Engineering a Tp0751-based Vaccine Scaffold: Applying Iterative Protein Design to Optimize Antigen Presentation

by

Lexie Thompson

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I acknowledge and respect the lək̓ʷəŋən peoples on whose traditional territory the university stands and the Songhees, Esquimalt and W̱SÁNEĆ peoples whose historical relationships with the land continue to this day.

Supervisory Committee

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Dr. Martin Boulanger, Department of Biochemistry and Microbiology
Co-Supervisor

Dr. Caroline Cameron, Department of Biochemistry and Microbiology
Co-Supervisor

Dr. Jeremy Wulff, Department of Chemistry
Outside Member

Abstract

Treponema pallidum subspecies *pallidum* is the causative organism of syphilis, a widespread sexually transmitted infection and re-emerging public health threat. The continued resurgence of syphilis, despite the discovery of effective penicillin treatment, highlights the need for preventative and affordable syphilis control. Previous efforts to develop a protective vaccine candidate have been hindered due to the limited discovery of protective antigens and the paucity of outer membrane proteins on the bacterial surface. A majority of the currently identified vaccine candidates are integral outer membrane proteins that, due to their highly insoluble nature, cannot be recombinantly produced in a properly folded and soluble state. Our focus is expressing the immunogenic epitopes from these large integral membrane proteins in a more sophisticated expression platform, allowing us to bypass the expression of these full-length proteins. Our designs focus on using Tp0751, a *T. pallidum* adhesin protein, as a scaffold on which to build our recombinant vaccine candidate. These studies aimed to engraft epitopes from a select few of these integral outer membrane proteins into the flexible loop and termini regions of Tp0751 to yield a stable and soluble candidate capable of recapitulating the immune response raised against each separate protein. Here we have successfully shown that not only is Tp0751 amenable to this engineering with up to four separate epitope engraftments, but that a lead chimeric candidate offers partial protection in a rabbit model. Altogether, the body of work summarized in this thesis highlights the success of Tp0751 as a vaccine platform and opens new avenues for future recombinant vaccine production.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Meaning
Ab	Antibody
CNS	Central nervous system
B. burgdorferi	*Borrelia burgdorferi*
CCAC	Canadian Council on Animal Care
CDC	Centres for Disease Control
CWS	Cell wall skeleton
DTH	Delayed type hypersensitivity
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
EU	Endotoxin unit
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HUVEC	Human umbilical vein endothelial cell
LAL	Limulus amebocyte lysate
LB	Lysogeny broth
MPL	Monophosphoryl lipid A
MS	Mass Spectrometry
MSM	Men who have sex with men
MW	Molecular weight
N. meningitidis	*Neisseria meningitidis*
NHBA	Neisserial Heparin Binding Antigen
NK	Natural Killer
NmfHbp	*Neisseria meningitidis* factor H binding protein
NRS	Normal rabbit serum
OMP	Outer membrane protein
PAMP	Pathogen-associated molecular pattern
PC	Post-challenge
PrEP	Pre-exposure prophylaxis
qPCR	Quantitative polymerase chain reaction
RIT	Rabbit infectivity test
SEC	Size-exclusion chromatography
SPUD	*Solanum tuberosum phyB* gene
T. pallidum	*Treponema pallidum*
TDM	Tetrahalose 6,6’-dimycolate
Tpr	*T. pallidum* repeat
UW	University of Washington
V regions	Variable regions
WT	Wild type

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Chapter 1: Introduction

1.1 Syphilis – a prominent global health concern

Syphilis is a chronic multi-stage sexually transmitted infection caused by *Treponema pallidum* subsp. *pallidum,* a Gram-negative-like spirochete bacterium. Despite continual sensitivity to penicillin, the global prevalence of the infection remains a public health concern for which no prophylactic vaccine is available (1).

1.1.1 Epidemiology

Syphilis is a widespread global health concern and research priority due to an estimated global burden of 50 million prevalent cases and 14 million new infections yearly (2). Though the infection remains endemic in most countries of Africa and Latin America, cases have risen dramatically in Canada, the United States, Europe, Australia, and China throughout the past decade (3–7). Between 2014 and 2019, the U.S. saw a 74% increase in primary and secondary syphilis cases and a 279% increase in the incidence of congenital syphilis (8). This resurgence has also been seen in Canada, where there were 9,126 cases reported in 2020, representing a 136% increase from 2016 (9). Infection rates for females of reproductive age in Canada increased by 740% between 2016 to 2020, resulting in a 1,150% increase in congenital syphilis rates over the same period (9). On a global scale, there are an estimated 1.4 million cases of syphilis among pregnant people each year, resulting in an estimated 500,000 adverse pregnancy outcomes such as low birth weight, premature delivery, stillbirth, and neonatal death (10, 11); consequently, syphilis remains the second leading cause of stillbirth worldwide (12). Based on the widespread resurgence of syphilis, it is apparent that current treatment and screening protocols are insufficient in controlling the spread of the disease, highlighting the need for preventative syphilis control.

1.1.2 Course of Infection

Syphilis is a chronic, multi-stage disease interspersed with periods of activity and latency, all of which can have diverse clinical manifestations (13). Infection is acquired by direct contact, usually sexual, with an active primary or secondary lesion, and vertically between a pregnant person and a developing fetus. Historical research shows that 16-30%

of people who have had sexual contact with an infected individual show signs of infection within 30 days (14, 15), though transmission rates may be much higher (16–18).

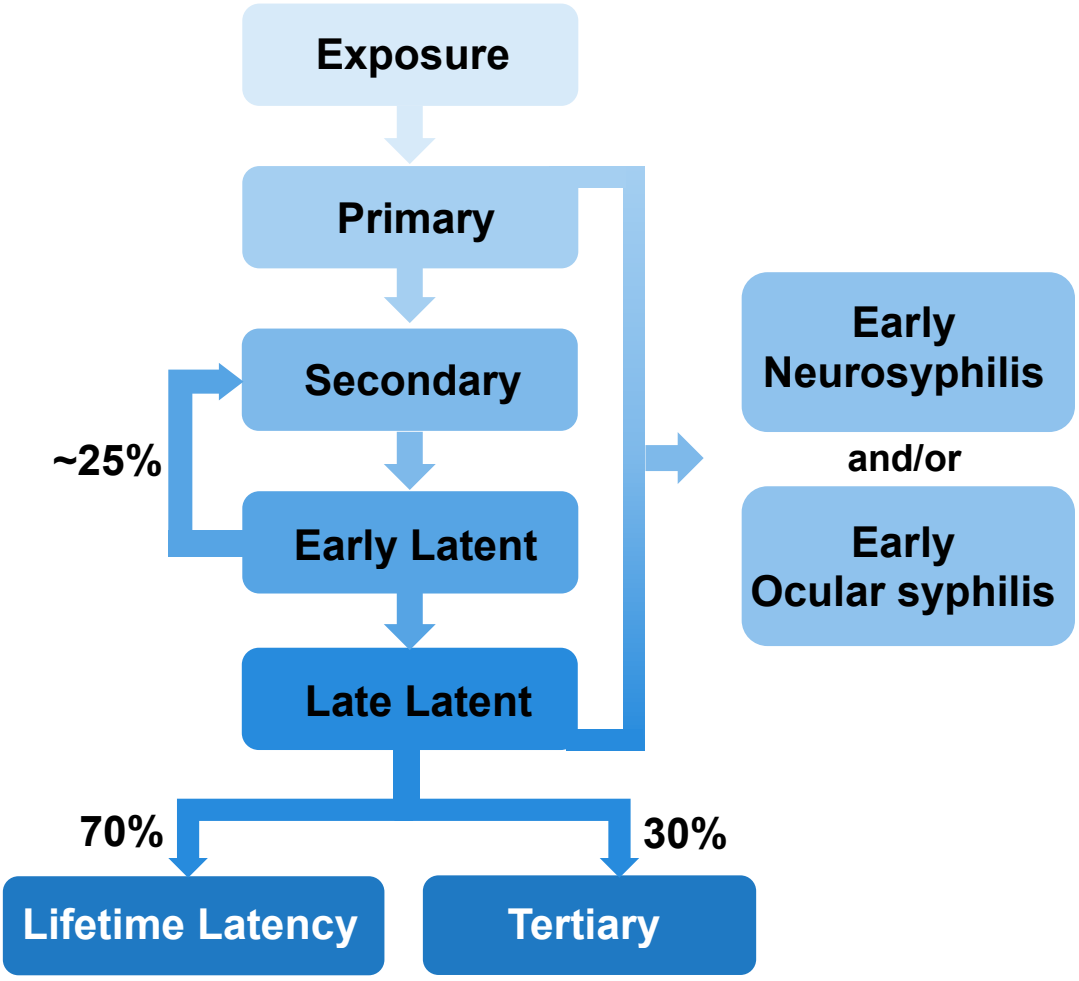


Figure 1. Disease progression of untreated syphilis. Following exposure, infected individuals will enter early syphilis, encompassing primary, secondary, and early latent (<2 years post-infection) disease stages. During early latency, up to 25% of individuals will re-enter symptomatic secondary syphilis. Following early latency, infected individuals will enter late latency (>2 years post-infection), where 70% of individuals will remain in latent disease for life. 30% of individuals will then progress to tertiary syphilis. At any point during disease progression, individuals can experience early ocular or early neurosyphilis by central nervous system (CNS) involvement.

1.1.2.1 Primary Syphilis

Initial infection occurs following *T. pallidum* penetration of mucous membrane or dermal abrasion, leading to an infectious chancre at the initial site of infection. These chancres are painless and can be located in inconspicuous sites, often delaying diagnosis until later disease manifestations appear. These chancres develop approximately three weeks following initial exposure, with higher inoculum doses resulting in more rapid development (19).

1.1.2.2 Secondary Syphilis

Within hours of initial primary infection, *T. pallidum* disseminates widely through the bloodstream, establishing infection in various distal tissues. This systemic dissemination leads to secondary syphilis manifestations, which generally occur within three months following the initial infection. Some manifestations, such as muscle aches, sore throat, and weight loss, represent the flu-like symptoms common during this stage of the disease (20). The migration of treponemes to the epithelium often results in a disseminated mucocutaneous rash commonly found on the palms and soles of the feet, which are the most common symptoms of this disease stage (20). These manifestations typically resolve within three months of their initial appearance, and the infection then enters a latency period for variable amounts of time, depending on the individual.

1.1.2.3 Central nervous system involvement

Historically, central nervous system (CNS) involvement has been associated with the tertiary stage of disease. However, more recent studies have shown that CNS penetration occurs in primary and secondary disease stages (21, 22). The symptoms of neurosyphilis stemming from CNS involvement may occur as early as primary infection, with common symptoms of fever, headache, nausea, vomiting, and neck stiffness. Involvement of the cranial nerve may result in visual abnormalities, as well as potential hearing loss and facial weakness (23). Roughly 40% of early syphilis patients and 25% of those with latent infection meet the criteria for neurosyphilis, which is increased protein and leukocyte levels in cerebrospinal fluid (21, 22). Most CNS invasion cases resolve on their own and clear the CNS treponemes, however, there are currently no known indicators for the development of symptomatic neurosyphilis (13).

1.1.2.4 Latent Syphilis

Following secondary syphilis, the asymptomatic latency period is divided into two stages based on the time of initial infection. Early latency is within the first year of infection, where up to 25% of individuals may have recurrent secondary manifestations (24). Late latency is defined as an asymptomatic infection lasting longer than one year. Sexual transmission during late latency is unlikely, though it is still possible to infect a developing fetus, resulting in congenital syphilis.

1.1.2.5 Tertiary Syphilis

Currently, late manifestations of syphilis are uncommon due to coincidental antibiotic therapy. In the pre-antibiotic era, however, the Oslo and Tuskegee studies have shed light on the disease progression and manifestations in late-stage tertiary syphilis cases (24, 25). In these studies, approximately 1/3 of individuals with latent syphilis developed clinical manifestations of tertiary syphilis, though these symptoms often appeared 20-40 years after initial infection. These serious symptoms appear in the forms of gummatous lesions, cardiovascular syphilis, and late neurosyphilis.

It is imperative to note that the Tuskegee study was morally and ethically abhorrent. The study involved 600 black men – 399 with syphilis and 201 without. The doctors and researchers conducting the study failed to disclose infection status to participants, failed to inform them that they were subjects of study, and failed to obtain informed consent. Researchers recruited participants under false pretenses of being treated for “bad blood”, a term coined for several ailments at the time. The study began in 1932 and concluded in 1972 after a recommendation by an Ad Hoc Advisory panel. By 1943, penicillin was used widely for syphilis treatment, but unfortunately, this treatment was purposefully kept from participants for the duration of the study. Despite the knowledge of tertiary disease manifestations obtained from this study, the moral and ethical implications far out shadow the scientific gain.

1.1.2.6 Congenital Syphilis

At any point during pregnancy, *T. pallidum* can be transmitted to the fetus through the placental barrier, though the risk of fetal transmission is much higher during the first

year of infection (26). Antibiotic treatment during the first two trimesters is generally sufficient in preventing negative fetal outcomes; however, lack of treatment can result in adverse outcomes such as low birth weight, premature delivery, neonatal death, or stillbirth (27–29). Soon after delivery, up to 4% of neonates will die due to pulmonary hemorrhage, secondary bacterial infection, and hepatitis (28, 29).

For the first two years after birth, symptoms similar to those seen in adult secondary syphilis, such as disseminated rash, gummatous lesions, and rhinitis can occur, usually becoming apparent within ten weeks of delivery. Beyond these two years, clinical manifestations generally worsen. Symptoms such as blindness from interstitial keratitis, neurosyphilis, deafness, and bone and tooth deformities represent the most common late manifestations (30).

