GROWTH AND CHARACTERIZATION OF

SULFOLOBUS TURRETED ICOSAHEDRAL VIRUS

by

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Hulda Björg Þórisdóttir

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ABSTRACT

Sulfolobus turreted icosahedral virus (STIV) and its infection cycle in *Sulfolobus solfataricus* P2 (*S. solfataricus* P2) was studied. A method to quantify the virus quickly was developed and optimized. The host-virus relationship between STIV and *S. solfataricus* P2 was studied and the transcripts of the virus were identified.

Cultures of *S. solfataricus* P2 were grown and infected with STIV. Cultures were infected with different amounts of the virus, at various temperatures and pHs, and virus production was monitored. ELISA protocol was developed to quantify the virus. Southern blots were performed to identify if the virus integrates into its host genome and Northern blots were carried out to identify viral transcripts in culture.

A condition was found where STIV could be produced consistently and monitored with Enzyme-Linked ImmunoSorbent Assay (ELISA). Viral production is at a maximum when cells were grown at pH 2.5 and in a temperature range of 75-85°C. The magnitude of virus production does not depend on amount of virus used to infect cultures, when the Multiplicity of Infection (MOI) is above 0.45. Southern blotting did show ideal banding pattern for a non-integrated virus, thus no evidence for integration of STIV into its host genome was found. A total of 9 viral transcripts were identified by Northern blotting of STIV infected *S. solfataricus* P2 cultures.

CHAPTER 1

INTRODUCTION

Archaea

Before the 1970's, the model by which all living things could be classified was accepted to be a system of five kingdoms, four eukaryotic kingdoms including plants, animals, fungi and protists and the fifth, the prokaryotic bacteria. This division recognized the characteristics eukaryotic organisms have in common such as cytoskeletons, nucleus and internal membranes. As molecular-based methods began to develop, relationships among the prokaryotes using DNA sequences were investigated [1] and it was discovered that Bacteria divided into two noticeably different groups. A division between prokaryotes that lived at high temperatures or produced methane and the typical prokaryotes and eukaryotes was observed. In 1977, these high temperature and methane producing organisms were recognized to be an entirely new group of organisms, later named Archaea [2].

Originally the Archaea was classified with Bacteria because of their appearance, but later it was recognized that Archaea are a completely different group of organisms and should be categorized separately. With the determination of the sequence of the archaeon *Mathanococcus jannaschii,* it became evident that the evolutionary lineage of Archaea is independent of both Bacteria and Eukarya [3]. Because of this distinct difference in genetic makeup, it was proposed that life should be divided into three domains: Eukarya (eukaryotes), Bacteria (initially Eubacteria), and Archaea (initially

Archaebacteria) [2]. The name Archaebacteria was changed to Archaea to prevent any misunderstanding because Archaea should certainly not be classified with Bacteria.

The three primary domains, Bacteria, Archaea, and Eukarya, can be characterized by a number of phenotypic properties. Some of these characteristics are unique to one domain, while others are found in two out of the three domains. The first distinct difference is in the structure of the cell wall. Virtually all Bacteria cell walls contain peptidoglycan with only two known exceptions. The members of the *Planctomyces-Pirella* group has cell walls composed of protein [4] and the *Chlamydia-Mycoplasma* groups lack cell walls altogether [5]. Cell walls containing peptidoglycan can be considered a signature for species of Bacteria, the domains Eukarya and Archaea lack peptidoglycan in their cell walls altogether. If cell walls are present in Eukarya, they are usually made of cellulose or chitin. The cell walls in Archaea can vary, from pseudopeptidoglycan to walls made of polysaccharide, protein, or glycoprotein [5].

The chemical nature of the membrane lipids is not the same in all three domains. Bacteria and Eukarya synthesize membrane lipids with a backbone consisting of fatty acids hooked in ester linkage to a molecule of glycerol. Archaea lipids on the other hand consist of ether-linked molecules [5].

A distinction between the three domains is also found within the transcription system. In all organisms transcription is carried out by DNA-dependent RNA polymerases where DNA is the template, and RNA is the product. In Bacteria, cells contain a single type of RNA polymerase containing four polypeptides, α, β, β', and σ, in the active polymerase [5]. Archaeal RNA polymerases are, on the other hand, more

complex than those of Bacteria. The RNA polymerases in methanogens and halophiles contain eight polypeptides [6, 7], and hyperthermophilic Archaea contain an even more complex RNA polymerase consisting of at least 10 distinct polypeptides [8]. The major RNA polymerase of eukaryotes contains 10-12 polypeptides, and the relative sizes of the peptides coincide most closely with those from the hyperthermophilic Archaea [5].

Since the ribosomal RNA sequences and several protein synthesis factors are different in the three domains it is not surprising that some aspects of the translation machinery differs also. Although the ribosomes of Archaea and Bacteria are the same size (70S, compared to 80S in the cytoplasm in eukaryotes), several steps in archaeal protein synthesis more strongly resemble those in eukaryotes than in Bacteria [5]. The translational start codon in Bacteria is AUG that calls for the incorporation of an initiator tRNA containing formylmethionine, while in eukaryotes and Archaea the initiator tRNA carries an unmodified methionine.

Under a microscope it is hard to distinguish between Bacteria and Archaea and the extreme conditions under which many Archaea live has made them difficult to culture, so their unique place among living organisms long went unrecognized. Archaea include inhabitants of some of the most extreme environments on the planet and are therefore labeled as “extremophiles”. In an anthropocentric view, environments hostile to man are designated as extreme. Some of these environments had been considered too extreme to support microbial life at all [9]. Examples include sites near rift vents in the deep sea with temperatures well over 100°C, terrestrial hot springs, waters with extreme

pH, and high salinity environments. Archaea live in the anoxic mud of marshes and at the bottom of the ocean, and even thrive in petroleum deposits deep underground [10].

One of the most important environmental factors controlling the activities and existence of an organism is temperature [11]. Cell components, including proteins, nucleic acid and membranes have adapted physiological and nutritional requirements to those high temperature environments. Due to the small size of a single cell, it is unlikely any of their cellular components can be insulated from the environment and therefore adaptation to high temperatures is necessary. One example is that the thermal resistance of the DNA double helix appears to be improved in hyperthermophiles by reverse gyrase. Reverse gyrase introduces positive supercoils into DNA that have been shown to greatly stabilize DNA to heat denaturation [5]. Additionally, Archaea have histones that are phylogenetically related to eukaryotic core histones. When histones are added to purified DNA *in vitro* its melting temperature increases dramatically [9].

Many different factors influence the growth of living organisms other than temperature. Factors such as pH, nutrient quality or availability, hydrostatic pressure, salinity and light intensity will influence temperature responses [11]. Growth in high temperature environments might be reduced due to continuous destruction of the more thermally sensitive molecules. Considerable energy would have to be spent in resynthesis of sensitive molecules and growth would be slowed. Even though many extremophiles are Archaea, not all Archaea are extremophiles. Many live in more ordinary temperatures and conditions. For example, Archaea can be found alongside bacteria and algae floating at the surface of the ocean.

The development of DNA sequence-based methods made it possible to detect evolutionary relationships connecting all life, resulting in a universal evolutionary tree, called the Tree of Life. The Tree of Life project is a global, collaborative effort documenting biodiversity and phylogeny of life. In the tree, all the events of biological evolutions are played out somewhere along the branches. Therefore it preserves traces of the historical evolutionary process that gave rise to the diversity of the species. The roots of the tree show that the common ancestor to all cellular life may have arisen more than 3 billion years ago [12]. In the Tree of Life the difference between two organisms is studied by aligning their 16S ribosomal RNA (rRNA) sequences and the dissimilarities in the alignment are counted and considered to be some measure of phylogenetic distance between the organisms. Only the change in nucleotide sequence is considered, not the passage of time. The difference between many pairs of organisms can be used to develop a phylogenetic tree [13]. rRNA analysis places many of the hyperthermophilic Archaea at the base of the universal tree of life, suggesting that thermophiles were among the first forms of life on earth [14]. Since many of these hyperthermophilic organisms live in unusual environments, such as very low or high pH, high salt or anaerobic environments, they very often have metabolisms adapted to conditions thought to be needed to survive on an early earth. Members of the deepest and shortest lineages of the tree exhibit the highest growth temperatures [9].

The domain Archaea is divided into two major kingdoms, the Crenarchaeota and the Euryarchaeota, defined as early as 1986 based on only a few cultured archaeal organisms [15]. The kingdom Euryarchaeota includes many different groups, but the

majority of them are either methanogens, organisms that produce methane, or extreme halophiles, organisms that inhabit environments with high salt concentrations. Most organisms of this kingdom are not thermophiles with a few exceptions [16]. The Crenarchaeota contains organisms that can either live at high temperatures in hot springs or low temperatures of the Artic Ocean. Two additional kingdoms have been proposed, the Korarchaeota and Nanoarchaeota. The Korarchaeota branches near the root of the archaeal branch of Tree of Life and may therefore display novel biological properties important to our understanding of ancient organisms. This proposed kingdom has no cultured isolates and is known only from environmental sequences. The Nanoarchaeota kingdom is composed of small symbiotic organisms that are larger then viruses, but smaller then any other described organism. The organisms in this kingdom are only found in combination with other species [17].

Until direct counts of microbes were introduced by Hobbie *et al*. in 1977, environmental microbiology consisted mainly of culturing microbes from different environments. From culturing it was thought that microbes probably had a minor role in global nutrient and carbon cycles. Later it was discovered that culturing had underestimated microbial abundances by at least 100 or even 1000 fold. Therefore it was evident that microbes are indeed major players in global carbon cycles [18].

Sulfolobus

Sulfolobus, a hyperthermophilic Crenarchaeota, grows in sulfur-rich acidic hot springs at temperatures up to 90°C (optimum around 80°C) and at pH values of 1-5

(optimum pH 3.0) [5]. *Sulfolobus* is an aerobic chemolithotroph that oxidizes hydrogen sulfide or elementary sulfur to sulfuric acid and fixes carbon dioxide as sole carbon source [5]. One of the best characterized members of the Archaea belongs to the *Sulfolobus* genera, *Sulfolobus solfataricus*, which has emerged as a model organism for the study of Archaea. This species can easily be cultivated in laboratories and its 3-MB genome has been sequenced [19]. Hot springs that meet these criteria and have been surveyed for *Sulfolobus* spp. have all been positive for the genera. So far *Sulfolobus* spp. has been isolated from geothermal areas from all over the globe, including from El Salvador, the Dominican Republic, Iceland, Italy, Japan, New Zealand, Russia and the United States of America.

Viruses

Viruses have been known as distinct biological entities for little more than a century. Consequently, efforts to understand and control these important agents of disease are phenomena of the 20th century. Where there are any living organisms, viruses are likely to be, so the viriosphere, the portion of the Earth where viruses interact with their hosts, probably spans all environments in which life occurs [20]. It seems that almost every species can be infected by a number of viruses [20, 21]. Viruses are not alive in the sense that they have no metabolism of their own, and they can reproduce only by infecting a living organism. When as some viruses inject their genome or RNA into a cell, they hijack that cells replication machinery and produce new viral particles [22]. By analyzing viruses, a better understanding of a particular cellular environment can be

achieved because viruses are molecular parasites of the cells in which they replicate. All viruses are dependent on the host cells' biochemical machinery to replicate so they adapt to their hosts' unique characteristics cellular environments [16]. The world of viruses is intimately linked with cellular life and possibly always has been, as it seems that viruses form lineages that extend from the root to all branches in the Tree of Life [21].

A viral genome is comprised of either DNA or RNA. Within an appropriate host cell, the viral genome is replicated and directs the synthesis of new virus particles by cellular systems or, in other cases, the virus itself has its own components for synthesis. All viral genomes that have been sequenced to date are densely covered with open reading frames (ORFs), showing that viruses very efficiently use all the space available. The viral genome is the nucleic acid-based repository of the information needed to build, replicate, and transmit a virus. Since most viruses depend on their hosts' machinery to replicate, the virus does not need to encode for its own machinery to carry out virus production [23]. The small size of the genome could be affected by the length of time needed to replicate where longer viral genomes might require unacceptably long periods to replicate, placing the virus at an evolutionary disadvantage. The size of the capsid might also limit the size of viral genomes [23]. Virions are formed by *de novo* assembly from newly synthesized components within the host cell. The virions are the vehicle for transmission of the viral genome to the next host cell where it infects the new host.

Classification of viruses is based on their shared properties but not the properties of the cells or organisms they infect. The classification is based on four principles: 1) The nature of the nucleic acid in the virion, whether it is RNA or linear or circular, single- or

double-stranded DNA. 2) The symmetry of the protein shell or the capsid. 3) The presence or absence of a lipid membrane, and 4) the dimensions of the virion and capsid [23].

It has become apparent in the last 30 years that viruses are abundant, ubiquitous and ecologically important in the environment. Surprisingly, little is known about viruses considering that they are the most abundant biological entities on the planet [18]. Unlike the ribosomal RNA (rRNA) gene sequences used in the three domains of life, there is no universal nucleic acid signature for viruses. This means it is not possible to identify viruses based on nucleic acid hybridization [24]. However, within certain viral taxonomic groups there are conserved genes shared amongst all the members that make whole genome comparisons possible, such as comparisons of their predicted proteome [25]. The diversity within known groups of viruses can be studied by comparing those conserved genes in cultured isolates as well as in the environment. Discovery of culture-independent methods has revealed that viruses are much more diverse in the environment than previously thought, since less then one percent of microbial hosts have been cultivated [18, 26].

Before the viruses of Archaea were discovered, 75 families of viruses were recognized, containing more than 4000 viruses. With the discovery of archaeal viruses many completely new families of viruses have been documented. Approximately 50 archaeal viruses have been described to date, half from each phyla of the Archaea [27]. The low number of archaeal viruses described so far is most likely due to the limited success in isolating them, since their isolation can often be problematic, and not a

reflection of any biological restriction limiting viral diversity and number in the domain [16]. Possible limitations of culturing viruses could be that the viruses may not stay outside their host cell for long periods of time, limiting the time being exposed to the environment. Also, only a few potential archaeal hosts have been cultivated to date and many of those require conditions that are not conducive to the isolation of viruses [16].

Geothermally heated environments with temperatures above 80°C, including hot springs, mud holes and deep-sea hydrothermal vents, have been screened for viruses [28]. These environments appear to be special habitats for viruses, where the morphological diversity of viruses and virus like particles (VLPs) infecting microorganisms in such environments, mainly hyperthermophiles Archaea, is extraordinary [29]. Viruses that infect the domain Archaea show an interesting distribution pattern. Only a minority of them show morphotypes, which are the most common among viruses that prey on prokaryotes living at moderate temperatures. All but two Euryarchaeota infecting viruses, His1, of the *Fuselloviridae* family that infects *Haloarcula hispanica* [30] and A3, a spindle-shaped particle isolated from *Methanococcus voltae* [31], have morphologies similar to the head-and-tail phage isolated from Bacteria, but sequence analysis shows little or no similarity to viruses previously isolated. The only viruses known to infect Crenarchaeota are filamentous or of varying unique morphologies that have not been observed among any other viruses [29, 32]. It is difficult to know whether this diversity in morphology is specific to the Crenarchaeal hosts or if it is the unusual habitat that they live in [29]. Since Crenarchaeota, but not viruses with the same unusual morphologies, can also be found in aquatic systems at moderate to cold temperatures of the Artic Ocean,

these morphologies of the viruses that infect the organisms of the domain seem to be habitat specific.

Many of the newly discovered viruses of the Crenarchaeota domain have led to new insights into the domain since they are morphologically and genetically unique. So far 12 different virus morphologies been described from the phyla [33]. Some of these morphologies have never been seen before, but others occur in all domains of life. For example, the spindle-shaped SSV-like viruses and double-tailed ATV morphologies have never been detected in any other domain of life. Analysis of most of these viruses' genomes shows little or no similarity to genes in public databases [16], and more than 90% of all open reading frames (ORFs) of the sequenced genomes have not yet been assigned functions and share no homolog to published sequences [34]. This suggests that the majority of viruses remain unknown, with most viruses belonging to novel groups that have not yet been cultured [18]. Morphology is the key to phylogeny. The relationships between closely related viruses can be obtained by comparing their genome, but when the separation is ancient, it becomes difficult to classify them. Since proteins only show convergence of function not the structure, it is clear that structural similarity is more likely to determine an evolutionary link [35].

All archaeal viruses isolated to date have a DNA nucleic acid, except for one that has been reported to contain an RNA component [36, 37]. Their genomes are double-stranded circular or linear that range in size from 12-230 kbp [16]. It has been suggested that RNA viruses can not exist in these hot or unusual environments since RNA is unstable at those temperatures [38], but this has yet to be confirmed.

The virus-host interaction of Crenarchaeal viruses is also special. While most double-stranded DNA (dsDNA) viruses of bacteria and euryarchaea kill the host cell during virus release, most of the viruses of the Crenarchaeaota establish a successful infection without killing or lysing the cell, a so-called carrier state [34]. This choice of a stable relationship with the host cell may reflect on the necessity to minimize or avoid direct exposure of the virus to the extreme and unstable conditions of the environment that these organisms live in. Only two hyperthermophilic viruses have shown to exhibit lytic properties. *Thermoproteus* virus 1 (TTV1) and *Acidianus* double-tailed virus (ATV) which infect *Thermoproteus tenax* and *Acidianus* sp. respectively, have been shown to lyse their hosts, with virus released as the cell bursts [28, 39].

All viruses of crenarchaeal Archaea to date have been found to infect only four different genera, *Sulfolobus, Acidianus, Thermoproteus* and *Pyrobaculum*. Most of these viruses have been ones that infect *Sulfolobales* [24, 27, 40, 41]. One reason is likely, that the aerobic *Sulfolobales* can be easily isolated and cultured compared to other anaerobic members of the phyla [16]. The viruses of *Sulfolobus* represent at least five additional viral families: Rudiviridae, Lipothrixviridae, Fuselloviridae, Guttaviridae and Globuloviridae, and two new families (BIC (ATV), and AMPUL (ABV)) have been proposed.

STIV

Sulfolobus turreted icosahedral virus (STIV) was originally isolated from a hot spring in the Rabbit Creek area which is located in the Midway Geyser Basin of Yellowstone

National Park, but can be found in various locations around the Park. It is a large icosahedral virus, like the name suggests, that infects *Sulfolobus*. The diameter of the virus capsid is approximately 70 nm with a 6.4 nm thick shell. The icosahedral asymmetric unit consists of five trimers (pseudo hexamers) of the major capsid protein (MCP) plus one additional minor capsid protein at the 5-fold vertex [14]. On all twelve fivefold vertices there are large turret-like appendages [42] which have been suggested to be involved in host recognition and/or attachment of the virus to the host [14]. The viral particle is composed of a 37-kDa MCP and three 10-, 12.5- and 25- minor capsid proteins and holds a 17,663 bp circular dsDNA genome [35]. The structural features of the capsid indicate that the virion is built on a pseudo-T = 31 icosahedral lattice. There also appears to be an internal lipid membrane sandwiched between the genome and the capsid shell host lipids [14]. Like the hosts' genome, the genome of the virus has a low GC content (36%). STIV's dsDNA genome has 36 predicted open reading frames, where only eleven have been confirmed in virus particles [14, 43].

With the discovery of STIV it was revealed that icosahedral non-tailed dsDNA viruses are present in all three domains of life, providing the first example of a double-barrel trimer lineage from the domain Archaea [10]. This discovery has led to speculation about a common viral ancestor that predates the divergence of the three domains, a suggestion that is supported by the shared general architecture of this group of viruses and the common fold of their MCP [43].

In this study STIV and its infection cycle in *S. solfataricus* P2 was studied. A condition was found where STIV could be produced consistently, along with a method to

detect and quantify the virus quickly. Timing and quantity of virus inoculation and propagation of virus in cultures were examined. The transcripts of the virus were identified and determined whether or not the STIV genome integrates into *S. solfataricus* P2.

CHAPTER 2

GROWTH OF *SULFOLOBUS SOLFATARICUS* INFECTED AND NON-INFECTED WITH SULFOLOBUS TURRETED ICOSAHEDRAL VIRUS

Introduction

The abundance of Sulfolobus turreted icosahedral virus (STIV) in hot springs is quite low. In order to have enough virus to carry out experiments, larger quantities of the virus are needed. Viruses rely on their host organism replication system for virus production, therefore, in order to produce the virus in the laboratory, the host organism for the virus needs to be cultured. Culturing microorganisms can be troublesome, since there are many different factors in the environment that influence growth. Factors, including temperature, pH, salinity, oxygen availability and carbon sources, need to be tested to determine optimum growth conditions for the host and its virus. Optimum conditions for growth of the host however, may not be the optimal for virus production.

Sulfolobus solfataricus isolate P2 has been shown to be a host for STIV. *S. solfataricus* grows in geothermal areas, at a temperature optimum of 80°C (with a range of 70°C to 87°C) and pH 3 (with a range of 1.5 to 5.5) [10]. The organism is an aerobic acidophile that grows heterotrophically, and has been shown to be able to use various carbon sources for growth [44]. The strain of *Sulfolobus solfataricus* used in this study is named P2, obtained from The American Type Culture Collection (ATCC). This strain was originally isolated from a hot spring in the solfataric field of Pisciarelli, Italy [45]. *S. solfataricus* ATCC strain P2 (*S. solfataricus* P2) has been shown to be virus free, and is therefore ideal for examining virus infection cycles. STIV was originally isolated from a

strain of *Sulfolobus islandicus*, isolated from Yellowstone National Park, YNP179. YNP179, in contrast to *S. solfataricus* P2, is not free of other virus infections and therefore *S. solfataricus* P2 is better to study new virus infection.

Three media were tested for growth of *S. solfataricus* P2, Media 88, Media 182 and media prepared in accordance with Zillig *et al.* (Zillig) [24]. The temperature range where the strain can be grown was tested, along with the pH range. The pH of the media has been shown to increase during growth of these organisms [46].

Production of STIV, with *S. solfataricus* P2 as a host was tested under different temperatures and pHs. Optimum conditions were determined for STIV production and these conditions were used for further experimentations. Multiplicity of infection for the virus to infect *S. solfataricus* P2 was determined by infecting virus free cultures with different amounts of virus, and determining the minimum amount of virus needed to establish a successful infection in the culture.

Timing of virus inoculation, early-log versus late-log, was examined along with the propagation of STIV in *S. solfataricus* P2 cultures. This was done to determine if STIV can establish a secondary round of infection in *S. solfataricus* P2, while so far only a single round of infection has been detected (B. Wiedener and D. Hansen, unpublished).

Materials and Methods

Starting Cultures

Cultures of *S. solfataricus* P2 are started by inoculating an aliquot of a glycerol-stocked ATCC strain into Medium 182 (http://www.dsmz.de/media/med182/) at pH 3.5, in a long-neck Erlenmeyer flask. Flasks are incubated in oil-bath shaker at temperatures close to the optimum growth temperature of *S. solfataricus* P2, ~80°C and shaken ~100 rpm to ensure aeration in the culture. Temperature in the culture was 2-5°C less than the incubator setting.

Growth in Different Media

A culture of *S. solfataricus* P2 was started as described above. At mid log-phase (OD = 0.250-0.500), 2 ml of the actively growing culture was diluted into 18 ml of three different media; Media 88 (http://www.dsmz.de/media/med88/), Media 182 and Zillig [24]. Additionally, growth was measured in the three media complemented with 1 g/L of carbon. All growth conditions were tested in duplicate by optical density measurements at 650 nm (OD_{650}), over a 10 day period.

Growth at Different Temperatures and pHs in the presence or absence of STIV

Three cultures of *S. solfataricus* P2 were started as described above. Following 2 days of growth, to reach mid-log growth, the cultures were diluted five fold in Media 182 at pH 3.0 and allowed to grow an additional 24 hours. The culture was diluted five to ten

fold in Media 182, again at pH 3.0. At mid log-phase, 2 ml of the actively growing culture was diluted into 18 ml of fresh Media 182 at pH 2.5, 3.0 and 3.5 and the cultures were grown at three different temperatures, 75, 80 and 85°C. When cultures were in early-log growth (OD = 0.150 – 0.200) they were infected with STIV from concentrated stock. Growth of *S. solfataricus* P2 was followed by measuring optical density every 12 hours and samples were taken every 24 hours to measure virus production by dot-blot, ELISA (Chapter 2) and Q-PCR (B. Widener, unpublished protocol). Briefly, dot-blots were prepared by spotting onto Hybond-C membranes with 1 μL of sample and allowing the membrane to dry. Membranes were blocked with 5% (w/v) skim milk powder in TBS (140 mM NaCl, 3 mM KCl, 25 mM Tris, pH 7.4 w/HCl) + 0.1% (v/v) Tween (TBST). Primary antibody, 0.04% (v/v), to the STIV coat protein was added to the blocking solution and incubated. Following washes in TBST, the membrane was incubated with the same concentration of a secondary antibody, a goat anti-rabbit antibody (Bio-Rad). The membrane was developed using an Opti-4CN Substrate Kit following the recommended protocol (Bio-Rad). The protocol for Q-PCR uses a SYBR Green Kit (QIAGEN). A 25 μl reaction is run with 2 μl of sample added. Forward and reverse primers were designed to amplify a 242 bp piece of the coat protein using a 4K fragment clone of the genome cloned into a TOPO A vector (Invitrogen) in *E. coli* (Fulton, unpublished) that includes the amplified portion of the genome as a positive control. The reaction is cycled 35 times with denaturation at 95°C, annealing at 50°C and extension at 72°C. A standard concentration curve is generated from a sample with a known concentration of STIV, which is used to calculate concentration of STIV in samples.

Multiplicity of Infection

A culture of *S. solfataricus* P2 was started as described above. Following 2 days of growth, the culture was diluted five fold in Media 182 at pH 2.5, and allowed to grow for additional 24 hours. The culture was diluted 5-10 fold in Media 182, again at pH 2.5. When the culture was in early log-phase it was divided into 24, 5 ml volumes in 20 ml glass test tubes. Serial dilutions, from 10^{0}–10^{-5} were made in Media 182 from concentrated stock of STIV. At t = 0, epifluorescent microscopy was used to estimate the number of virus particles in each dilution. Briefly, 100 μl of culture were fixed for 30 minutes at 4°C, with 10 μl of glutaraldehyde. Fixed cultures were transferred to Anodisk 25 filters, with 0.02 μm pour size (Whatman) and stained with 1xSYBR gold (Invitrogen) for 15 minutes in dark. Virus particles were then directly counted under epifluorescent microscopy.

The tubes were then infected in triplicate with 50 μl or 5 μl aliquots of dilutions of STIV. Three cultures were left uninfected as a negative control. The linear relationship between the optical density and cell numbers per mL of culture is

$$\text{Cells/mL} = 6.73 \cdot 10^{9} \times \text{OD}_{650} - 7.96 \cdot 10^{8} \quad (1)$$

The number of host cells per culture was estimated by measuring the OD of the culture, then using equation (1) to calculate the number of cells in the cultures (A.Ortmann, unpublished). Multiplicity of infection (MOI) measures the minimum amount of virus added to a culture to successfully establish an infection, reported in virus particles added per host cell.

Growth of the cultures was monitored with optical density every 12 hours over a 72 hour period post infection. Virus production was followed by Q-PCR and ELISA every 24 hrs.

Rediluting cultures and reinfection with STIV

Two cultures of *S. solfataricus* P2 were started as previously described. Following 2 days of growth, the cultures were diluted five fold in Media 182 at pH 2.5 and allowed to grow an additional 24 hours. Further five- to ten-fold dilutions of the cultures were performed on the fourth day in Media 182, pH 2.5. At mid log-phase, the actively growing cultures were split in half, and then one was infected with STIV (STIV) while the other was uninfected (P2). The cultures were grown for 72 hours then split according to Tables 2.1 and 2.2, with triplicates of each culture. Growth of infected and uninfected cultures was monitored by optical density and samples were taken every 24 hours and stored at 4°C for Q-PCR analysis, to determine virus production. Slides for epifluorescent microscopy based virus counts were prepared immediately.

Table 2.1. Experimental set up of growth experiment 1.
Following 72 hours of growth the initial cultures P2 (uninfected) and STIV (infected) were split into 6 different treatments. Each treatment was carried out in triplicate.

P2	20 mL of P2 culture, no treatment
STIV	20 mL of STIV culture, no treatment
P2 + STIV	20 mL of P2 culture, infected with virus at t = 72 h
STIV + STIV	20 mL of STIV culture, reinfected with virus at t = 72 h
P2 – 182	2 mL of P2 culture diluted into Media 182, pH 2.5 at t = 72 h
STIV – 182	2 mL of STIV culture, diluted into Media 182, pH 2.5 at t = 72 h

Table 2.2. Experimental set up of growth experiment 2.
Following 72 hours of growth the initial cultures P2 (uninfected) and STIV (infected) were split into 6 different treatments. Each treatment was carried out in triplicate.

P2 – P2	2 mL of P2 culture diluted into 18 mL of P2 culture in early log-phase, at t = 72 h
STIV – P2	2 mL of STIV culture diluted into 18 mL of P2 culture in early log-phase, at t = 72 h
P2 – P2 + STIV	2 mL of P2 culture diluted into 18 mL of P2 culture in early log-phase and infected with virus, at t = 72 h
STIV – P2 + STIV	2 mL of STIV culture diluted into 18 mL of P2 culture in early log-phase and reinfected with virus, at t = 72 h
P2 – 182 + STIV	2 mL of P2 culture diluted into Media 182, pH 2.5 and infected with virus at t = 72 h
STIV – 182 + STIV	2 mL of STIV culture at t = 0 h, diluted into Media 182, pH 2.5 and reinfected with virus at t = 72 h

Results

Growth in Different Media

Growth of *S. solfataricus* P2 in three different media, supplemented and non-supplemented with 1 g/L glucose, was monitored over time and plotted (Figure 2.1). *S. solfataricus* P2 did not grow considerably different in the three media tested. When the glucose was added to the media, the growth of *S. solfataricus* P2 improved noticeably, increasing the yields of biomass 30 – 80%. Showing that *S. solfataricus* P2 can efficiently use glucose as a carbon source for its growth.

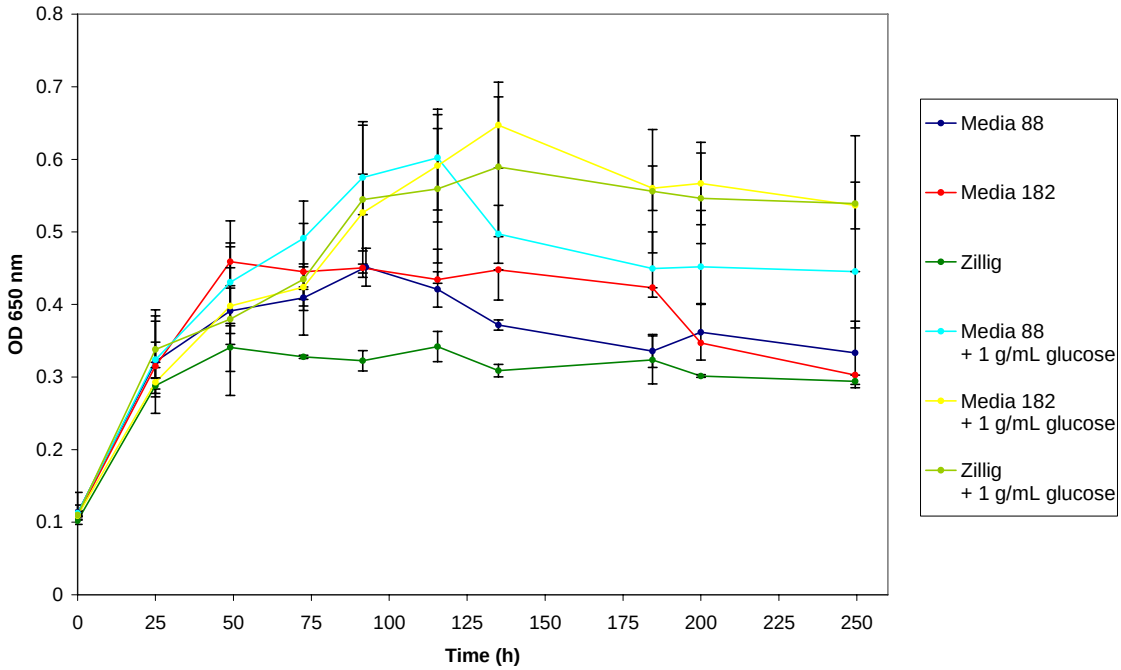


Figure 2.1. *S. solfataricus* P2 grown in three different media, with and without 1 g/mL glucose. Cultures supplemented with glucose grow considerably better than cultures without glucose. Difference between growth in Media 88, Media 182 and Zillig is not significantly different.

