel was a more advanced prototype. The system consists of an aluminum box with a bolted acrylic lid. The metal construction allows for higher baseline conductance, which could be mitigated with insulation (Figure 4-3). Electrical connections were facilitated using a similar design to the previous vessel iteration, implemented into a modular Teflon connector. The modular design of the connector space led to the development of additional connectors for increasing amounts of wiring. This vessel was able to successfully hold pressure for over 24 hours, potentially more, and while the pressure vessel is not necessarily the final SETG instrument design, it is similar enough that it can be used to model the SETG system.

Figure 4-3: Pressure Vessel, Version 2, with high density connector
4.3 Sequencing in Martian Conditions

A milestone on our path to TRL6 was our successful "Sequencing at Mars" test. The development of our thermal vacuum, the design and assembly of a Pre-TRL6 pressure vessel, and an understanding of our thermal requirements all contributed to a successful demonstration that the MinION sequencer, paired with a portable computer, could sequence while enclosed in a vessel subjected to Mars-like temperature, pressure, and power conditions.

4.3.1 Hardware Setup

A basic Martian condition sequencing setup was designed to consist of two basic elements: a MinION Sequencer and an Intel Compute Stick. The Compute Stick was a low power, small footprint computer that could run the MinKNOW software package as well as interface with the sequencer. Both would be powered through the DC electrical connections in the pressure vessel through the thermal vacuum to the Compute Stick power supply, plugged into an AC electrical outlet. A sensor was placed on the power supply to track the power draw of the system.

Pressure vessel V1 was designed with sequencing at Mars as an immediate goal; as such, it was designed to house the MinION and Compute Stick alone, with just enough space for a basic barometer and temperature sensor. Due to our previous overheating failure, discussed earlier, this vessel had to be designed to account for thermal control. Both the Stick and MinION were designed to run at room temperature; the MinION can control its internal temperature to 32° C, the normal sequencing temperature, if exposed to room air. The next pressure vessel was built mostly out of aluminum, which would exchange heat with the environment better than the acrylic and Teflon that the first version was constructed with. Insulation could then be implemented to control how quickly the interior would cool down. A platinum resistance temperature detector (Honeywell HRTS-5760-B-U-0-12) was placed inside the system to track the internal air temperature every 15 seconds, while a 10 Watt, 12 Volt heater was used with an on-off controller to provide additional heat when needed.
4.3.2 Sequencing Experiment

Using this setup, we attempted to conduct a low-input sequencing test using CarrierSeq. CarrierSeq is a protocol and sequence analysis work-flow designed for low input nanopore sequencing. The protocol involves preparing target DNA with a sample of carrier DNA, which allows for library preparation with ideal stoichiometry; this protocol allows us to detect small amounts of DNA without using whole genome or targeted amplification, which may bias population results or decrease taxonomic resolution, respectively [35].

The input to the sequencing system was 0.2 ng of *B. subtilis* DNA with 1000 ng of Lambda phage DNA as a carrier, extracted and prepared by Angel Mojarro and Julie Hachey. Upon loading the MinION, it and the Intel Compute Stick were placed inside the pressure vessel inside the thermal vacuum, which was then commanded to reach and maintain Mars temperature and pressure: −60° C and between 400 and 500 Pascals. Upon stabilization at those conditions, the MinION and Compute Stick were powered on.

Successful sequencing was finally achieved with this experiment. Sequencing occurred in the chamber for the full thermal vacuum test of 2.4 hours. Additionally, the sequencing experiment on the Compute Stick was continued through termination of the test to allow for analysis of any changes caused by returning the system to room conditions. The MinION continued operating using the same flow cell on the bench with a desktop computer for an additional 37.9 hours, for a total of 40.3 hours.

The system was able to sequence 470 million bases the course of 2.4 hours (Table 4.1). Over the full 40.3 hours, 5.4 billion bases were read. The system was able to maintain 20° C successfully over the course of 2.4 hours inside the vacuum chamber; the MinION and Intel Compute Stick drew 15 Watts of power through the test, with the heater drawing 10 Watts when active. Judging by the fact that the heater was running constantly by the end of the test, however, we can make the case that more heating or insulation may be required for sequencing for extended periods of time at that temperature.
Run Results for Sequencing on Mars

| Input: 0.5 ng *B. subtilis* DNA + 1000 ng Lambda DNA |
|---------------------------------------------|-----------|
| **Vacuum Test** | **Benchtop Test** |
| Total Reads | 59,649 | 547,478 |
| *B. subtilis* Reads | 148 | 590 |
| Total Bases | 474,275,308 | 4,914,693,436 |
| *B. subtilis* Bases | 122,509 | 826,783 |

Table 4.1: Sequencing on Mars results, enabled by CarrierSeq

### 4.4 Simulation

These pressure vessels, while effective, confirmed that managing the thermal input and output of the pressure vessel would be key to future sequencing endeavours. Rather than test designs blindly, we turned to simulation as a concordant solution to explore heat as a failure mode. Thermal simulation is a valuable tool in constraining the thermal requirements for the SETG instrument. The average temperature on Mars is roughly $-60^\circ C$, with daily variations on the order of plus or minus $40^\circ C$. The reactions of library preparation require temperatures of mostly room temperature, with several reagents being heated to $37^\circ C$ or $70^\circ C$. Thus, the structure and thermal management subsystems of the SETG instrument have to maintain a potentially large temperature difference over long time scales, since a sequencing run could take up to 48 hours. When designing a system, then, simulating its response to these thermal gradients and its effort to maintain temperature is important.