1.1.3 Difficulties in treating and diagnosing syphilis

Despite penicillin remaining a highly effective treatment with no cases of resistance in the nearly 80 years since its discovery, syphilis remains a very prevalent global health threat. In high-income settings, the burden is most prominent among the men who have sex with men (MSM) population (4, 31). This asymmetry in syphilis infections is a cause for concern, being that syphilis infections can cause an estimated three-fold increase in human immunodeficiency virus (HIV) transmission and acquisition (32–36). Thankfully, the growing use of preventative therapies such as pre-exposure prophylaxis (PrEP), a daily anti-retroviral drug, has shown to be highly effective in reducing the disease incidence of HIV among high-risk MSM populations (37). However, the growing use of these preventative therapies, as well as non-adherence to condom use, are predicted to be contributing to the increasing rates of other sexually transmitted infections (STIs), such as syphilis. A 2016 meta-analysis found that individuals taking PrEP were 44.6 times more likely to acquire syphilis than those not taking PrEP (38). Similarly, previous research also found that in the U.S., 50% of patients were diagnosed with an STI within 12 months of beginning PrEP usage (39, 40).

In low- and middle-income settings, a major contributing factor to the incidence of syphilis infection is the limited access to screening and treatment. Historically, diagnostic treatment required that testing be done offsite due to the requirement of specialized

equipment, causing a delay in diagnosis and treatment (41, 42). More recently, the development of improved point-of-care testing has ameliorated diagnosis and testing and has helped to reduce the rates of congenital syphilis due to the advent of antenatal screening (1, 43).

Current treatment guidelines from the U.S. Centres for Disease Control and Prevention (CDC) recommend parenterally administered penicillin G to treat all stages of syphilis infections, including infected pregnant individuals (44). The treatment regimen varies depending on disease progression and clinical manifestation, with patients in latent and tertiary stages of the disease undergoing a more prolonged penicillin G treatment compared to those in primary and secondary stages. This prolonged treatment regimen and the necessity for the penicillin G to be injected can pose barriers to those in areas of the world with limited and infrequent access to healthcare. In addition to these barriers, manufacturing of penicillin is very inexpensive, causing many manufacturers to halt production due to limited profit margins. A recent study outlining global shortages reported that 39/91 surveyed countries faced penicillin G shortages, including six high-income countries. Eighteen of these countries reported having no stock at all (45). Given that penicillin is the only antibiotic currently known to safely treat congenital syphilis, worldwide and equitable access to this antibiotic is crucial for the global reduction of congenital syphilis (46).

Current syphilis diagnostic assays have very low sensitivity for detecting early primary stages of infections, causing concern as this stage of infection is the most highly infectious, and poses the highest risk for vertical transmission of *T. pallidum* during pregnancy (47–49). In addition to this difficulty, primary chancres are often found in inconspicuous places on the body, such as the cervix, labia, anal canal, rectum, and oral cavity. This challenge, in combination with the painless nature of the lesion, often allows the initial stage of infection to go unnoticed, undiagnosed, and untreated (13, 50).

1.1.4 The need for a syphilis vaccine

Despite showing no signs of penicillin resistance over the past 75 years of continual use, we continue to see the spread of infection worldwide, indicating that our current methods for treatment and prevention are insufficient to control the spread of disease. Great

efforts have been made with public health control initiatives, including the CDC's 1999 National Plans to Eliminate Syphilis from the United States and the 2006 Syphilis Elimination Plan (51, 52). These initiatives were crucial in raising awareness amongst the public and healthcare providers, as well as increasing funding and services for controlling infection spread (51, 52). Despite the success of these initiatives, we continue to see a rise in cases worldwide, highlighting the need for complementary preventative biomedical interventions, such as vaccines. A protective vaccine would aid in overcoming many of the current challenges faced by public-health targeted syphilis control, including the difficulties associated with clinical diagnoses.

1.2 *Treponema pallidum* subspecies *pallidum*

1.2.1 *T. pallidum* biology

T. pallidum belongs to a family of spiral-shaped bacteria known as *Spirochaetaceae*, the same family as the well-known bacterium *Borrelia burgdorferi*, the causative agent of Lyme disease, and *Leptospira interrogans*, the causative agent of leptospirosis. *T. pallidum* varies from 6 to 16 µm in length and 0.2 µm in diameter. The bacterium's body is surrounded by a cytoplasmic membrane, enclosed by a loosely associated and fragile outer membrane. Endoflagella, the organelles responsible for its corkscrew motility, are located below the outer membrane in the periplasmic space (53).

Despite our longstanding knowledge of *T. pallidum*, efforts to uncover molecular mechanisms of pathogenesis have been hindered due to its historical inability to be cultured *in vitro*. The inability to culture *T. pallidum in vitro* has restricted research solely to those organisms propagated *in vivo* within outbred rabbits, creating ethical and economic implications for continued research (54). Due to the bacteria's inability to be cultured, the field has been unable to develop the commonly used genetic engineering tools employed in the research of most pathogens. This difficulty has led to a very limited understanding of the virulence factors and molecular mechanisms behind *T. pallidum*'s pathogenicity. Researchers have spent decades investigating cell culture optimization (55–61), which has recently led to long-term *in vitro* culture using rabbit epithelial cells (62). This breakthrough led to the first demonstration of successful genetic engineering, paving the way for future research (63).

1.2.1.1 The limited genome

By establishing residence in the relatively homeostatic and rich environment of its host, *T. pallidum* has been able to dispense the genes required for *de novo* synthesis of fatty acids, amino acids, and nucleotides, as well as genes involved in the TCA cycle and oxidative phosphorylation (64–66). This process has resulted in a genome of only 1.14 Mb, one of the smallest genomes known of any pathogenic bacteria, encoding only 1,041 putative protein genes (64). Comparatively, the genome of the well-known bacterium *Escherichia coli* K-12 is 4.6 Mb, several times larger.

1.2.2. Systemic dissemination

Systemic dissemination is a strategy many pathogens use to invade distal tissues, and *T. pallidum*'s ability for systemic dissemination is central to disease progression. The bacteria rapidly enter the bloodstream within minutes of initial infection, allowing widespread infection to distal tissue and organs (67–70). This systemic dissemination is evidenced by the numerous disease manifestations that can occur in later stages of the disease, such as disseminated lesions in secondary syphilis, gummatous lesions, cardiovascular complications, and bone destruction during tertiary syphilis (13, 20, 24, 25, 71–76).

Outer membrane proteins such as Tp0751, Tp0155, and Tp0483 mediate treponemal attachment to endothelial cells through interactions with various components of the host vasculature such as laminin, fibrinogen, and fibronectin (77–82). It is speculated that these interactions are implicated in basement membrane adhesion, which could facilitate pathogen-host attachment through bridged integrin interactions, a common strategy for pathogen-host cell adhesion (77, 79, 81, 83).

1.2.3 Macrophage-dependent opsonophagocytosis of *T. pallidum* is critical for the clearance of syphilis lesions

Initial suggestions for the importance of macrophage-dependent immune clearance were mentioned following reports where both intact and degraded *T. pallidum* were found inside phagocytic vacuoles of macrophages (53, 84). The presence of IFNγ and macrophages in both primary and secondary syphilis lesions is consistent with numerous

reports in the experimental rabbit model suggesting that IFNγ-activated macrophages play a key role in *T. pallidum* clearance (85–90). It has since been shown that *in vitro* macrophage-dependent phagocytosis of *T. pallidum* increases when organisms are preincubated with serum from rabbits infected with *T. pallidum*, indicating that opsonization plays a key role in phagocytosis (91, 92). Previous research has highlighted that this opsonization is accomplished by both immunoglobulin G (IgG) and immunoglobulin M (IgM), showing that antibody-mediated immune responses are required for effective lesion clearance (93, 94).

1.2.4 Both T- and B- cell-driven immune responses are imperative for protection against *T. pallidum*

During syphilis infection, the resolution of primary and secondary lesions is associated with T-cell and macrophage infiltration (88, 95–99). Primary lesions in humans predominantly contain CD4+ T-cells, macrophages, and natural killer (NK) cells, whereas secondary lesions in humans contain a higher abundance of CD8+ T-cells (99, 100). It has also been shown that a robust delayed-type hypersensitivity (DTH) response is imperative in the clearance of *T. pallidum* from lesion sites (101). The DTH response is typified as a beneficial cell-mediated response involving the expansion of antigen-specific T-cell populations, producing cytokines to recruit and activate additional lymphocytes and macrophages (102). This type of response requires the production of Th1 cytokines such as IFN-gamma, IL-2, and IL-12 (90, 97, 103–106). Opsonophagocytosis mediated by the activated macrophages is thought to be the major mechanism of *T. pallidum* clearance in lesion sites (91, 100, 104, 107).

Though these cell-mediated responses are important in the hosts' ability to clear the infection, it is clear that the humoral response plays a critical role as well. It has been shown that opsonophagocytosis of *T. pallidum* by macrophages is dependent upon the presence of immune serum, highlighting the importance of an effective humoral response to complement cell-mediated immunity (104, 107).

1.2.5 Immune evasion and persistence - The stealth nature of *T. pallidum*

Despite its sensitivity to environmental factors, *T. pallidum* is remarkably invasive and causes chronic infections in its host, displaying exquisite adaptation to its niche. Interestingly, the *T. pallidum* genome lacks classical virulence factors that could account for the observed clinical manifestations of syphilis. Though *T. pallidum* is known as a Gram-negative-like bacterium, it completely lacks any lipopolysaccharide layer characteristic of Gram-negative bacteria (64). Furthermore, though the bacterium does have many lipoproteins capable of activating dendritic cells and macrophages, they are generally found below the cell surface, and are thereby hidden from most circulating immune cells (108, 109). This paucity of pathogen-associated molecular patterns (PAMPs) allows the treponemes to disseminate virtually undetected by the innate immune system, leading to the lack of inflammatory symptoms characteristic of syphilis (110).

T. pallidum disseminates to distal sites throughout the host, including immune-privileged sites such as the central nervous system and eyes, where innate immune surveillance occurs less frequently. This strategy may allow the bacteria to replicate slowly and periodically reinfect other tissues throughout disease progression. By maintaining cell density below a critical antigen mass, *T. pallidum* may survive undetected by failing to trigger a significant host immune response (111, 112).

1.2.5.1 Scarcity of outer membrane proteins

Studying the surface of *T. pallidum* has historically been a very arduous process (109, 113). Demonstrating the sparsity of outer membrane antigens, freeze-fracture and scanning probe microscopy analyses demonstrate that the surface of *T. pallidum* contains approximately 100-fold fewer outer membrane proteins (OMPs) than that of *E. coli* (114, 115). Though much is still to be learned about how *T. pallidum* accomplishes this immune evasion, studies have shown that surface reactive antibodies are generated against *T. pallidum* during infection (116, 117). However, due to the scarcity of OMPs as well as antigenic variation, *T. pallidum* evades high titers of circulating anti-treponemal antibodies. Consequently, the few surface-exposed proteins are believed to be key candidates for vaccine-based research (13, 118–120). Interestingly, opsonophagocytosis and immunolabelling assays have revealed that populations of *T. pallidum* harvested from an

active infection are heterogenous, with sub-populations of both antibody-reactive and non-reactive bacteria (100, 121, 122). This heterogeneity may explain the observation that both clearance and persistence of treponemes occur simultaneously (100).

There are several potential protein candidates responsible for the observed difference in surface antigenicity, primarily within the *T. pallidum* repeat (Tpr) family of proteins, though there are a select few where sufficient research has been conducted. For example, antibodies against both BamA and TprC, two rare outer membrane proteins, are raised primarily against the proteins' periplasmic domains (123–125). For the remaining opsonic antibodies raised against surface-exposed epitopes, the limited copy number of these antigens on the bacterial surface may severely hinder their effectiveness (125, 126).

1.3 *T. pallidum* repeat (Tpr) proteins are key targets for vaccine development

Several candidate OMPs belong to the Tpr gene family, which encodes 12 proteins divided into three subfamilies, Tpr subfamily I, II, and III. TprC, TprD, TprF, and TprI belong to subfamily I; TprE, TprG, and TprJ belong to subfamily II; and TprA, TprB, TprH, and TprK belong to subfamily III. Many of these proteins are predicted to be outer membrane localized, with subfamily I and TprK predicted to have cleavable signal peptides (77, 127). The Tpr proteins in *T. pallidum* are homologous to the outer membrane localized sheath protein in *Treponema denticola*, which has adhesive and porin functions (128, 129). These integral OMPs are large β-barrel containing proteins consisting of antiparallel β-strands connected by flexible loop regions protruding from either the cell surface or into the periplasmic space (130). The Tpr proteins have been of great interest for vaccine design as many of these proteins induce an immune response during syphilitic infection (131–133). Because initial infection clearance is achieved through macrophage-dependent opsonophagocytosis, identifying surface-exposed epitopes to induce these opsonic antibodies is integral for vaccine development (91, 107).

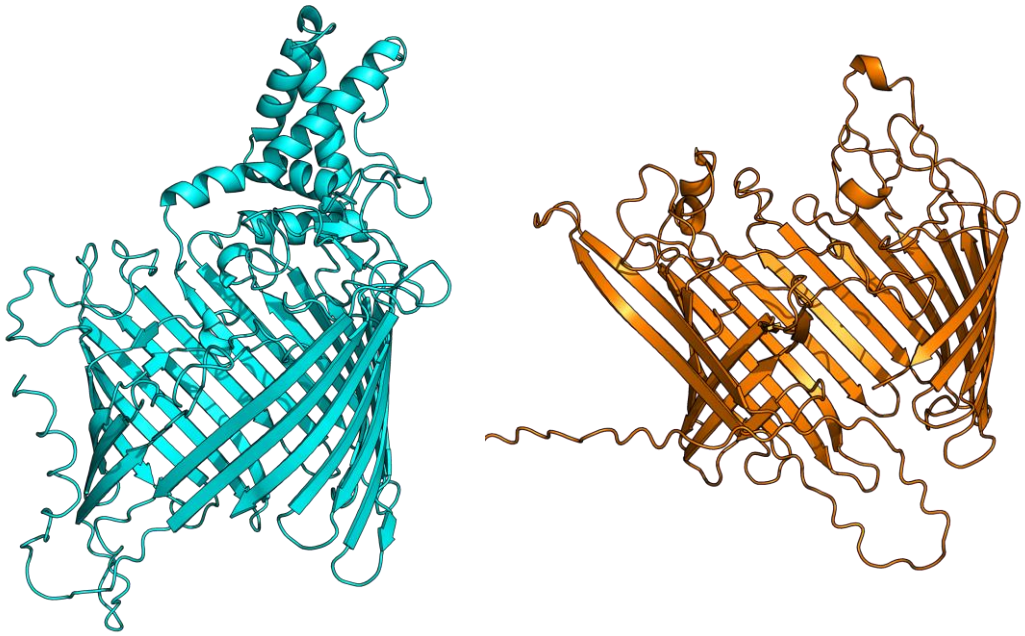


Figure 2. AlphaFold structural predictions of TprC (cyan) and TprK (orange). Sequences used for structural predictions were found at O88138_TREPA (Uniprot) for TprC and AIP85494.1 (GenBank) for TprK.