Growth at Different Temperatures and pH

Growing *S. solfataricus* P2, and infecting the culture with STIV, at three different temperatures shows that the growth of the cultures does not change significantly between temperatures (Figure 2.2). This suggests that *S. solfataricus* P2 has a wide temperature at which it can efficiently grow. Infecting *S. solfataricus* P2 with STIV does not overly affect its growth since cultures that were not infected did not show better growth than ones that were infected with the virus. Virus production in the cultures starts earlier at higher temperatures according to Q-PCR and ELISA measurements, but higher yields of virus are not detected (Growth curves, Q-PCR and ELISA data in Appendix A).

Growing *S. solfataricus* P2, and infecting the culture with STIV, at three different pHs shows that the growth of the cultures does not change significantly at those different pHs. Virus production, however, is dependent on the initial pH the cultures are started at. At pH 2.5 and 3.0 production of virus is similar, while no virus was detected at pH 3.5. The pH of the cultures was monitored over time (Figure 2.3). The pH of cultures that started at pH 3.5 increase greatly over the time. After 72 hours, the pH has increased to slightly less then pH 6, which is three orders of magnitude higher than it started at. The pH in cultures that were started in media with pH 3.0 rise slower than cultures at pH 3.5, but still increase dramatically, up to pH 4.5 over a 100 hour period. Those cultures which started at pH 2.5 remain at that pH throughout the whole time course. The compound most likely to be buffering the media is the sulfate, SO_4, since the pKa value of SO_4 is 2.0. The reason for no virus production is probably this drastic increase of pH over the time, since the virus is more stable at lower pHs.

Plotting the growth rate for all temperature and pHs measured (Figure 2.2), shows, considering the standard deviation of the growth rate, μ, that the growth rate at different temperature and pH is very similar.

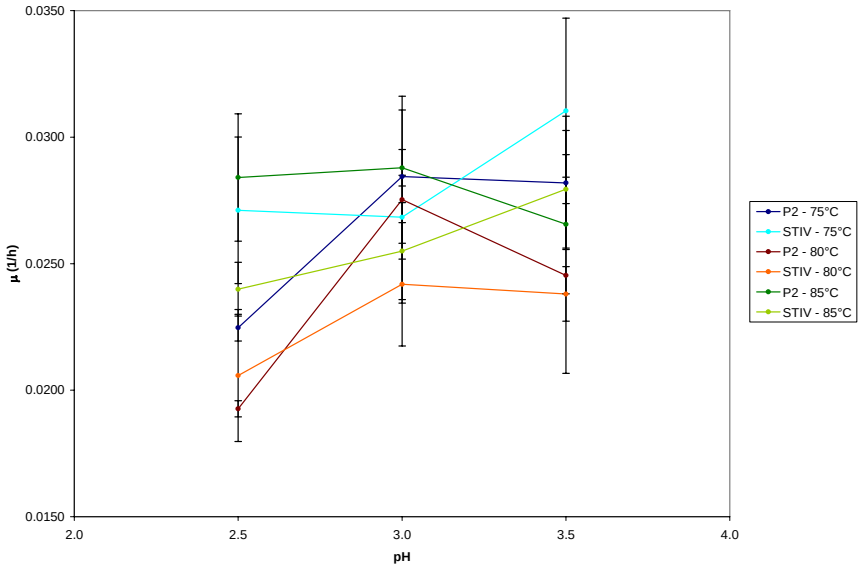


Figure 2.2. Growth rate of *S. solfataricus* P2 infected and uninfected with STIV
Growth rate, μ (h^{-1}), of *S. solfataricus* P2 infected and uninfected with STIV at 75, 80 and 85°C, pH 2.5, 3.0 and 3.5.

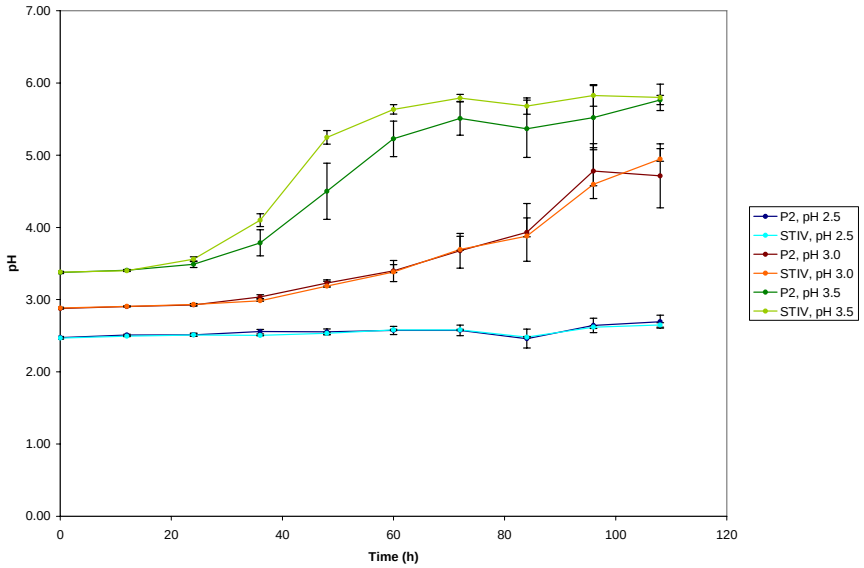


Figure 2.3. pH of the *S. solfataricus* P2 cultures over time.
Cultures that start at pH 2.5 stay at that pH throughout the time course while pH of cultures that start at higher pHs rise as time passes. Values shown are means of triplicate cultures with error bars showing standard deviation.

Multiplicity of Infection

Eight independent MOI experiments were performed and data from all eight are summarized in Figure 2.4. These eight experiments tested MOIs ranging from 0.003 to 16000 and used 3 different virus stocks. From these eight experiments, it can be shown that an infection will be established if a MOI above 0.45 is used, meaning that not all cells in a culture are getting infected. A MOI below 0.04 never results in an infection being established, while cultures infected with a MOI between 0.04 and 0.45 resulted in an infection in 53% of the cultures.

One complete experiment (Experiment 8, Figure 2.5), in which all 7 dilutions established infection, reveals that a wide range of STIV was added to the cultures (Table 2.3), but cultures with more virus added do not produce more virus than those where less virus was added. The only difference between virus production in the cultures when different amounts of virus was added, is that when more virus was added, virus production occurs earlier than when less virus is added (Figures 2.6.B and C, Q-PCR and ELISA respectively). Q-PCR detects virus infection earlier than ELISA since Q-PCR is detecting the viral genome produced while ELISA measures the production of the major capsid protein (MCP). Only one infection cycle was detected, regardless of when the peak production of virus occurred.

Table 2.3. MOI's of Experiment 8.
Concentrated stock of STIV diluted and used to infect *S. solfataricus* P2 cultures at mid log-phase.

Dilutions	MOI
$1:10^0$	3148.061
$1:10^1$	314.806
$1:10^2$	39.959
$1:10^3$	4.373
$1:10^4$	0.856
$1:10^5$	0.636
$1:10^6$	0.298

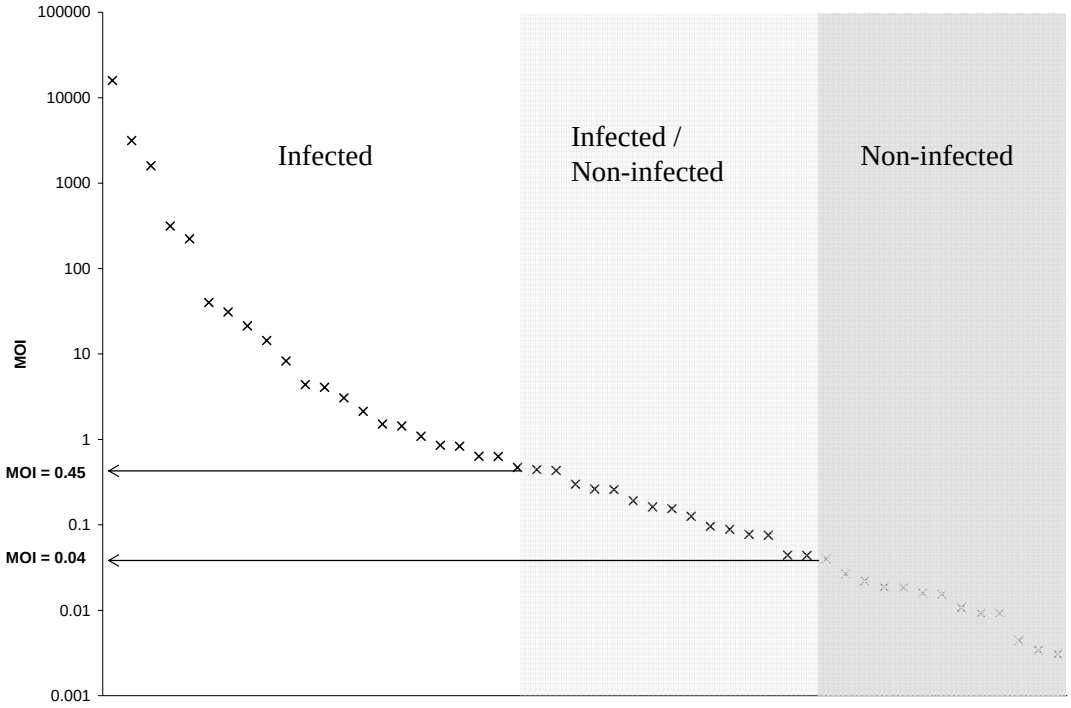
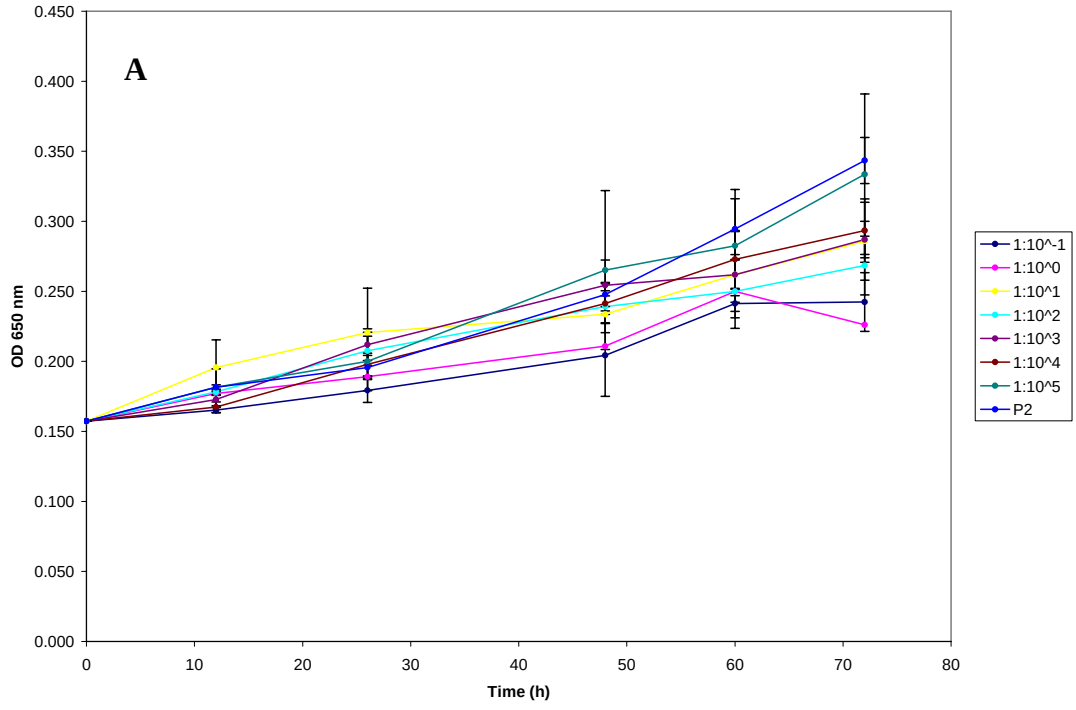


Figure 2.4. MOI of eight different experiments.
Showing whether or not an infection was established in the culture at different MOI's. The white region represents cultures where virus infection was established, the light grey region where 53% of cultures were infected and the dark grey region where virus infection was never established.



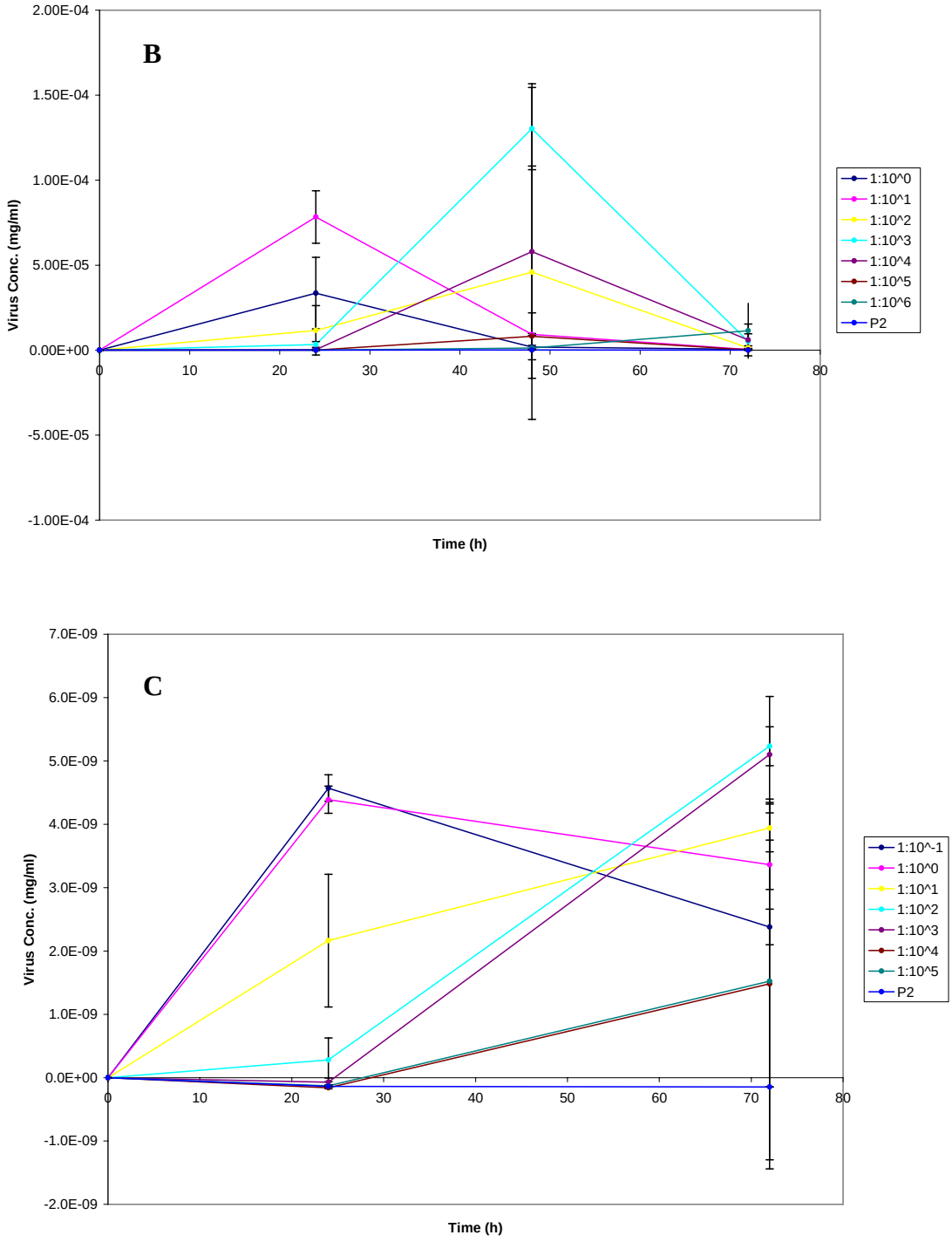


Figure 2.5. MOI experiment 8.
S. solfataricus P2 infected with various concentrations of STIV. A) Growth of cultures measure with optical density. B) and C) show the concentration of STIV over time measured with Q-PCR and ELISA, respectively. Values shown are means of triplicate cultures with error bars showing standard deviation.

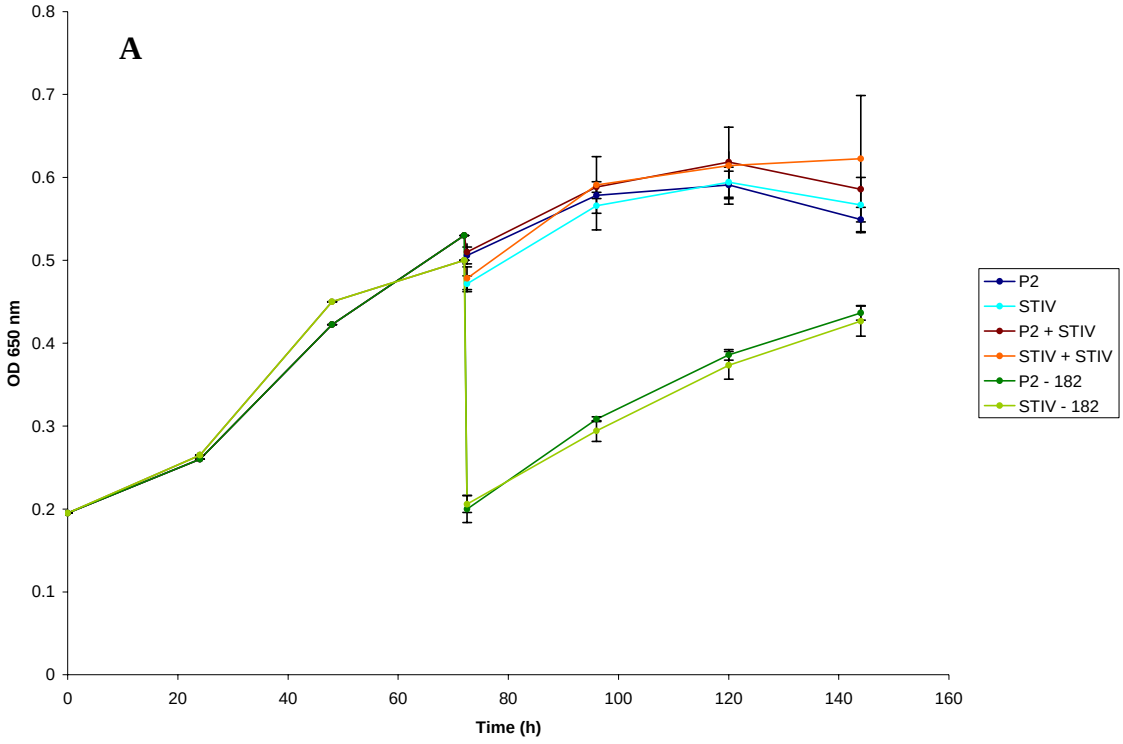
Diluting cultures and re-infecting with STIV

Cultures of *S. solfataricus* P2, infected and non-infected, were grown for 144 hours to see if second STIV infection cycle would occur. The infected (STIV) and uninfected (P2) cultures were grown up for 72 hours and then split up into different experiments. Some were left untouched while others were diluted into fresh Media 182 at pH 2.5 or *S. solfataricus* P2 cultures in early log-phase growth. At t = 72 h half of the cultures were also infected with STIV, some being infected with the virus for the first time in their stationary phase, while others were being re-infected (Figure 2.6 and 2.7).

Virus infection was established in P2 cultures that were infected with STIV in early log-phase, while cultures that did not get infected until stationary phase (t = 72 h) did not establish infection (Figures 2.6 and 2.7, A and B). When cultures that had been previously infected in early log-phase were re-infected at 72 h, they did not produce more virus compared to cultures that had only been infected in early log-phase. In cultures that were infected in early log-phase, a second cycle of virus infection was not detected when diluted into fresh Media 182 at 72 h, even though the host continued to grow. Cultures that were initially infected with STIV, were also diluted into fresh P2 in early log-phase of growth. In these cultures a second virus infection cycle occurred, possibly due to infection of the fresh P2 cells in the culture, but not from the ones that were already in stationary phase. Infected cultures that were diluted into fresh P2 cultures in early log-phase were also reinfected with STIV. A second virus production cycle was also seen in these cultures. Comparing that to uninfected cultures that were infected with STIV for the first time at t = 72 h when diluted into fresh P2 cells in early log-phase, shows that the

same magnitude of virus production occurs, suggesting again that the cells in the early log-phase are getting infected. Another experiment that supports that idea is when STIV infected cultures were diluted into fresh Media 182, pH 2.5 and re-infected with STIV at t = 72 h, second peak of virus production is not seen.

In experiment 2, virus production is observed in STIV 24 h post infection while in experiment 1 is not detectable until after 72 h. Viral infection in the cultures was observed at the same time with both epiflourecent microscopy and Q-PCR. The two methods correlate well with each other, although the peak of virus production is steeper when measured with Q-PCR.



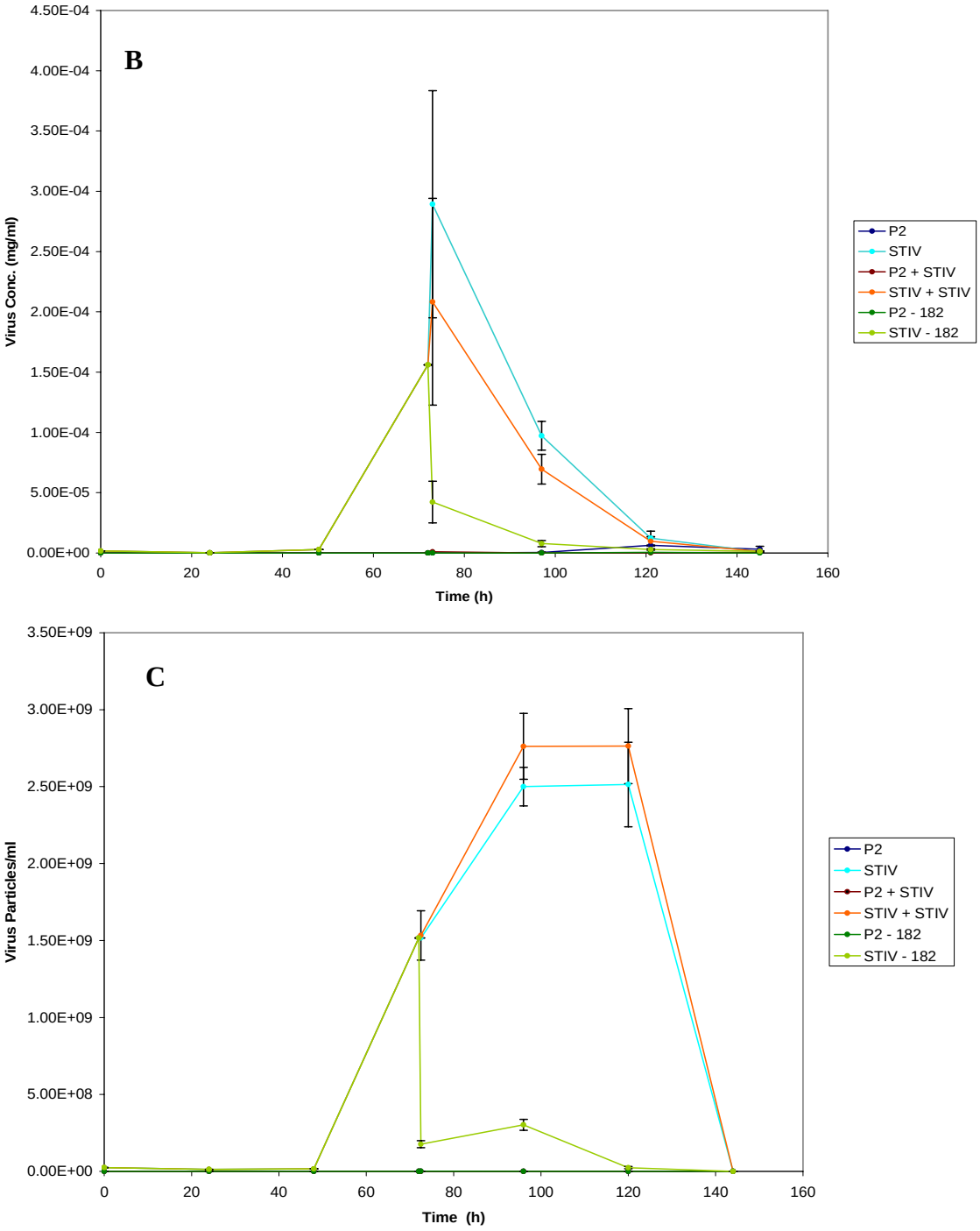
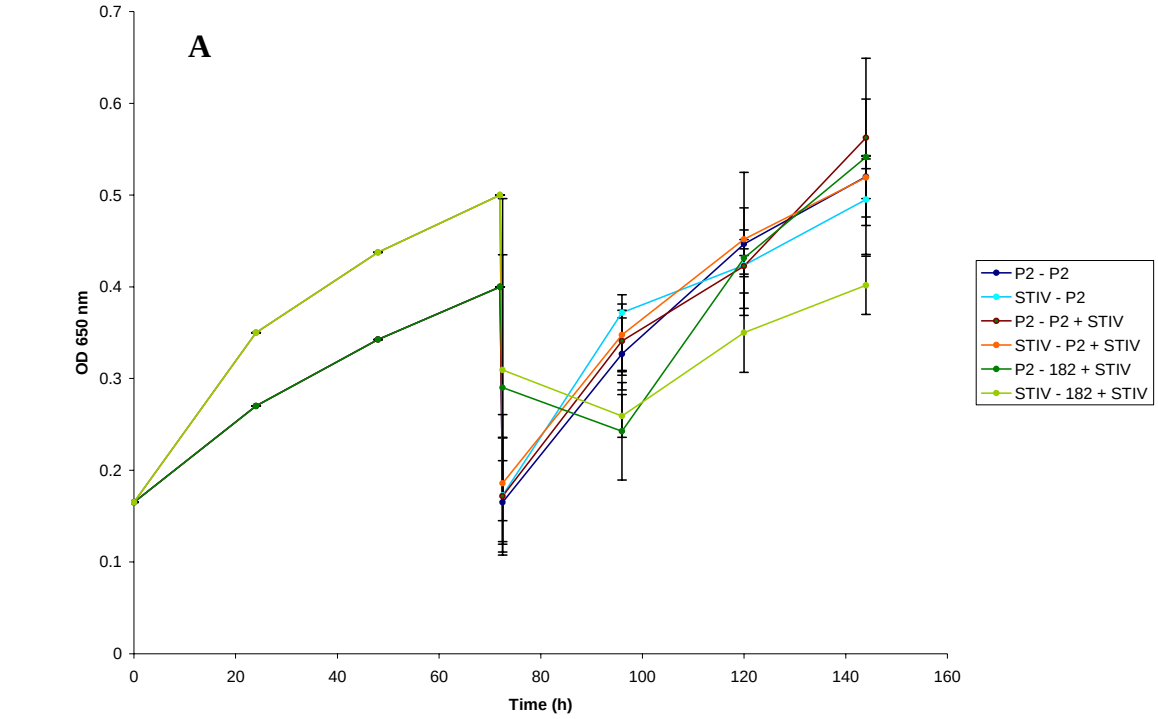
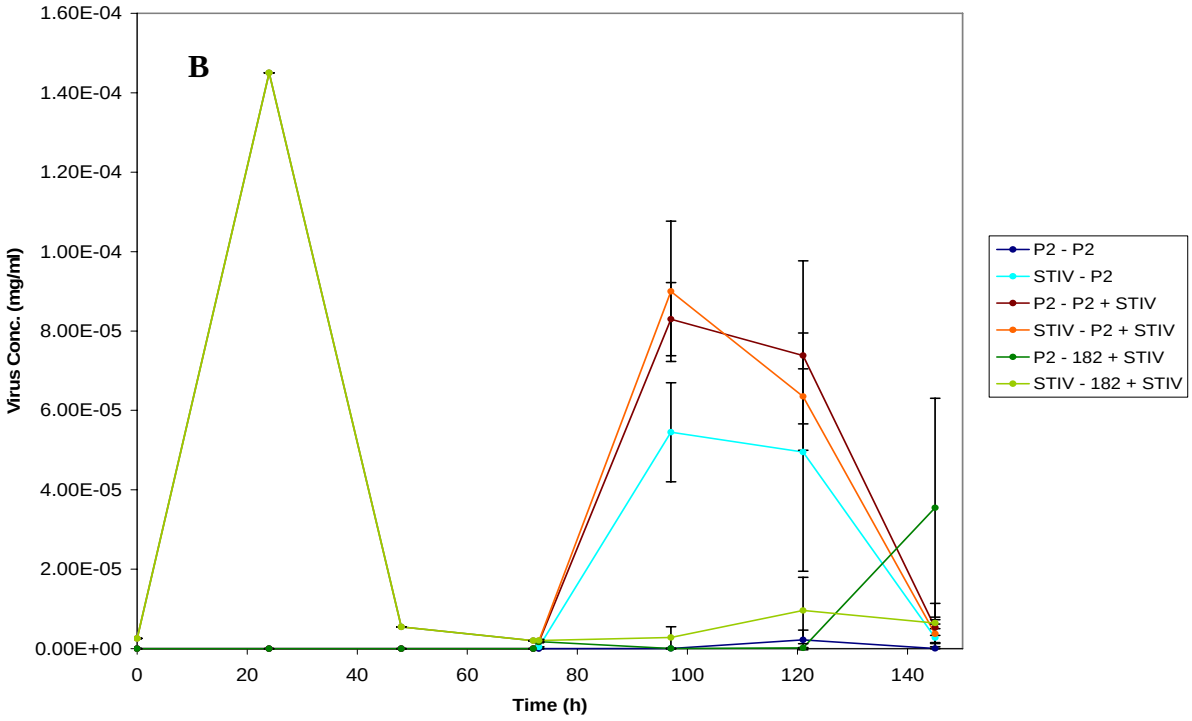


Figure 2.6. Experiment 1.
P2 and STIV cultures were grown for 72 h. At t = 72 h, 20 mL cultures in triplicate were allowed to grow for additional 72 h without any changes (P2, STIV), 20 mL cultures in triplicate were infected with virus and grown for additional 72 h (P2+STIV, STIV+STIV) and 2 mL of each culture is diluted in 18 mL Media 182, pH 2.5 (P2-182, STIV-182), again allowed to grow for additional 72 hours. A) Growth curve B) and C) Virus production measured with Q-PCR and ELISA, respectively. Values shown are means of triplicate cultures with error bars showing standard deviation.