As a current work in progress, simulation of the SETG system is being done via Simulink, the same graphical programming platform used in the automated SETG testbed. There are other thermal modeling packages available, such as FloTherm and ANSYS; however, these software packages require or rely on computer aided design (CAD) models to analyze transient heat flow. Simulink allows for a block-based design system that can be updated more rapidly than a CAD based modelling solution. Additionally, Simulink integrates easily with MATLAB, and can take advantage of MATLAB for its ease of programming and extensive libraries for mathematical modeling. There are tools already in place in Simulink for conducting large-scale thermal simulations of buildings.
Adapting those tools, it becomes possible to begin to model the SETG pre-TRL6 system through its thermal transfer with the outside environment. This code can be updated for varying amounts of insulation and heat flux without having to modify a CAD model. The current SETG thermal model is derived from a thermal model of a building, with heat entering and leaving the system through the walls, the underside, and the acrylic lid. The air inside the thermal vacuum and inside the pressure vessel are treated as two separate thermal masses, with the vacuum chamber's heat plate acting as a constant temperature source. Heat flow is then modeled through conduction, convection, and radiation. The heater is modeled as it is currently controlled: it switches on when the internal temperature is sensed to be lower than some threshold temperature. Through this initial work, it will be possible to start to constrain our thermal requirements even further.

Both the sequencing run and leak test runs provided data on the chamber pressure and temperature, vessel temperature and pressure, and system power draw. Using those data sets, we were able to work on designing the Simulink model to emulate the behavior of the pressure vessel over time, if subjected to similar conditions. Due to noisy temperature sensor measurements, it is more useful to track how often a heater has to be turned on within a given time frame. In our thermal data from a sequencing run, it was clear that the heater had to be turned on frequently initially, eventually having to be constantly active after 1-2 hours. Our initial simulation data indicates that we can design a pressure vessel to roughly emulate this behavior. By no means does this imply an accurate model as of yet; we believe, however, that pursuing this direction of simulation will allow us to effectively emulate a pressure vessel in the future via a thermal model.

A qualitatively similar thermal model can allow us to rapidly modify input weather profiles, heater output, thermal controller designs, insulation levels, and heat loss amounts to the atmosphere or ground. While the model will be simple, these simulations will help inform future structural and thermal subsystems quickly, allowing us to perform testing, verification, and validation on a smaller subset of potential designs. With a future model, we can design thermal requirements to fit the needs
of the system. Without accurate data or simulation, our thermal system could be insufficient or excessive; a thermal system that was under-designed would not be functional, while an over-designed system would add unnecessary mass and complexity to the SETG instrument. Knowledge of how our system would react to thermal inputs can allow us to design the exact thermal system we need with an appropriate amount of margin. The work with this model is still quite new, but looking ahead to TRL6, it could lead to more well-written and precise requirements for a final flight model, and would thus be an important avenue of research to pursue.

4.5 Continued Work

Simulation and thermal vacuum testing are only the early steps of TRL6 development. The data derived from both experiments will help to put limits on the final design of the SETG instrument; assuming the system can contain all necessary subsystems, the size, shape, and material of the overall structure will be determined by survivability conditions on Mars. In particular, the thermal simulation of the instrument has only just been designed, and the virtual model has not been subjected to a full range of potential thermal conditions.

Certain subsystems also need specific modeling. Continued study will need to be conducted, for example, on the library preparation subsystem. While sequencing involves maintaining one temperature over a long period of time, library preparation involves the maintenance of different temperatures for different reagents at different moments for different periods of time. While our current vessel design can maintain its overall internal temperature, selectively heating a reagent in an enclosed volume presents further challenges. The exploration of this subsystem is dependent on the continuation of work from the automated testbed.

Both testbed research and rapid TRL6 development will be crucial for success. Building off of the sequencing on Mars run, it will be possible to pursue designs from the pressure vessel side while conducting experiments on the automated testbed; both studies will allow us to meet in the middle for an integrated SETG instrument.
Chapter 5

Analysis and Conclusions

5.1 Technology Readiness Level of Instrument

At the close of this thesis, the SETG system is close to TRL5. The system will still require more design, testing, verification, and validation before reaching the TRL6 level. Most subsystems have not been tested inside an analog environment, i.e. a thermal vacuum chamber. Transitioning the automated testbed to a more compact and robust form factor will be the next stage of development, alongside increasing the accuracy and efficiency of the nanopore sequencer. Many NASA flight proposals require or encourage systems to be roughly at a TRL6 level to allow for sufficient lead time to refine the instrument for an upcoming launch date. As such, reaching TRL6 is the primary goal of SETG development for the foreseeable future.