1.3.1 TprK

Characterized by seven discrete variable (V) regions separated by stretches of conserved residues, varying TprK sequences have been observed in every studied *T. pallidum* strain (134–136). Throughout the course of infection, various subpopulations of *T. pallidum* accumulate and are differentiated through their divergent TprK sequences (137). DNA cassettes corresponding to the various V regions of TprK are found in a portion of the *T. pallidum* chromosome, located separately from the *tprK* gene, and are presumed to replace the V regions of TprK by gene conversion throughout infection (137). This gene recombination is observed during rabbit passage and in *tprK* sequenced from patients with primary and secondary infections (135, 137, 138). Collectively, the *tprK* gene site and donor regions comprise ~4,000 bp of the 1.14 Mb genome, indicating that this gene variability plays a key role in the survival of *T. pallidum*.

Antibodies raised against TprK are predominantly targeted to V regions, while T-cells predominantly recognize the conserved sequence stretches, suggesting that the conserved regions may be largely intramembrane domains (132, 139). Because these

antibodies are strictly raised against the V regions, treponemes expressing new variants of TprK throughout infection can likely escape immune recognition.

Immunizations with portions of TprK have been shown to significantly attenuate both lesion formation and ulceration in a rabbit infection model (127, 140). Similarly, animals challenged with *T. pallidum* expressing the same TprK variant used in the immunizations have better protection than those challenged with a different strain variant (127, 139, 140). These findings indicate that TprK may be an important candidate for vaccine design as it can protect against lesion development during early syphilis. The focus for vaccine design should be on incorporating only conserved regions of TprK to prevent the immune response from targeting variants of TprK that may change throughout infection. If such a candidate were to be used in a vaccine, it could presumably lower the transmissibility from host to host by preventing the formation and ulceration of infectious primary chancres.

1.3.2 Tpr Subfamily I – TprC/D

Reported to be localized to the outer membrane and have porin activity, TprC/D is another Tpr of interest for vaccine development (123, 141). All Tpr subfamily I proteins (TprC, TprD, TprF, and TprI) share conserved N-terminal and C-terminal sequences, broken up by non-conserved regions that define each protein. Similarly, sequence analysis has shown that in most *T. pallidum* strains, the *tprC* and *tprD* alleles are identical in sequence. Previous research has shown that not only do the N-terminal conserved regions of Tpr Subfamily I elicit strong antibody and T-cell responses during infection, but that immunization with the same region attenuates lesion formation and ulceration during challenge (133). Interestingly, peak antibody responses are not observed until day 60 of naïve infections (133). This delay in response poses a problem, as most treponemes are cleared from lesions within 30 days post-challenge (91). This significant lag in antibody response indicates that the immune system likely is not exposed to sufficient amounts of Tpr Subfamily I during early infection to mount an immediate response to these proteins. It has been postulated that this delay may be due to low copy numbers on the cell surface or potentially that the Tpr proteins are not expressed until later in the infection course (133).

1.4 Tp0751 - A surface-exposed *T. pallidum* lipocalin domain-containing protein offers an intriguing profile for vaccine development

Another protein of great interest for vaccine development is Tp0751, a lipid-anchored outer membrane protein. This protein promotes the adhesion of the bacterium to endothelial cells and enables dissemination within the host. Structural data reveals that Tp0751 adopts a lipocalin-like fold comprised of an 8-stranded β-barrel connected by a network of flexible loops. Tp0751 is a primary target of opsonic antibodies and thus is predicted to be surface exposed on *T. pallidum* (142). Tp0751 has also been shown to participate in the degradation of host components encountered by *T. pallidum* during dissemination (77, 82, 83, 142). In particular, Tp0751 has the propensity to bind host molecules that are in close proximity to the vasculature, including extracellular matrix components found within the sub-endothelial matrix (laminin) and associated with the glycocalyx on the apical surface of endothelial cells (fibronectin and fibrinogen) (77, 79, 81, 83, 142). This model is further supported by heterologous expression of Tp0751 in the model spirochetes *Treponema phagedenis* and *B. burgdorferi*, where expression of Tp0751 conferred binding of the spirochetes to vascular cells (79, 81). These proposed functional roles support the findings by Lithgow *et al.*, revealing that immunization of rabbits with Tp0751 (24-237) reduced treponemal burden to multiple distal organ sites compared to unimmunized control rabbits. Additionally, this study demonstrated that lymph nodes recovered from a proportion of Tp0751 (24-237) immunized rabbits were unable to induce a productive infection when injected into naïve recipient rabbits, further supporting that Tp0751 inhibits dissemination in an immunization model (143).

1.4.1 Lipocalins – promising engineering targets with innate structural stability

Lipocalins are a superfamily of small extracellular binding proteins found in many diverse phyla of life, from bacteria to humans (144–147). Despite exhibiting substantial sequence diversity, lipocalins retain a highly conserved structure consisting of a rigid 8-stranded beta-barrel central folding motif incorporating four hyper-flexible loops that serve as structural gates controlling access to a ligand binding site (148). Both structural and biochemical observations support the idea that lipocalins are extremely stable and can support loops with highly divergent sequences and structures (144). Both natural and

engineered lipocalins exhibit notable thermal stability, with many exhibiting denaturation (Tm) above 70°C, making them promising candidates for protein engineering studies (149). In addition to their overall structural stability, these proteins are easily expressed in *E. coli*, meaning they are relatively cost-effective and tractable to recombinant production, greatly facilitating the GMP manufacturing process (150).

1.4.1.1 Anticalins – modular binding proteins engineered from the lipocalin scaffold

Motivated by their structural integrity and sequence diversity, initial protein engineering experiments using lipocalins were conducted to assess their inherent ability to be structurally manipulated (144, 151). Using combinatorial genetic engineering, researchers aimed to engineer the binding pockets of native lipocalins to create novel ligand-binding functions, similar to current antibody therapies. These engineered lipocalins have since been termed "Anticalins" and have greatly interested the pharmaceutical industry over the past 25 years (152–157). Anticalin engineering involves the random mutagenesis of residues in the four structurally variable loops of the lipocalin binding regions. Through target-specific selection via phage display, Anticalins are engineered to recognize and bind various targets such as enzymes, small molecules, and protein antigens such as soluble peptides and receptors (152–157).

1.5 Research hypothesis and objectives

Tp0751 plays an active role in *T. pallidum* pathogenesis, and preliminary vaccine studies have shown the potential to inhibit treponemal dissemination, however, it is insufficient as a single protein vaccine candidate to prevent both lesion formation and reinfection (143). Multi-component vaccine cocktails are often used to overcome such limitations by stimulating the necessary B- and T-cell immune responses to drive protection. The development of a multi-component syphilis vaccine has historically been hindered by the lack of characterized *T. pallidum* proteins, particularly surface-exposed proteins. Many currently characterized surface-exposed proteins adopt transmembrane β-barrel structures, which are recalcitrant to recombinant protein production, making them unable to be used in a classical multi-component vaccine. However, Tp0751 offers an innovative solution through its compact lipocalin-like fold that incorporates a highly stable

core of eight beta strands connected by a network of loops that we postulate are amenable to substitution (Figure 3) (81, 83). Using Tp0751 as a scaffold, we can engineer a multi-component syphilis vaccine using a single protein that is both stable, easily produced, and targets both lesion formation and dissemination.

Our labs have been at the forefront of the structural and functional characterization of Tp0751 (81). Analysis of the Tp0751 structure revealed a lipocalin-like fold that we hypothesized would be amenable to protein engineering and, more specifically, allow for immune targeting of several important *T. pallidum* OMP epitopes in a single antigen (81). This thesis aims to demonstrate that Tp0751 is a highly stable scaffold that can accommodate substitutions of loops from other proteins to yield a multi-component single-protein chimeric vaccine. The Tp0751 vaccine platform has been designed to be agnostic to the epitope source, meaning that an engineered chimera could display multiple *T. pallidum* epitopes for a protective syphilis vaccine.

A critical feature of the Tp0751 scaffold is the ability to expand beyond simply decorating a scaffold protein with multiple antigens, but to also present epitopes in their native conformation to elicit the appropriate immune response. Many of the most suitable epitopes are canonically displayed in the loops of OMPs presented on the bacterial surface. By engrafting these epitopes into the permissive loops of Tp0751, the native structures of these loop epitopes are accurately recapitulated, thereby improving the efficacy of the engineered chimeras.

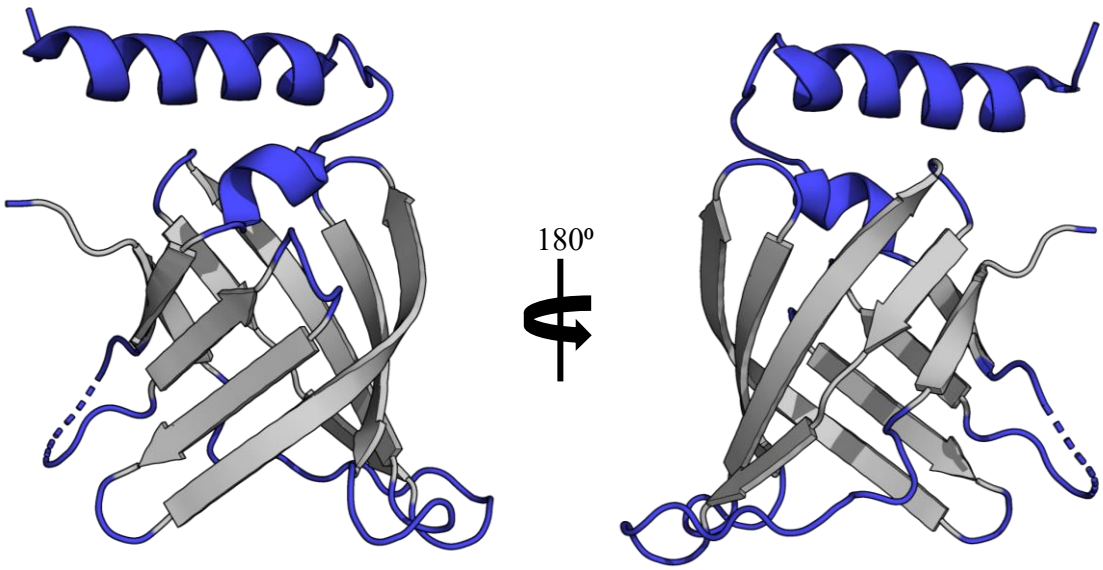


Figure 3. Structure of the Tp0751 lipocalin-like domain (96-237) (PDB: 5JK2). Tp0751 lipocalin-like domain with beta-strands shown in grey and flexible loops and termini shown in blue.

1.6 Experimental approach

Here I describe an innovative protein engineering strategy that leverages the core structural features of the lipocalin-like domain of Tp0751 to develop a highly stable, protein-based vaccine platform that can accommodate multiple epitopes from TprC and TprK. Using structure-guided principles, we initially identified and prioritized regions of Tp0751 (99-237) that would be most amenable to substitution. Chimeric constructs were generated and tested in lower resolution moderate throughput followed by higher resolution approaches to evaluate various biochemical and biophysical parameters, including expression levels, protein folding, and stability relative to wild type (WT) Tp0751 (99-237). A key chimeric candidate was then moved into protection trials in an animal model to substantiate Tp0751 as a stable scaffold on which to build a recombinant vaccine.

It is important to mention the highly collaborative nature of this project, pulling expertise from various labs, including both the Boulanger and Cameron labs at the University of Victoria (UVic), and the Giacani lab at the University of Washington (UW). Our overarching goal is to develop a vaccine candidate with the potential to be deployed in a clinical setting, meaning that input from various experts is paramount to this project's success.

Chapter 2: Materials and Methods

Materials

All basic chemicals were purchased from Sigma Aldrich (Oakville, ON).

Epitope mapping

TprK/TprC epitopes

All epitope mapping was carried out by collaborators in the Giacani lab at UW (158). *TprK epitopes - unpublished data*.

Cloning

Focusing on the lipocalin-like domain (99-237) to aid in overall construct stability and purification, clones encoding all Tp0751 (99-237) constructs were codon optimized for *E. coli* expression and synthesized by GenScript. Synthesized genes were subcloned into an engineered vector including a TEV protease cleavable N-terminal hexahistidine tag. See Figure A1 for a full vector map. See Table A1 for full vector sequence. Restriction enzymes used for cloning are NcoI and NotI. Lyophilized construct vector DNA was resuspended in 80µL of DNase-free dH_20 and stored at -20ºC.

Transformation

1µL of resuspended vector DNA solution was added to 50µL competent *E. coli* (DH5α for MiniPrep, BL21 Star (DE3) for protein expression) cells and left on ice for 30 minutes. Cells were then heat shocked at 42ºC for 45 seconds before being returned to ice for 2 minutes. From there, 500 µL of Lysogeny Broth (LB) media with no antibiotic was added to the cell mixture and incubated at 37ºC for 1 hour with shaking. Cells were spun at 5000 rpm for 1 minute. Following centrifugation, 400 µL of supernatant was discarded before gently resuspending the cells in the remaining media. The remaining 150 µL was then spread on pre-warmed agar plates with ampicillin to select for successfully transformed colonies. Plates were incubated at 37ºC overnight.

MiniPrep

A single colony from a successfully transformed *E. coli* DH5α plate was grown in 5 mL LB + ampicillin overnight at 37ºC with shaking. QIAprep Spin Miniprep Kit protocol (Cat. ID: 27104) was followed to extract vector DNA. Following extraction, DNA was stored at -20ºC.