A
OD 650 nm
Time (h)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
140
160
P2 - P2
STIV - P2
P2 - P2 + STIV
STIV - P2 + STIV
P2 - 182 + STIV
STIV - 182 + STIV


B
Virus Conc. (mg/ml)
Time (h)
1.60E-04
1.40E-04
1.20E-04
1.00E-04
8.00E-05
6.00E-05
4.00E-05
2.00E-05
0.00E+00
0
20
40
60
80
100
120
140
P2 - P2
STIV - P2
P2 - P2 + STIV
STIV - P2 + STIV
P2 - 182 + STIV
STIV - 182 + STIV

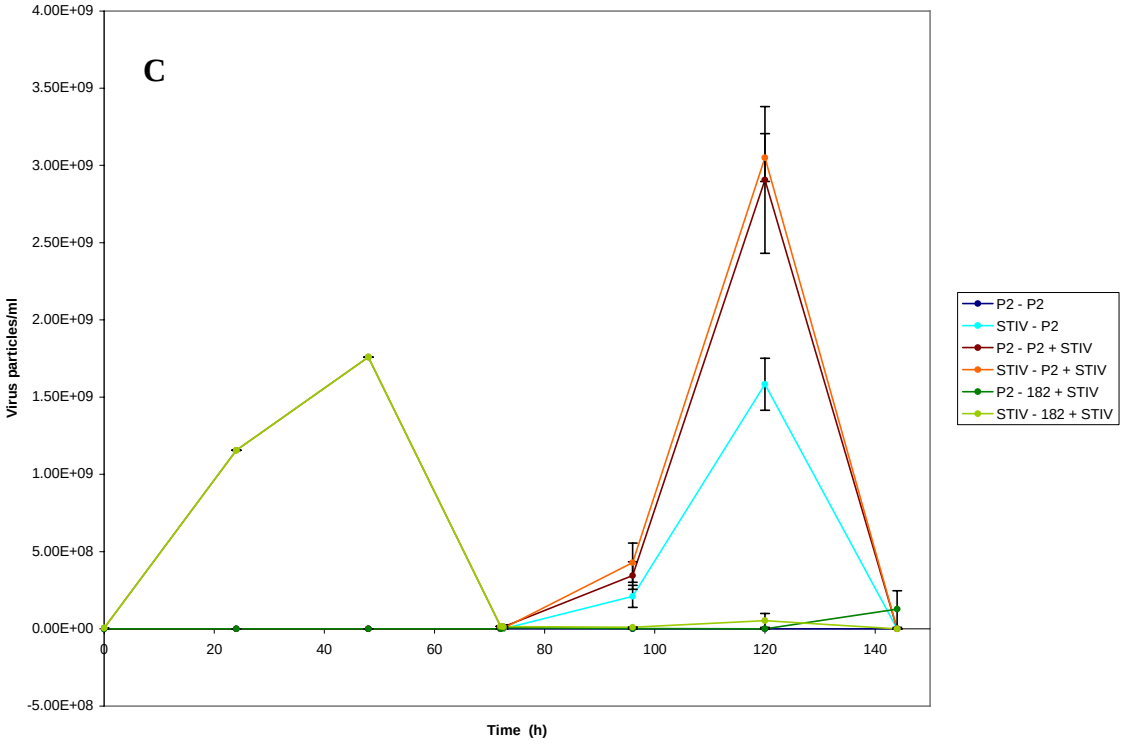


Figure 2.7. Experiment 2.
P2 and STIV cultures were grown for 72 h. At t = 72 h, 2 mL of P2 and STIV were diluted into 18 mL of P2 in early log-phase and allowed to grow for additional 72 h (P2 – P2, STIV – P2), 2 mL of P2 and STIV were diluted into 18 mL of P2 culture in early log-phase, infected with virus and grown 72 h (P2 – P2 + STIV, STIV – P2 + STIV) and 2 mL of P2 and STIV were diluted into 18 mL of Media 182, pH 2.5, infected with virus and grown 72 h (P2 – 182 + STIV, STIV – 182 + STIV). A) Growth curve B) and C) Virus production measured with Q-PCR and ELISA, respectively. Values shown are means of triplicate cultures with error bars showing standard deviation.

Discussion

Growth of *S. solfataricus* P2 in the three media considered in this study is not considerably different. When the media are supplemented with 1 g/ml glucose, *S. solfataricus* P2 grows better in all three media. Cultures grew well at all three temperatures considered, 75, 80 and 85°C and all pHs, 2.5, 3.0 and 3.5 that were tested. Growth rate of the cultures was not dependent on temperature or pH.

The allowable temperature and pH range was not as wide for virus production. STIV was produced at all three temperatures considered, but virus production and release started earlier at higher temperatures. No virus production was detected at pH 3.5 while STIV was produced at both pH 2.5 and 3.0 in similar magnitudes. The likely reason for this is the dramatic increase in the pH when cultures are started at pH 3.5. After only 24 hours of growth, the pH increases to almost pH 6.0. The pH of cultures started at 3.0 does not start increasing until after about 40 hours of growth, is much slower, and is not as drastic as in cultures that are started at pH 3.5. Cultures started at pH 2.5 manage to stay at pH 2.5 throughout the whole time course, making the environment more favorable for STIV. The reason for no virus production in cultures started at pH 3.5 is probably this drastic increase of pH over the time, since the virus is more stable at lower pHs.

A wide range of multiplicities of infections was tested for STIV infecting *S. solfataricus* P2 to ensure that the virus was not a limiting factor. An MOI of at least 0.45 was needed to establish an infection in a *S. solfataricus* P2 culture, or that maximum of about half of the cells in a culture are getting infected with STIV. Viral infection is, in some cases, established with MOIs between 0.04 and 0.45, but at MOIs lower than 0.04

infections were never documented. The magnitude of virus produced is not dependent on the amount of virus added to culture while above MOI 0.45. That suggests that not all cells are susceptible for infection of STIV in the culture. Thin sections of STIV-infected *S. solfataricus* P2 cultures analyzed with transmission electron microscope (TEM), revealed that STIV was seen inside only approximately 1 in every 50 *S. solfataricus* P2 cells, or 2% (S. Brumfield, unpublished).

Not much is known about the virus-host relationship between STIV and *S. solfataricus* P2. To interpret the data, three interactions must be considered. The three cases are: 1) cells susceptible to virus infection in the culture are limiting, 2) virus added to cultures is limiting, and 3) neither one is limiting. In the first case two possibilities exist. First there may only be a small percentage of the total number of host cells which the virus can attach to and or infect. Second, only few cells may be capable of producing virus particles. If susceptible cells are limiting, the amount of virus produced would be independent of MOI since all susceptible cells would be infected at the lowest dilution of virus that caused an infection. The second case is where the virus is limiting. Then, not enough viruses have been added for every receptive cell. In that case the amount of virus produced would have a direct linear relationship to the MOI used to infect the culture. In the third case, where neither is limiting (hence every virus is infective and every cell is receptive), would also produce amount of virus directly related to the MOI.

Virus produced in a culture of *S. solfataricus* P2 was not dependent on the amount of virus added. Based on this observation, the possibility that the virus is limiting is not likely, since the range of MOIs used to infect cultures was very wide, from 16000 down

to 0.003. It does suggest that the number of susceptible host cells is the limiting factor in the amount of virus that is produced. Similar magnitude of infection is seen when the MOI is between 0.45 and 16000.

Only a single cycle of virus infection were seen in the MOI experiments. To determine if secondary infection could be established, *S. solfataricus* P2 cultures were grown for long time periods and various different experiments were carried out. At early log-phase of time courses, t = 0 h, half of the cultures were infected with STIV while the other half was left uninfected. 72 h post-infection the infected and uninfected cultures were left untouched, diluted into Media 182, pH 2.5 and *S. solfataricus* P2 in early log-phase growth. Half of the cultures were infected while the other half was infected at t = 72 h. Cultures infected at t = 0 that produced virus in the first 72 h of growth did not establish a secondary infection in the 144 h that the cultures were grown for. When these same cultures were diluted into Media 182, pH 2.5, at t = 72 h, a secondary cycle of infection was not recorded either, even though the host cells were growing efficiently. Addition of STIV at t = 72 h to cultures that had been diluted into Media 182 did not establish an infection either. Diluting *S. solfataricus* P2 cells that had been infected at t = 0 h after 72 h of growth into actively growing cells in early log-phase did establish a second round of infection. When additional virus was added to cultures that had been infected at t = 0 h, and diluted into actively growing cells in early log-phase at t = 72 h, virus production was more then when no additional virus was added. *S. solfataricus* P2 cultures that were grown for 72 h before being infected with STIV, then in stationary phase, did not produce virus.

These results suggest that once a *S. solfataricus* P2 culture has been infected with STIV it cannot be infected again for additional virus production. A secondary viral infection is not detected in cultures grown for an extended time period. Only when virus producing cultures are diluted into fresh actively growing *S. solfataricus* P2 cultures in early log-phase of growth is there a secondary cycle of infection detected. Comparing that to the same cultures diluted into fresh media, where a secondary cycle of infection is not detected, suggests that once *S. solfataricus* P2 cells have been infected with STIV they are capable of defending themselves from a secondary infection of the virus with a possible immune response, not yet understood.

CHAPTER 3

ELISA TO QUANTIFY THE VIRUS

Introduction

A tool to quantify STIV straight from environmental samples from Yellowstone National Park was something that was needed, but was not available. Being able to quantify the virus in samples straight from the environment was important, since valuable time could be saved by not having to culture the environmental samples. First, this tool had to be very specific, not recognizing any Archaea present in the hot springs nor any archaeal viruses other then STIV. Second, it was important for this tool to be highly sensitive, since the abundance of the virus in the hot springs was thought to be very low. Third, this tool would preferably be easy to use and relatively fast. The range of the tool needed to be broad enough to quantify virus from environmental samples as well as from cultured samples, where the abundance of STIV is orders of magnitude higher than in the environment. One assay that fulfills all these requirements is a method called ELISA or Enzyme-Linked ImmunoSorbent Assay. ELISA is a powerful method for estimating the quantity of an antigen in a sample, where an antigen is a substance capable of inducing a specific immune response [5]. Viruses have many antigenic sites and therefore ELISA can be used to quantify viruses. The method is highly specific since antibodies, which are used in the assay, recognize and bind to only specific antigens. Antibodies are proteins of the immune system called immunoglobulins (Ig), which are composed of four

polypeptide chains, two heavy chains and two light chains, connected to each other by disulfide bonds [47] (Figure 3.1).

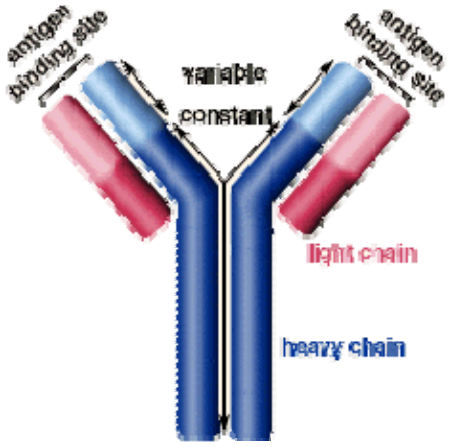


Figure 3.1. Basic structure of an antibody [48].

These four chains join to form a flexible “Y” shaped molecule, with a hinge region. The stalk of the “Y” is termed the crystallizable fragment (Fc) and contains the site at which the antibody molecule can bind to a cell. The top of the “Y” consists of two antigen-binding fragments (Fab), which bind with compatible antigenic determinant sites. The ends of the light and the heavy chain at the tip of the “Y” form the variable region of the antibody. This variable region (V_L and V_H; L for light chain, H for heavy chain), is the part of the antibody that gives it its specific binding to an antigen, and is different for all antibodies. The remaining part of the antibody, the constant region (C_L and C_H; L and H stands for light and heavy chain, respectively), is the same within each of the five classes of antibodies [47].

ELISA uses antibodies to detect antigens, such as virus particles, from samples. The samples are added to the wells of a microtiter plate previously coated with antibodies specific for the antigen to be detected. If the antigen is present in the sample, it will bind to the antigen-binding sites on the antibodies. Following a wash, secondary antibody, conjugated to an enzyme, is added. The second antibody, like the first, is specific for the antigen, thus it binds to any remaining exposed antigen sites. After washing unbound material away, the enzyme activity of the bound material in each well of the microtiter plate is determined by adding the substrate of the enzyme. The color formed in the wells is proportional to the amount of antigen present [5].

Materials and Methods

The major capsid protein (MCP) of the virus was cloned and purified from an *E. coli* expression system. This allowed production of large quantities of the antigen sites to establish a response in an immune system (E. Larson). A sample of the cloned coat protein was injected into a rabbit. After the immune system had approximately a week to respond to the antigen a pre-bleed was taken from the rabbit. That pre-bleed was tested to evaluate if it had antibodies to the desired coat protein and further, if it had antibodies to other viruses and microorganisms from the same environment as the virus. At the same time, the rabbit was given another injection of the same antigen. A week after the second injection, a final bleed was taken from the rabbit. The antibody was then purified from the serum with ImmunoPure® (G) IgG Protein G Purification kit (Pierce) following the suggested protocol.

Protocol for ELISA was developed by combining three previously published protocols (Appendix C) [49-51]. Four different types of microtiter plates were tested for antibody binding capacities and background noise, MaxiSorp™ (Nunc) and Immulon 1, 2 and 4 (Dynatech). Buffers tested to coat the plates were PBS (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.1 w/HCl) and carbonate coating buffer (15 mM Na_2CO_2, 35 mM mM $NaHCO_3$, 3 mM NaN_3). Blocking conditions tested were, no blocking, 3% BSA (bovine serum albumin) and 2% PVP (polyvinylpyrrolidone) + 0.2% ovalbumin.

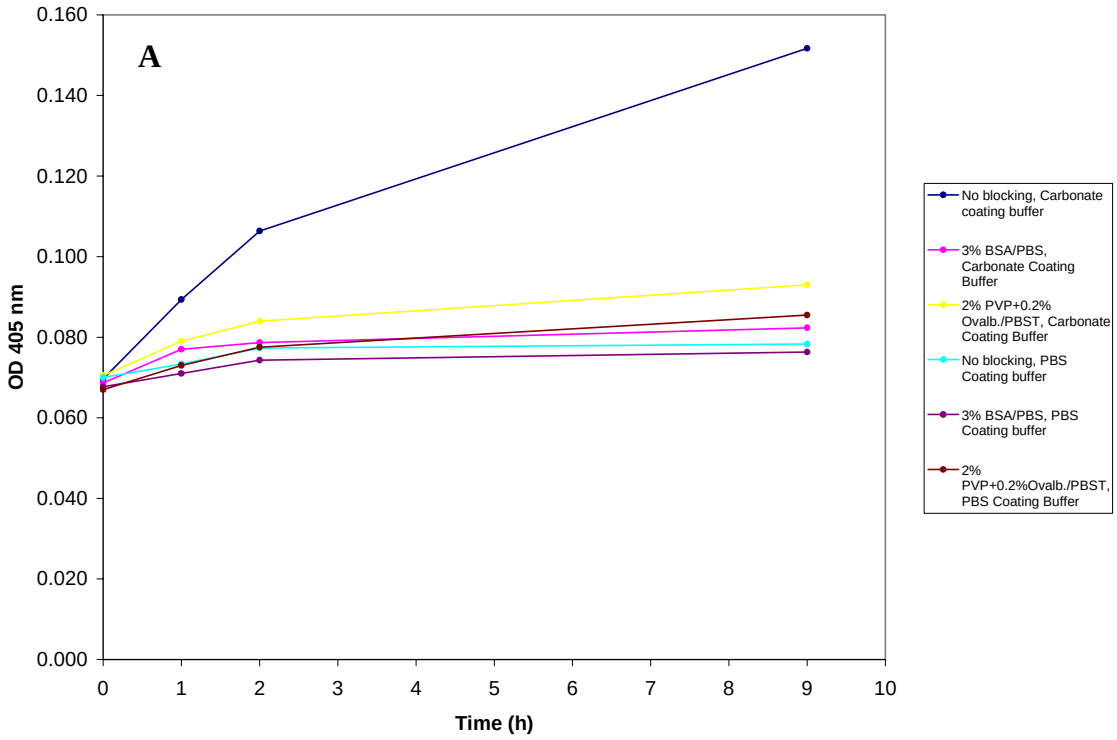
Alkaline phosphatase (AP) activated (Roche), was conjugated to the antibody following the manufacturer's suggested protocol. Substrate used for detection of antibody was p-nitrophenylphosphate in substrate buffer (15% (w/v) 85% diethanolamine, 3.8 mM NaN_3, pH 9.8 w/HCl) at room temperature. The development of the ELISA was followed by reading the absorbance at 405 nm. Positive controls on plates were obtained by quantifying total protein with Bradford assay (Bio-Rad), from concentrated stock of STIV.

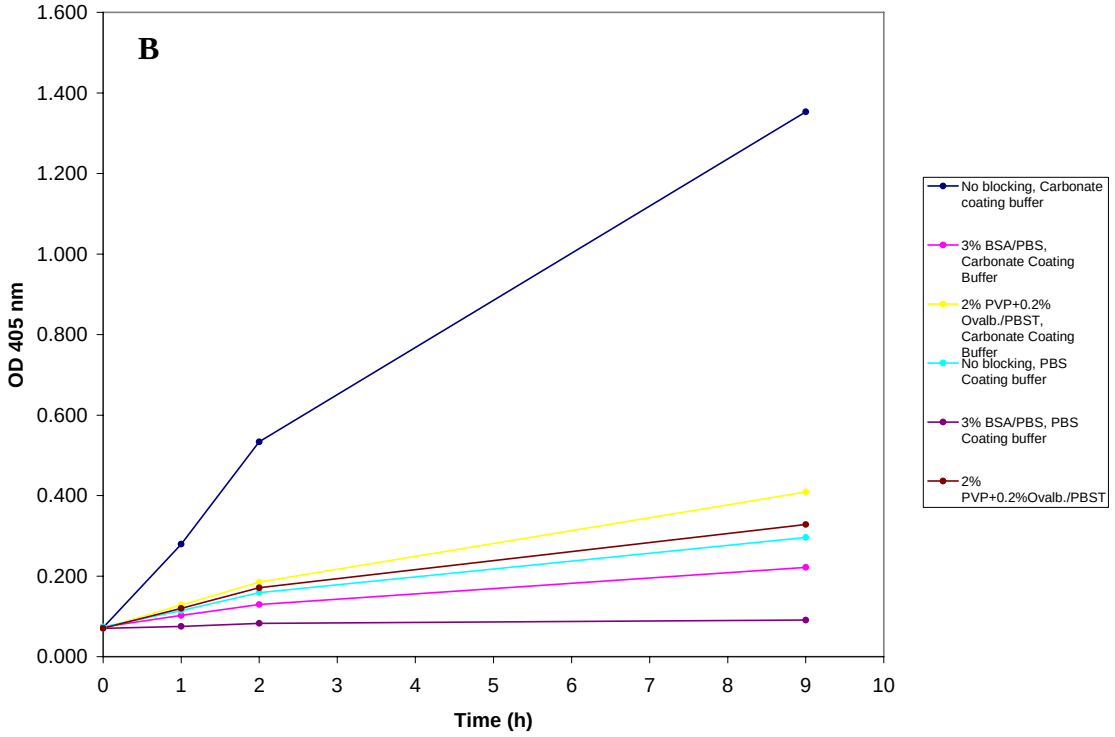
Results

Plates Immulon 1 and 2 were considered unacceptable for ELISA because of their low binding affinities (Figure 3.2, A and B). While binding of antibody to both MaxiSorp and Immulon 4 plates was higher compared to the other two plates tested and fairly equal, MaxiSorp was chosen to be used for ELISA. Even though unspecific binding of antibody was similar using both types of plates, specific binding was much higher in the MaxiSorp plates.

The coating buffer chosen to be used for the ELISA was the carbonate coating buffer. That selection was made since the binding of antibody was higher in the carbonate buffer than it was in the PBS buffer (Figure 3.2 C).

Blocking of sites not occupied by antibodies in wells on the microtiter plates was shown to be necessary since, when no blocking was used in the protocol, unspecific binding was unacceptably high. Of the two blocking conditions tested, 3% BSA and 2% PVP + 0.2% Ovalbumin, less unspecific binding is detected with the latter. The 2% PVP + 0.2% Ovalbumin was therefore chosen to be used in the ELISA (Figure 3.2 C).


A
OD 405 nm
Time (h)
0.160
0.140
0.120
0.100
0.080
0.060
0.040
0.020
0.000
0
1
2
3
4
5
6
7
8
9
10
No blocking, Carbonate coating buffer
3% BSA/PBS, Carbonate Coating Buffer
2% PVP+0.2% Ovalb./PBST, Carbonate Coating Buffer
No blocking, PBS Coating buffer
3% BSA/PBS, PBS Coating buffer
2% PVP+0.2%Ovalb./PBST, PBS Coating Buffer


B
OD 405 nm
Time (h)
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
1
2
3
4
5
6
7
8
9
10
No blocking, Carbonate coating buffer
3% BSA/PBS, Carbonate Coating Buffer
2% PVP+0.2% Ovalb./PBST, Carbonate Coating Buffer
No blocking, PBS Coating buffer
3% BSA/PBS, PBS Coating buffer
2% PVP+0.2%Ovalb./PBST

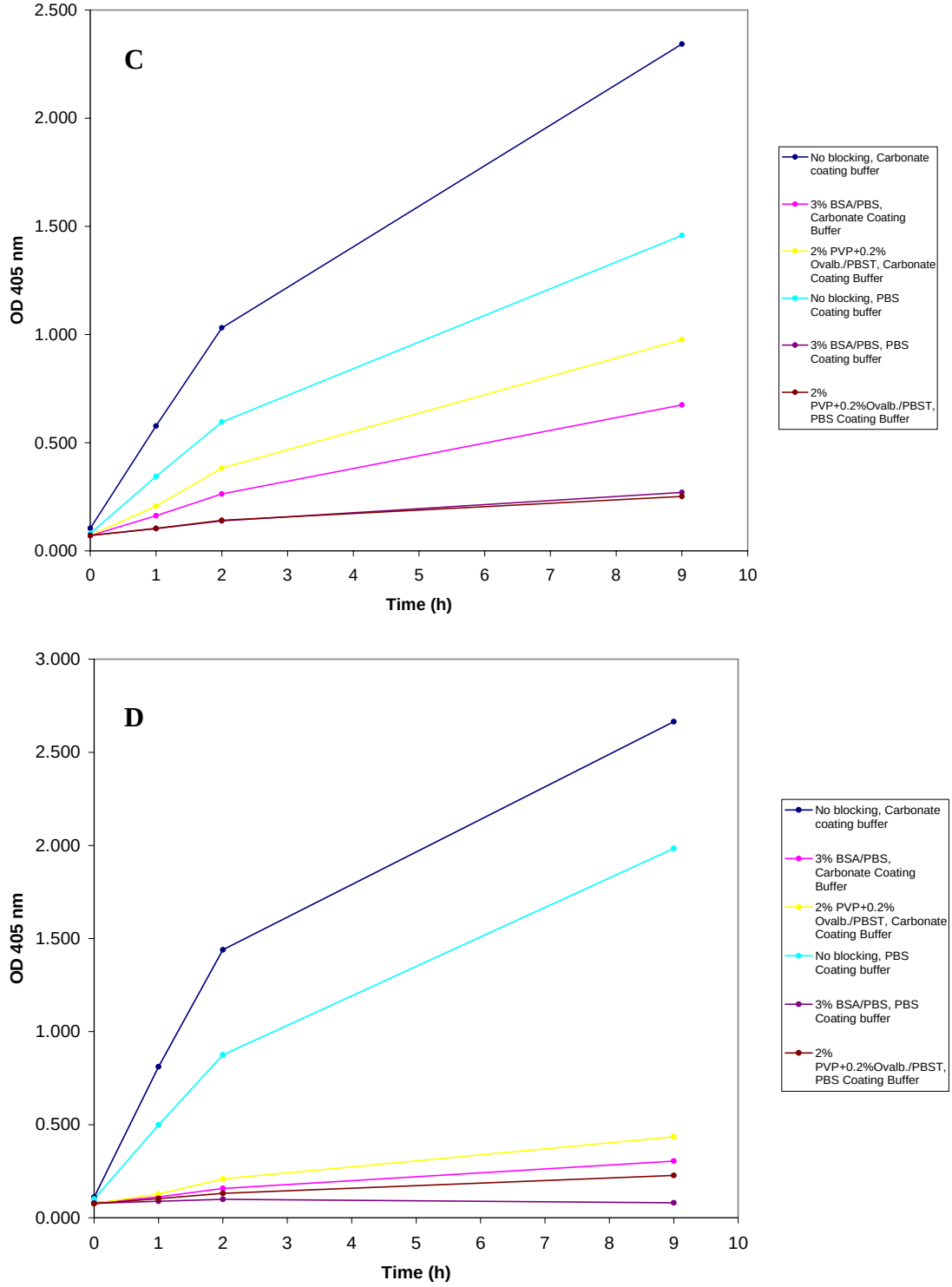


Figure 3.2. ELISA conditions tested.
Blocking conditions tested; no blocking, 3% BSA and 2%PVP + 0.2% Ovalbumin. Buffers tested; Carbonate and PBS Coating Buffer. Absorbance measured at t = 0, 1, 2 and 9 h. A) Immulon 1 plate. B) Immulon 2 plate. C) MaxiSorp plate. D) Immulon 4 plate.

ELISA for STIV detection

ELISA was used to quantify STIV in environmental samples from Yellowstone National Park (YNP). Being able to quantify the virus in samples straight from the environment is important, since valuable time will be saved by not having to culture the environmental samples. Culturing the samples from the environment is both time consuming and culturing bias has been observed [52].

Twenty four hot springs in Rabbit Creek area of Midway Geyser basin of YNP were sampled. Temperature of the hot springs varied from 66-92°C and the pH from 3.0-5.3. Samples were screened for STIV virus with ELISA, in triplicate (Figure 3.3).

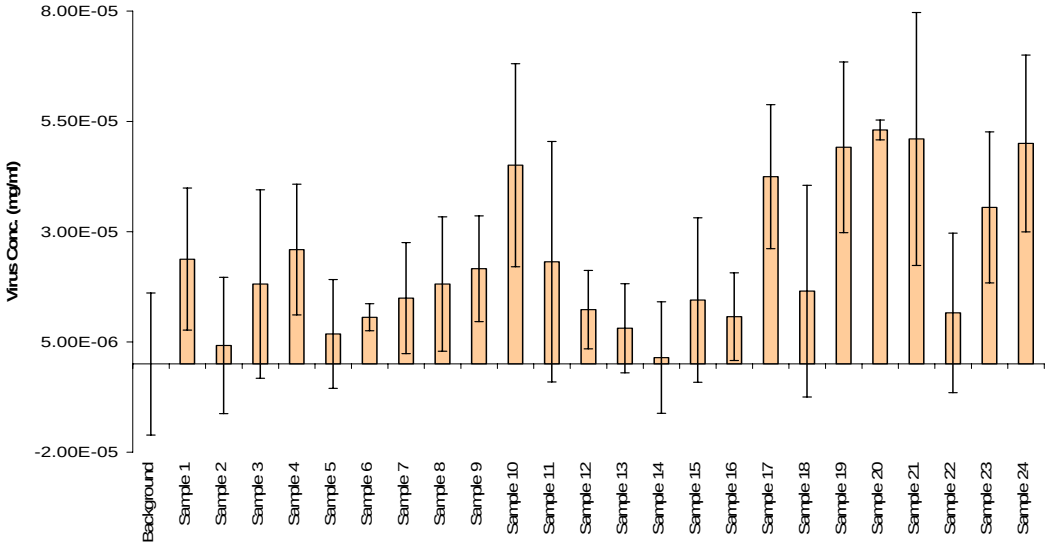


Figure 3.3. Virus concentration in environmental samples from YNP.
Values shown are means of triplicate tests with error bars showing standard deviation.

Fifteen samples were positive for STIV in all 3 tests and the standard deviation is above what the background is in the environment. Nine samples were not positive for the virus, combining all three tests for those samples.

Discussion

ELISA, Enzyme-linked immunosorbent assay, was developed to be used to quantify virus in environmental and cultured samples. Three different protocols were used to develop the new protocol, optimizing conditions for STIV. Four different microtiter plates were tested, and the type of plate best suitable for use was MaxiSorp, based on binding affinities. Coating buffers tested were carbonate coating buffer and PBS. Carbonate coating buffer was chosen to be used since binding affinities of antibody to wells of microtiter plates was higher with the buffer. Blocking the unoccupied sites in wells was proven to be necessary and blocking conditions 3% BSA and 2% PVP + 0.2% Ovalbumin were tested. 2% PVP and 0.2% Ovalbumin showed to be better to use for blocking in the ELISA since the unspecific binding was less using that condition than 3% BSA.

CHAPTER 4

SOUTHERN ANALYSIS OF SULFOLOBUS TURRETED ICOSAHEDRAL VIRUS INFECTED *SULFOLOBUS SOLFATARICUS*

Introduction

Southern blotting was named after Edward M. Southern who developed this procedure at Edinburgh University in the 1970s [53]. The method is designed to locate a particular sequence of DNA within a complex mixture. In the case of a virus infecting its host, the form of the viral genome during the viral replication can be analyzed. Southern blotting can be used to determine if the virus genome remains free in the cytoplasm or is integrated into the host genome.

By understanding how the virus replicates in its host, virus production can be optimized according to the replication method. Production of viruses, where the viral genome integrates into the host genome, can often be induced with external methods, such as UV-induction, Mitomycin C, or sudden change in environment [54]. These methods do not generally work for viruses where the genome does not integrate. So far, only two viruses of Archaea are known to integrate into its host's genome, SSV1 and ATV [55-57]. We chose to use Southern blotting to determine if the viral genome of STIV integrates into the chromosomal of its hosts DNA.

In the STIV genome, ORF A510 was observed to contain a region that could possibly belong to the Int family of site-specific recombinases. Recombinases of the Int

family have various functions; for example integrative and excisive recombination of viral and plasmid DNA into and out of the host chromosome, conjugative transposition, resolution of catenated DNA circles, regulation of plasmid copy number, DNA excision to control gene expression and DNA inversion controlling expression of cell surface proteins or DNA replication [58]. Therefore, finding a possible recombinase in the genome does not dictate that the viral genome integrates into the host genome.

On a Southern blot, a circular genome that does not integrate into its host genome will show the same banding pattern as a restriction digest of the pure genome, while on a Southern where the virus integrates, one band will be missing and two additional bands will be noticed (Figure 4.1)

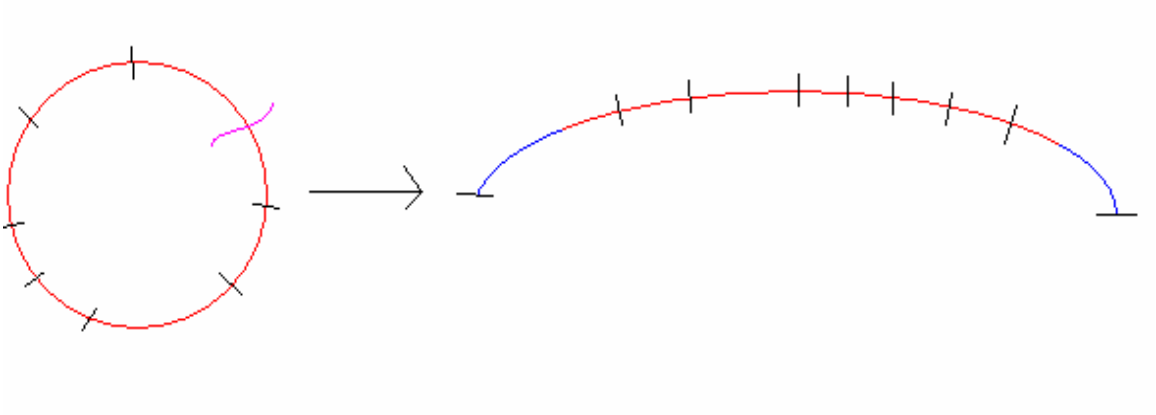


Figure 4.1. Demonstration of banding pattern generated by a virus integrating into a host genome.
Pink = Supposed site where virus plasmid opens up to integrate, Red = Viral plasmid, Blue = Host chromosome, Black = Restriction enzyme digest sites.

Materials and Methods

Two cultures were started by inoculating aliquots of glycerol-stocked ATCC strain of *S. solfataricus* P2 and glycerol-stocked strain of the original *Sulfolobus* isolate from which STIV was isolated, YNP179 from Yellowstone National Park (P2/P2 and P2/179, respectively), into Medium 182 at pH 3.5, in a long-neck Erlenmeyer flasks. Cultures were incubated in an oil-bath shaker at a temperature close to the optimum growth temperature of *S. solfataricus* P2, ~80°C and shaken ~100 rpm. Following 2 days of growth, the cultures were diluted five-fold in Media 182 at pH 2.5 and allowed to grow an additional day. Further five- to ten-fold dilution of the cultures was performed on the fourth day in Media 182, pH 2.5. At early log-phase the actively growing cultures were infected with a concentrated stock of STIV particles. Cultures were monitored for STIV production with ELISA (Chapter 3) and dot-blots as previously described in Chapter 2. When virus production was believed to be at maximum value, estimated from previous experiments, total DNA was extracted from cultures with UltraClean™ Microbial DNA Kit (Mo Bio).