5.2 Future Work

As such, in the short term, the immediate goal will be to fully automate the instrument and bridge the gap between a bench environment and an analog environment. In order to create a sufficiently sensitive instrument that can perform on Mars, there are two primary challenges: increasing sensitivity and designing components to function at Mars temperature and pressure. While the sensitivity challenge is outside the scope of this thesis, designing and testing a system to function inside the vacuum chamber
is a natural progression of the systems and testbed work discussed previously.

5.2.1 Challenges and Knowledge Gaps

Some subsystems are further along in environmental development than others. Several pressure vessels have been machined and tested inside the thermal vacuum chamber, and have been designed to be able to accommodate the full SETG system. The final TRL6 design could potentially borrow some or all of its elements from the latest pressure vessel model. The sample preparation subsystem is being worked on with Claremont BioSystems, and is already a capable device that could be adapted for vacuum with some changes. From a power conditioning and communication standpoint, we have already designed connectors for the pressure vessel that can transfer power and data from external devices. Other subsystems, however, will require far more work. The inlet is still underdeveloped as a concept, although work is currently being done in the lab on its design [36]. Waste management is simple for the testbed but will be more complicated in a sealed environment; we will need to develop ways to limit issues caused by freeze-out of waste reagents. Library preparation is still complicated due to its many steps and many reagents, and we have yet to design an integrated component for that subsystem.

In addition, there are gaps in our knowledge that we will have to design around. One is the nature of how sample will be provided to the system. Our current assumption is that the rover will deliver a regolith sample, ground to a certain granularity, to the inlet of our device. This was the case on Curiosity, so we believe it is a valid assumption moving forward. If that assumption does not hold true, however, the design of the inlet at minimum would have to change. A different mechanism on the arm, whether it is a scoop, a core drill, or a gripper, would interface differently with our inlet. As such, while our inlet design is not very defined, the requirements for that design could change between now and a flight proposal. Another gap is a lack of knowledge of final mission location. The temperatures on Mars vary greatly from the equator to the poles, and thus the thermal requirements will be different based on location. Additionally, Mars has a number of different soils, all of which would react
differently on a chemical level with spores and nucleic acids, from binding competitively to the DNA to outright nucleic acid destruction. For now, we have developed a semi-universal protocol so that nucleic acids can be extracted irrespective of the regolith that the instrument would encounter \cite{28}. Knowing our location, however, will help us optimize and refine the relevant protocols for our instrument. Additionally, more accurate thermal requirements would help in constraining the power draw of the system.

5.3 Potential Solutions

In working with the design, testing, and validation of this instrument, however, it becomes possible to identify potential avenues of research that will refine the system further and meet some of the challenges ahead in development.

5.3.1 Simulations and Models

Simulations allow us a way to rapidly test our system before building, and can provide key insights that would otherwise require extensive testing, which could incur large scheduling risks. While we have already started looking at computer aided simulations in the previous chapter, additional modeling will be required to further develop the instrument. The current thermal model can be refined to account for operational power modes of the system and for potentially maintaining varying temperatures inside the instrument. Mechanical modeling will also be required to test for structural and functional sensitivity to static and dynamic loads; the system's unique payload will have to survive the accelerations and vibrations associated with launch, transit, reentry, and operation. Finally, simulation can offer us insights into how our reagents will move in a microfluidic system, and how that might change with temperature and gravity.
5.3.2 Future Tests

Paired with the aforementioned modeling will be extensive testing. To achieve TRL6, the SETG instrument will have to be tested in an analog environment; simulation will not be enough. Initially, this will involve the thermal vacuum chamber subjecting the instrument to Mars-like temperatures and pressures, though the chamber could be adapted to simulate Martian humidity as well.

Later on, however, these tests would be paired with environmental field testing. Environmental testing is a key part of developing SETG to TRL6, aiding in understanding how the instrument would handle actual samples with confounding factors, such as non uniform particle size or the presence of clay. The SETG lab has already been testing some subsystems, such as the OmniLyse system and the MinION, on Volcán Copahue and at Devon Island. Being able to successfully test the full instrument with environmental samples in the lab, as well as testing the instrument in the environment itself, would be a valuable form of validation for a Martian instrument.

There are even more additional tests that would be helpful for understanding the system’s performance under off-nominal conditions. For example, operating the instrument on slopes of varying grades would be relevant, if the instrument was on a rover going over tough terrain. Vibrational testing is important to understand how the system will handle launch loads as well as vibrations from rover operations. Even just cooling the system to low temperatures and allowing it to heat back up would be relevant to gauging how the system would handle the journey to Mars. Testing is already an important part of the development of the SETG instrument, and it will only become a more important factor in the work to follow.

5.4 Conclusion

The SETG instrument still has years of engineering ahead before a flight. In that time, requirements will be refined and new designs will be implemented. The development of the system such that it can successfully carry out a life-detection mission is still ongoing. These initial steps outlined in this thesis, however, are vital for laying
a good foundation for future research. Systematically and rigorously pursuing the
design, testing, and validation of the SETG instrument ensures that it can become a
viable payload for space flight, and a valuable system for planetary science.
Appendix A

Flowcharts
Figure A.1: Reservoir to Reservoir Flowchart
Bibliography