Small-scale protein expression trials

Tp0751 (99-237) constructs were expressed in *E. coli* BL21 Star (DE3) cells and grown in 5 mL 2XYT media + ampicillin overnight at 37˚C. Following overnight growth, 50 mL of 2XYT media was inoculated with 0.5 mL of starter culture and grown at 37˚C until an OD_{600} of 1.6 - 1.8 was reached. 2 mL of culture was removed as a negative control, and cultures were then induced with 0.4 mM IPTG and left to express overnight at 16˚C. Following expression, 2 mL of culture was removed and centrifuged at 8000 rpm for 4 minutes at 4ºC. The resulting pellets were then lysed for 8-10 minutes with 150 µL of BugBuster® Protein Extraction Reagent (Millipore Cat. ID: 70584-4) and 1 µL 2.5 mg/mL DNase. Following lysis, samples were centrifuged at 13,000 rpm for 10 minutes at 4ºC, and supernatant transferred to a fresh tube. For the insoluble fraction, pellets were resuspended in 130 µL dH_20, and 45 µL of 4X SDS-PAGE sample buffer was added. For the soluble fraction, 20 µL of supernatant was transferred to a fresh tube, and 6.7 µL of 4X SDS-PAGE sample buffer was added. The remaining supernatant was purified using nickel affinity chromatography.

30 µL bed volume of nickel beads per sample were equilibrated by washing with 1X Binding Buffer (20 mM HEPES pH 8.0, 1M NaCl, 30 mM imidazole). 30 µL bed volume of equilibrated nickel beads were added to each supernatant sample and rotated at 4ºC for 1 hour. Samples were then centrifuged at 2300 rpm for 3 minutes at 4ºC, and supernatant was removed. 300 µL of 1X binding buffer was added to beads before being centrifuged as before. Supernatant was removed. Bound protein was eluted for 2-3 minutes using 30 µL of 1X Stripping Buffer (20mM HEPES pH 8.0, 1M NaCl, 500 mM imidazole). Samples were centrifuged as before, and 20 µL of supernatant was transferred to a new tube for the purified sample. 6.7 µL of 4X SDS-PAGE sample buffer was added.

Samples taken from multiple stages of purification (insoluble pellet, lysis supernatant, purified sample) were separated on a 12% Bolt 12% Bis-Tris Plus gel at 200 V for 30 minutes, and expression was assessed by the presence or absence of a strong band at the expected molecular weight (dependent on the construct being assessed).

Protein production and purification

Tp0751 (99-237) constructs were expressed in *E. coli* BL21 Star (DE3) cells, and starter cultures were grown in 50 mL of 2XYT media overnight at 37˚C. Following overnight growth, 2 L of 2XYT media was inoculated with 2 mL of starter culture and grown at 37˚C until an OD_{600} of 1.6 – 1.8 was reached. Cultures were then induced with 0.4 mM IPTG and left to express overnight at 16˚C. Following overnight expression, cells were centrifuged at 6300 rpm for 12 minutes at 4ºC, and pellets were resuspended with 20 mL 1X Binding Buffer and 30 µL Millipore Protease Inhibitor Cocktail Set III, EDTA-Free (Cat. ID: 539134-1SET) before being stored at –80ºC. Cells were then lysed using 80 mL lysis buffer (10 mM Tris-HCl pH 8.8, 130 mM NaCl, 10 mM NaF, 0.1% Triton-X-100) and 50 µL 2.5 mg/mL DNase per liter of cell culture, and protein was purified using Ni-affinity chromatography.

For nickel purification, lysed cells were centrifuged at 13,000 rpm for 30 minutes at 4ºC. 2 mL bed volume of nickel beads were equilibrated using 1X Binding Buffer + 1% glycerol. Following centrifugation, supernatant was transferred to an Erlenmeyer flask, and sufficient 4X Binding Buffer + 4% glycerol was added to a final concentration of 1X. A batch bind was performed by adding beads to the supernatant and incubating with a stir bar at 4ºC for 1 hour. Following incubation, the solution was filtered through a vacuum filtration set up, with care taken to not let the beads run dry. Beads were then washed with ~80 mL of 1X Binding Buffer and transferred to a disposable BioRad column on ice. The remaining wash was pushed through the column and kept as the “Wash” fraction. Beads were eluted for 5 minutes with 7mL of 1X Elution Buffer +1% glycerol (20 mM HEPES pH 8.0, 1M NaCl, 250 mM imidazole) 5-6 times, until A_{280} was around 1/5 of initial elution.

Once all protein was eluted, the beads were then stripped with 1X Stripping Buffer. Nickel purification and elutions were then repeated a second time.

Wash, elution, and strip fractions were then separated on a 12% Bolt 12% Bis-Tris Plus gel at 200 V for 30 minutes, and protein-containing elution fractions were then pooled and concentrated in a 10 kDa cut-off centrifugal filter.

TEV cut proteins were cut at a ratio of 60:1 protein:TEV overnight at 4ºC in 20mM HEPES pH 8.0, 150mM NaCl +1% glycerol. Proteins were further purified using SEC on either an ENrich™ SEC 70 or a Superdex™ 75 column in 20 mM HEPES pH 8.0, 150 mM NaCl + 1% glycerol. The final samples were flash-frozen using liquid nitrogen and stored at -80˚C for future use.

Thermal shift stability trials

Samples of each construct were prepared to a final concentration of 0.125 mg/mL protein and 5X SYPRO Orange dye. 20 µL of each sample was run in triplicate using an Applied Biosystems™ StepOnePlus™ Real-Time PCR System. Protein denaturation was measured using a melt curve with a range from 25˚C to 99˚C.

Limited Proteolysis

Trypsin was added to a mixture of 0.4 mg/mL protein in SEC buffer to a final concentration of 0.004 mg/mL and incubated at room temperature. 30 µL aliquots were removed at 0, 1, 5, 10, 20, 30, 40, 60, and 90 minutes and inactivated with 0.3 µL of Millipore Protease Inhibitor Cocktail Set III, EDTA-Free (Cat. ID: 539134-1SET). Each timepoint was then separated on a 12% Bolt 12% Bis-Tris Plus gel at 200 V for 30 minutes for visualization.

Mass Spectrometry

Samples were prepared for mass spec and analyzed by the UVic Genome BC Proteomics Centre. All protocols were developed and carried out by the UVic Genome BC Proteomics Centre.

2µL of each protein sample was diluted 10-fold with 0.1% formic acid before desalting over a POROS R2 reversed-phase column (50µM, 2000 Å pore size, PerSeptive Biosystems). After elution with 50% acetonitrile/0.1% formic acid, a 5µL aliquot was placed in a NanoES spray capillary (ThermoFischer Scientific) positioned at the orifice of a Fusion Tribid Orbitrap mass spectrometer (ThermoFischer Scientific) equipped with a Nanospray Flex source (ThermoFischer Scientific). The Fusion Tribid Orbitrap instrument acquisition parameters were as follows: Nano-electrospray ion source with spray voltage 1.4kV, capillary temperature 275 ºC. Survey MS1 scan m/z range 350-2000 profile mode, resolution 120,000 FWHM@225m/z, RF lens setting of 60, one microscan with maximum injection time 50 ms. The automatic gain control (AGC) target value for FTMS was 400,000 counts.

Raw files were visualized with FreeStyle 1.7 SP2 software (ThermoFischer Scientific) and masses were determined with the Xtract deconvolution feature. The Xtract settings were monoisotopic (M) uncharged output mass, H+ adduct element, charge range 5-40, relative abundance threshold 3% with protein isotope table, and minimum 5 charges detected.

Sterile filtration

Purified protein solutions were sterile filtered using a Millex ® GV 0.22µM Hydrophilic Durapore (PVDF) Membrane.

Endotoxin Assay

Endotoxin levels were tested using the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (Genscript Cat. No. L00350).

Rabbits and Treponema pallidum

Outbred male New Zealand White rabbits (3.0-3.5kg, Charles River Laboratories, Ontario, Canada) with nonreactive VDLR and FTA-ABS serologies were used for *T. pallidum* propagation and for immunization studies. All *T. pallidum* used during propagation and challenge studies were Nichols strain. All rabbits were fed antibiotic-free food and water, and were housed between 18-20ºC. All animal studies were approved by the UVic institutional review board. Studies were conducted in strict accordance with standard

accepted principles as set forth by the Canadian Council on Animal Care (CCAC) in facilities accredited by the CCAC. Institutional biosafety approval was obtained under biosafety certificate 13170-010.

Adjuvants

RIBI Natural adjuvant, prepared by PAI LifeSciences, was used in immunization studies. RIBI Natural contains the same components as the original RIBI adjuvant (RIBI Adjuvant System®, Corixa Corporation, Seattle, WA): monophosphoryl lipid A (MPL) + tetrahalose 6,6'-dimycolate (TDM) + cell wall skeleton from *Mycobacterium phlei* (CWS) (159).

Immunization and infectious challenge

Rabbits were immunized 5 times at 3-week intervals with 125µg of Construct 3.2 per rabbit mixed with RIBI Natural adjuvant reconstituted to a 1:1 adjuvant:protein ratio. Three weeks following final immunizations, 8 immunized rabbits and 8 naïve rabbits were challenged at 10 sites on their shaved backs with 10^5 *T. pallidum* Nichols strain per site, for a total of 10^6 *T. pallidum* per rabbit. Challenge sites were monitored daily for lesion formation by diameter and height of induration. Lesion volume was determined as described at https://www.sangakoo.com/en/unit/the-spherical-dome-surface-area-and-volume.

T. pallidum quantitation by quantitative polymerase chain reaction (qPCR)

At day 19 post-challenge (PC), lesions were aspirated for darkfield microscopic determination and qPCR quantitation of *T. pallidum*. qPCR protocol was followed as previously described (160). qPCR was performed on gDNA extractions of *T. pallidum* using SsoFast™ EvaGreen® Supermix (Bio-Rad, Mississauga, ON, Canada). Quantification of *T. pallidum* gDNA was determined using high-performance liquid chromatography (HPLC)-purified primers (Integrated DNA Technologies, Coraville, IA, United States) targeting the *flaA* gene (GenBank accession number M63142.1), as previously described (143, 161). The sense primer (5′-AACGCAAACGCAATGATAAA-3′) anneals to bases 475 to 494, and the antisense primer (5′-CCAGGAGTCGAACAGGA GATAC-3′) anneals to bases 738 to 759 of *flaA*. As previously described, *Solanum*

tuberosum phyB gene (GenBank Y14572) or 'SPUD,' encodes a species-specific gene that can be spiked into experimental samples (162). SPUD has previously been used as an internal amplification control (162). In these experiments, SPUD plasmid DNA was spiked into samples prior to DNA extraction to normalize DNA extraction efficiency across all samples. HPLC-purified primers (Integrated DNA Technologies) targeting SPUD included the sense primer (5′-AACTTGGCTTTAATGGACCTCCA-3′) and antisense primer (5′-ACATTCATCCTTACATGGCACCA-3′) (162); SPUD standard curves were generated by 10-fold serial dilution of SPUD plasmid from 10^7 to 10^2 copies/µl. All reactions (20 µl) were performed in triplicate with SsoFastTM EvaGreen® Supermix, 500 nM primers, and 1 µl of template (extracted gDNA). Assays were run on a Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Mississauga, ON, Canada) using twin.tecTM skirted 96-well plates (Eppendorf, Mississauga, ON, Canada) sealed with microseal B film (Bio-Rad Laboratories). PCR conditions were as follows: 95°C for 2 min, followed by 40 amplification cycles of 95°C for 10 s, and 15 s at the appropriate annealing temperature (*flaA*: 65°C, SPUD: 65°C). Following 40 cycles, there was a final denaturation step for 10 s at 95°C and melt curve analysis from 65 to 95°C in 0.5°C increments for 5 s each (*flaA*, SPUD). Each assay was run with the following controls: negative controls including no template control and no primer control, and positive control including linearized *flaA* plasmid as the template. Data was analyzed using Bio-Rad Maestro software, version 4.1.2. Standard curves produced for *flaA* and SPUD were used to determine sample DNA concentrations, and concentrations were normalized within biological replicates. The SPUD normalization factor was determined by dividing the calculated SPUD copies measured in the extraction by the expected SPUD copies (106/extraction). The calculated *flaA* copies per extraction were divided by the SPUD normalization factor to account for differences in extraction efficiency between experiments and replicates.

T. pallidum dissemination

At day 22 PC, challenge rabbits were euthanized, and their popliteal lymph nodes harvested for the Rabbit Infectivity Test (RIT) to determine the presence and relative quantity of viable *T. pallidum* in these distant sites as a metric of dissemination. The nodes were

minced on stainless steel mesh in 10% normal rabbit serum (NRS) in 0.9% NaCl (10% serum saline) and injected intratesticularly into one testis of naïve seronegative rabbits. The recipient rabbits were monitored daily for visual signs of infection (orchitis) for up to 180 days or until orchitis appeared. Semi-quantitation of viable *T. pallidum* in the injected sample was determined by length of time to orchitis development, using both historical data and recently published literature (112, 163).

Antibody titers

Blood was collected from each rabbit prior to immunization and prior to infectious challenge for determination of serum antibody titers to Tp0751 and to the engrafted TprC and TprK epitopes. ELISA protocol followed as listed below.

Enzyme-linked immunosorbent assay (ELISA)

Blood was collected from rabbits prior to infectious challenge for determination of serum antibody titers. A 96-well plate was coated with 15pmol protein or peptide (WT Tp0751 (99-237), Construct 3.2, TprK loop 3, or TprC loop 6) in 1X TBS and incubated overnight at 4ºC. Wells were then emptied and 200µL of blocking buffer (4% skim milk powder + 1X TBS) was added per well and left to incubate at room temperature for 2.5 hours. The plate was then washed 4 times with wash buffer (0.2% Tween 20 + 1X TBS pH 7.4), and 50µL of diluted sera (dilution range 1:10K-1:5120K) was added to each well and incubated for 1 hour at room temperature. The plate wash was repeated 4 times, then a 1:1000 dilution of horseradish peroxidase goat anti-rabbit IgG (H+L; Sigma-Aldrich Canada Ltd.) was added to each well and incubated for 1 hour at room temperature. The plate wash was repeated 4 times, then 100uL of 1:1 TMB2-component microwell peroxidase substrate (SeraCare Product Number: 5120-0047) was added to each well and incubated for 10 minutes at room temperature. The plate was then read on a BioTek Synergy HT plate reader at 600nm.