For the time course experiment, a culture was started in the same way as previously described, except after inoculating the culture with virus, multiple samples were taken for DNA extraction. Samples were taken approximately every 12 hours, starting at t = 0, although more frequently around the time when viral transcript in the culture was expected to be at maximum, determined from previous experiments. Production of virus in the culture was monitored with ELISA and dot-blots. DNA was extracted from equal volumes of culture at each time point of the time course, with

previously mentioned UltraClean™ Microbial DNA Kit (Mo Bio). The DNA was quantified by measuring the absorbance with a UV/VIS spectrometer.

DNA extracted from P2/P2 and P2/179 was purified on CsCl gradients following standard protocol [50]. Only one chromosomal band formed (Figure 4.2), which was collected and EtBr was extracted from the sample with butanol [50]. DNA was dialyzed into 1xTE (10mM Tris, 1mM EDTA, pH 7.5 w/HCl).

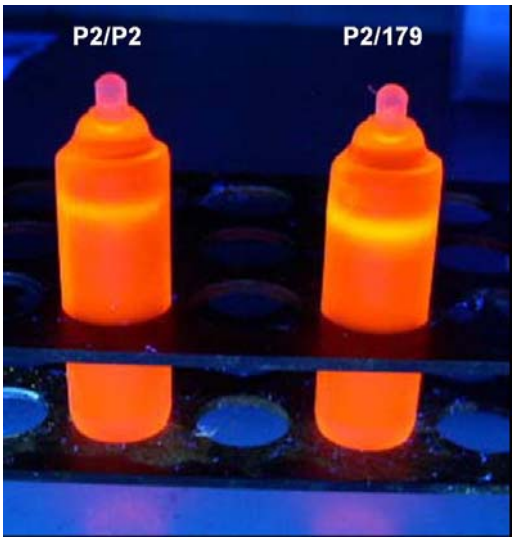


Figure 4.2. P2/P2 and P2/179 purified on CsCl gradient.

Equal quantities of DNA, estimated with UV/VIS spectrometer, were digested with five restriction enzymes; AccI, BanI, EcoRI, HindIII and PstI, following the manufacturers' suggested protocol (Promega). Equal volumes of DNA extracted from the time course was digested with EcoRI, MspA1I and PsiI restriction enzymes according to suggested protocols (EcoRI; Promega, MspA1I and PsiI; New England Biolabs).

Restriction fragments were resolved by electrophoresis on 1% (w/v) agarose gels in 1xTBE buffer (90 mM Tris, 90 mM Boric Acid, 10 mM EDTA pH 8.0). After the DNA was separated by size, it was transferred to positively charged nylon membranes (Immunobilon – NY+, Millipore) with upward capillary transfer overnight in 0.4M NaOH (approximately 16 hours). After transfer, the nucleic acid was crosslinked to the membranes with UV crosslinker with 120,000 microjoules/cm^2 energy (Stratagene). The membranes were then incubated in pre-hybridization solution (5xSSC (20xSSC; 3M NaCl, 0.3M sodium citrate dehydrate), 5xDenhardt's solution (100xDenhardt's solution; 2% ficoll 400, 2% polyvinylpyrrolidone (MW 40,000), 2% bovine serum albumin), 50% deionized formamide, 1% SDS and 100 μg/ml denatured Herring sperm DNA) for 1 hour, with rotation, at 43°C. The Herring sperm DNA was included to block unoccupied sites on the membrane.

The DNA template used to make DNA probes for STIV was the full length clone of the STIV genome (J. Fulton, unpublished). The DNA probes were made by randomly priming 1 μg of the full length STIV clone with Ready-To-Go™ DNA Labeling Beads (-dCTP) Kit (Amersham Biosciences) according to the manual, using (α-^{32}P)-dCTP's from MP Biomedicals with specific activity of 800 Ci/mmol. The probes were incubated for 1 hour at 37°C, and then the unincorporated nucleotides were separated from the product using sepharose columns (Sepharose CL-6B, Sigma). Finally, the probes were denatured by incubation at 95°C for 2 minutes, followed by a 2 minute incubation on ice.

Denatured probes were added to the pre-hybridization buffer and the membrane was incubated overnight, with rotation, at 43°C. Membranes were then washed twice in

2xSSC + 0.1% SDS (for 1xSSC = 150 mM NaCl, and 15 mM $Na_3C_6H_5O_7$, pH 7.0 with 1M HCl) at room temperature for five minutes and twice in 0.1xSSC + 0.1% SDS for 15 minutes at 45°C. After washing, the membranes were exposed to a high-performance chemiluminescence film (Hyperfilm ECL; Amersham) in film cassettes with intensifying screens to increase the signal from the membranes. The film cassettes were stored at -80°C to increase signal. About 24 hours later, the films were developed and results were analyzed. If a membrane needed more exposure for the signal to be seen, new films were put on the membrane and more time was allowed to pass before film was developed again.

Portions of the STIV genome were amplified with polymerase chain reaction (PCR). Three different sets of primers were used to amplify parts of the genome. The first set was designed to amplify an 800 bp fragment, starting at basepair 15084 and ending at 15917 of ORF B345 (primers BF and BR), the second set started at 16641 and ended at basepair 17447, amplifying a portion of C557 (primers CF and CR), and the third amplified a bigger piece, starting at basepair 14969 of the STIV genome and ending at 17552, covering both ORFs B345 and C557 (primers BbF and CbR). When amplifying with primer sets BF-BR and CF-CR, the template was denatured at 94°C for 30 seconds, primers annealed to the template at 55°C for 1 minute, and extension with GoTaq DNA polymerase (Promega) at 72°C for a minute and a half, for 35 cycles. In the reaction amplifying the larger region, using primer set BbF-CbR, the template was denatured at 98°C for 10 seconds, annealing was carried out at 55°C for 30 seconds, and the extension at 72°C for 1 minute with iProof™ DNA Polymerase (Bio-Rad Laboratories), for 30

cycles. Templates used for the PCR reaction were P2/P2 and P2/179 mentioned previously and viral DNA extracted from STIV infected *S. solfataricus* P2 cultures with Plasmid Midi Kit (QIAGEN).

DNA extracted with Perfectprep® Gel Cleanup Kit (Eppendorf) from purified STIV was digested with enzymes Sau3AI and DpnI (Promega) following manufacturers' suggested protocol. Sau3AI cuts where sequence GATC is found whether the genome is methylated or not, while DpnI cuts the same sequence only if the sequence is methylated. Restriction fragments were resolved by electrophoresis on 1% (w/v) agarose gels in 1xTBE buffer.

Primers were designed that spanned ORFs B345 and C557, a 2612 bp piece, starting at basepair 14967 and ending at 17579 in the STIV genome. PCR was performed, denaturing at 98°C of 10 seconds, annealing at 55°C for 30 seconds, and extending at 72°C for 1 minute, cycling 35 times using iProof™ DNA Polymerase (Bio-Rad Laboratories). Templates for the PCR were P2/P2, P2/179 and DNA extracted from a STIV infected *S. solfataricus* P2 culture with UltraClean™ Microbial DNA Kit (Mo Bio).

PCR products were the digested with restriction enzymes, AccI, AflIII, EcoRI and NdeI (AccI, EcoRI and NdeI; Promega, AflIII; New England Biolabs), following manufacturers' protocols. Digested pieces were separated by sizes on 1% agarose gel in 1xTBE buffer.

Results

Cultures of *S. solfataricus* P2 were infected with STIV and samples for DNA extraction were taken, first every 12 hours, and then more frequently around the time when virus production was at maximum around 36 hours post-infection. The DNA was digested with three different enzymes, EcoR1, MspA1I and PsiI. The virus genome is first detected 34 hours post-infection and is at a maximum 40 hours after STIV is added to the *S. solfataricus* P2 cultures (Figure 4.4). The digestion pattern of the STIV genome theoretically has eight bands when EcoR1 is used (Figure 4.3, Table 4.1), while on a Southern blot of the time course, only seven bands can be resolved (Figure 4.4). No additional bands were noticed, as would be expected if the virus integrates into its hosts' genome. The intensity of the 2127 bp band is higher than expected, suggesting that about 300 bp addition of sequence has occurred in that part of the STIV genome, and therefore the 1820 bp band that is missing is overlapping with the 2127 bp band. Additionally, the intensity of the 1688 kb band is lower than expected of a band of that size.

Table 4.1. EcoRI cutting sites.
Position in the genome and fragment sizes generated cutting with EcoRI. Generated with computer program pDRAW [59].

Digestion site no.	Cuts at positions	Fragment sizes
1	6354	1688
2	8042	3139
3	11181	2127
4	13308	583
5	13891	1402
6	15293	1820
7	17113	333
8	17446	6571

The Southern blot of DNA extracted from the time course and digested with MspA1I has eight bands (Figure 4.5), same number of bands as expected from a non-integrated virus. The 1499 bp band (Table 4.2) is slightly bigger than expected, or closer to 1800 bp. This band is from the same area of the STIV genome that the proposed addition of sequence noticed on Southerns, digested with EcoR1. Again, no additional bands were observed on the Southern blot, as would be expected if the virus was integrated into its host genome.

Table 4.2. MspA1I cutting sites
Position in the genome and fragment sizes generated cutting with MspA1I. Generated with pDRAW.

Digestion site no.	Cuts at positions	Fragment sizes
1	125	439
2	564	2535
3	3099	2004
4	5103	587
5	5690	1299
6	6989	8097
7	15086	1499
8	16585	1203

Seven bands are detected when PsiI digested DNA is Southern blotted (Figure 3.6). All bands are consistent with a non-integrated form of the virus (Figure 3.3, Table 3.3), and no bands are bigger or missing as seen when EcoRI and MspA1I are used to digest the DNA. Although the intensity of the 4453 kb band is lower then expected, it is the right size.

Table 4.3. PsiI cutting sites.
Position in the genome and fragment sizes generated cutting with PsiI. Generated with pDRAW.

Digestion site no.	Cuts at positions	Fragment sizes
1	2056	3351
2	5407	1080
3	6487	4453
4	10940	658
5	11598	1352
6	12950	1470
7	14420	5299

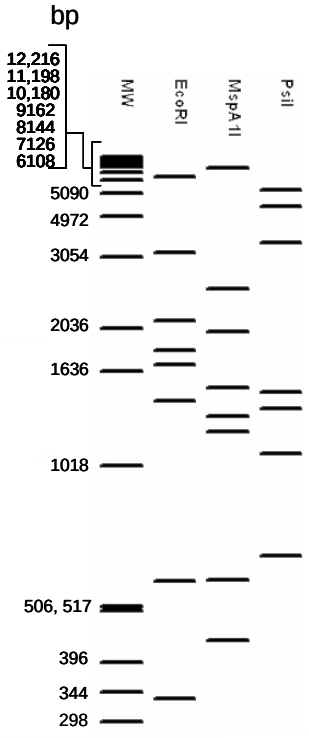


Figure 4.3. Ideal restriction digestion of STIV with EcoRI, MspA1I and PsiI. Generated with pDRAW.

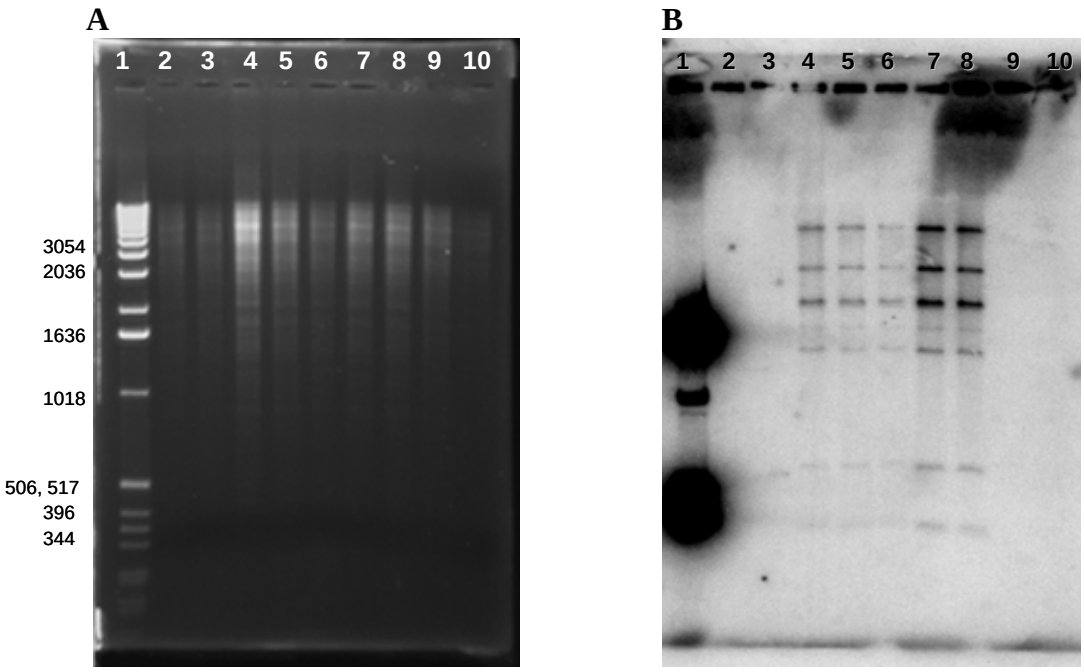


Figure 4.4. Southern of a time course digested with EcoRI.
A) Gel of DNA extracted from a time course and digested with EcoRI. Lane 1; Ladder, Lane 2; t = 12h. Lane 3; t = 24h. Lane 4; t = 34h. Lane 5; t = 36h. Lane 6; t = 38h. Lane 7; t = 40h. Lane 8; t = 47h. Lane 9; *S. solfataricus* P2. Lane 10; 100 pg STIV DNA. B) Southern of gel, probed with a full length DNA probe.

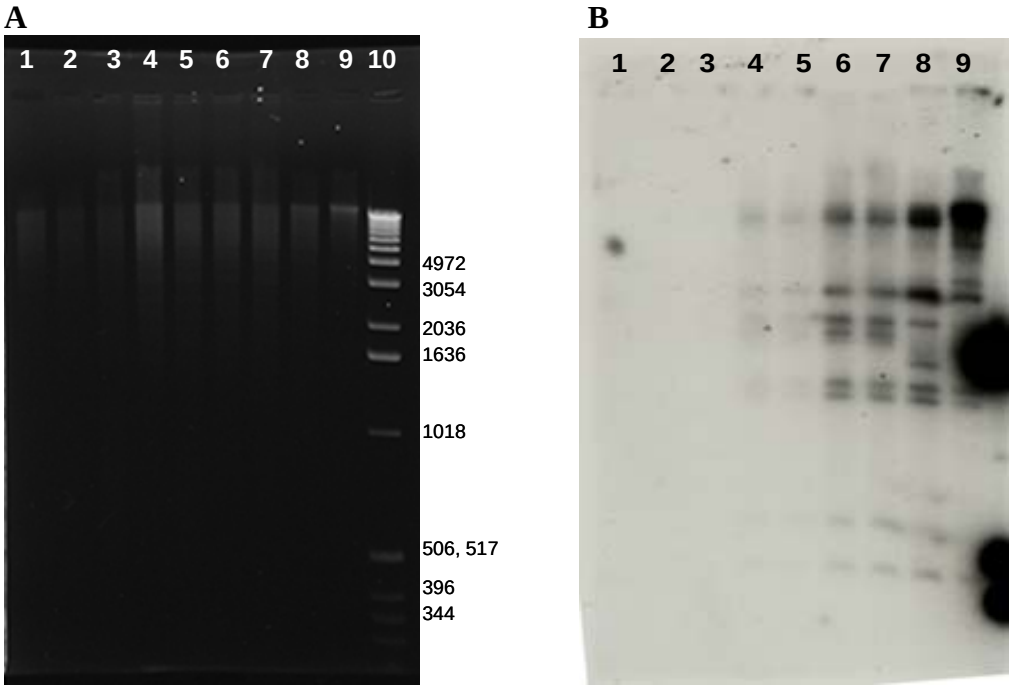


Figure 4.5. Southern of a time course digested with MspA1I.
A) Gel of DNA extracted from a time course and digested with MspA1I. Lane 1; *S. solfataricus* P2, Lane 2; t = 12h. Lane 3; t = 24h. Lane 4; t = 34h. Lane 5; t = 36h. Lane 6; t = 40h. Lane 7; t = 47h. Lane 8; P2/P2. Lane 9; P2/179. Lane 10; Ladder B) Southern of gel, probed with a full length DNA probe.

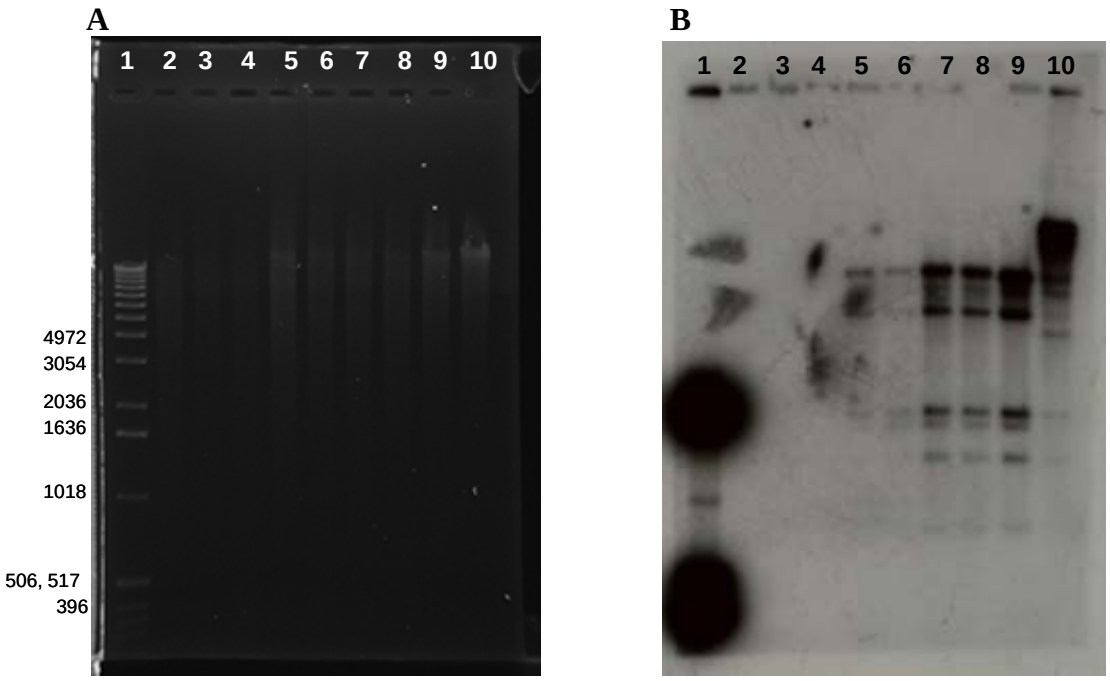


Figure 4.6. Southern of a time course digested with PsiI.
A) Gel of DNA extracted from a time course and digested with PsiI. Lane 1; Ladder. Lane 2; *S. solfataricus* P2, Lane 3; t = 12h. Lane 4; t = 24h. Lane 5; t = 34h. Lane 6; t = 36h. Lane 7; t = 40h. Lane 8; t = 47h. Lane 9; P2/P2. Lane 10; P2/179. B) Southern of gel, probed with a full length DNA probe.

Four additional restriction enzymes were used to digest the DNA with AccI, BanI, HindIII and PsiI before running a Southern blot on the digestions, along with a repeat with EcoRI and a combination of two enzymes, AccI and EcoR1. These additional digestions were intended to determine if the digestion of STIV with these enzymes would be as expected for the non-integrated form of the virus or if STIV integrates into its host genome.

Southern blot of DNA digested with AccI, BanI, PsI and HindII show the digestion patterns (Figures 4.9 and 4.10) that would be expected from a non-integrated form of STIV (Figures 4.7 and 4.8, Tables 4.4 and 4.5). The smallest fragments cannot be seen easily on the Southern blot since their small size means less probe is targeted to

those fragments. The DNA from P2/179 cultures did not completely digest with HindIII. As before with EcoRI digestions, one band is missing, although no additional bands are apparent. The intensity of the 2422 bp band in the PsiI digestion of P2/P2 is lower than expected. The 2422 bp band starts at nucleotide 14992 and ends at 17414, the same part of the genome where the missing band in the EcoRI digestion is.

Table 4.4. Cutting sites of enzymes AccI, BanI and AccI+EcoRI.
Cutting sites of restriction digestion enzymes AccI, BanI and combined digestion of AccI and EcoRI, position in the genome and fragment sizes generated. Generated with pDRAW.

	AccI		BanI		AccI + EcoR1	
Digestion site no.	Cuts at positions	Fragment sizes	Cuts at positions	Fragment sizes	Cuts at positions	Fragment sizes
1	2271	2807	135	217	2271	2807
2	5078	6398	353	606	5078	1276
3	11478	102	959	4672	6354	1688
4	11578	2819	5631	9730	8042	3139
5	14397	1461	15361	2438	11181	295
6	15858	4076			11476	102
7					11578	1730
8					13308	583
9					13891	506
10					14397	896
11					15293	565
12					15858	1255
13					17113	333
14					17446	2488

Table 4.5. Cutting sites of restriction digestion enzymes HindIII and PstI.
Position in the genome and fragment sizes generated. Generated with pDRAW.

	HindIII		PstI	
Digestion site no.	Cuts at positions	Fragment sizes	Cuts at positions	Fragment sizes
1	11026	828	925	1781
2	11854	240	2706	8125
3	12094	164	10841	2390
4	12258	830	13231	1761
5	13088	1180	14992	2422
6	14286	2122	17414	1174
7	16390	12299		

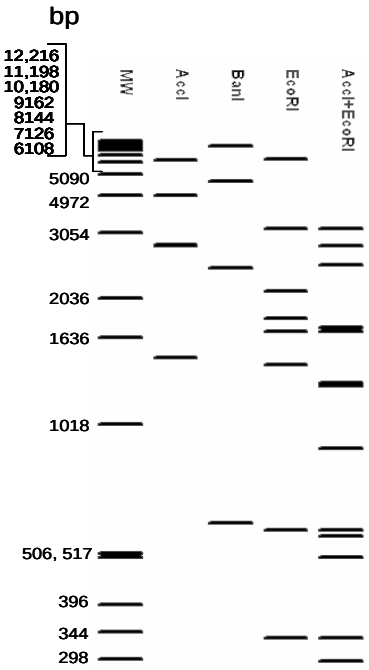


Figure 4.7. Digestion of STIV with AccI, BanI, EcoRI and AccI+EcoRI
Generated with pDRAW.

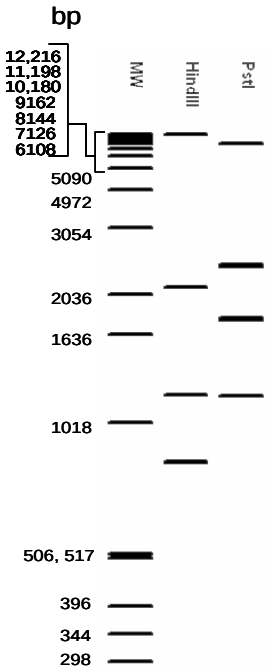


Figure 4.8. Restriction Digestion of STIV with Hind III and PstI.
Generated with pDRAW.

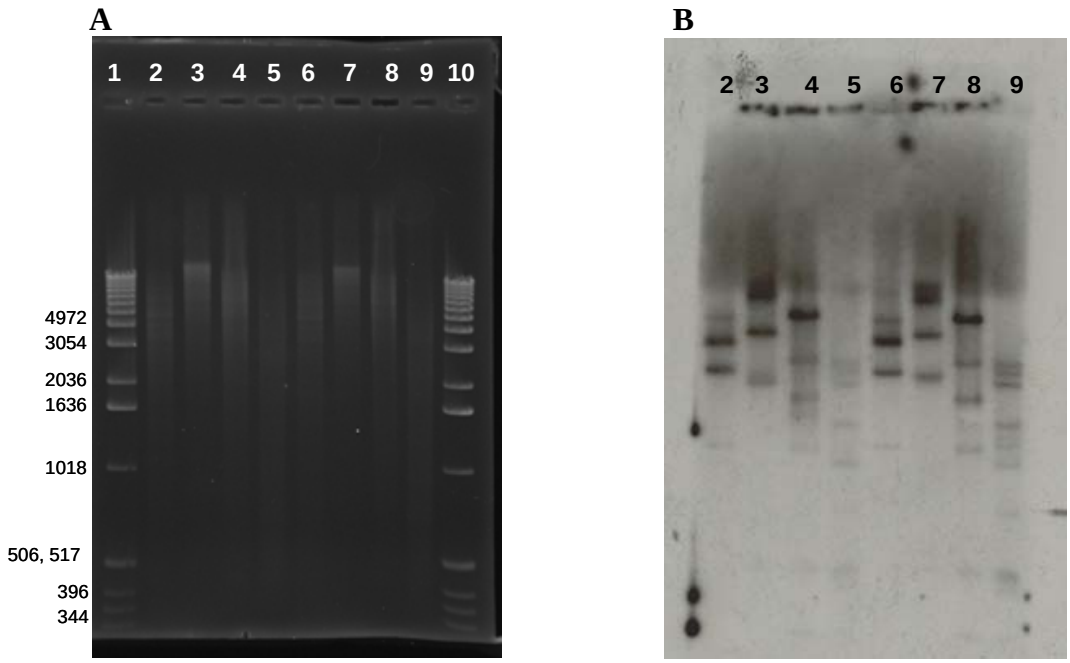


Figure 4.9. Southern of a P2/P2 and P2/179 digested with various enzymes 1.
A) Gel of DNA extracted from P2/P2 and P2/179, digested with different enzymes. Lane 1; Ladder. Lane 2; P2/P2, AccI, Lane 3; P2/P2, BanI. Lane 4; P2/P2, EcoRI. Lane 5; P2/P2, AccI + EcoRI. Lane 6; P2/179, AccI. Lane 7; P2/179, BanI. Lane 8; P2/179, EcoRI. Lane 9; P2/179, AccI + EcoRI. Lane 10; Ladder. B) Southern of gel, probed with a full length DNA probe.

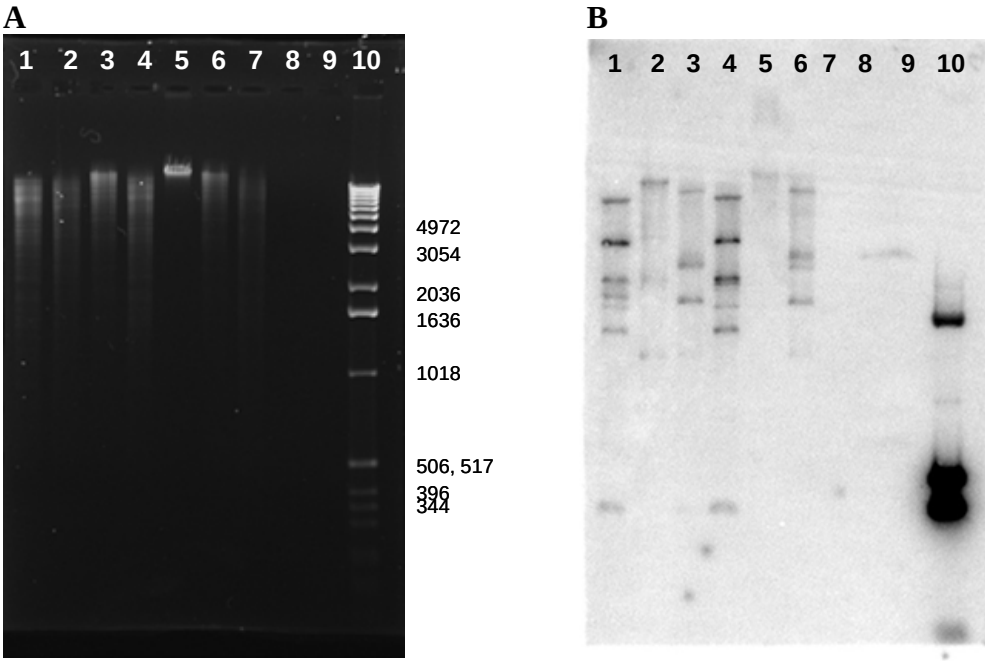


Figure 4.10. Southern of a P2/P2 and P2/179 digested with various enzymes 2.
A) Gel of DNA extracted from P2/P2 and P2/179, digested with different enzymes. Lane 1; P2/P2, EcoRI, Lane 2; P2/P2, HindIII. Lane 3; P2/P2, PstI. Lane 4; P2/179, EcoRI. Lane 5; P2/179, HindIII. Lane 6; P2/179, PstI. Lane 7; P2, EcoRI. Lane 8; 10 ng STIV DNA. Lane 9; 100 ng STIV DNA. Lane 10; Ladder.
B) Southern of gel, probed with a full length DNA probe.

To determine why the EcoRI digest looks different than expected for a virus that does not integrate into its hosts' genome, DNA from different sources was digested with the enzyme and a Southern blot was carried out (Figure 4.11). Three different sources of YNP179 cultures that produced STIV, P2/P2 and P2/179, a *S. solfataricus* P2 culture that had been infected with STIV in early log-phase, two different sources of STIV DNA extracted from purified virus, and the full length clone of STIV were used. No bands were seen in two of the YNP179 sources or in the DNA purified from purified STIV particles. The most probable reason is that the concentration of STIV DNA is very low in those the samples. The banding patterns in lanes 4 and 6 (YNP179 sample 3 and P2/179 respectively), and of the full length clone of the genome (lane 10), look like what would

be expected if STIV does not integrate into the host, while the 1820 bp band is missing in lanes 5 and 7 (P2/P2 and STIV infected *S. solfataricus* P2 culture) as seen on previous Southern blots.

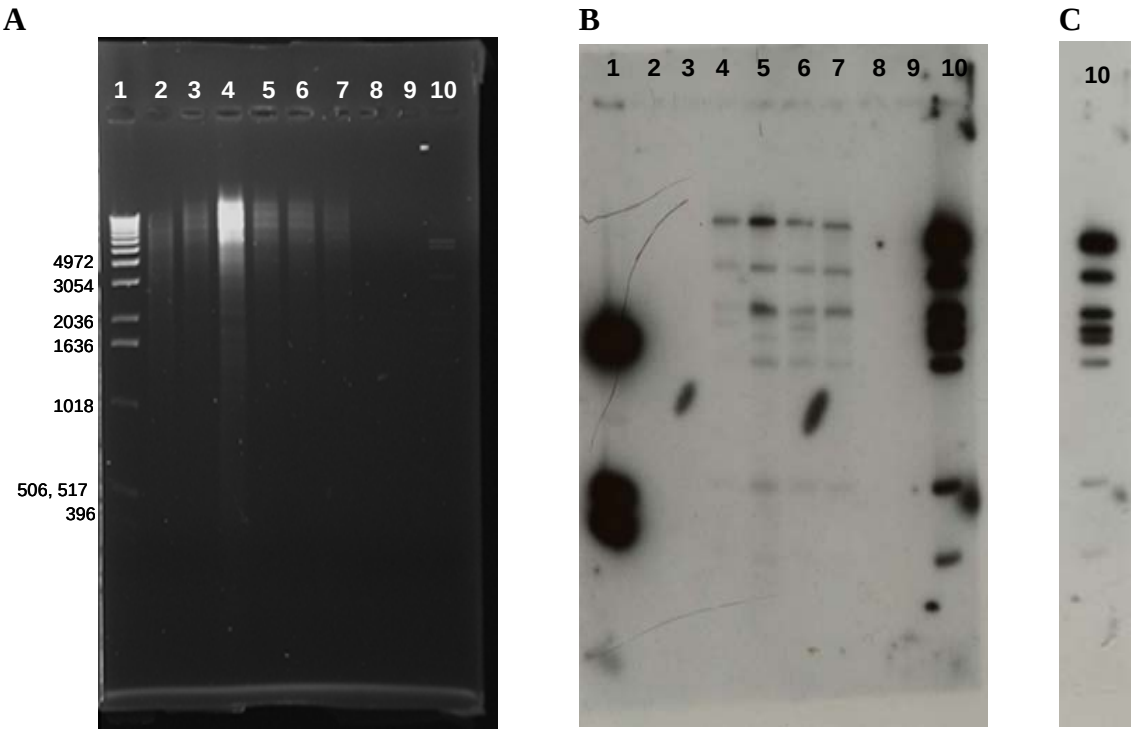


Figure 4.11. Southern of samples from various sources digested with EcoRI.
A) Gel of DNA extracted from different sources, digested with EcoRI. Lane 1; Ladder, Lane 2; YNP179, sample 1. Lane 3; YNP179, sample 2. Lane 4; YNP179, sample 3. Lane 5; P2/179. Lane 6; P2/P2. Lane 7; P2-STIV infected, 60h. Lane 8; DNA extracted from purified virus, sample 1. Lane 9; DNA extracted from purified virus, sample 2. Lane 10; Full length clone. B) Southern of gel, probed with a full length DNA probe. C) Lane 10, Full length clone after less exposure to film.

To understand why the 1820 bp band in EcoRI digestions is missing on Southern blots, a part of the STIV genome that includes the area where the fragment comes from was amplified with PCR from DNA extracted from purified virus samples and P2/P2 and P2/179 (Figures 4.12a and 4.13a). The area amplified starts at bp 16641 and ends at 17447 (primers CF-CR), including the EcoR1 restriction cutting site at bp 17113, which is suspected to not being cut efficiently with the enzyme. The amplification shows that

this part of the genome of STIV is the correct size, and when digested with EcoRI post amplification, the restriction enzyme cutting site is there and cuts (Figure 4.12b and 4.13b). This suggests that the EcoRI site is not accessible or in some way protected in the genome.

DNA extracted from purified virus was digested with two different enzymes, DpnI and Sau3AI (Figure 4.14). Sau3AI digests where the sequence GATC is found, whether or not the DNA is methylated, while DpnI digests only if nucleotide A in the GATC sequence is methylated. DNA was not digested with DpnI, while it was digested with Sau3AI, hence it can be determined that the DNA of STIV is not methylated.

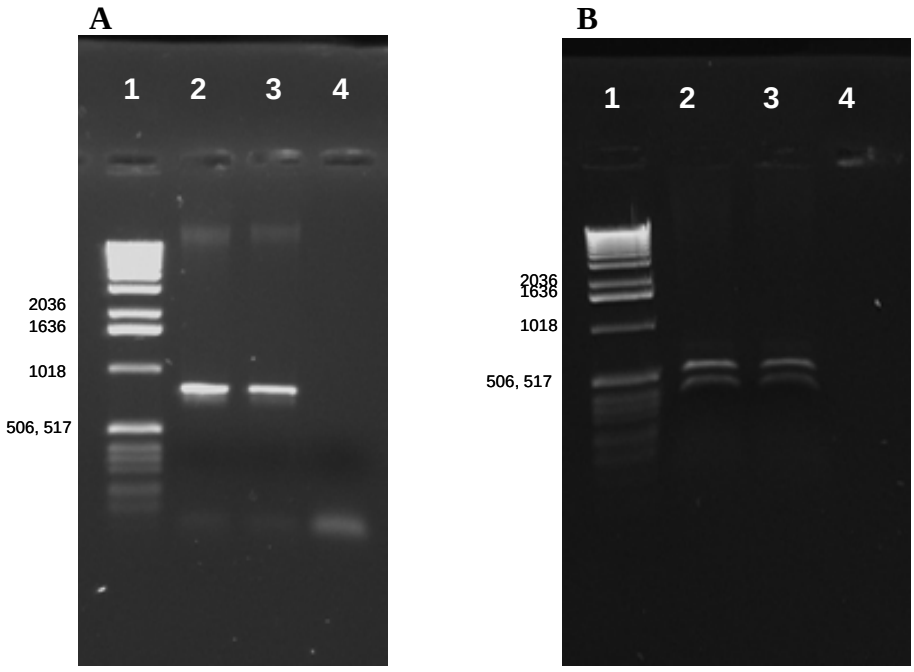


Figure 4.12. bp 16641-17447 amplified with PCR and digested with EcoRI 1.
A) Gel of PCR product. Lane 1; Ladder, Lane 2; P2/P2. Lane 3; P2/179. Lane 4; Negative control. B) Gel of PCR product digested with restriction enzyme EcoR1.

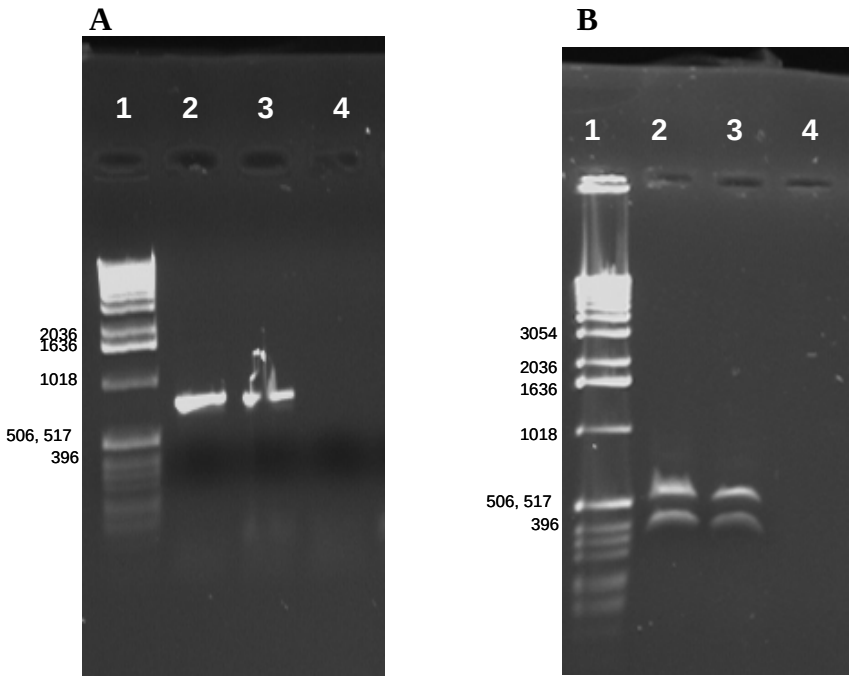


Figure 4.13. bp 16641-17447 amplified with PCR and digested with EcoRI 2.
A) Gel of PCR product. Lane 1; Ladder, Lane 2; Purified virus sample 1, Lane 3; Purified virus sample 2. Lane 4; Negative control. B) PCR product digested with restriction enzyme EcoR1.

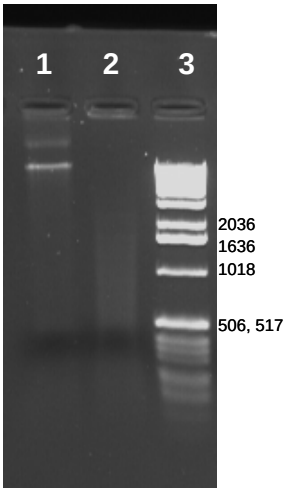


Figure 4.14. Methylation test.
Gel of DNA extracted from purified virus, digested with Sau3AI and DpnI. Lane 1; DNA digested with DpnI, Lane 2; DNA digested with Sau3AI, Lane 3; Ladder.

PCR was performed using primers BbF and CbR spanning ORFs B345 and C557 (Figure 4.16). PCR products were the digested with restriction enzymes AccI, AflIII, EcoRI and NdeI (Figure 4.17). AflIII did not digest the genome, but the enzyme was eight years beyond expiration date. Enzymes AccI, EcoRI and Nde digested the PCR pieces like would be prospected by the sequence of the area (Figure 4.15, Table 4.6).

Table 4.6. Cutting sites in of enzymes AccI, AflIII, EcoRI and NdeI of PCR spanning B345 and C557.
Cutting sites in of restriction digestion enzymes AccI, AflIII, EcoRI and NdeI of PCR spanning B345 and C557, position in the genome and fragment sizes generated. Generated with pDRAW.

AccI		AflIII		EcoR1		NdeI	
Cuts at positions	Fragment sizes	Cuts at positions	Fragment sizes	Cuts at positions	Fragment sizes	Cuts at positions	Fragment sizes
891	891	577	577	326	326	442	442
	1721		2053	2146	1820		2170
				2479	333		
					133		

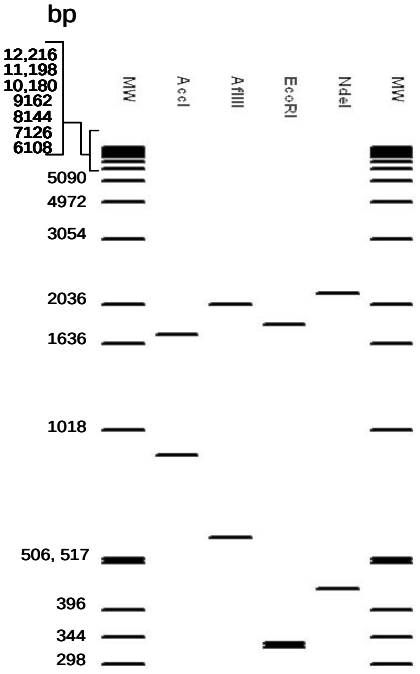


Figure 4.15. Restriction digestion of STIV with AccI, AflIII, EcoRI and NdeI.
Generated with pDRAW.

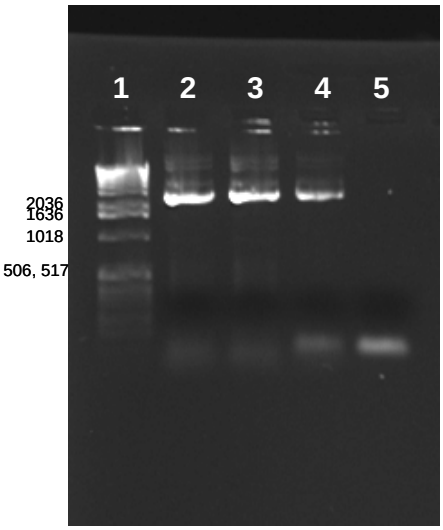


Figure 4.16. ORFs B345 and C557 of STIV amplified.
PCR product using primers BbF and CbR. Lane 1; ladder. Lane 2; P2/P2. Lane 3; P2/179. Lane 4; STIV producing *S. solfataricus* P2 culture.

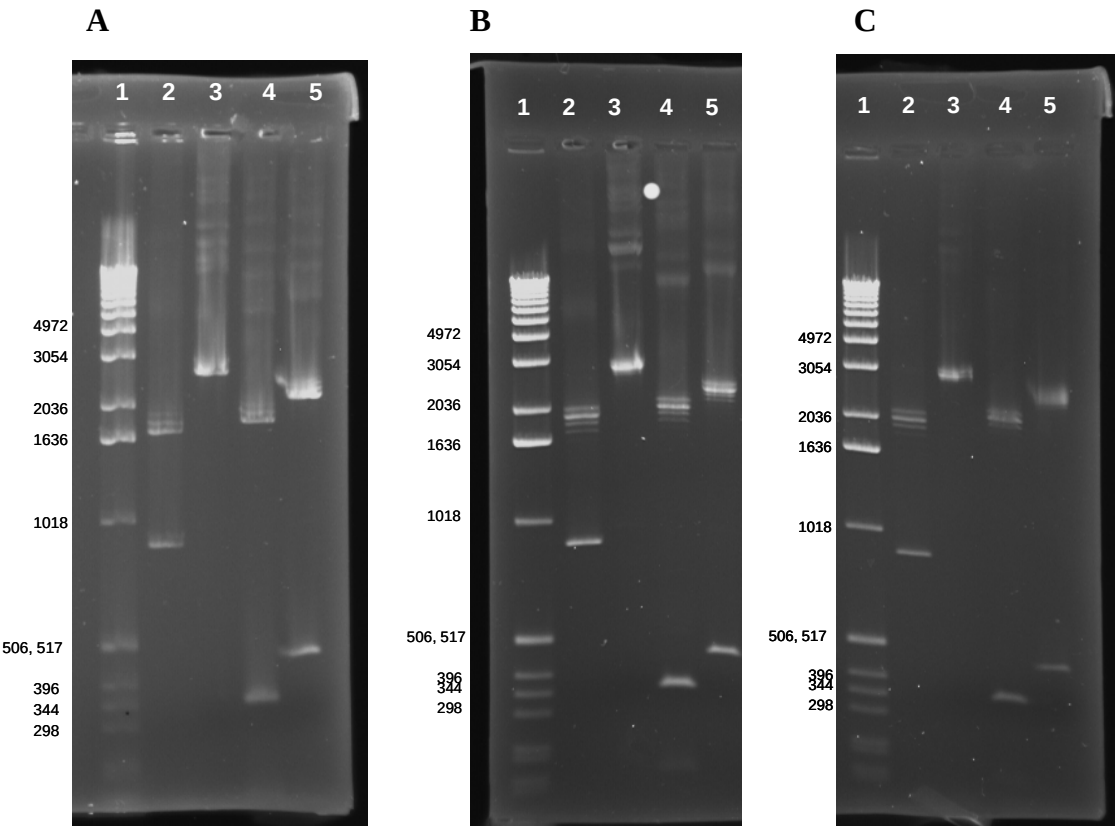


Figure 4.17. PCR amplified ORFs B345 and C557 digested with AccI, AflIII, EcoRI and NdeI.
Restriction enzyme digestions product using primers BbF and CbR. Lane 1; ladder. Lane 2; AccI. Lane 3; AflIII. Lane 4; EcoRI. Lane 5; NdeI. A) P2/P2. B) P2/179. C) STIV producing *S. solfataricus* P2 culture.

Discussion

Southern blotting of STIV infected *S. solfataricus* P2 cultures, suggests that the virus probably does not integrate into its host's genome. Restriction enzyme digestions with enzymes AccI, BanI, HindIII, PsiI and PstI show the same digestion patterns as would be expected from a virus that does not integrate into its host genome. The banding pattern of Southern blots where EcoRI was used to digest the DNA shows that there is one band missing from the non-integrated form of the viral DNA, and the intensity of one is lower then expected. On Southerns where the DNA had been digested with MspA1I, the 1499 bp band was larger then expected. The addition to the band was approximately 300 bp. Interestingly, this band is from the same area of the genome of STIV where the 1820 bp EcoRI band was missing. Southern analysis, using PsiI to digest the time course, shows the ideal banding pattern for a virus that does not integrate into the hosts' genome, although one band is less intense than expected for a band that size. This suggests that there is a mixed population of STIV being produced in the cultures. One mutation of the virus has a 300 bp addition of sequence somewhere in the 15300 to 17000 area, while the other mutation has suffered a deletion of sequence between base pairs 8000 and 10000 in the STIV genome.

The area covering ORFs B345 and C557 was amplified and digested with three different enzymes. All enzymes digested as predicted from the sequence of STIV. No insertions or deletions were apparent. The template for the PCR amplification was from a different source than the template for Southern blots.

In the genome, ORF A510 was thought to potentially be an integrase recombinase based on the tertiary structure. Despite the large diversity in integrase recombinase primary amino acid sequences, the resolved structures reveal that the integrases have almost identical three-dimensional peptide folds [60]. Performing a thread analysis on the ORF with program HHpred (Max-Planck institute for Developing Biology, Germany) [61, 62] revealed that the most similar protein based on the possible tertiary structure of A510 is indeed a site-specific recombinase from *E.Coli*, XerD, but the probability for the hit is only 49% (e-value of 2.7). Other matches based on possible tertiary structure, with 29-36% likelihood of match (e-values from 300-410), include a conserved hypothetical protein from *Staphylococcus aureus*, the Spectrin alpha chain in chicken brains, and the gamma and beta chains of Fibrinogen.

Based on these results it is highly unlikely that the genome of STIV contains an integrase recombinase, and that it integrates into the *S. solfataricus* P2 genome. Even though a single band is shifted on each Southern blot when digested with two out of seven enzymes used in the analysis, amplifying the region with the unexpected banding pattern with PCR and digesting with EcoRI shows that the site is present and the bands are of the right size.

CHAPTER 5

NORTHERN ANALYSIS OF SULFOLOBUS TURRETED ICOSAHEDRAL VIRUS INFECTED *SULFOLOBUS SOLFATARICUS* CULTURES.

Introduction

Northern blotting is a method that can be used to visualize the transcripts of the virus. When DNA is replicated, the sequence of the bases in the DNA are recorded as a sequence of complementary bases in a single stranded RNA molecule, also called a transcript [63] which is then used to synthesize proteins. RNA of virus-infected cultures can be extracted and probed with the genome of the virus itself to identify the viral transcripts. Northerns are used to visualize which parts of the STIV genome are transcribed.

In STIV there are 37 predicted open reading frames (ORFs) >50 amino acids associated with TATA-like elements. These 37 ORFs potentially encode for proteins ranging in size from 5.1 to 57 kDa. A search of the genome for TATA-like elements associated with the Sulfolobales [TTTTTAAA [64]] found three exact matches and 15 other TATA-like elements similar to the Sulfolobales consensus sequence [14, 43] (Figure 5.1). Since these ORFs are merely putative, Northern analysis gives valuable information about which open reading frames are being transcribed and which are not. The important role of a TATA motif is that it indicates the site of the initiator element where transcription is indicated, and is usually located at position of about -25 bp upstream from the transcription start site [63].

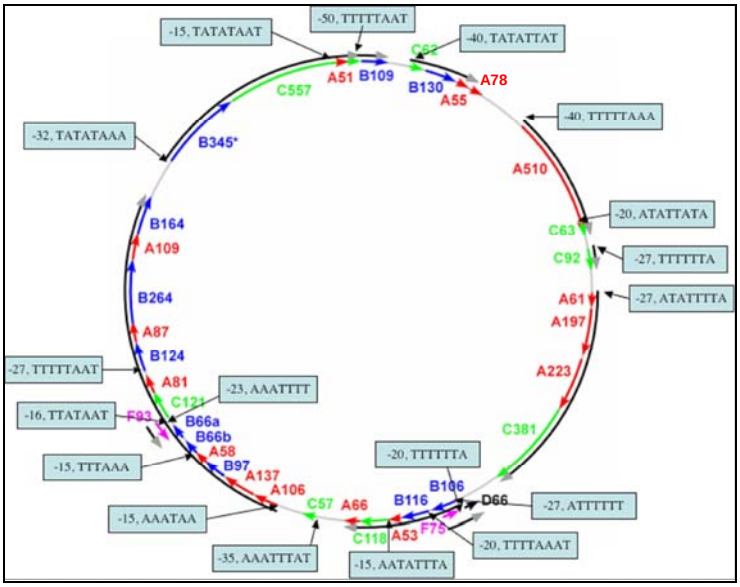


Figure 5.1. TATA-like elements found in the STIV genome [14].

Cultures of *S. solfataricus* P2 were grown and infected with STIV. RNA was extracted from the cultures when virus production was at maximum and separated by sizes utilizing electrophoresis and transferred to a membrane. The membranes were subsequently probed with three different probes; two of them RNA probes specific to certain areas of the STIV genome, while the third was a random hexamer DNA probe made from the full length clone of STIV. RNA probes can be designed to probe for a certain region of the genome, single strand at a time, while DNA probes are made to both strands of the template. Transcripts of STIV were visualized and analyzed.

Materials and Methods

Two cultures were started by inoculating aliquots of glycerol-stocked ATCC strain of *S. solfataricus* P2 and glycerol-stocked strain of the original *Sulfolobus* isolate from which STIV was isolated, YNP179 from Yellowstone National Park (P2/P2 and P2/179, respectively), into Medium 182 at pH 3.5, in a long-neck Erlenmeyer flasks. Cultures were incubated in an oil-bath shaker at temperature close to the optimum growth temperature of *S. solfataricus* P2, ~80°C and shaken ~100 rpm. Following 2 days of growth, the cultures were diluted five fold in Media 182 at pH 2.5 and allowed to grow an additional day. Further five- to ten-fold dilutions of the cultures were performed on the fourth day in Media 182, pH 2.5. At early log-phase the actively growing cultures were infected with a concentrated stock of STIV particles. Cultures were monitored for STIV production by ELISA and immuno dot-blots. When virus production was at maximum value, total RNA was extracted from cultures with RNeasy Mini Kit (Qiagen). The RNA was quantified by measuring the absorbance with a UV/VIS spectrometer.

RNA was separated by size with electrophoresis on a 0.7% formaldehyde agarose gel in 1xMOPS buffer (0.04 Morpholinoproanesulfonic acid, 0.01M Sodium Acetate·$3H_2O$,1mM EDTA, pH 7.2 with NaOH). After the RNA was separated by sizes it was transferred to a positively charged nylon membrane (Immunobilon – NY+, Millipore) with upward capillary transfer overnight (approximately 16 hours) in 10XSSPE (1.5M NaCl, 0.1M $NaH_2PO_4 \cdot H_2O$, 0.01M EDTA, pH 7.4 with NaOH). After transfer, the nucleic acid was crosslinked to the membrane with a UV crosslinker with 120,000 microjoules/cm^2 energy (Stratagene). The membrane was then incubated in two

different pre-hybridization solutions, depending on the nature of the probe, with rotation, at 43°C, with Herring sperm DNA added to block unoccupied sites on the membrane. Pre-hybridization solution for RNA probe consisted of 5xSSPE, 50% deionized formamide, 5xDenhardt's solution (100xDenhardt's solution; 2% polyvinylpyrrolidone (MW 40,000), 2% bovine serum albumin and 2% ficoll 400), 1% SDS ,10% dextran sulphate, Na salt (MW 500,000) and 100 μg/ml denatured Herring sperm DNA. For DNA probes the pre-hybridization solution included 5xSSC (20x SSC; 3M NaCl, 0.3M sodium citrate dihydrate), 5xDenhardt's solution, 50% deionized formamide 1% SDS and 100 μg/ml denatured Herring sperm DNA.

Three different probes were made. The template for the first probe was a full-length clone of the STIV genome (J. Fulton, unpublished). A DNA probe was made by randomly priming 1 μg of the full length clone with Ready-To-Go™ DNA Labeling Beads (-dCTP) Kit (Amersham Biosciences) according to manual, using (α-^{32}P) – dCTP's from MP Biomedicals with specific activity of 800 Ci/mmol. The reaction was incubated for 1 hour at 37°C, and then the unincorporated nucleotides were separated from the product on a sepharose column (Sepharose CL-6B, Sigma). Finally the probe was denatured by incubation at 95°C for 2 minutes, followed by 2 minute incubation on ice.

The templates for the other two probes were 4 and 5 kilobasepair (kbp) fractions of the STIV genome in TOPO 2.1 vector (Invitrogen). The 4 kbp probe started at basepair 14634 and spanned 4405 base pairs, while the 5 kbp fraction started at 1410 and ended at basepair 7188. The 4 and 5 kbp pieces were linearized with restriction enzymes SacI and

EcoRV, respectively. Probes were made with MAXIscript® SP6/T7 Kit (Ambion) following the suggested protocol, from the transcribing strand of the genome, using (α-^{32}P)-CTP's from MP Biomedicals. Unincorporated nucleotides were separated from the product and denatured as described above.

Probes were added to the pre-hybridization buffer following 2 hours of blocking and incubated over night, with rotation, at 43°C. The membrane was washed twice in 2XSSC + 0.1% SDS (for 1XSSC = 150 mM NaCl and 15 mM $Na_3C_6H_5O_7$, pH 7.0 with 1M HCl) at room temperature for five minutes, and twice in 0.1XSSC + 0.1%SDS for 15 minutes at 45°C and 60°C for the DNA and RNA probes, respectively. After washing the membrane, the membrane was exposed to a high-performance chemiluminescence film (Hyperfilm ECL; Amersham) in a film cassette with an intensifying screen to increase the signal from the membrane. The film cassette was stored in at -80°C while developing to increase signal. About 24 hours later, the film was developed and the results were analyzed. If the membrane needed more exposure for the signal to be seen, a new film was put on the membrane and more time was allowed to pass before film was developed again.

Results

4 kbp RNA probe

Probing the total RNA extracted from the infected *S. solfataricus* P2 cultures along with RNA extracted from cultures that had not been infected with STIV show that the infected cultures have a different banding pattern than the uninfected ones (Figure 5.2). In all lanes the ribosomal bands of *S. solfataricus* P2 are detectable, but in the infected lanes two additional bands are observed. The sizes of the additional bands are 1.2 and 1.8 kb, which could result in protein sequence of about 400 and 600 amino acids.

If open reading frames (ORFs) C62, B130, A55 and A78 are transcribed together, the transcript would be approximately 1000 bp in size, matching to the approximately 1200 bp transcript observed on the Northern. On the other hand, it would also be the size if ORF B345 was transcribed separately, but what would result in a 1035 kb transcript. The 1800 bp transcript corresponds to the size of the transcript that would be seen if ORF C557 was transcribed separately.

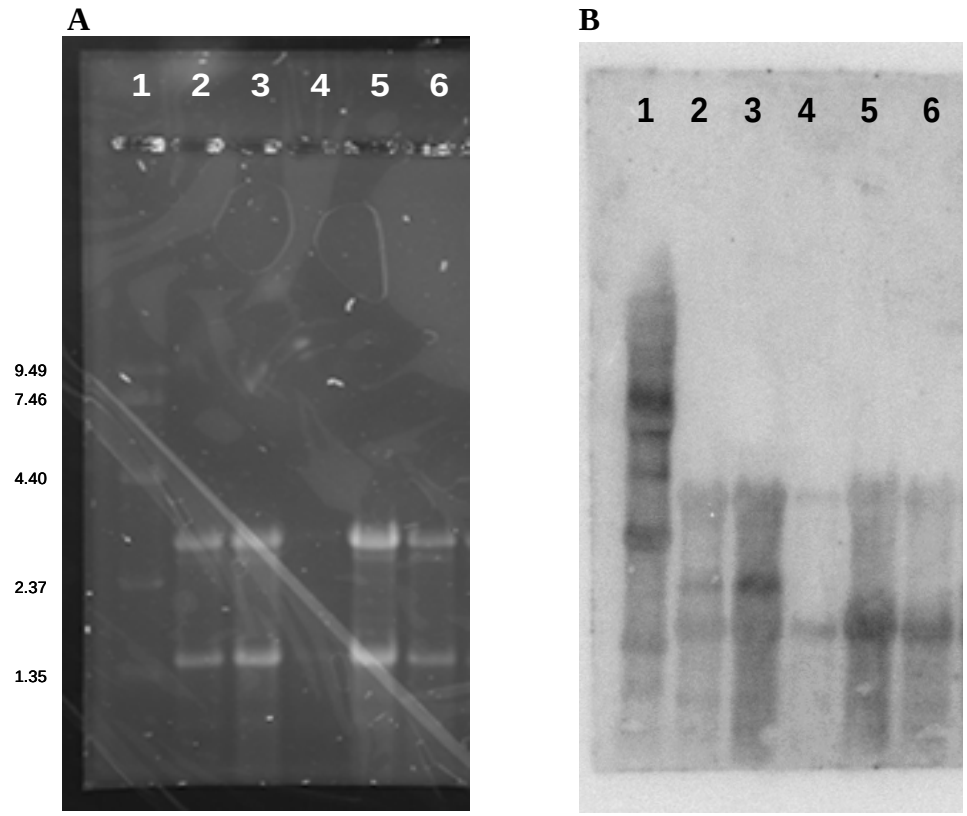


Figure 5.2. Northern probed with 4 kbp probe.
A) RNA gel showing in Lane 1: Ladder. Lane 2: P2/P2 STIV. Lane 3: P2/179. Lane 4: P2/P2 DNased. Lane 5: P2. Lane 6: P2 DNased. B) Northern of that same gel probed with a 4 kbp RNA probe.

5 kbp RNA probe

With the 5 kbp probe, in addition to the ribosomal bands, three transcripts were observed in the cultures that were infected compared to the non-infected cultures (Figure 5.3). The sizes of those three transcripts were approximately 1.9, 1.3 and 0.4 kb. The biggest transcript, the 1900 bp, could result from transcription of two different parts of the genome. The first possibility is that ORFs A510 and C63 are transcribed together, making a 1719 bp transcript. The second possibility is that ORF A223 and ORF C381 are transcribed on the same transcript, resulting in a transcript of 1812 bp. The 1300 bp

transcript seen on the Northerns could also possibly be transcribed from two different areas. The first possibility is that the transcript results from ORF C381 being transcribed by itself, which creates a 1142 bp transcript, but it could also possibly result from ORFs A61, A197 and A223 being transcribed together. Those three ORFs being transcribed together would make a transcript of 1443 bp. The smallest transcript of the Northern is only about 400 bp, most likely from C92 which has been seen to be transcribed by itself with microarray experiments (A. C. Ortmann, unpublished) at very high levels.

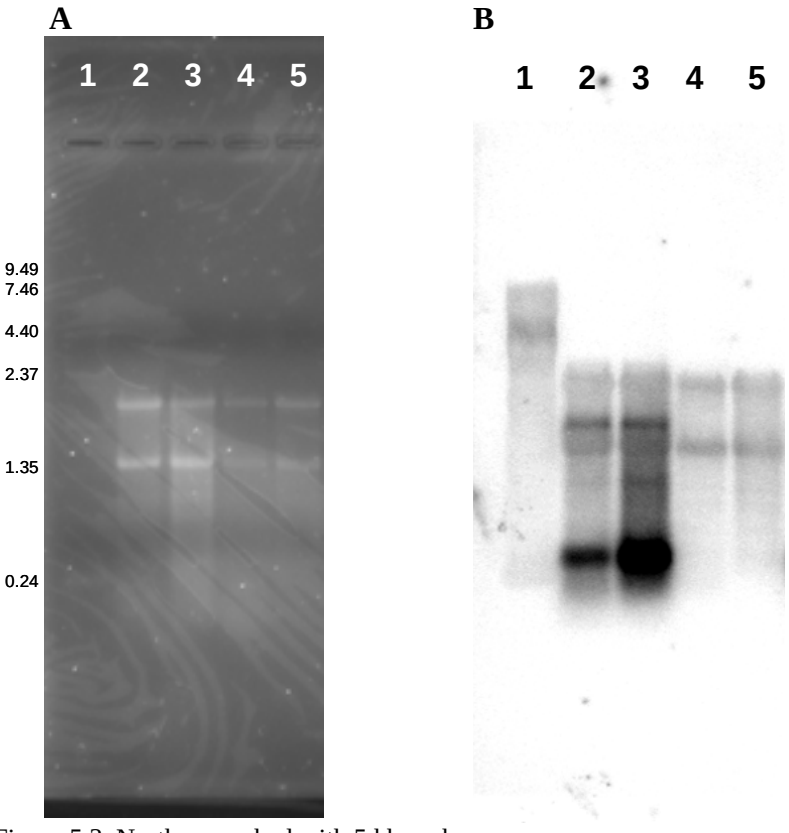


Figure 5.3. Northern probed with 5 kb probe.
A) RNA gel showing in Lane 1: Ladder. Lane 2: P2/P2. Lane 3: P2/179. Lane 4: P2 DNased. Lane 5: P2.
B) Northern of that same gel probed with a 5 kbp RNA probe.

Whole Genome DNA probe

Probing the RNA on the Northerns with a DNA probe made from the full length clone of the genome yields nine bands (Figure 5.4). The biggest two bands are the genomic DNA of the virus while the other seven are virus transcripts. The sizes of these seven transcripts are 1, 1.3, 1.4, 1.8, 2.3, 3.1 and 3.8 kb. The 1.8, 1.4 and 1.3 kb transcripts most likely correspond to the 1.9, 1.8, 1.3 and 1.2 transcripts seen on the Northerns probed with the 4 and 5 kb probes. The smallest transcript seen on the Northern probed with the 5 kb probe, the 0.4 kb transcript, cannot be seen on this Northern since the smallest band that would be seen on this Northern would be about 600 b. The gel transferred to the membrane was allowed to run further than the previous gels to get a better separation of the bigger transcripts in order to get a better size estimate.