Chapter 3: The design and validation of Tp0751-based chimeras

Contributions: M.J. Boulanger, R. Ramaswamy and N. Funk performed initial design work and experiments relating to all proof-of-concept experiments. L. Thompson performed all chimeric Tpr/Tp0751 construct designs. L. Thompson, E. Baker, and T. Hill performed all protein purification related to chimeric Tpr/Tp0751 constructs. L. Thompson performed all protein analyses, including thermal shift and limited proteolysis. This data chapter and Chapter 4 form the basis of a recently filed provisional patent (Patent application number 63/499,007).

Chapter 3 will cover the general design framework and protein assessment workflow used for each engineered chimera. This design framework begins with a proof-of-concept engineering experiment to assess the amenability of each Tp0751 loop to engraftment using the loops of structurally similar lipocalins *Neisseria meningitidis* Factor H binding protein (NmfHbp) and Neisserial Heparin Binding Antigen (NHBA). From there, I present the complete evaluation data sets of four key chimeric candidates, one protein from each of Generations 1 through 4. These key candidates cover single engraftment chimeras to quadruple engraftment chimeras.

3.1 Introduction

3.1.1 Scientific Challenge

The continued resurgence of syphilis worldwide, despite the availability of effective penicillin treatment, highlights the need for preventative syphilis control. To date, no protein-based immunizations have been able to confer full protection against this infection. This research aims to engineer a single soluble protein scaffold on which to build a protective syphilis vaccine.

Previous efforts to develop a protective vaccine have been hindered due to the limited discovery of protective antigens and the paucity of outer membrane proteins present on the bacterial surface. This presents one of the key challenges with using classical recombinant protein vaccines; the majority of *T. pallidum* vaccine candidates are integral membrane proteins. These membrane proteins are extremely difficult to express in a

soluble form, and methods to confirm proper folding, such as circular dichroism, are unreliable when working with these large structures. To develop a vaccine capable of expressing these integral membrane antigens, we need a sophisticated expression platform that allows for the soluble expression of these immunogenic epitopes. Our approach to solving this problem is to engineer a vaccine scaffold using a protein known as Tp0751, a *T. pallidum* adhesin protein. Tp0751 is one of the only proteins currently implicated in treponemal dissemination and binding to host vasculature, making it a priority in vaccine development. Previous efforts in our lab have solved the crystal structure of Tp0751, revealing a non-canonical lipocalin structure (81). Lipocalins are comprised of an 8-stranded beta-barrel connected by four structurally flexible loop regions. These lipocalins have been of great interest in the pharmaceutical industry and are used as templates to engineer novel proteins dubbed Anticalins (152–157). Anticalins have been engineered for use as biologic therapies, whereby mutations in their flexible loop regions allow for specificity to various targets, such as small molecules and proteins of interest.

3.1.2 Goals

Using the same principles used in Anticalin engineering, here I show the refinement of a sophisticated engineering workflow using Tp0751 as a flexible and soluble protein scaffold. This workflow was initially developed during our proof-of-concept engineering strategy, which was used to probe the engineering potential of four loops of Tp0751, loops 2, 3, 6, and 8 (Figure 5). Using structurally similar lipocalins, *Neisseria meningitidis* Factor H binding protein (NmfHbp) and Neisseria Heparin Binding Antigen (NHBA), each of the targeted loops of Tp0751 were excised and replaced with the corresponding loops from NmfHbp and NHBA. Next, each newly designed construct followed a workflow consisting of small-scale expression trials to ensure soluble protein expression, large-scale purification to ensure protein viability and ease of purification, size exclusion chromatography to ensure size and shape uniformity, and thermal shift analyses to ensure thermal stability similar to that of WT Tp0751 (99-237).

Once the Tp0751 scaffold was assessed for loop permissiveness through these proof-of-concept engineering experiments, we began engineering epitopes from both TprK and TprC into the permissive loops of Tp0751. The engineering began with single-loop

engraftments, where each newly designed chimeric protein followed the same workflow used in our proof-of-concept engineering. Lead candidates were then used as the base design for double engraftment chimeras, which were then used as the base design for triple and subsequently quadruple loop engraftments.

3.2 Methods

See Chapter 2: Materials and Methods for all general methods used throughout the remainder of this chapter.

3.3 Results

3.3.1 Chimeric engineering workflow development and refinement

An initial draft of our engineering workflow was developed in the early stages of the project during the proof-of-concept engineering stage and further refined during the chimeric construct engineering portion of the project. The final workflow (Figure 4) was used throughout experiments as a guide by which to evaluate the success of each engineered construct. To be deemed successful, an engineered construct must meet each of the success criteria outlined and listed within the workflow.

3.3.2 Scaffold and substitution site evaluation: Bioinformatic analysis provides evidence for flexibility in the loops of Tp0751

The first step in the engineering process relied on in-depth structural analysis of the Tp0751 (99-237) lipocalin-like domain (PDB: 5JK2) to assess the engineering potential of each flexible region as defined by a region that is predicted to be dispensable to the core stability of the lipocalin-like domain. Structural data, including thermal motion information (b-factor) analysis, were used to generate a preliminary map of permissive regions. For example, loop 8, the longest loop in Tp0751, showed the highest b-factor overall (Figure 5) and was prioritized for substitution. Additional regions, including the C-terminus and loop 2, could not be fully modelled in the structure consistent with inherent flexibility and greater potential to accommodate substitutions (Figure 5). Additionally, each loop's length was considered, with the hypothesis that larger loops would be more aptly suited to

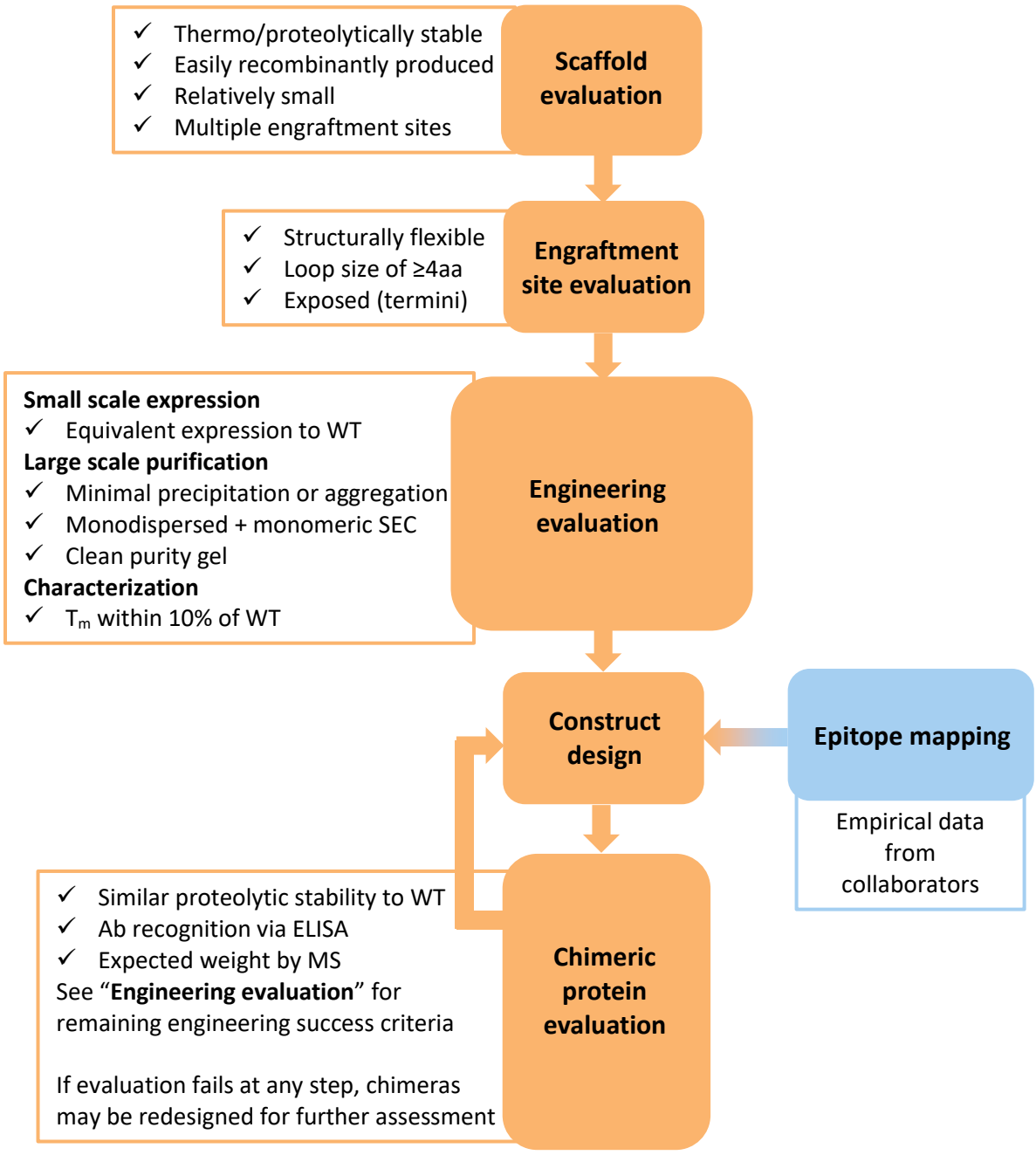


Figure 4. Chimera engineering workflow and success criteria. This figure demonstrates the general workflow followed from the initial scaffold evaluation to the final chimeric protein evaluation. Success criteria are outlined for each step.

epitope engraftment. Using this hypothesis, the four largest loops were chosen for proof-of-concept engraftment studies, loops 2, 3, 6, and 8.

Next, the boundaries of each flexible region (loops and termini) of Tp0751 (99-237) were delineated using structural analysis. Using the WT Tp0751 crystallography data, each of the loop residues were assessed for their interactions with the core β-barrel structure, ensuring that the residues chosen for removal were not participating in backbone hydrogen bonding between β-strands, which would, in turn, affect structural stability. The final residues chosen for removal can be seen in Table 1.

Table 1. Tp0751 (99-237) loop sequences and the residues chosen for removal in proof-of-concept constructs.

Loop name	Loop sequence (residues chosen for removal shown in **bold+underline**)
Loop 2	QTE**ISPNS**DIHP
Loop 3	R**EH**A
Loop 6	TA**IS**SI
Loop 8	VAR**LKIGSTS**MW

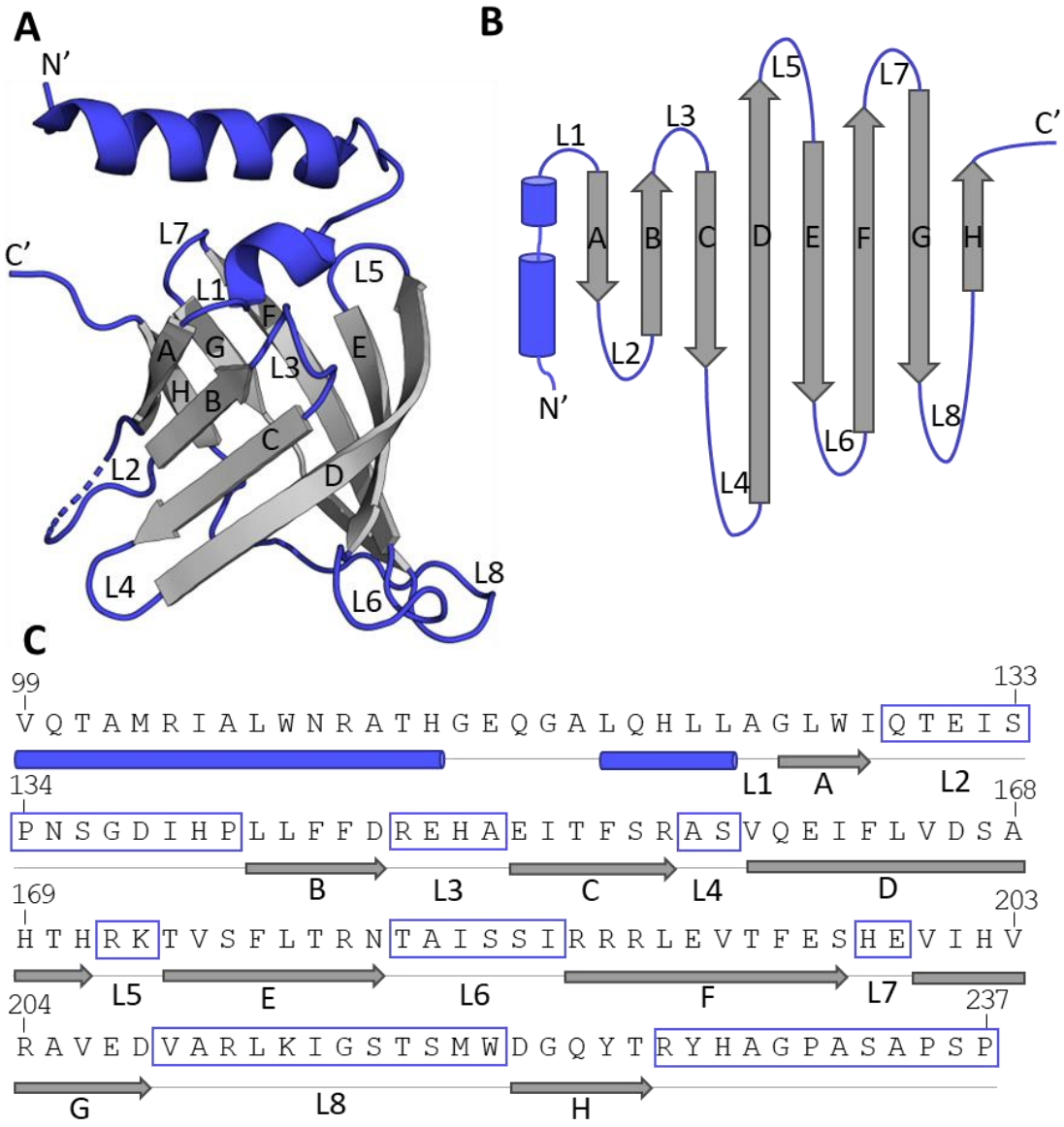


Figure 4. Structure and sequence data for Tp0751 (99-237) lipocalin-like domain. A) Ribbon structure of WT Tp0751 (99-237) (PDB: 5JK2). **B)** Topographical depiction of Tp0751, including β-strands shown as grey arrows (A-H), loop regions shown as blue lines (L1-L8), and the N-terminal helices shown as blue cylinders. C) Protein sequence of Tp0751 (99-237) depicting secondary structures, β-strands depicted as grey arrows, loops regions depicted with blue boxes, and helices depicted as blue cylinders.