If the 1.8, 1.4 and 1.3 transcripts represent the previously discussed transcripts, the remaining transcripts are most likely from the part of the genome that the 4 and the 5 kbp probes do not cover. In that area there are a lot of small ORFs clustered together, therefore it is hard to predict which ORFs will be transcribed on the same transcript. Many guesses can be made, but one is not more likely then the others. To be able to say for sure which transcript comes from with ORFs the start sites of the transcripts would have to be mapped by primer extension.

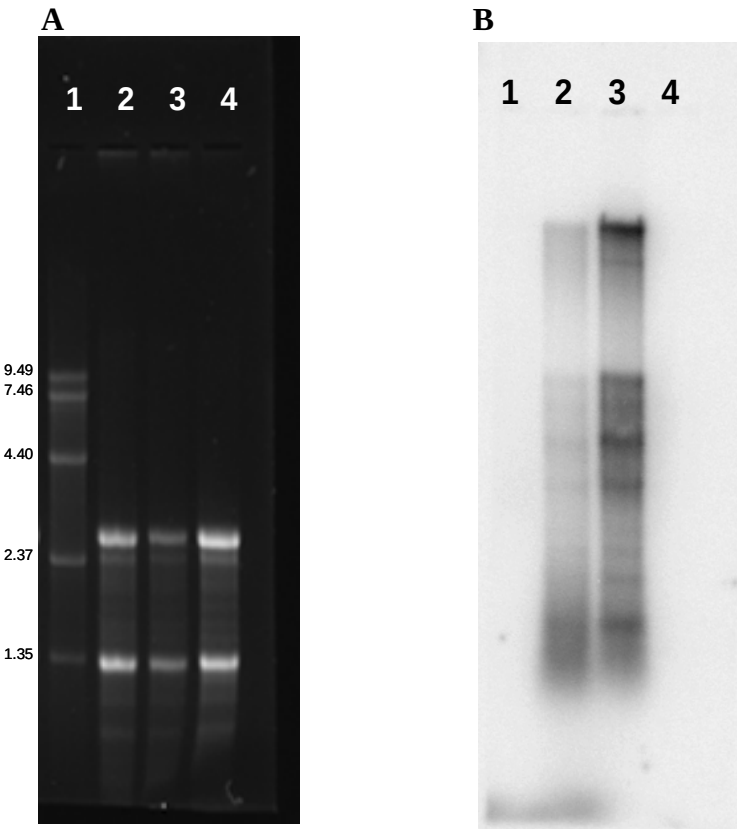


Figure 5.4. Northern probed with whole genome DNA probe.
A) RNA gel showing in Lane 1: Ladder. Lane 2: P2/P2. Lane 3: P2/179. Lane 4: P2 B) Northern of that same gel probed with a full length probe of the STIV genome.

Discussion

Three different Northerns were probed with 4 and 5 kbp RNA probes and a full length DNA probe. Common bands were seen on all three Northerns. The Northern probed with the 4 kbp RNA probe had 4 different bands, two of them the ribosomal bands of the host. The other two bands, which were only seen in infected cultures, were of the sizes 1.2 and 1.8 kbp. The smaller transcript could be transcribing two different areas. First it could be a polycistronic transcript, transcribing ORFs C62, B130, A55 and A78, but it could also be a monocistronic transcript, transcribing only ORF B345. The 1800 bp transcript corresponds to the size of the transcript what would be seen if ORF C557 was transcribed separately.

The Northern probed with the 5 kbp RNA probe had 5 bands. Two of them were the ribosomal bands of the host also seen in the non-infected negative control. Sizes of the additional bands were, 0.4, 1.3 and 1.9 kbp. The smallest transcript is most likely monocistronic, transcribing ORF C92 only. This transcript has been shown to be transcribed by itself with microarray experiment. It is not as obvious what ORFs are transcribed on the 1.3 and 1.9 kpb transcripts. The 1.3 kbp transcript could be only transcribing one ORF, C381, but it would also be transcribing ORFs A61, A197 and A223. The biggest transcript in this area, the 1.9 kbp transcript, could also be transcribing for two different areas of the genome. The possibilities are that ORFs A223 and C381 are being transcribed together, or ORF A510 and ORF C63. Since ORFs A223 and C382 are both found in the virus particle it is highly likely that they would transcribe together. A

method to distinguish which is right would be to map the start of the transcripts by primer extension.

The Northern in which the RNA was probed with a DNA probe made randomly from a clone of the full length genome produced total of 9 bands. The biggest two bands correspond to the genomic DNA of the STIV, while the other seven are transcripts of the virus. Three of these transcripts have been seen when RNA from cultures infected with STIV were probed with the 4 and 5 kbp probes. The other four transcripts were not seen on Northerns probed with the other two probes, therefore it can be concluded that they come from the genome that those probes do not cover. It is not possible to make reasonable guesses which ORFs are transcribed on any of those transcripts since the area is tightly packed with small ORFs. Mapping the starting points of the transcripts would reveal which transcript transcribes for which area.

CHAPTER 6

CONCLUSIONS

The work described in this thesis has increased our understanding of STIV and its infection cycle in *S. solfataricus* P2. We are now able to grow *S. solfataricus* P2 and produce STIV consistently, and detect the virus quickly. A better understanding has been established of its replications and transcription that will hopefully lead to further discoveries of the replication cycle of STIV in its hosts.

Growth of *S. solfataricus* P2 in the three media considered in this study is not considerably different, although when media are supplemented with 1 g/ml glucose, *S. solfataricus* P2 grows significantly better. Cultures grew well at all three temperatures and pHs considered, showing that the range of temperature and pH where *S. solfataricus* P2 can be grown at is very wide.

The allowable temperature and pH ranges investigated for virus production were not as wide. STIV was produced at all three temperatures considered, but virus production and release started earlier at higher temperatures. No virus production was detected at an initial culture pH of 3.5, while STIV was produced at lower pHs in similar abundances. One likely reason for this is the dramatic increase in the media pH when cultures are started at higher pHs. The pH of cultures started at 3.0 does not start increasing until after about 40 hours of growth and is much slower and is not as drastic as when cultures are started at pH 3.5, which rapidly increases to almost pH 6.0. Cultures started at pH 2.5 remain at pH 2.5 throughout the whole virus life cycle, making the

environment more favorable for STIV, although not necessarily producing more viruses. The compound most likely to be buffering the media is the sulfate, SO_4, since the pKa value of SO_4 is 2.0.

A wide range of multiplicities of infections were tested for STIV infecting *S. solfataricus* P2. An MOI of at least 0.28 is needed to establish an infection in a *S. solfataricus* P2 culture. Viral infection is, in some cases, established when MOIs between 0.04 and 0.45, but at lower MOIs infections were never documented. The magnitude of virus production is not dependent on the amount of virus added to culture when the MOI is above 0.45. This suggests that not all cells in the culture are susceptible to infection by STIV. Thin sections of STIV infected *S. solfataricus* P2 cultures analyzed with transmission electron microscope (TEM) revealed that STIV was seen inside only approximately 1 in every 50 *S. solfataricus* P2 cells, or 2%.

S. solfataricus P2 cultures were grown and some infected with STIV while other were left uninfected. Cultures were infected at various stages of growth and diluted into both fresh media and actively growing cultures of *S. solfataricus* P2 in early log-phase. Cultures that were infected after early log-phase did not manage to establish a measurable infection of STIV, while ones infected earlier did. Secondary infection of virus was not detected if cultures were re-infected with the virus, nor if cultures were diluted into fresh media even though *S. solfataricus* P2 was still actively growing. On the other hand, if cultures that are producing virus are diluted into fresh cultures in early log-phase of growth, a second cycle of infection is established. From these it can be concluded that the

time frame that *S. solfataricus* P2 cultures can be infected is fairly narrow. Moreover, cultures that have been previously infected cannot be infected again.

Enzyme-linked immunosorbent assay, ELISA, was developed to be used to quantify the virus in environmental and cultured samples. Virus can be quantified straight from the environment, while cultured samples need to be diluted since the concentration of the virus is above the linear range of the assay. With the method, production of the virus STIV can be monitored over time. The assay is fairly inexpensive and relatively easy to carry out.

Southern blotting of STIV infected *S. solfataricus* P2 cultures, suggests that the virus most likely does not integrate into its hosts' genome. Using various restriction enzymes to digest the DNA, five out of seven show the correct banding pattern for a non-integrated form of the virus. Although two digestions do show an atypical banding pattern, PCR amplification of the part of the genome where the wrong size bands are from shows that the region is present and the right size. A potential integrase recombinase was thought to be in the STIV genome, however thread analysis of the tertiary structure of ORF A510 showed that a protein translated from the ORF would only have 49% structural similarities with published structures of integrase recombinases. Considering the above data, it is highly unlikely that the DNA of STIV integrates into *S. solfataricus* P2.

Three different Northern blots were probed with 4 and 5 kbp RNA probes and a full-length DNA probe. The number of viral transcripts obtained from the 4-, 5 kbp RNA probes and full length DNA probe were 2, 3 and 7, respectively. Total number of viral

transcripts determined from these three probes was 9 transcripts, although the precise mapping of the transcripts could not be determined. In order to accurately map the transcripts, a mapping of the transcription starting sites needs to be done.

Other additional studies that could be done following this work would be to sequence the region spanning ORFs B345 and C557 to determine if there is an insertion of sequence in the area, explaining the increased size of bands from the area.

Also it would be interesting to grow *S. solfataricus* P2 at pH 3.5 in a media with better buffering capacity that would contain the culture at that pH, the pH of the hot spring that STIV was first isolated from. Under that condition more STIV might be produced and it might be stable outside the host cell.

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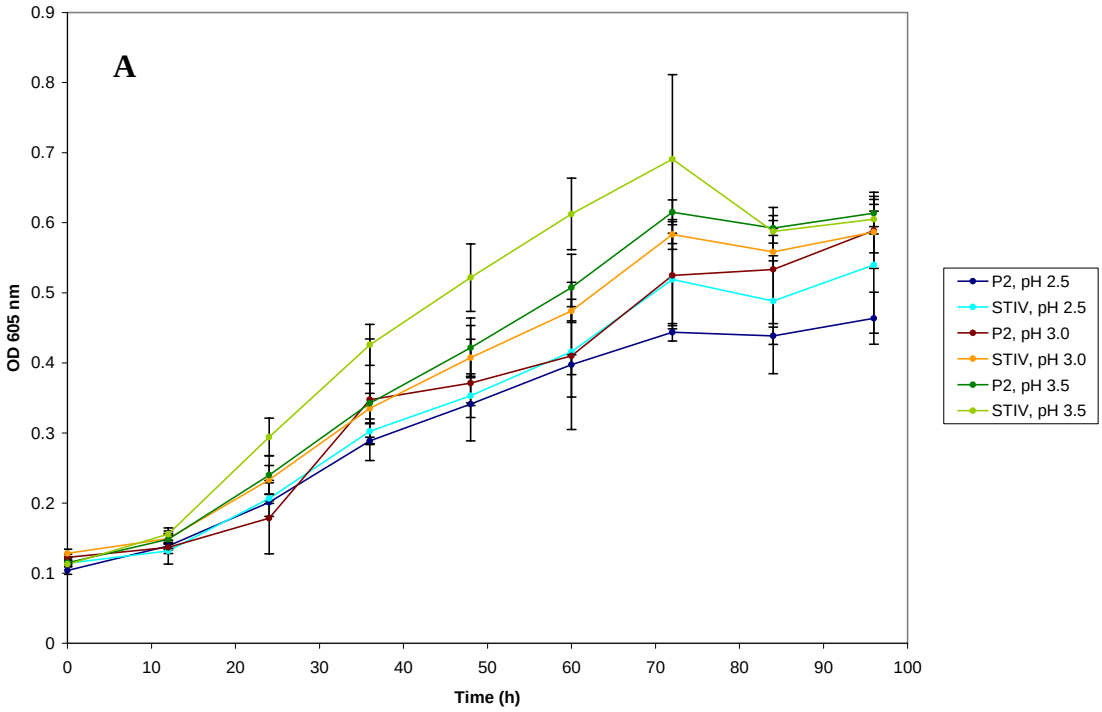
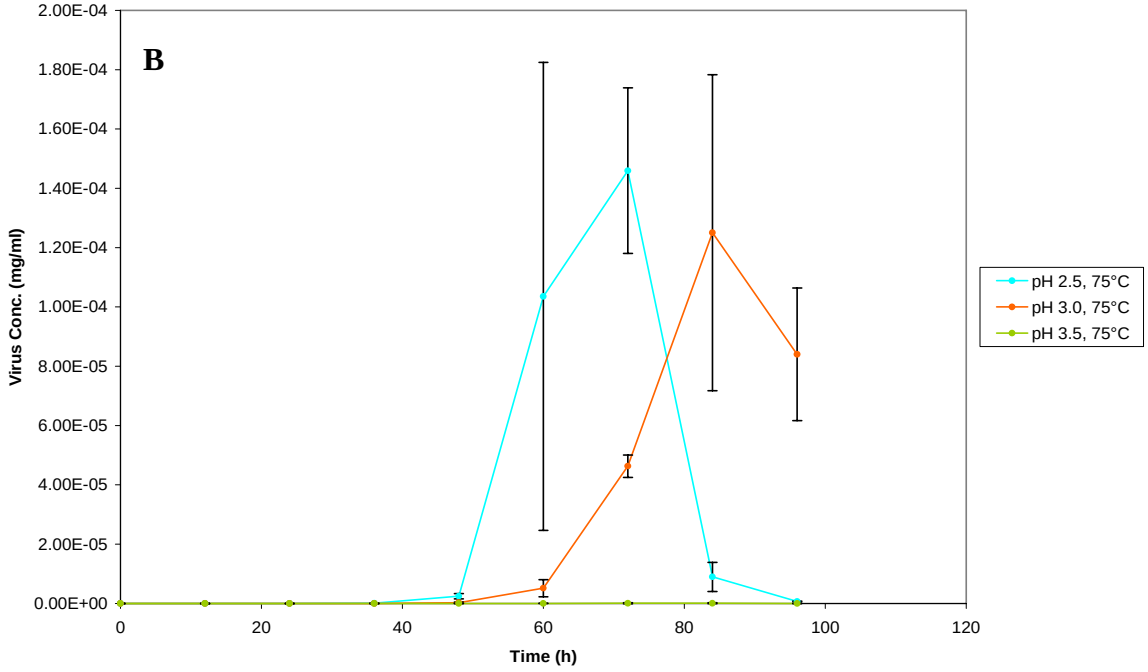
APPENDICES

APPENDIX A

GROWTH DATA FROM DIFFERENT TEMPERATURES AND pHs

Temperature 75°C





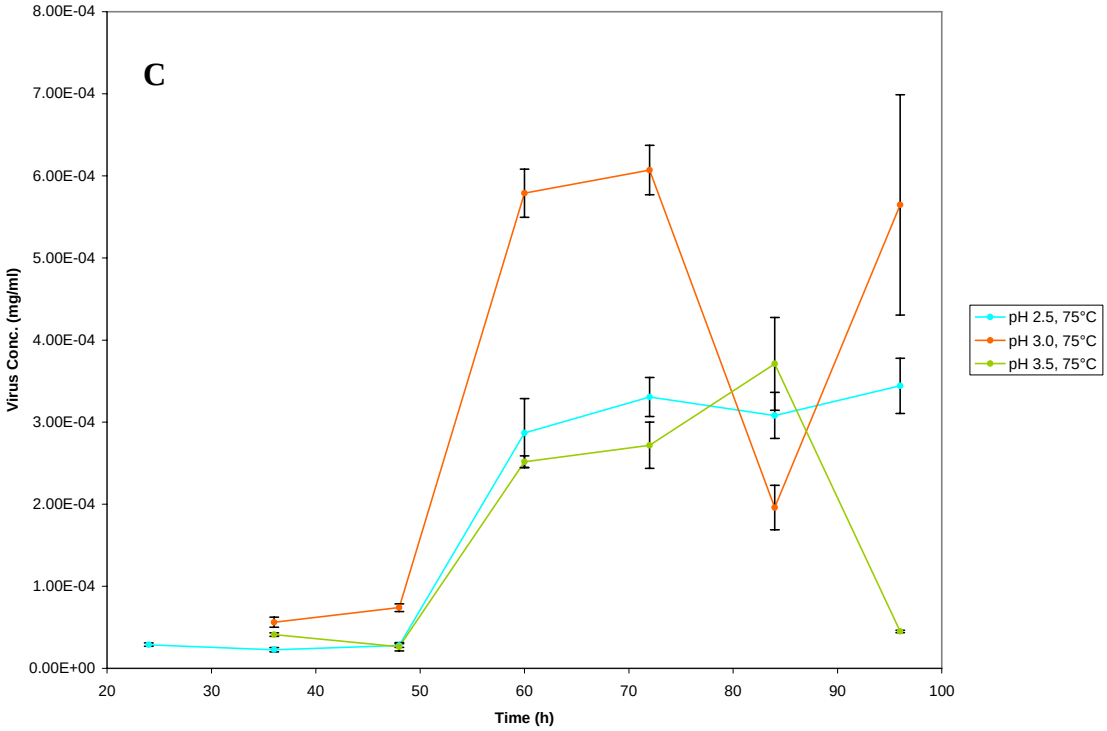
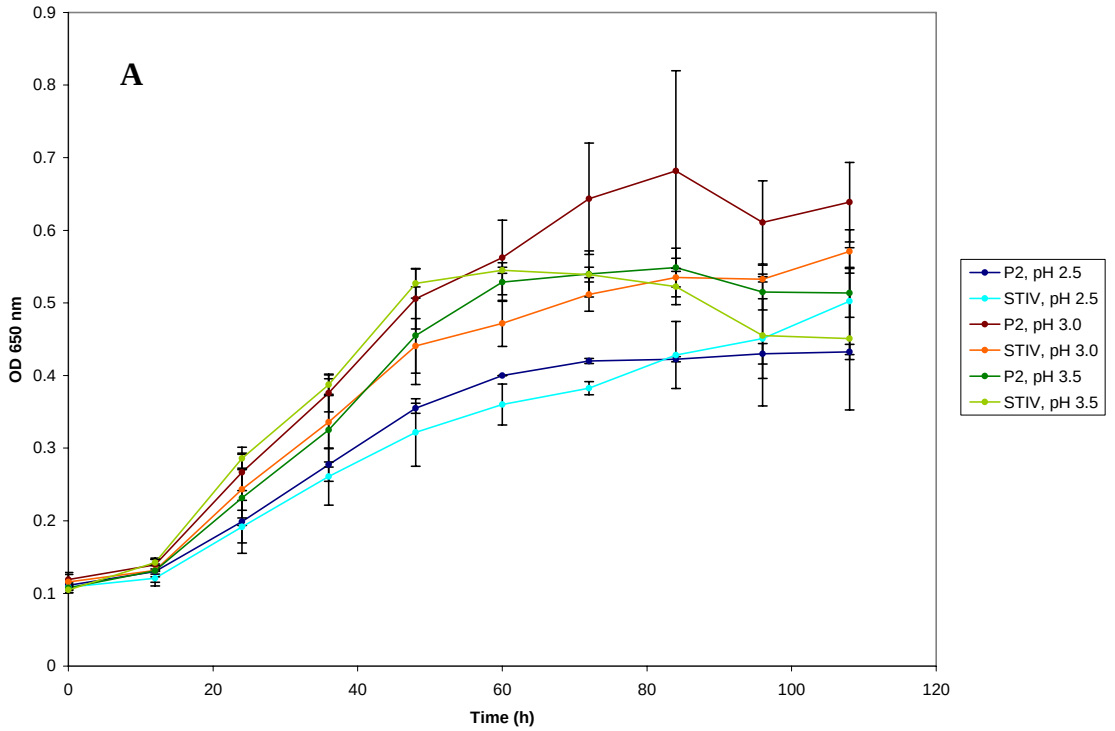
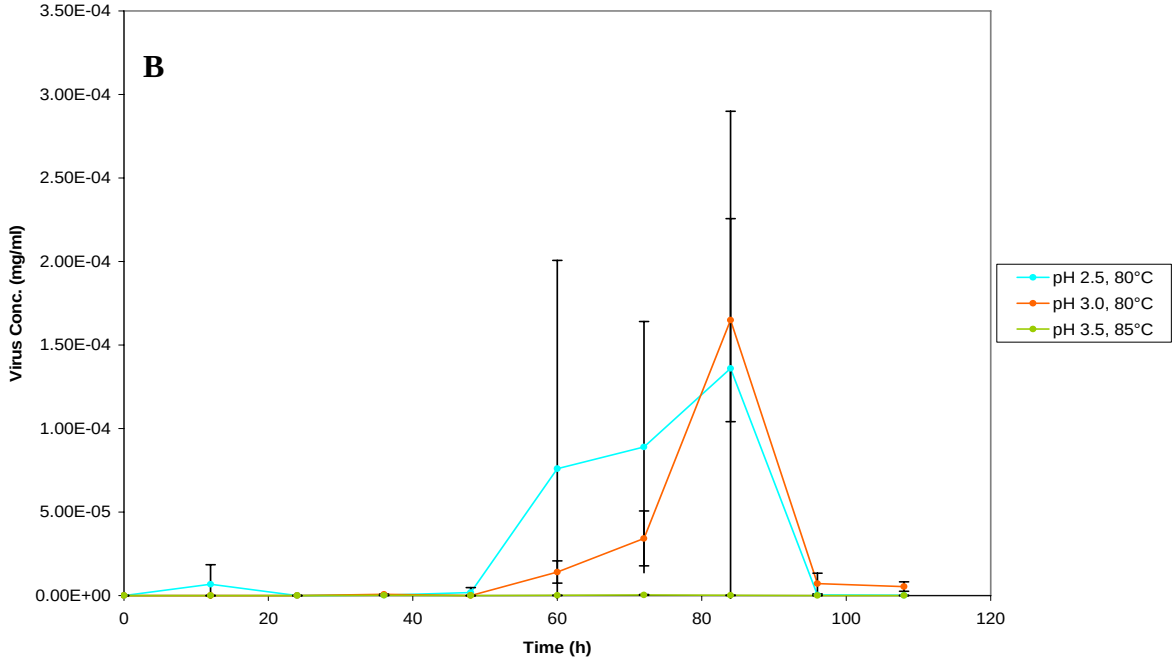


Figure A.1. Growth study, pHs: 2.5, 3.0 and 3.5 at 75°C.
P2, infected and uninfected with STIV, grown at three different pHs: 2.5, 3.0 and 3.5 at 75°C.
A) Growth curve of cultures measured by optical density. B) and C) show virus concentration (mg/ml) over the time course using Q-PCR and ELISA, respectively. Values shown are means of triplicate cultures with error bars showing standard deviation.

Temperature 80°C





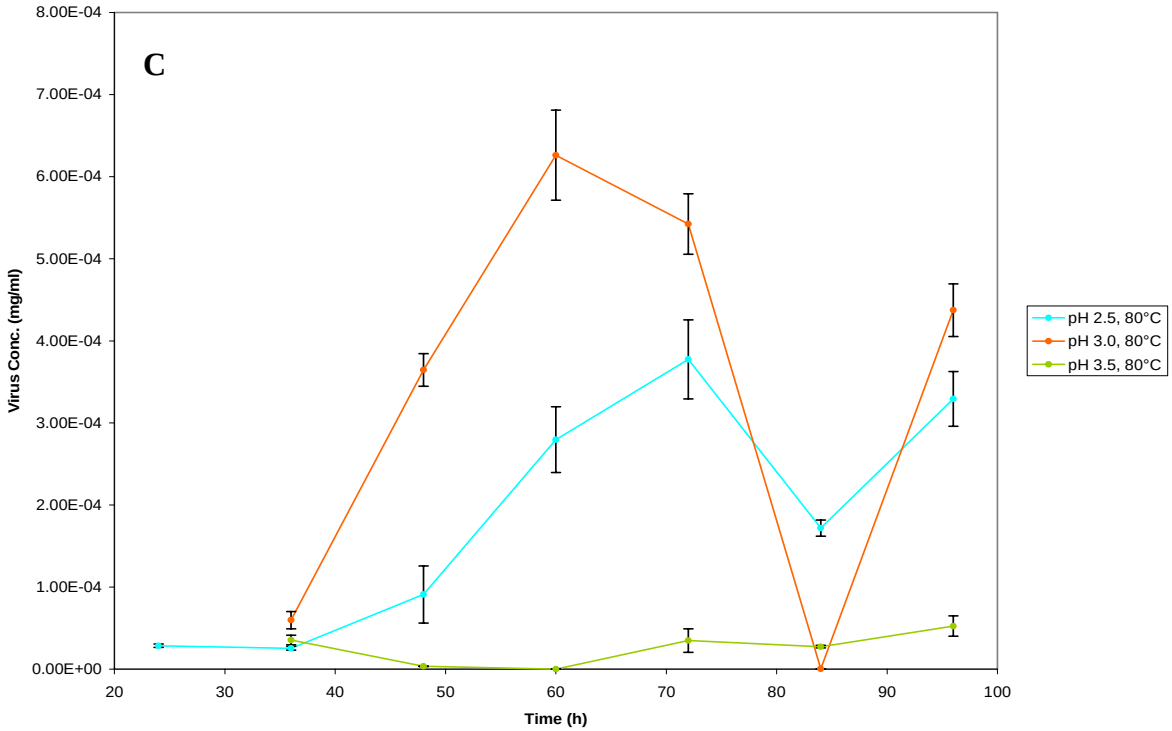
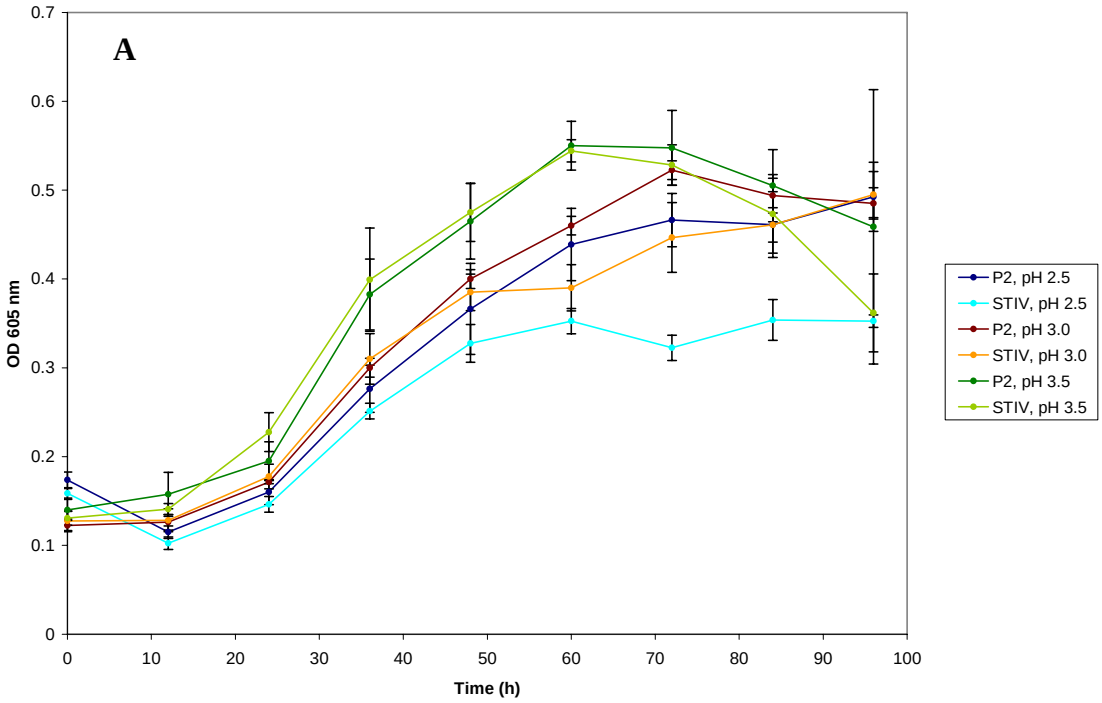
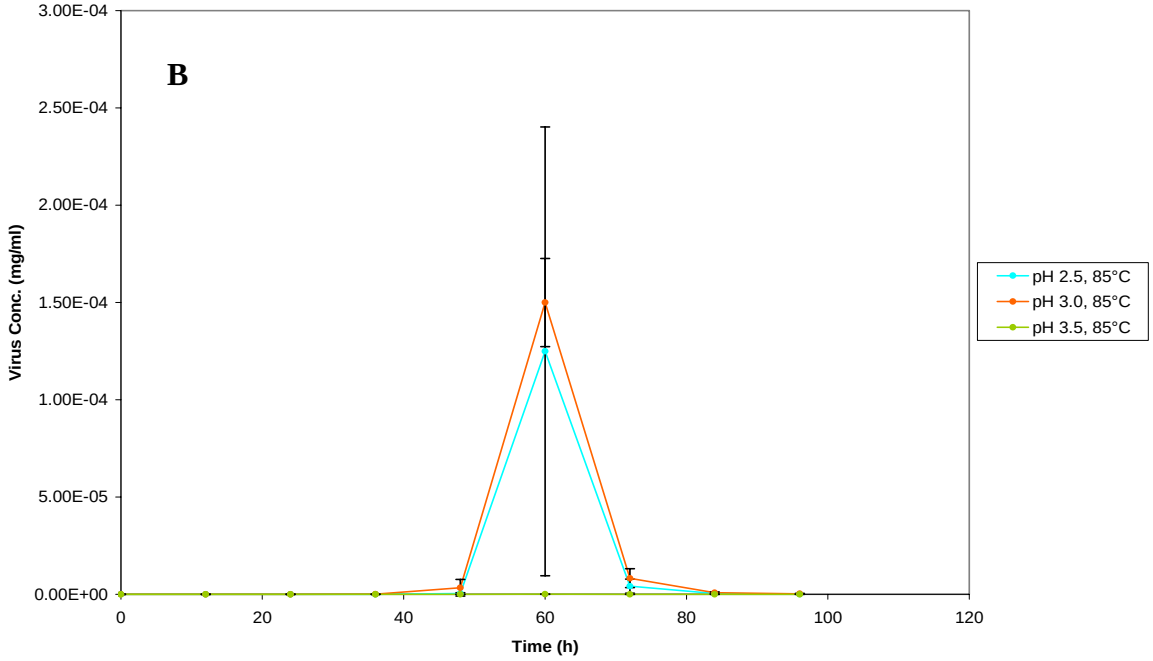


Figure A.2. Growth study, pHs: 2.5, 3.0 and 3.5 at 80°C.
P2, infected and uninfected with STIV, grown at three different pHs: 2.5, 3.0 and 3.5 at 80°C.
A) Growth curve of cultures measured by optical density. B) and C) show virus concentration (mg/ml) over the time course using Q-PCR and ELISA, respectively. Values shown are means of triplicate cultures with error bars showing standard deviation.