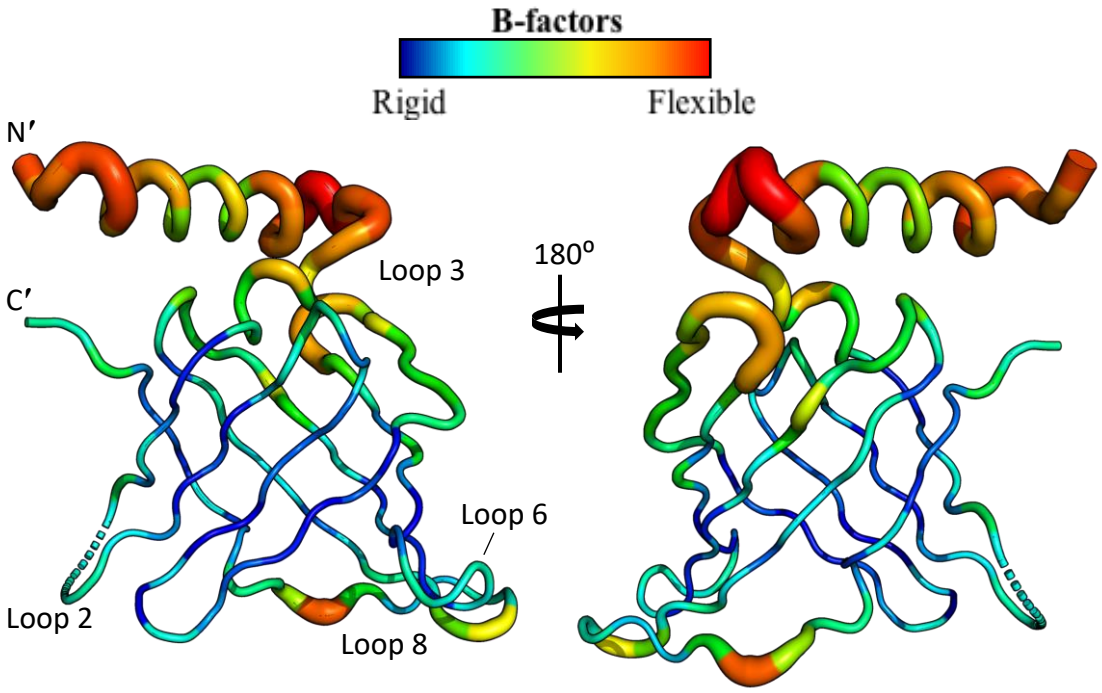


Figure 5. Thermal parameter (B-factor) distribution in Tp0751. The b-factor is represented in both thickness and colour gradient, where thin blue lines depict structural rigidity, and thick red lines depict structural flexibility.

3.3.3 Proof-of-concept engineering evaluation

To test our hypotheses on the amenability of each Tp0751 loop to substitution, we first began with a proof-of-concept engineering strategy using loop substitutions from structurally similar proteins. Two *N. meningitidis* lipocalins were chosen as the loop engraftment sources, NmfHbp and NHBA (Figure 6). Both proteins are 8-stranded β-barrels connected by a network of flexible loop regions, as seen in Tp0751. Our aim with these experiments was to determine if the native Tp0751 loops residues are required for inherent structural stability. We hypothesized that if the loops of Tp0751 were amenable to substitution, they would likely be the most stable with loop substitutions from similar-sized and shaped proteins. Our first step focused on engrafting three loops from these two proteins, two from NmfHbp and one from NHBA, into loops 2, 3, 6, and 8 of Tp0751. A

total of 11 constructs were designed (Table 2) for further analysis. Full protein sequences are listed in Table A2.

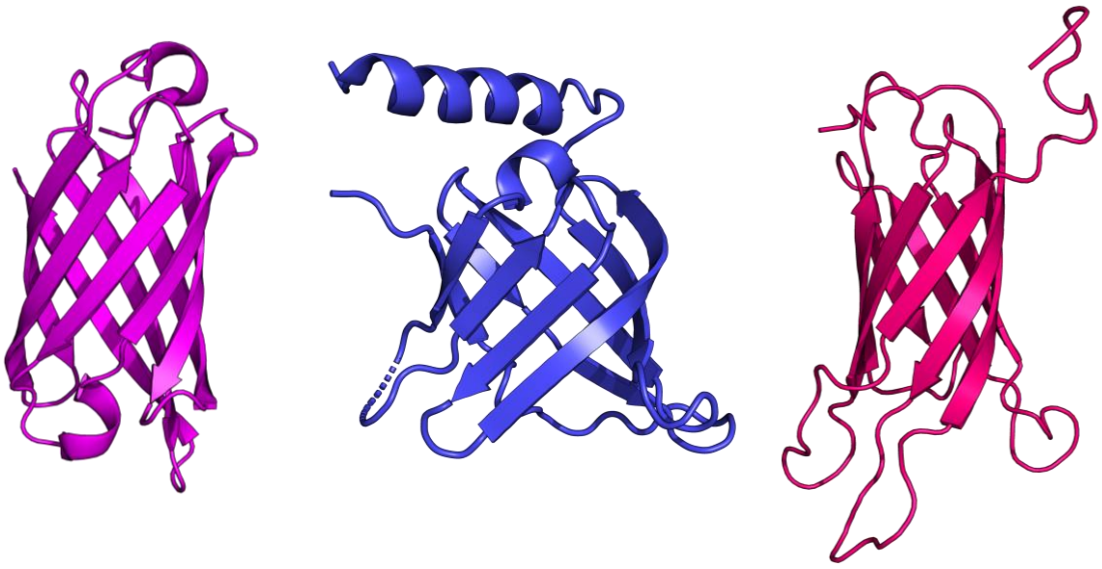


Figure 6. Structures of NmfHbp (purple), Tp0751 (blue), and NHBA (pink). Structural data from both NmfHbp (PDB:3KVD) and NHBA (PDB: 2LFU) lipocalins next to Tp0751 (blue) to show their size and structural similarity.

Table 2. Tp0751 (99-237)-neisseria chimera IDs and sequence descriptions. 0.X construct IDs refer to chimeras with one loop engraftment from a structurally similar protein, either NmfHbp or NHBA. Sequences of replaced Tp0751 loops and subsequently engrafted epitopes are shown in bold. The nomenclature under the "Sequence Description" column is as follows: Tp0751 sequence removed → engrafted sequence.

Construct ID	Sequence Description
0.1	Tp0751 ΔLoop 2 **ISPNS → GDDLHMG** (NHBA)
0.2	Tp0751 ΔLoop 2 **ISPNS → KTVNGIRH** (NmfHbp)
0.3	Tp0751 ΔLoop 2 **ISPNS → VLYNQAEKGS** (NmfHbp)
0.4	Tp0751 ΔLoop 3 **EH → GDDLHMG** (NHBA)
0.5	Tp0751 ΔLoop 3 **EH → VLYNQAEKGS** (NmfHbp)
0.6	Tp0751 ΔLoop 6 **IS → GDDLHMG** (NHBA)
0.7	Tp0751 ΔLoop 6 **IS → KTVNGIRH** (NmfHbp)
0.8	Tp0751 ΔLoop 6 **IS → VLYNQAEKGS** (NmfHbp)
0.9	Tp0751 ΔLoop 8 **LKIGSTS → GDDLHMG** (NHBA)
0.10	Tp0751 ΔLoop 8 **LKIGSTS → KTVNGIRH** (NmfHbp)
0.11	Tp0751 ΔLoop 8 **LKIGSTS → VLYNQAEKGS** (NmfHbp)

3.3.3.1 Small-scale expression trials show soluble expression for the Tp0751-Neisseria epitope constructs

To assess the proper expression and solubility of each newly designed construct, small-scale expression trials were conducted using 2 mL of an induced 50mL *E. coli* BL21 Star (DE3) culture. Each sample was purified using nickel affinity before analysis using gel electrophoresis. Wild type (WT) Tp0751 (99-237) was used as a positive control following previous studies showing soluble expression (81). Of the 11 newly designed constructs, medium-to-strong soluble expression was seen in all but three constructs, Constructs 0.5, 0.6, and 0.8, where decreased amounts of expression were observed compared to the WT protein (Figure 7.). Though the expression levels in these three constructs are lower than their counterparts, these experiments still demonstrate successful soluble expression of the loop constructs, indicating successful engraftment.

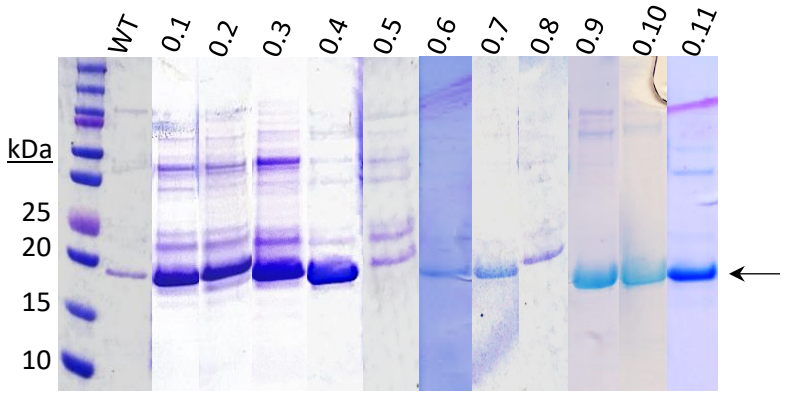


Figure 7. Small-scale expression trials of Tp0751 chimeras. Samples taken from multiple stages of purification (insoluble pellet, lysis supernatant, purified sample) from *E. coli* cultures were evaluated on a 12% Bolt Bis-Tris Plus gel at 200 V for 30 minutes, and expression was assessed by the presence or absence of a strong band at the expected molecular weight (designated by arrows). Only nickel-enriched samples are shown above. Chimeric constructs that showed expression equivalent to wild type Tp0751 (99-237) and that could be enriched by nickel pulldown indicated correctly folded protein and were advanced to large-scale expression trials.

3.3.3.2 Large-scale purification and size exclusion chromatography demonstrates successful purification for the Tp0751-Neisseria epitope constructs

To assess stability and protein folding through large-scale purification, each construct was grown in 2L of *E. coli* BL21 Star (DE3), followed by nickel affinity purification and size exclusion chromatography (SEC). Throughout the large-scale purification, three metrics (benchmarked to WT Tp0751 (99-237)) were used to assess the amenability of each loop: minimal protein precipitation and aggregation during concentration, a monodispersed and monomeric SEC trace, and sufficient yield and purity following SEC. Substantial amounts of precipitation during purification would indicate protein instability, signalling that the engineering strategy used for that loop was insufficient or potentially that the epitope used for engraftment was unstable in that specific Tp0751 loop. Next, each resulting SEC trace was assessed to ensure a monomeric and monodispersed SEC trace was obtained, indicating monomeric protein expression and uniform protein folding. The final metric of a successfully purified protein is a purity gel, which must show a strong single band at the expected molecular weight (MW), indicating high purity and minimal protein degradation.

We established that each new chimeric protein, excluding Construct 0.5, was successfully purified with little to no precipitation or aggregation. Each successful protein was purified as a single-peak on size-exclusion chromatography, indicating proper folding and monomeric expression (Figure A2). All resulting purity gels display a single strong band at the expected MW, indicating minimal to no protein degradation (Figure A4).

3.3.3.3 Thermal shift data indicates that loops 2, 6, and 8 are amenable to engraftment

Thermal shift analyses were used to determine denaturation temperatures as a metric to compare construct stability of engineered chimeras compared to WT Tp0751 (99-237). From these analyses, it was determined that loops 2, 6, and 8 and their resulting constructs are stable and amenable to engraftment using the loops of NmfHbp and NHBA. Construct 0.4, representing a loop 3 engraftment, showed a significant reduction in T_m (>10% WT T_m), indicating that this loop 3 engraftment had a destabilizing effect on protein thermal stability (Figure 8). This decrease in stability is likely due to the relatively small

insertion site (2 amino acids for loop 3) compared to loops 2 and 8 (5 and 8 amino acids, respectively).