Temperature 85°C





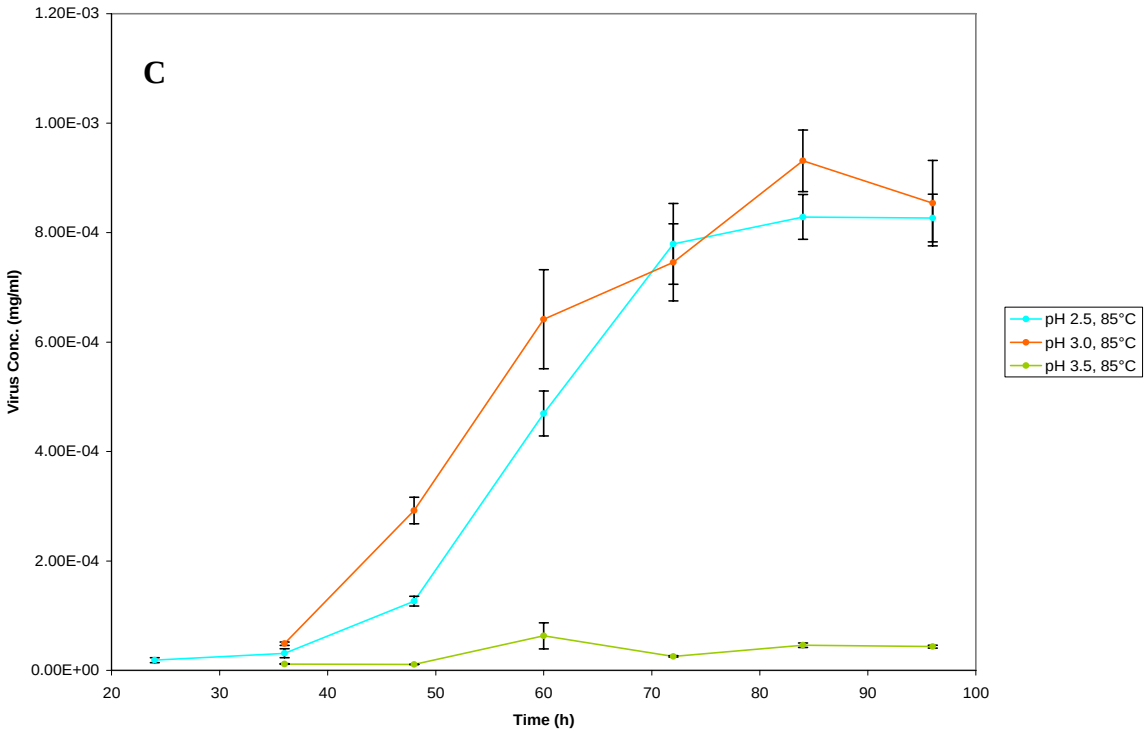
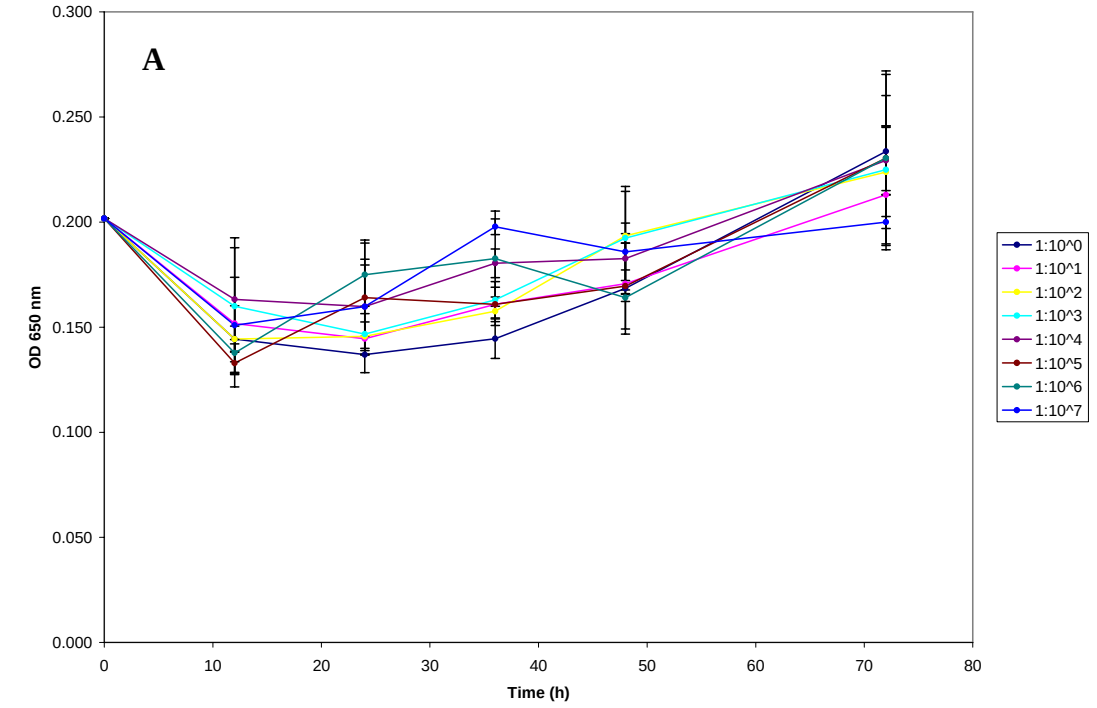


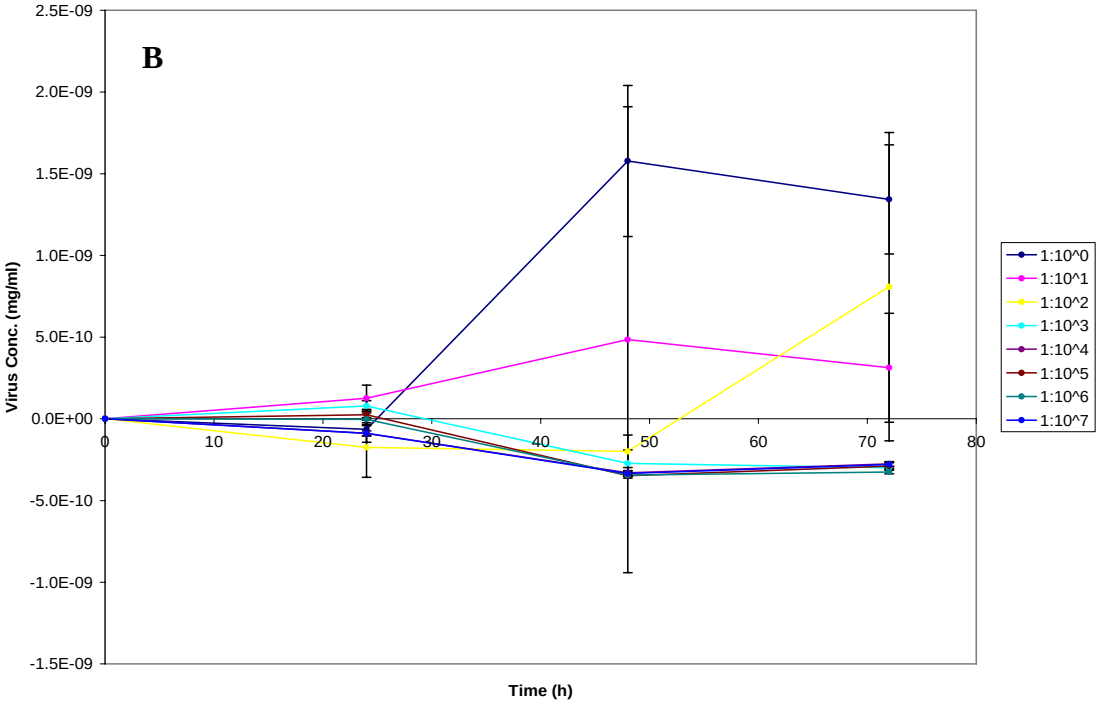
Figure A.3. Growth study, pH2: 2.5, 3.0 and 3.5 at 85°C.
P2, infected and uninfected with STIV, grown at three different pH2: 2.5, 3.0 and 3.5 at 85°C.
A) Growth curve of cultures measured by optical density. B) and C) show virus concentration (mg/ml) over the time course using Q-PCR and ELISA, respectively. Values shown are means of triplicate cultures with error bars showing standard deviation.

APPENDIX B

MOI DATA

Experiment 1





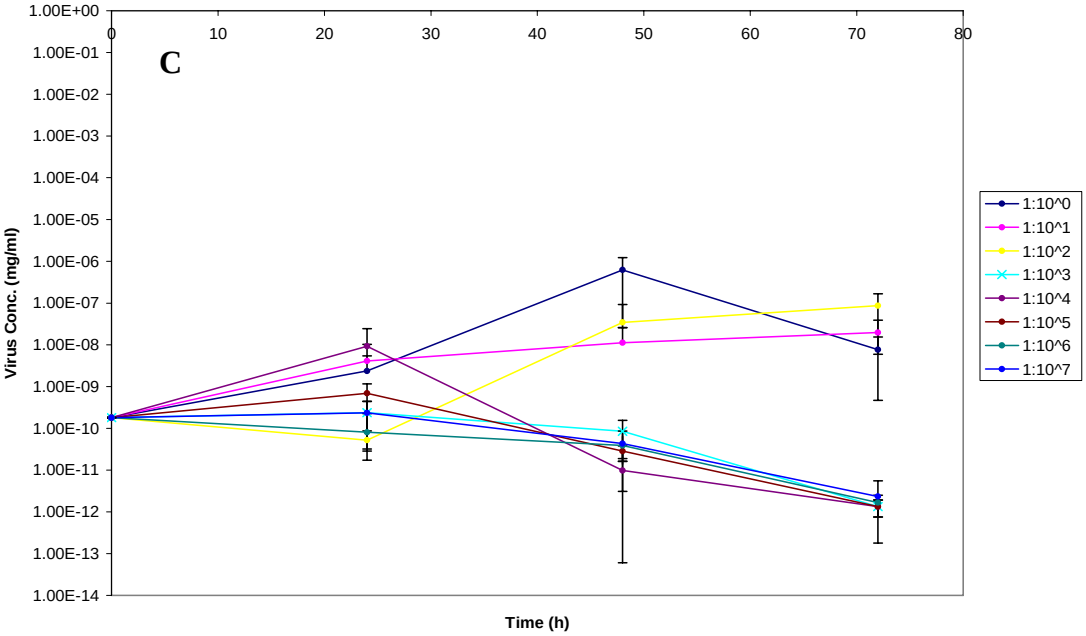
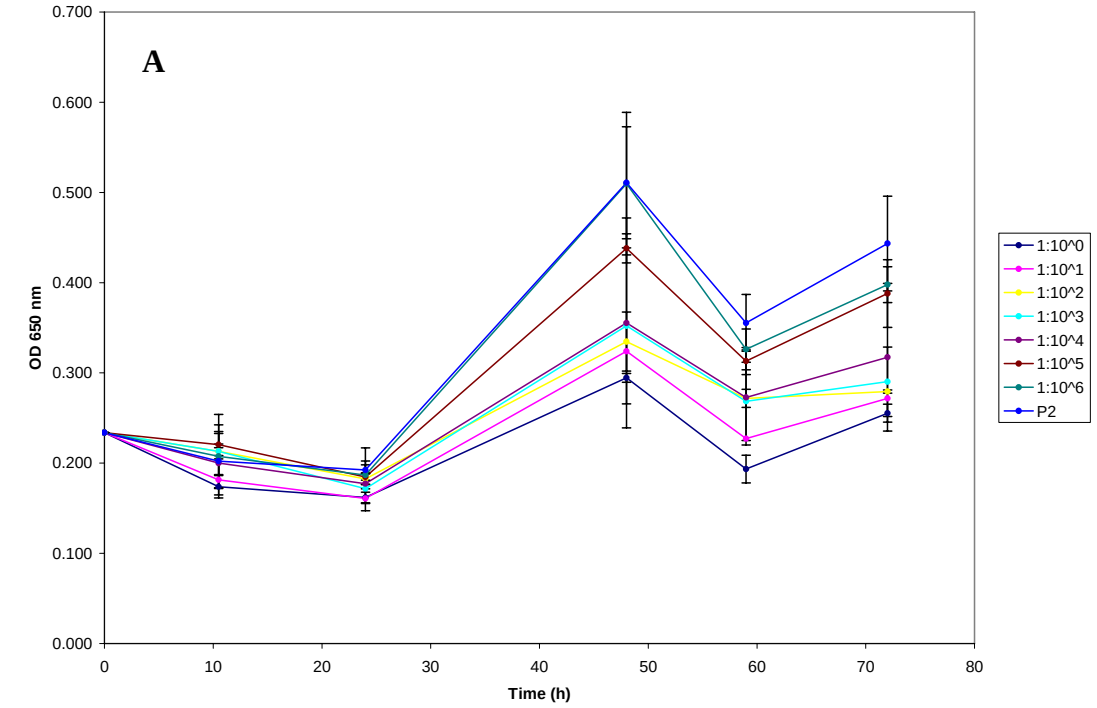
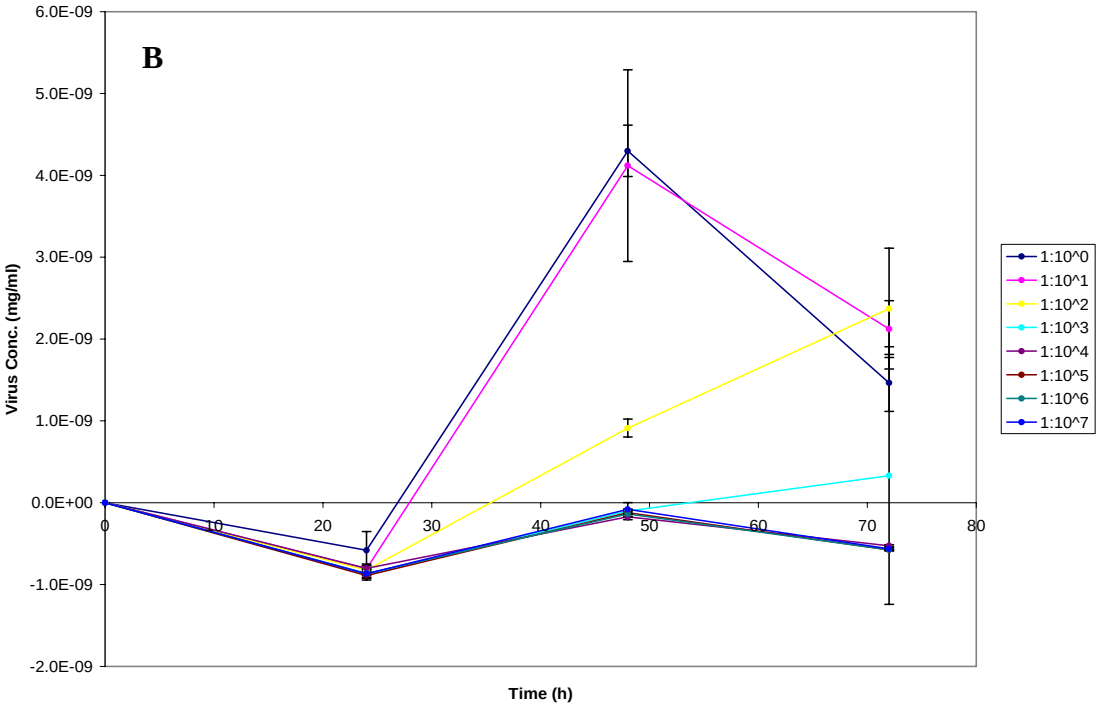


Figure B.1. MOI experiment 1.
P2 infected with various concentrations of STIV. A) Growth of cultures measure with optical density. B) and C) show the concentration of STIV over time measured with Q-PCR and ELISA, respectively.

Experiment 2





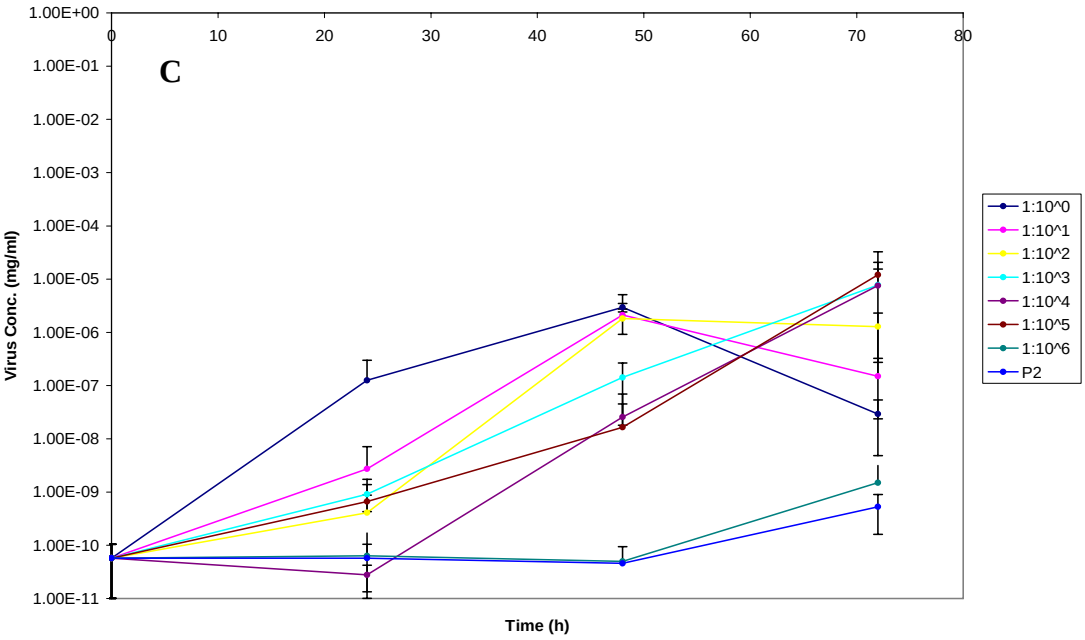
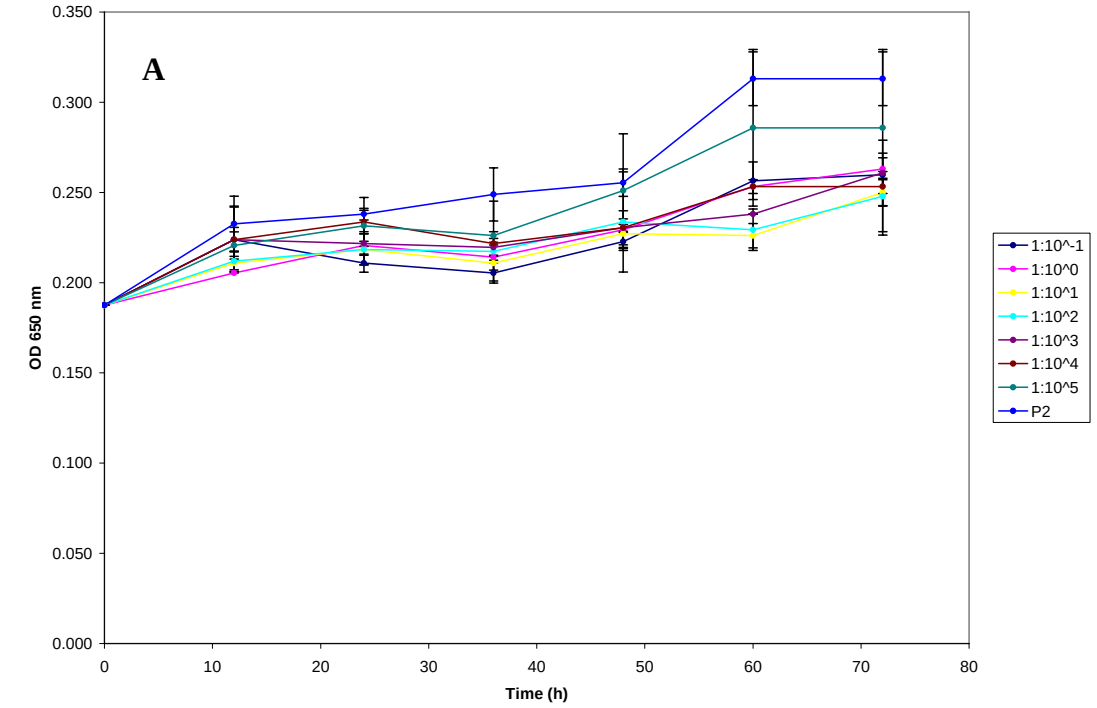
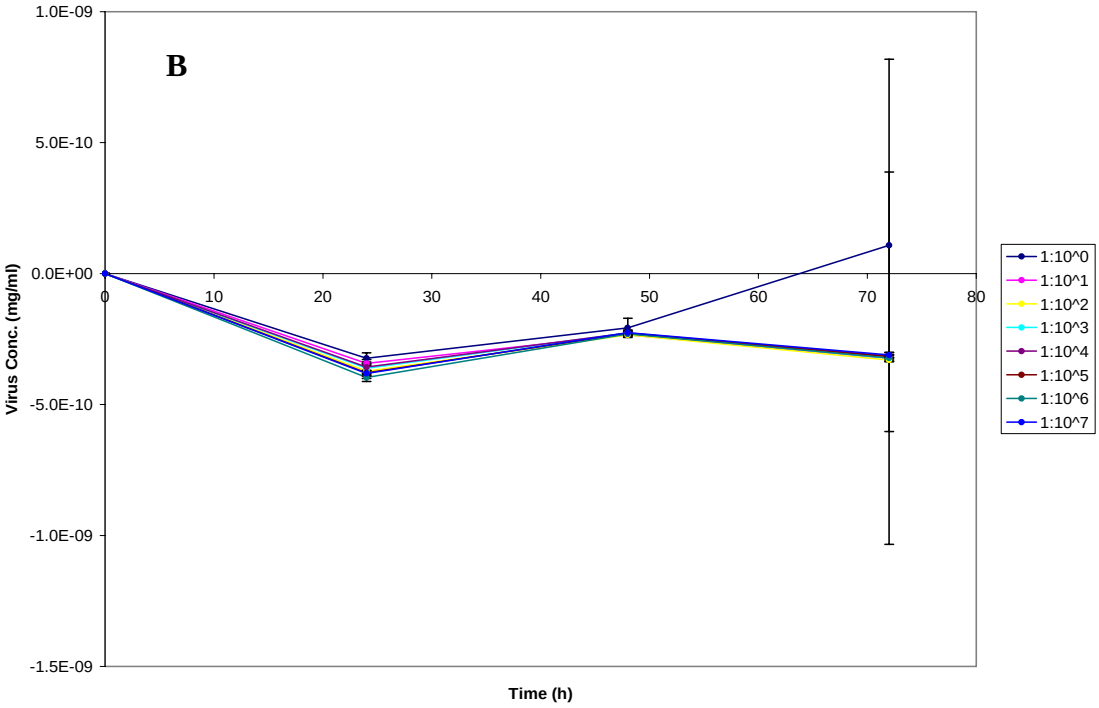


Figure B.2. MOI experiment 2.
P2 infected with various concentrations of STIV. A) Growth of cultures measure with optical density. B) and C) show the concentration of STIV over time measured with Q-PCR and ELISA, respectively

Experiment 3





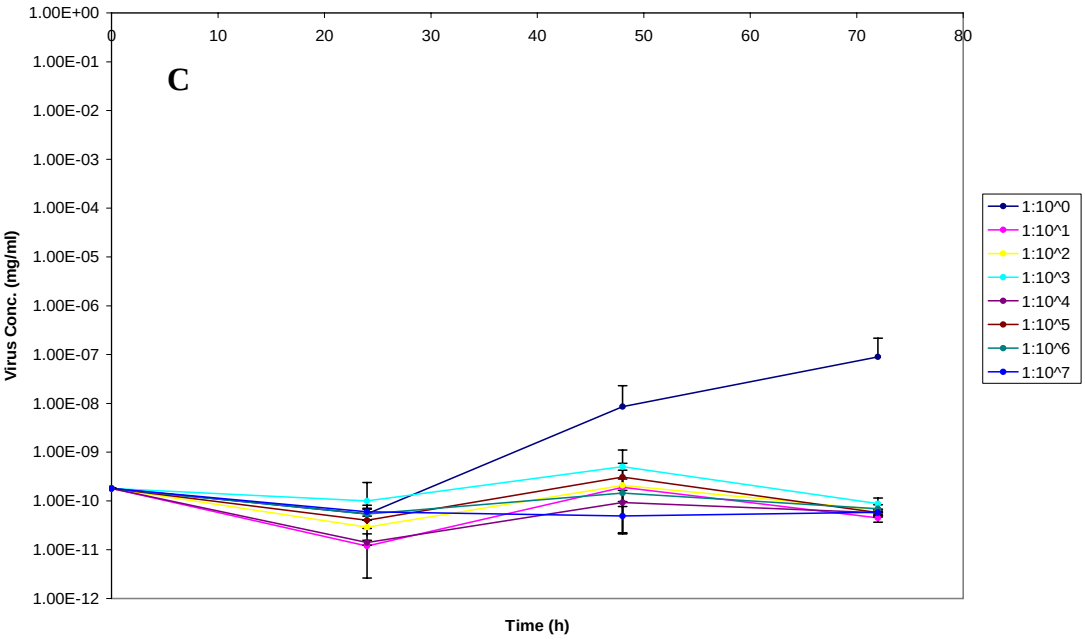
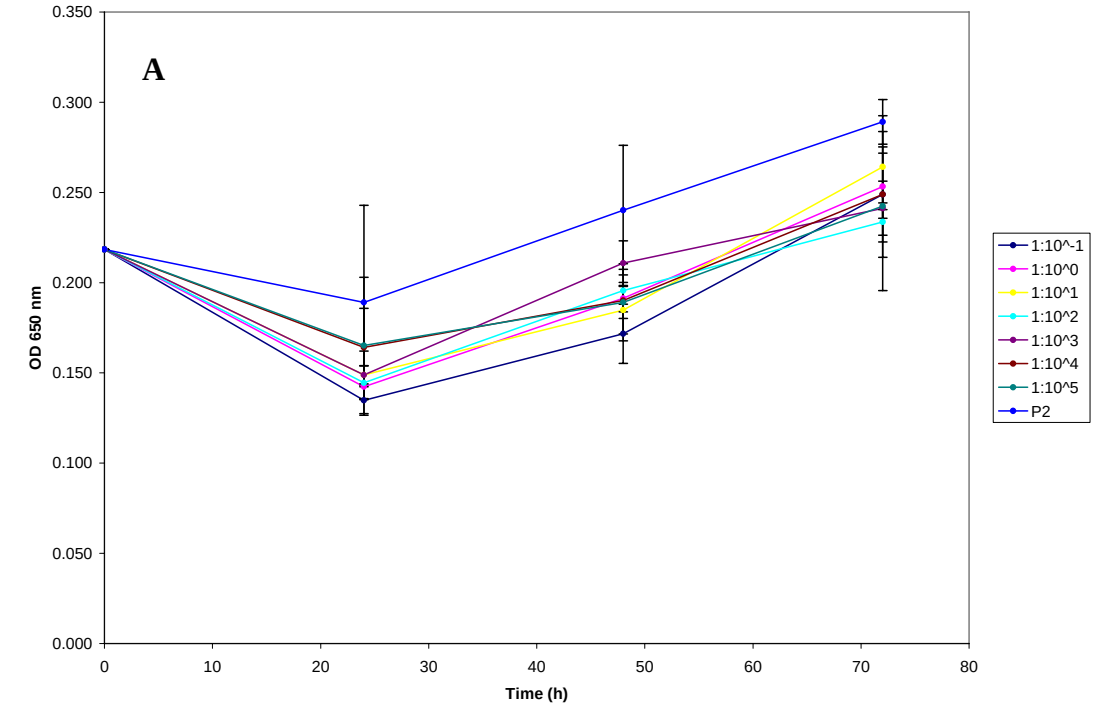
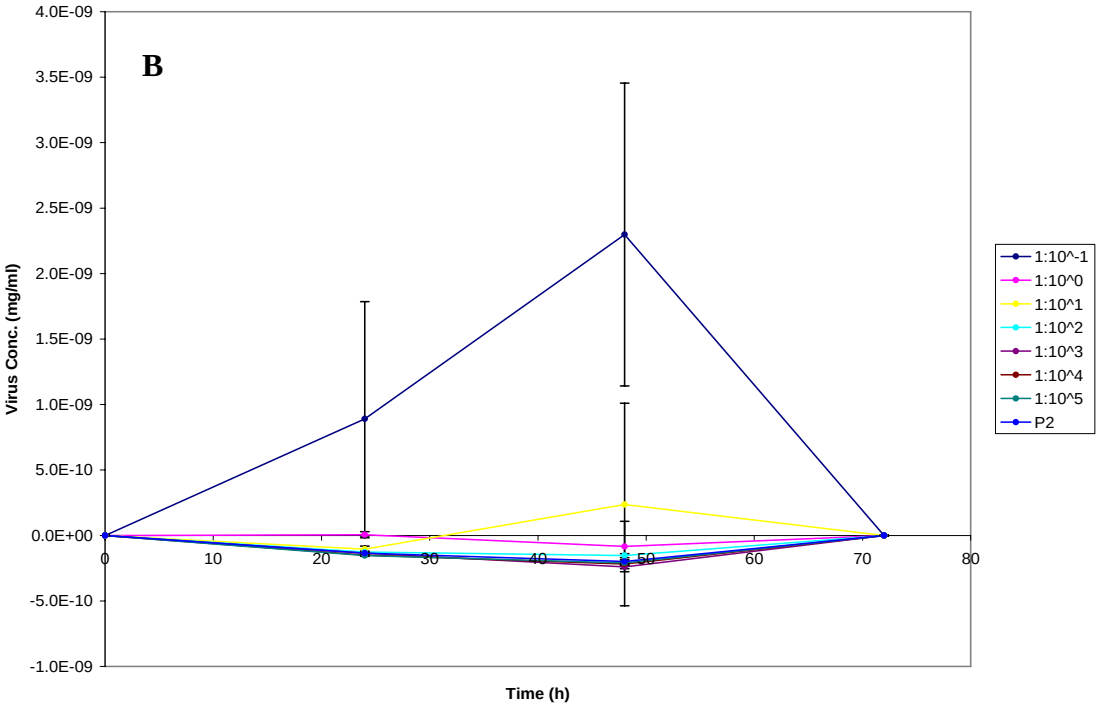


Figure B.3. MOI experiment 3.
P2 infected with various concentrations of STIV. A) Growth of cultures measure with optical density. B) and C) show the concentration of STIV over time measured with Q-PCR and ELISA, respectively

Experiment 4





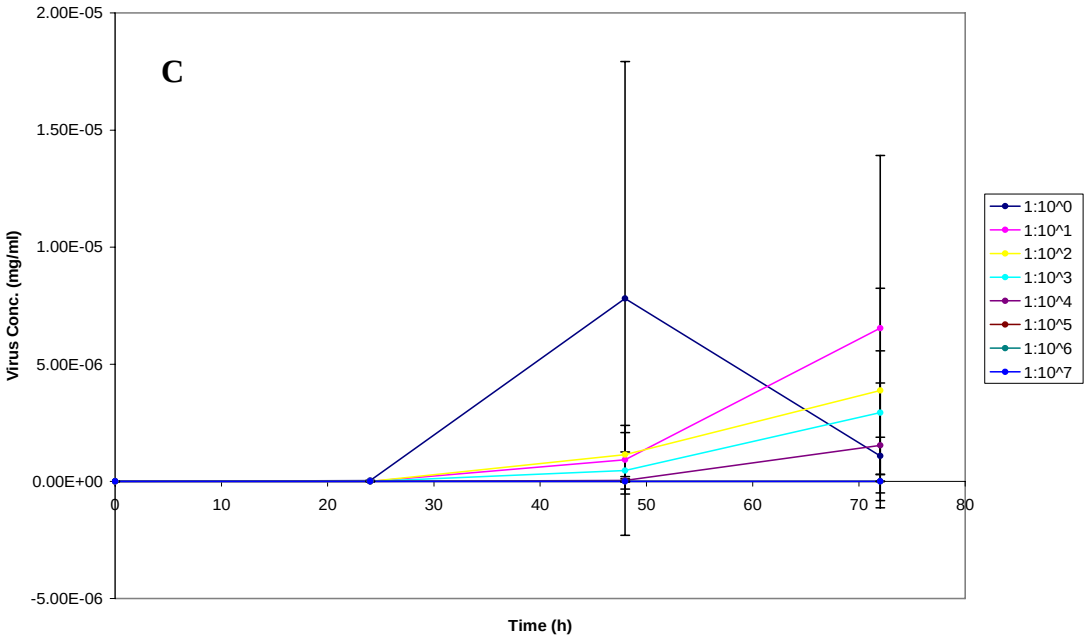
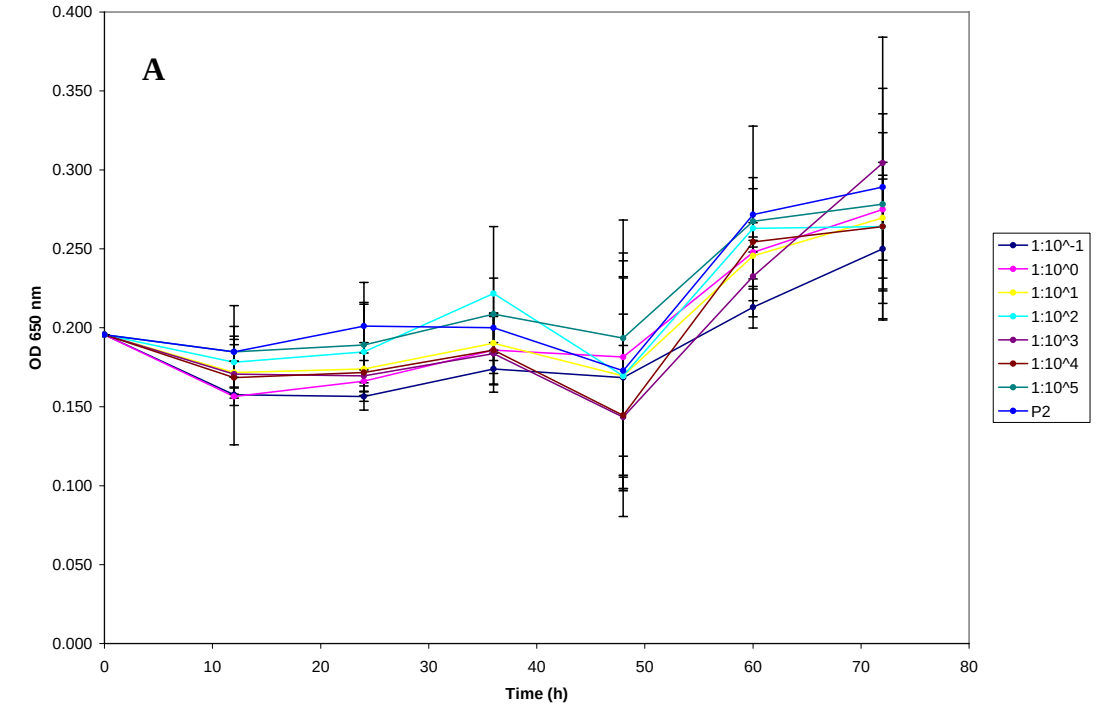
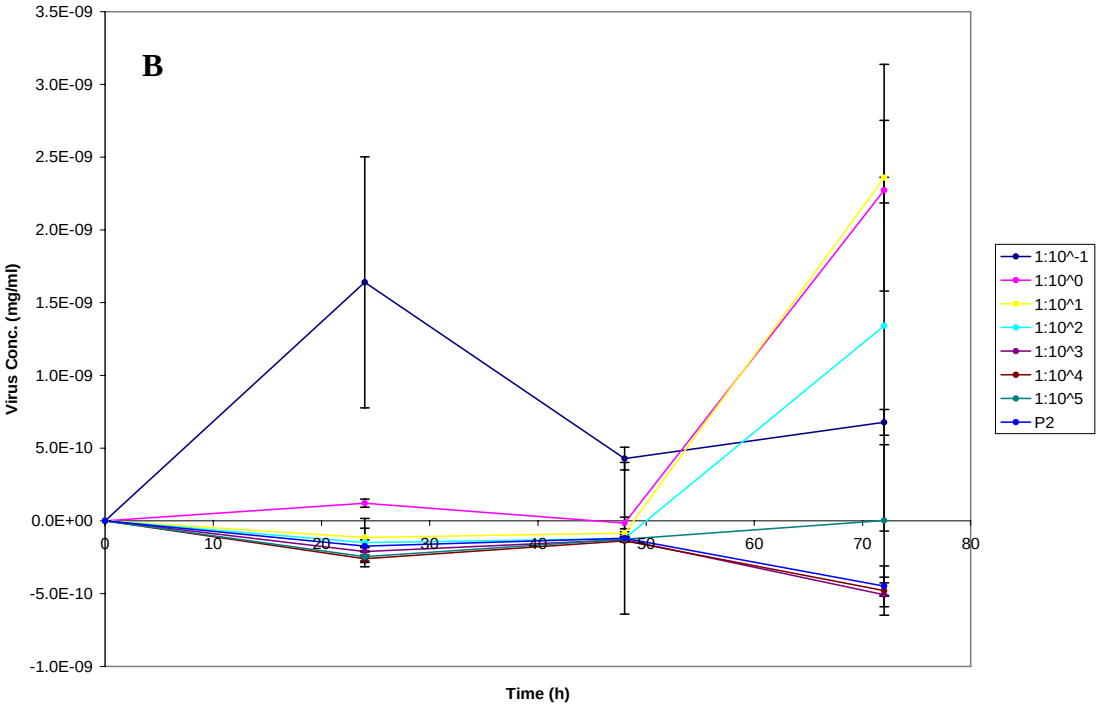


Figure B.4. MOI experiment 4.
P2 infected with various concentrations of STIV. A) Growth of cultures measure with optical density. B) and C) show the concentration of STIV over time measured with Q-PCR and ELISA, respectively

Experiment 5





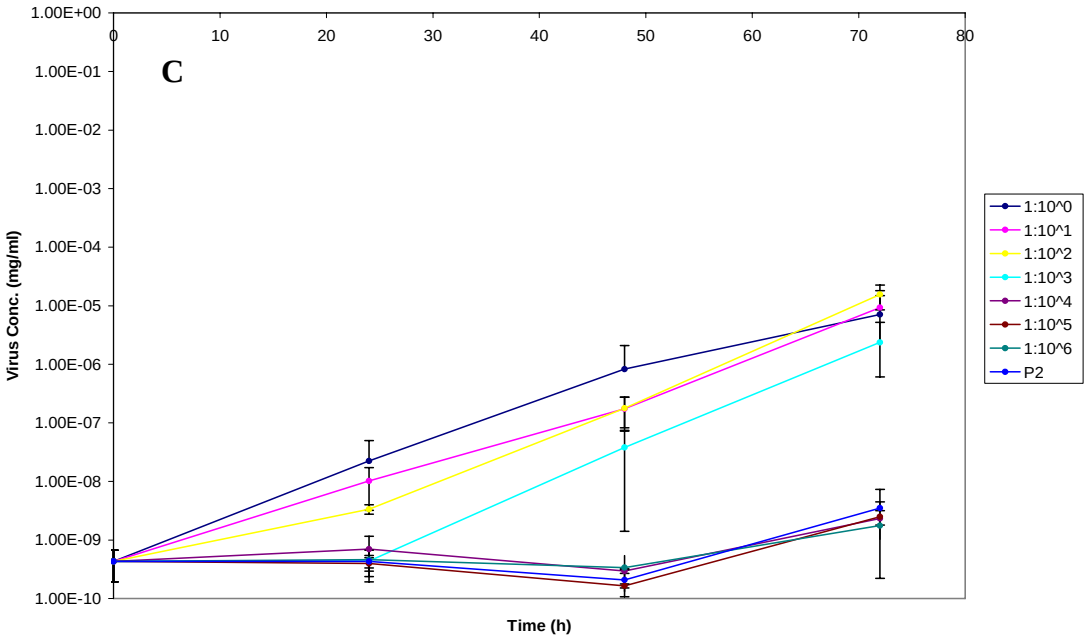
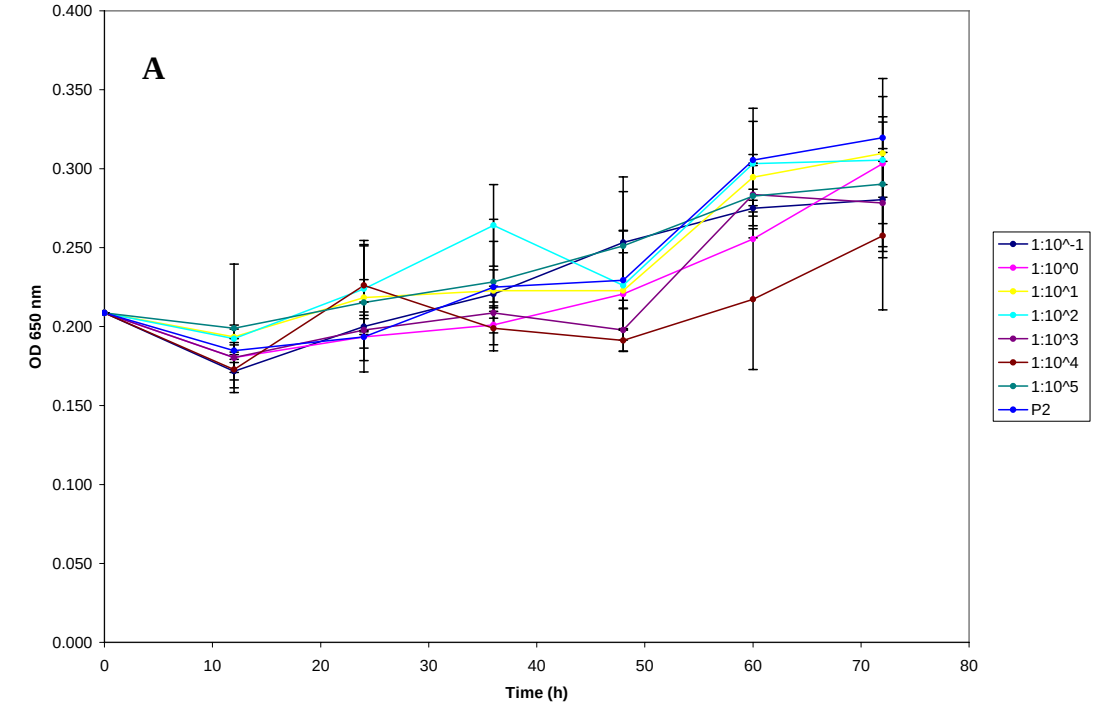
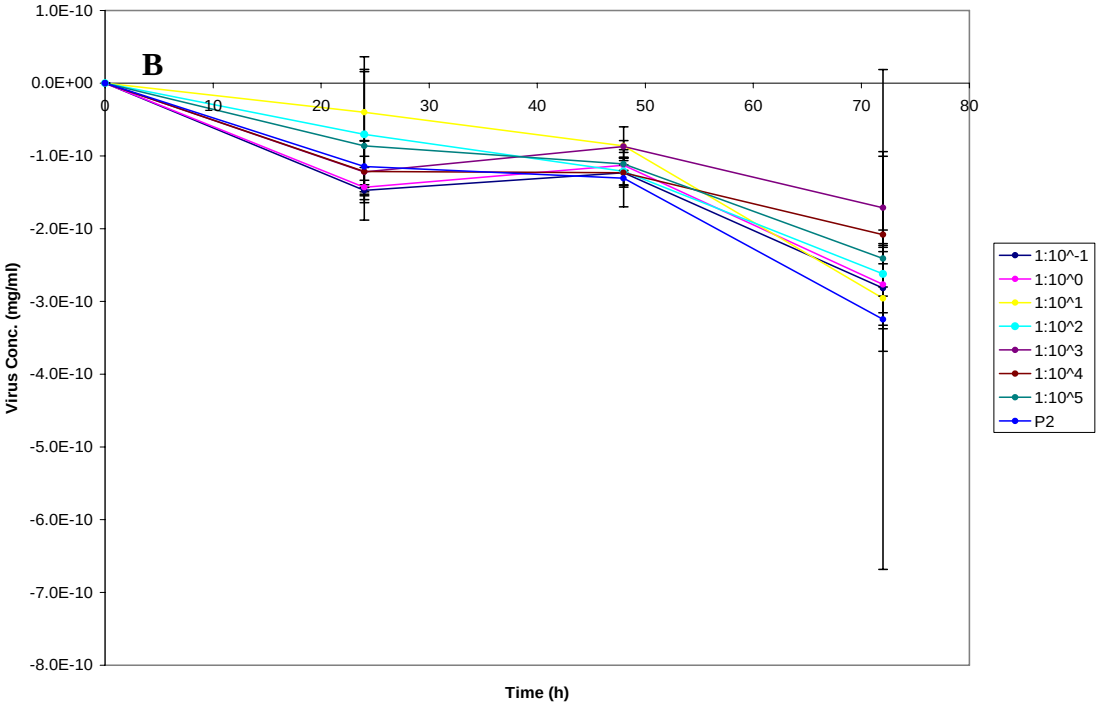


Figure B.5. MOI experiment 5.
P2 infected with various concentrations of STIV. A) Growth of cultures measure with optical density. B) and C) show the concentration of STIV over time measured with Q-PCR and ELISA, respectively

Experiment 6





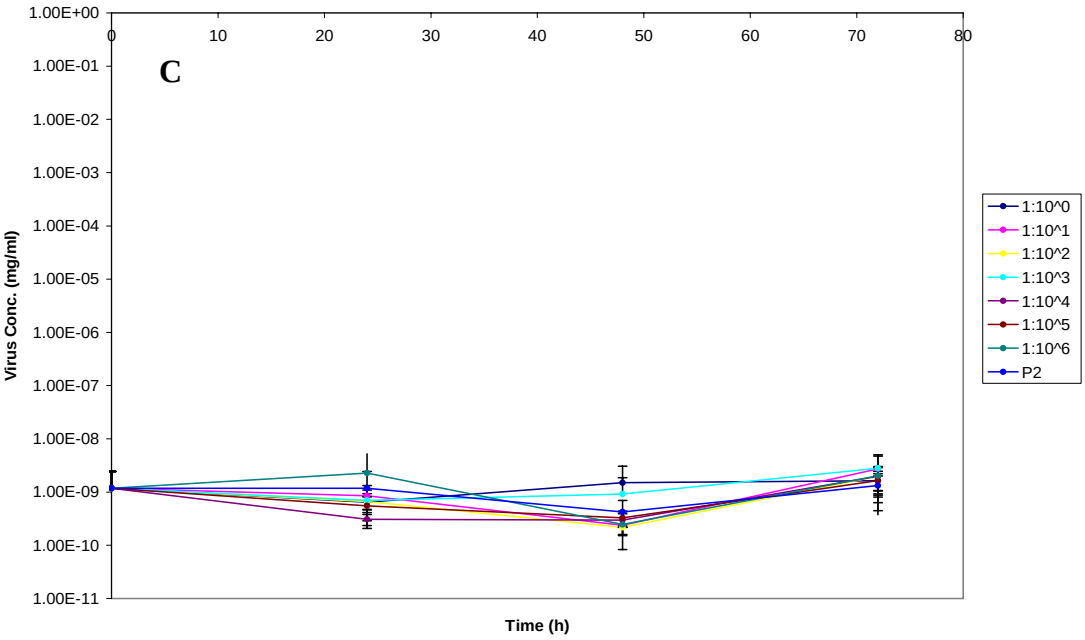
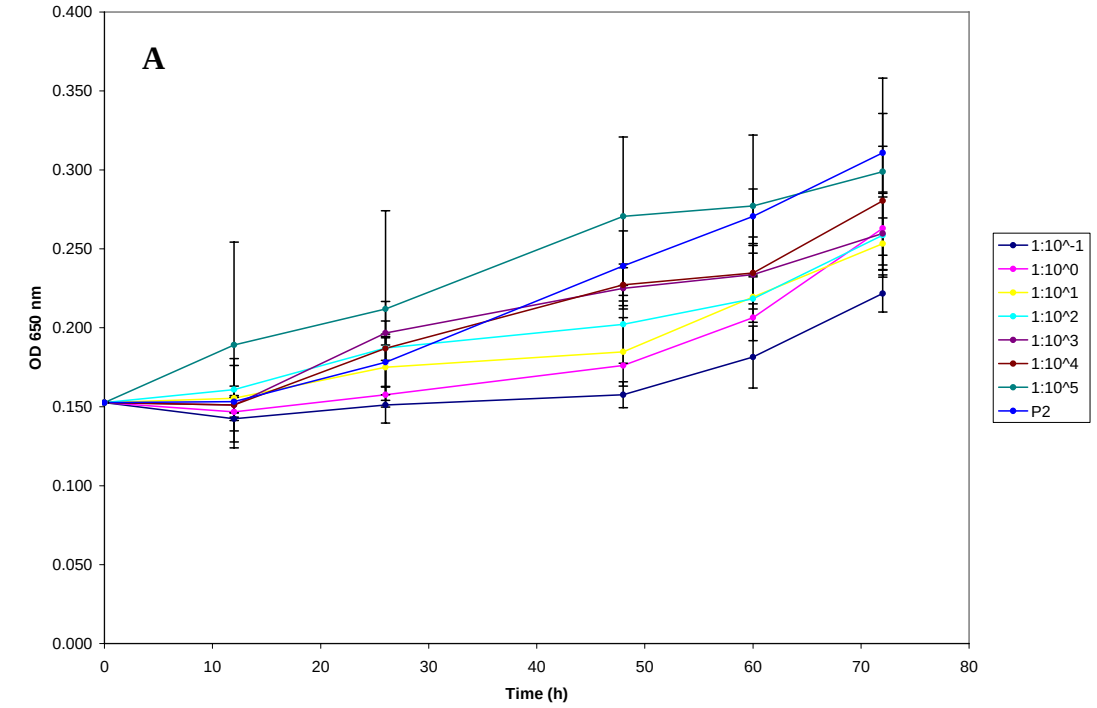
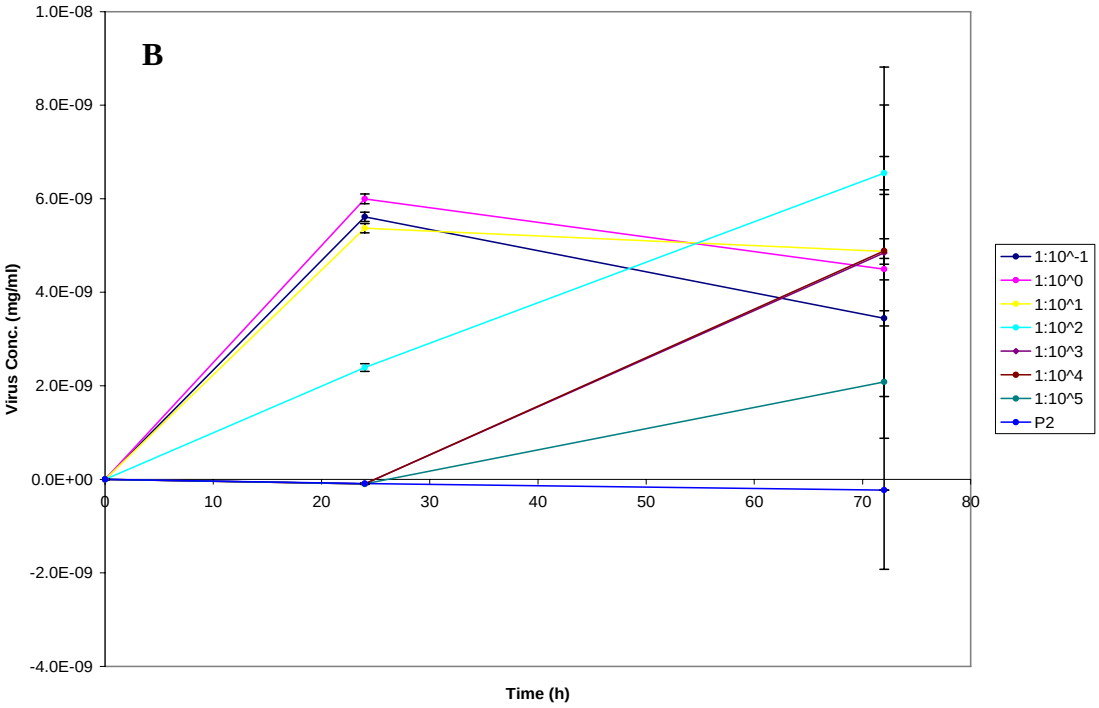


Figure B.6. MOI experiment 6.
P2 infected with various concentrations of STIV. A) Growth of cultures measure with optical density. B) and C) show the concentration of STIV over time measured with Q-PCR and ELISA, respectively

Experiment 7





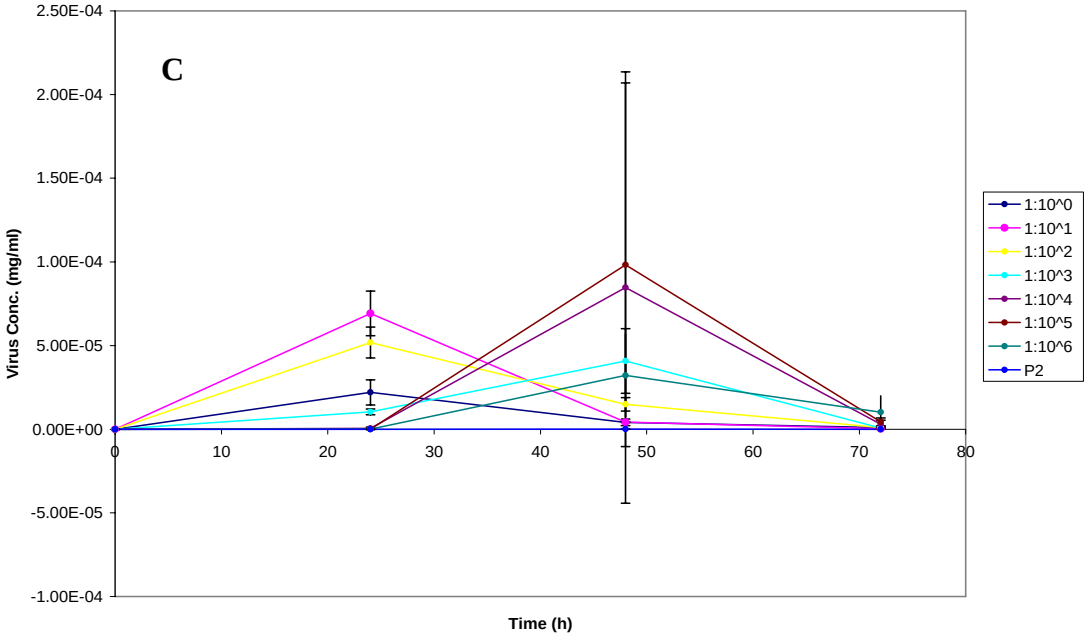
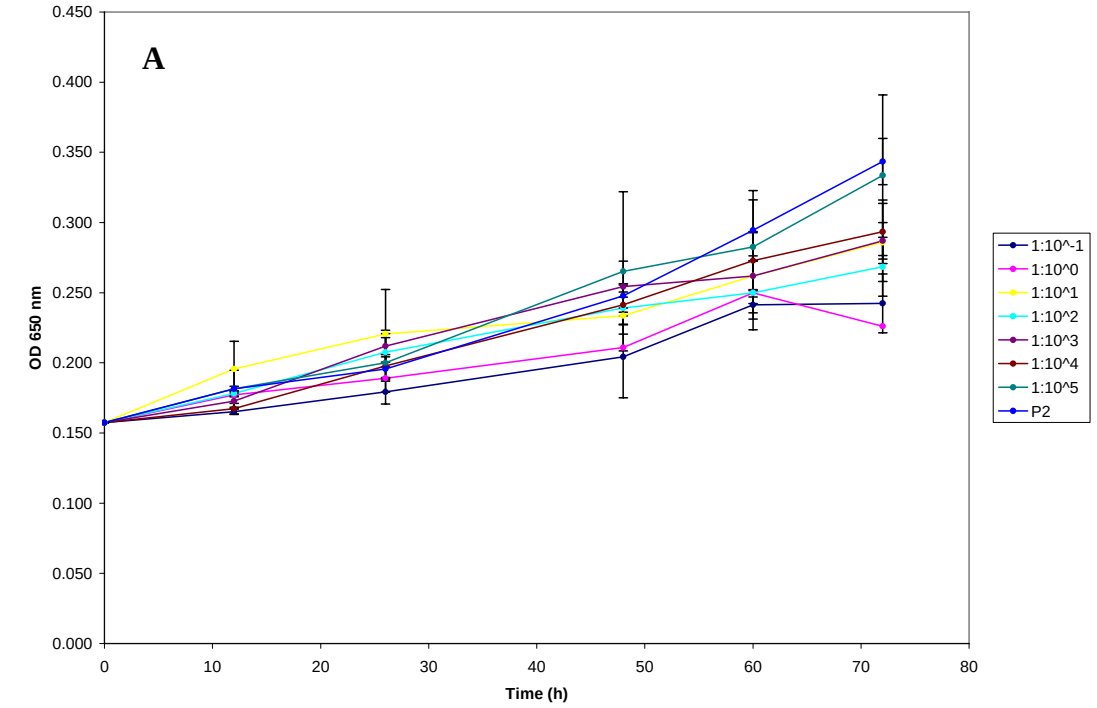
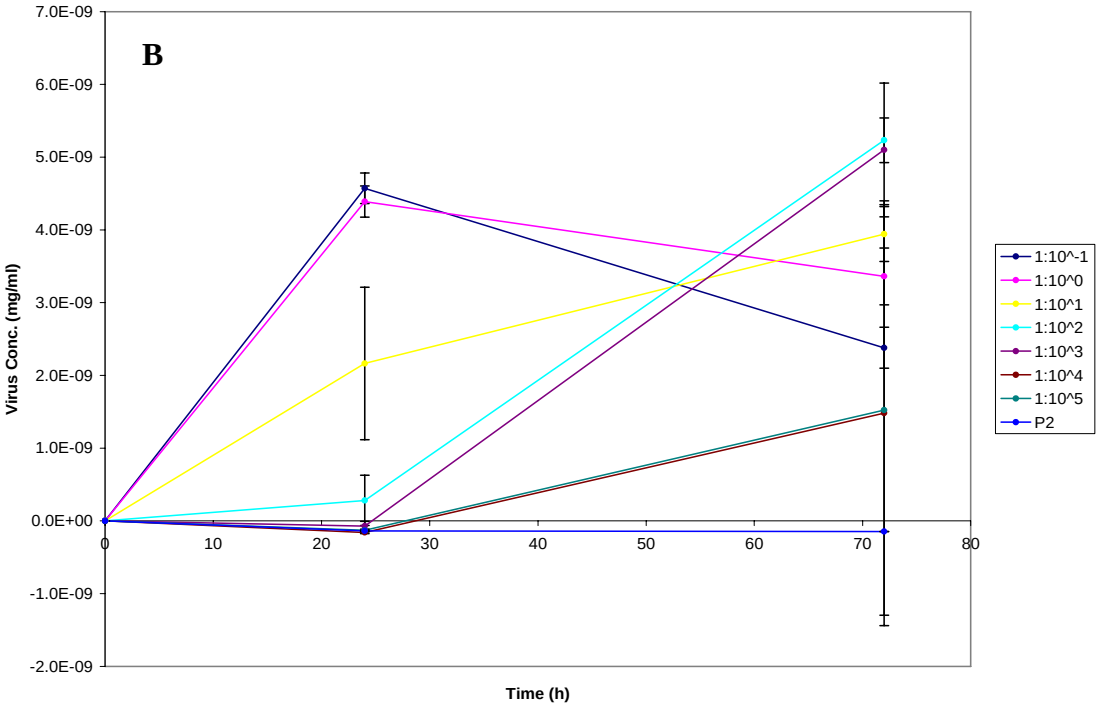


Figure B.7. MOI experiment 7.
P2 infected with various concentrations of STIV. A) Growth of cultures measure with optical density. B) and C) show the concentration of STIV over time measured with Q-PCR and ELISA, respectively

Experiment 8





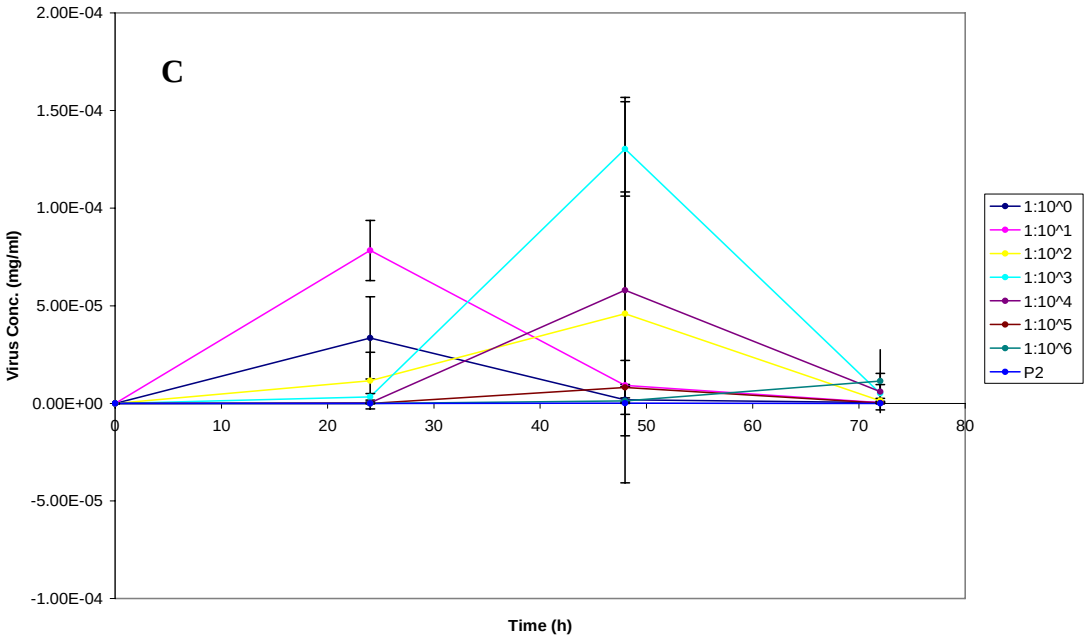


Figure B.8. MOI experiment 8.
P2 infected with various concentrations of STIV. A) Growth of cultures measure with optical density. B) and C) show the concentration of STIV over time measured with Q-PCR and ELISA, respectively

APPENDIX C

ELISA PROTOCOL

STIV ELISA

This protocol assumes that the concentration of the IgG is about 7 mg/ml.

Coating the plate

- Dilute the IgG 1:10,000 in **Coating Buffer** (all buffers listed after the protocol).
- Load 100 μL in each well of a 96 well plate.
- Cover the plate and incubate for 2h at 37°C.
- Wash three times with ~150 μL of **PBS** + 0.1% Tween (PBST). Leave wash in wells for 3 min each time.

When the plates have been coated and washed, they can either be used right away or saved frozen at -20°C.

Antigen

Make a standard curve from a sample where the virus concentration is known, 1-100 ng of virus pr. well is appropriate. Buffer used to make standard curve and to dilute samples in is PBST + 2% PVP + 0.2% Ovalbumin (4°C) that has to be made fresh right before use.

- Dilute your samples 1:1 in buffer to obtain the right pH; you will need about 400 μL of the final solution of each sample.
- Load standard curve and samples to plate, replicate of three, 100 μL per well. Avoid using the wells on the edges. If the whole plate is used 24 different samples can be tested at the same time.
- Cover the plate and incubate overnight at 4°C.
- Wash as before.

Conjugate

- Dilute the conjugate (IgG with enzyme linked to it) to 1:5000 with PBST + 2% PVP + 0.2% Ovalbumin (again make fresh before use).
- Load 100 μL into the wells that you used of the plate.
- Cover and incubate for 2h at 37°C.
- Wash as before.

Substrate

- Dissolve 1 mg/mL of substrate, p-nitrophenylphosphate (at room temperature) in **substrate buffer** (RT again). Load 100 μL in wells that were used.
- Take an OD reading at 405 nm every 30 min to 1 h (also at t = 0). The ELISA should be fully developed after about 2-5 hours when developed at room temperature.

Data analysis

- Take the average of the 3 samples and plot the standard curve. From the equation of the trend line, the amount of virus can be calculated (remembering that the samples were diluted).

Buffers used in the protocol

Coating Buffer

1.59 g Na_2CO_3

2.93 g $NaHCO_3$

0.2 g NaN_3

1L ddH2O

Measure the pH, it should be around 9.60. The buffer should be kept at 4°C.

PBS

~800 mL dH_2O

8 g NaCl

0.2 g KCl

1.44 g Na_2HPO_4

0.24 g KH_2PO_4

Set the pH to 7.4 w/conc. HCl

Dilute to 1L, then autoclave.

Substrate Buffer

6.08 g 85% diethanolamine (1.45 M)

0.01 g NaN_3 (3.8 mM)

40 mL ddH_2O

Set to pH 9.8 w/conc. HCl.

The substrate buffer is stable at 4°C for about a week, but should be at RT when used.