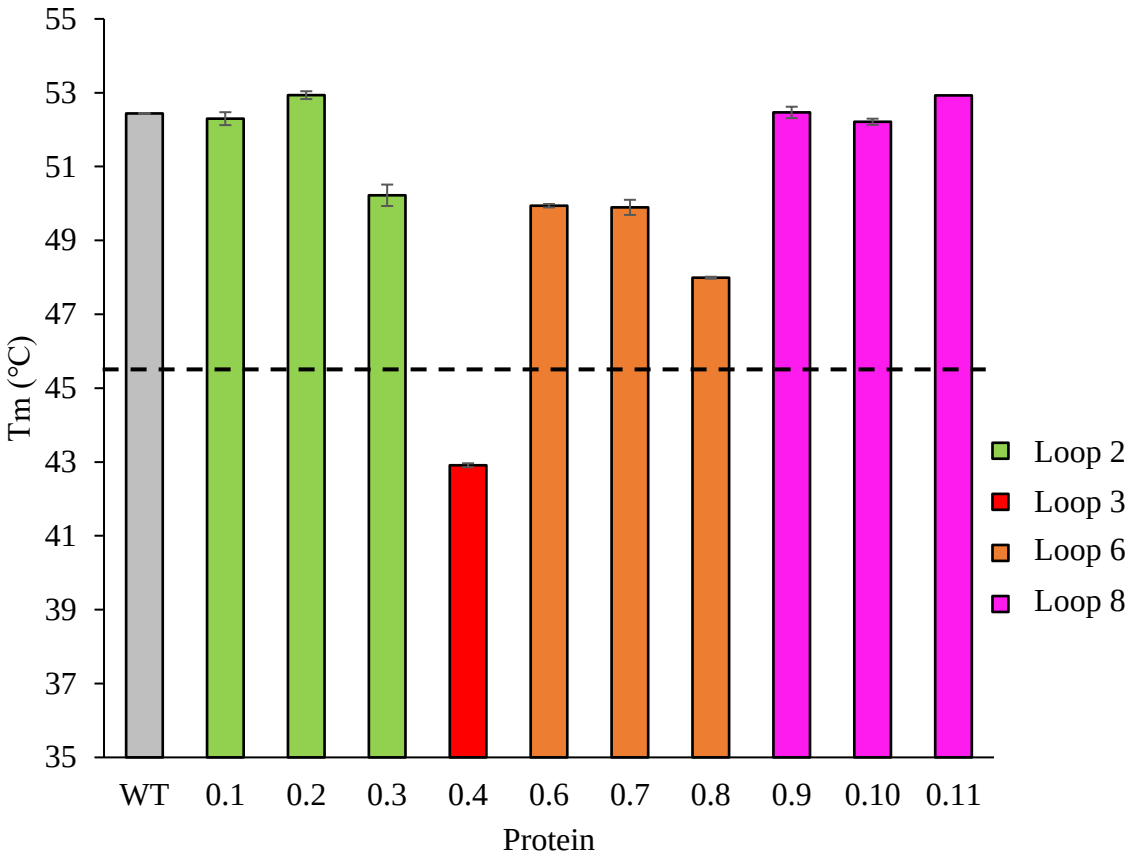


Figure 8. Loops 2, 6, and 8 are amenable to engineering. Thermal shift data shows that loop 2 (green), loop 6 (orange), and loop 8 (magenta) constructs exhibit no significant decrease in thermal stability when compared to WT Tp0751 (99-237) (grey) (stable = T_m shift < 10% WT Tm). Loop 3 exhibits a marked decrease in T_m, indicating a loss in stability. The dashed line indicates a 10% decrease in T_m from the WT protein.

3.3.3.4 Proof-of-concept engineering assessments indicate that Tp0751 is amenable to loop substitution

As shown by the successful purification and thermal stability profiles mentioned in previous sections, loops 2, 6, and 8 of Tp0751 were amenable to engraftment using the loops of structurally similar lipocalins. These findings show that these native Tp0751 loop residues are not required to maintain structural stability, leading us to hypothesize that these

engraftment locations may be amenable to engraftment using epitopes derived from various other *T. pallidum* OMPs. The following sections outline my efforts to test this hypothesis by iteratively designing and assessing many Tp0751-TprC-TprK chimeric proteins.

3.3.4 Chimeric construct design

Following the proof-of-concept engineering assessments, we began the chimeric protein engineering by focusing on engrafting various epitopes from TprC and TprK into the loops and C-terminus of Tp0751 (99-237). The TprK- and TprC-derived epitopes were mapped out by collaborators in the Giacani lab at UW.

This engineering process began by designing and producing several proteins with single epitope engraftments into various loops of WT Tp0751 (99-237). Each engineered single epitope chimera followed the workflow outlined in Figure 4, and successful candidates were prioritized for future iterative designs. Successfully engineered single epitope candidates were then used as the base scaffolds for double loop engraftment chimeras, which were then evaluated in the same fashion as their single epitope predecessors. This iterative design process continued into triple and quadruple epitope engraftments. A comprehensive list of Tp0751 chimeras can be seen in Table 3. An example schematic of this design iteration is shown in Figure 9. This workflow reflects an iterative process where information gained on targeted loops and engrafted sequences are used to refine a Tp0751 chimera that satisfies our established criteria.

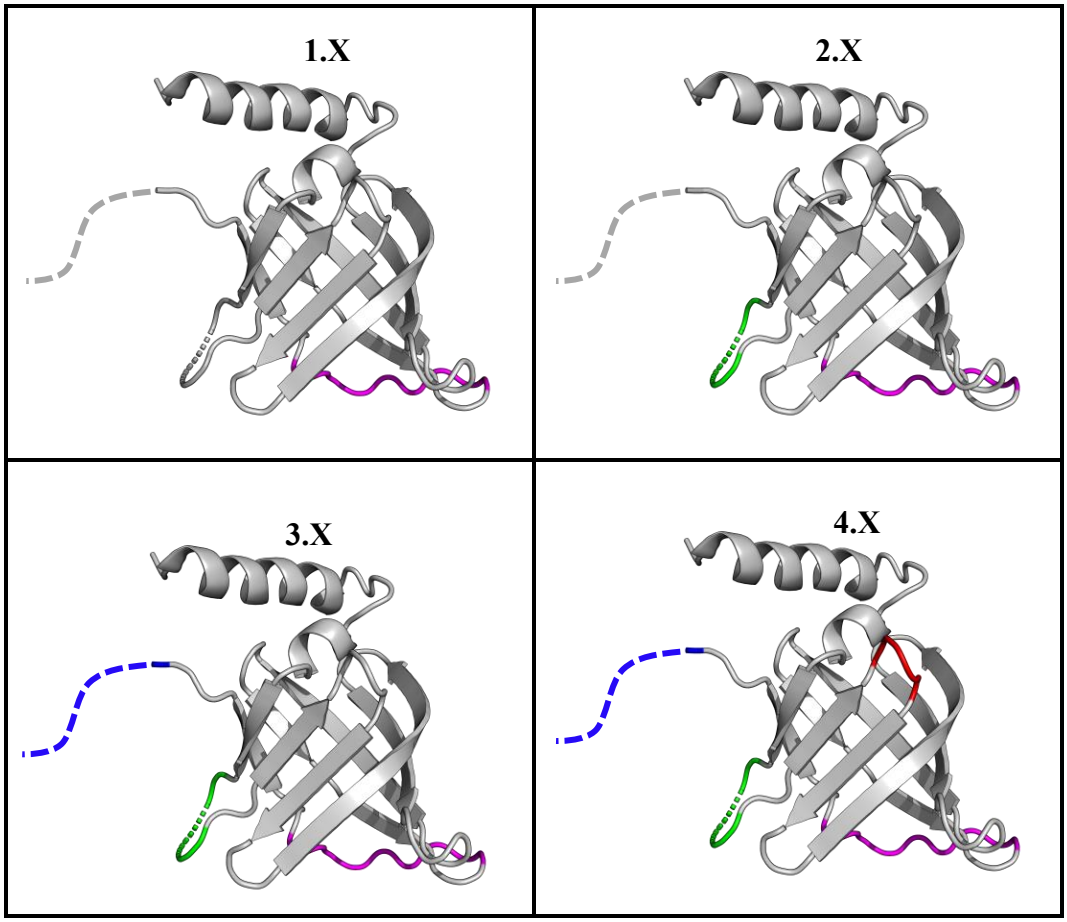


Figure 9. Tp0751 chimeric protein engineering follows an iterative design process. 1.X, 2.X, 3.X, and 4.X generation designations refer to chimeras with one, two, three, or four loop engraftments from TprC/TprK, respectively.

3.3.4.1 Chimeric construct loop selection and delineation

In addition to engineering constructs using the same loop delineations as used in the proof-of-construct experiments, I also wanted to test whether it was possible to remove more of the native loop residues prior to engraftment. By removing more of the native loop residues, I aimed to accommodate larger epitope engraftments and increase the stability of existing engraftment regions. Multiple constructs in Table 2 highlight this in the notation "Expanded ΔLoop X", where X denotes any loop number. An expanded loop engraftment was deemed successful if all success criteria listed in Figure 4 were met. Using this engineering strategy, I re-assessed the loop 3 residues chosen for removal in an effort to make it a stable engraftment location. In the initial proof-of-concept experiments, two out

of four loop residues were chosen for removal (corresponding to amino acids in bold – R**EH**A), which resulted in unstable constructs that either could not be purified (Construct 0.5) or had insufficient thermal stability (Construct 0.4). By removing all four loop residues (**REHA**), we successfully used loop 3 as an engraftment site, highlighted in Construct 4.1. Though we achieved success with this engraftment location, it is important to note that only relatively small epitopes (<7 amino acids) were able to be engrafted into loop 3 with success. Any attempts to engraft larger epitopes of 10 (Construct 1.8) and 13 (Construct 1.10) amino acids were unsuccessful, indicating that epitope size plays an important role in the resulting construct stability.

To maximize the number of engraftment locations within Tp0751, we investigated the engraftment amenability of the C-terminal region in addition to loops 2, 3, and 8. Structural data could not model the C-terminal region, consistent with high inherent flexibility. We hypothesized that this inherent flexibility, alongside the diminished structural constraints compared to the loop regions, would allow us to engraft a large epitope (17 amino acids) onto the terminal region. The success of this engineering strategy is highlighted in Construct 3.2 and 4.1 in the following sections.

It is important to note that despite loop 6 showing promise as an engraftment site during proof-of-concept experiments, we have decided against using this site during the engineering of the Tpr chimeras. When mapping functional Tp0751 peptides to the various loop sites, a functional peptide known as peptide 10 was shown to encompass the entirety of loop 6. Peptide 10 has been implicated in the binding of laminin and its receptor LamR, fibrinogen, fibronectin, and collagen IV, all important components of the host vasculature (81, 83, 161). Peptide 10 has also been shown to play an important role in the adhesion of Tp0751-expressing *B. burgdorferi* to human umbilical vein endothelial cells (HUVECs) (81). In order to retain all potential immunogenic and functional epitopes in our Tp0751 scaffold, we have decided against engrafting epitopes within the residues mapped to peptide 10, including loop 6.

Table 3. Tp0751 (99-237) chimera IDs and sequence descriptions. 1.X, 2.X, 3.X and 4.X construct IDs refer to chimeras with one, two, three, or four loop engraftments from TprC/TprK, respectively. Sequences of replaced Tp0751 loops and subsequently engrafted epitopes are shown in bold. The nomenclature under the "Sequence Description" column is as follows: Tp0751 sequence removed → engrafted sequence.

Construct ID	Sequence Description
1.1	Tp0751 Expanded ΔLoop 2 **EISPNSGDI** → TprC Loop 3 **RAYSEKDTRYA**
1.2	Tp0751 Expanded ΔLoop 2 **EISPNSGDI** → TprC Loop 6 **GKLVQNLPNIMMPPGIT**
1.3	Tp0751 ΔLoop 2 **ISPNS** → TprK Loop 2 **AKTIEATLH**
1.4	Tp0751 Expanded ΔLoop 2 **EISPNSGDI** → TprK Loop 2 **AKTIEATLH**
1.5	Tp0751 ΔLoop 2 **ISPNS** → TprK New Loop 2 **ESNGGAK**
1.6	Tp0751 ΔLoop 2 **ISPNS** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE**
1.7	Tp0751 Expanded ΔLoop 2 **ISPNSGD** →TprK Loop 10 **LQENSNVVIEKNV**
1.8	Tp0751 Expanded ΔLoop 3 **REHA** → BamA1 **DNNQPFDLTV**
1.9	Tp0751 Expanded ΔLoop 3 **REHA** → TprK New Loop 2 **ESNGGAK**
1.10	Tp0751 Expanded ΔLoop 3 **REHA** → TprK Loop 10 **LQENSNVVIEKNV**
1.11	Tp0751 Expanded ΔLoop 6 **TAISS** → BamA1 **DNNQPFDLTV**
1.12	Tp0751 Expanded ΔLoop 6 **TAISS** → TprC Loop 3 **RAYSEKDTRYA**
1.13	Tp0751 Expanded ΔLoop 6 **TAISS** → TprK New Loop 2 **ESNGGAK**
1.14	Tp0751 Expanded ΔLoop 6 **TAISS** → TprK Loop 10 **LQENSNVVIEKNV**
1.15	Tp0751 New Expanded ΔLoop 8 **RLKIGSTSM** → BamA1 **DNNQPFDLTV**
1.16	Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprC Loop 1 **FQKNPRTGPGKHTHGFRTTN**
1.17	Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprC Loop 2 **LAVELASSKSSTALSFTKPTAS**
1.18	Tp0751 Expanded ΔLoop 8 **RLKIGSTSM** → TprC Loop 3 **RAYSEKDTRYA**
1.19	Tp0751 Expanded ΔLoop 8 **RLKIGSTSM** → TprC Loop 10 **GTTNRFNIINAAGNLLN**
1.20	Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 **NGDYKSKGDKPVYEPG**
1.21	Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE**
1.22	Tp0751 New Expanded ΔLoop 8 **RLKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE**
1.23	Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 2 **DYKSKGDKP**
1.24	Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 3 **SKGDKPVYE**
1.25	Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 4 **KSKGDKPVY**
2.1	Tp0751 ΔLoop 2 **ISPNS** → TprC Loop 3 **RAYSEKDTRYA** Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE**
2.2	Tp0751 ΔLoop 2 **ISPNS** → TprC Loop 6 **GKLVQNLPNIMMPPGIT** Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE**
2.3	Tp0751 ΔLoop 2 **ISPNS** → TprC Loop 10 **GTTNRFNIINAAGNLLN** Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE**
2.4	Tp0751 ΔLoop 2 **ISPNS** → TprC Loop 10 (Nichols Strain) **GTTNRFNIINPAGNLLN** Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE**
2.5	Tp0751 ΔLoop 2 **ISPNS** → TprC Loop 11 **QGVLADAPYMGIAESIW**

	Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE**
2.6	Tp0751 ΔLoop 2 **ISPNS** → TprK Loop 1 **KYRGEGNVYA** Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE**
2.7	Tp0751 ΔLoop 2 **ISPNS** → TprK Loop 2 **AKTIEATLH** Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE**
2.8	Tp0751 ΔLoop 2 **ISPNS** → TprK Loop 4 **GKLQENSNVV** Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE**
3.1	Tp0751 ΔLoop 2 **ISPNS** → TprK Loop 2 **AKTIEATLH** Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE** Tp0751 C-term + TprC Loop 6 **GKLVQNLPNIMMPPGIT**
3.2	Tp0751 ΔLoop 2 **ISPNS** → TprK Loop 2 **AKTIEATLH** Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE** Tp0751 ΔC-term **AGPASAPSP** → TprC Loop 6 **GKLVQNLPNIMMPPGIT**
3.3	Tp0751 ΔLoop 2 **ISPNS** → TprK Loop 2 **AKTIEATLH** Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE** Tp0751 ΔC-term **AGPASAPSP** → TprC Loop 6 Truncation 1 **LVQNLPNIM**
3.4	Tp0751 ΔLoop 2 **ISPNS** → TprK Loop 2 **AKTIEATLH** Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE** Tp0751 ΔC-term **AGPASAPSP** → TprC Loop 6 Truncation 2 **NLPNIMMPP**
3.5	Tp0751 ΔLoop 2 **ISPNS** → TprK Loop 2 **AKTIEATLH** Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE** Tp0751 ΔC-term **AGPASAPSP** → TprC Loop 6 Truncation 3 **NIMMPPGIT**
3.6	Tp0751 ΔLoop 2 **ISPNS** → TprK New Loop 2 **ESNGGAK** Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE** Tp0751 C-term **AGPASAPSP** + TprC Loop 6 **GKLVQNLPNIMMPPGIT**
3.7	Tp0751 ΔLoop 2 **ISPNS** → TprK Loop 10 **GKLQENSNVV** Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE** Tp0751 ΔC-term **AGPASAPSP** → TprC Loop 6 **GKLVQNLPNIMMPPGIT**
4.1	Tp0751 Expanded ΔLoop 2 **ISPNSGD** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE** Tp0751 Expanded ΔLoop 3 **REHA** → TprK New Loop 2 **ESNGGAK** Tp0751 Expanded ΔLoop 8 **RLKIGSTSM** → TprC Loop 3 **RAYSEKDTRYA** Tp0751 ΔC-term **AGPASAPSP** → TprC Loop 6 **GKLVQNLPNIMMPPGIT**
4.2	Tp0751 Expanded ΔLoop 2 **ISPNSGD** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE** Tp0751 Expanded ΔLoop 3 **REHA** → TprK Loop 10 **LQENSNVVIEKNV** Tp0751 New Expanded ΔLoop 8 **RLKIGSTSM** → TprC Loop 3 **RAYSEKDTRYA** Tp0751 ΔC-term **AGPASAPSP** → TprC Loop 6 **GKLVQNLPNIMMPPGIT**
4.3	Tp0751 Expanded ΔLoop 2 **ISPNSGD** → TprK Loop 10 **LQENSNVVIEKNV** Tp0751 Expanded ΔLoop 3 **REHA** → TprK New Loop 2 **ESNGGAK** Tp0751 Expanded ΔLoop 8 **RLKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE** Tp0751 ΔC-term **AGPASAPSP** → TprC Loop 6 **GKLVQNLPNIMMPPGIT**

3.3.5 Chimeric construct evaluation

Due to the high number of engineered chimeras, as seen in Table 2, in the following sections I will highlight a key protein candidate from Generations 1 through 4 to demonstrate the efficacy of the workflow outlined in Figure 4. Full data sets including SEC traces, small-scale expression trials, purity gels, and thermal shift data for all engineered constructs are shown in Figures A2, A3, A4 and Table A3, respectively. In addition to the success criteria outlined in the proof-of-concept experiments, successful TprC/TprK/Tp0751 chimeras were also tested for proteolytic stability using time-course trypsin digestion. To be considered proteolytically stable, successful chimeras would show a similar degradation profile over time to WT Tp0751 (99-237). The highlighted protein candidates will again demonstrate the iterative design process followed throughout the engineering process.

3.3.5.1 Construct 1.21 highlights the amenability of Loop 8 to Tpr epitope engraftment

The first step in our engineering process began by investigating single-loop engraftments using various Tpr epitopes. Here I have highlighted a top single engraftment chimera, Construct 1.21. This chimera displays a single TprK epitope engraftment in Loop 8 of Tp0751, as shown in Figure 10A. In the remainder of Figure 10, we outline the readouts for each step of our workflow, and the full sequence descriptions are listed in Table 3.

The small-scale expression trial of Construct 1.21 displays an expression band at the expected molecular weight (MW) of 19.5 kDa, with similar expression levels to WT Tp0751 (99-237) (Figure 10B). Further, in the large-scale purification and SEC, we see a strong monomeric and monodispersed peak on the SEC chromatogram and a clean band in the resulting purity gel, indicating proper protein folding and minimal protein degradation (Figure 10C). It is important to note that during large-scale purification, this protein underwent an N-terminal hexahistidine tag cleavage, demonstrated by the perceived decrease in MW in the purity gel (Figure 10C) compared to the small-scale expression gel (Figure 10B). The final MW of the protein following cleavage was 16.8 kDa. Both the

thermal shift data and limited proteolysis data display similar stability profiles compared to WT Tp0751 (99-237) (Figures 10D and F).

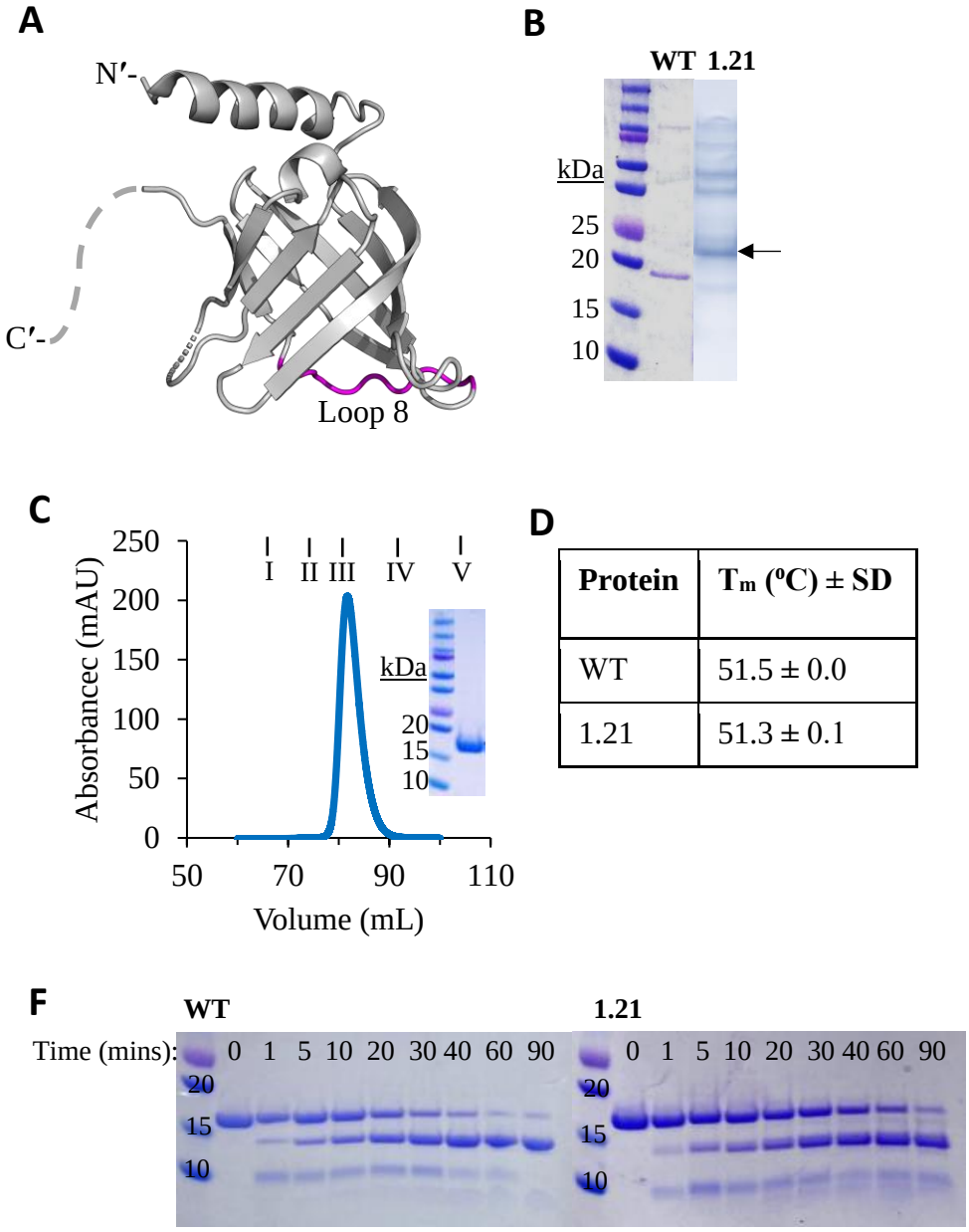


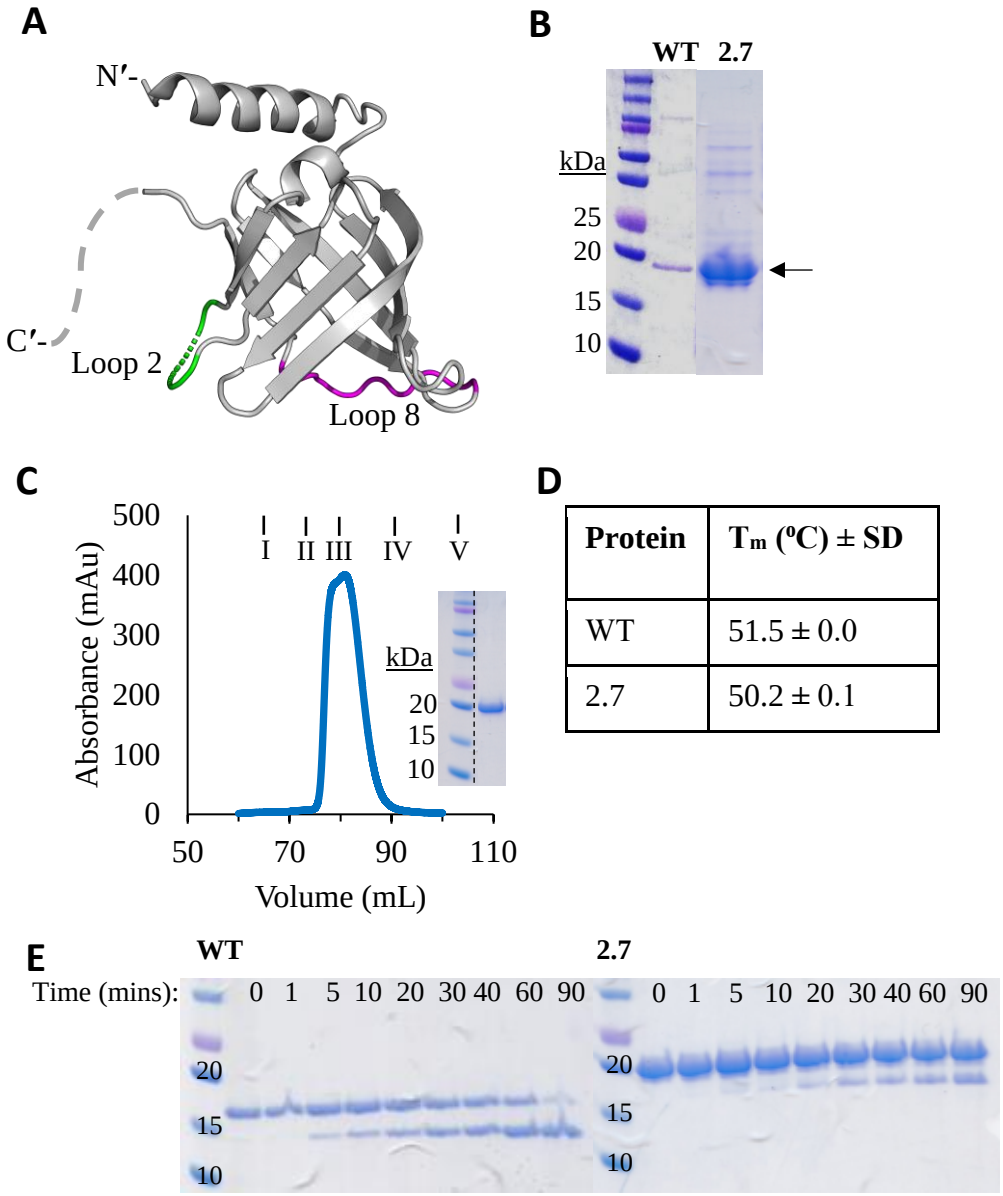
Figure 10. Purification data set for Tp0751 Construct 1.21 (Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE). A)** Structural schematic showing targeted Tp0751 loop 8 (magenta). **B)** Small-scale soluble expression of WT Tp0751 (99-237) and 1.21, showing that 1.21 has similar expression to WT Tp0751 (99-237). **C)** SEC trace and corresponding purity gel indicating successful purification of

1.21. Protein standards are listed as I-V above the chromatogram: I – conalbumin (75 kDa), II – ovalbumin (44 kDa), III – carbonic anhydrase (29 kDa), IV – ribonuclease (13.7 kDa), V – aprotinin (6.5 kDa). **D)** Melting temperature (T_m) for both WT Tp0751 (99-237) and 1.21, showing that 1.21 is equivalent to WT Tp0751 (99-237). **E)** Proteolytic stability profiles of WT Tp0751 (99-237) and 1.21 following trypsin digestions at various time points, showing that 1.21 is equivalent to WT Tp0751 (99-237).

3.3.5.2 Construct 2.7 demonstrates the amenability of Tp0751 to double epitope engraftments in Loop 2 and Loop 8

Using Construct 1.21 as the base scaffold on which we engineered Construct 2.7, this section highlights Construct 2.7 as a successfully engineered double engraftment protein. Construct 2.7 retains the same Loop 8 epitope engraftment seen in Construct 1.21 and has an additional TprK engraftment in Loop 2, as seen in Figure 11A. This double engraftment chimera followed the same workflow as Construct 1.21 to assess the stability profile of this newly designed protein.

Like Construct 1.21, Construct 2.7 met all success criteria outlined in Figure 4. Starting with the small-scale expression trials, Construct 2.7 displayed an expression band at the expected MW of 19.9 kDa, showing similar expression levels to WT Tp0751 (99-237) (Figure 11B). Following large-scale purification, the resulting SEC chromatogram displayed a strong monomeric and monodispersed peak on the SEC chromatogram and a clean band in the resulting purity gel, indicating proper protein folding and minimal protein degradation (Figure 11C). The resulting thermal and proteolytic stability profiles closely match those of WT Tp0751 (99-237) (Figures 11D and F).



Protein	T_m (°C) ± SD
WT	51.5 ± 0.0
2.7	50.2 ± 0.1

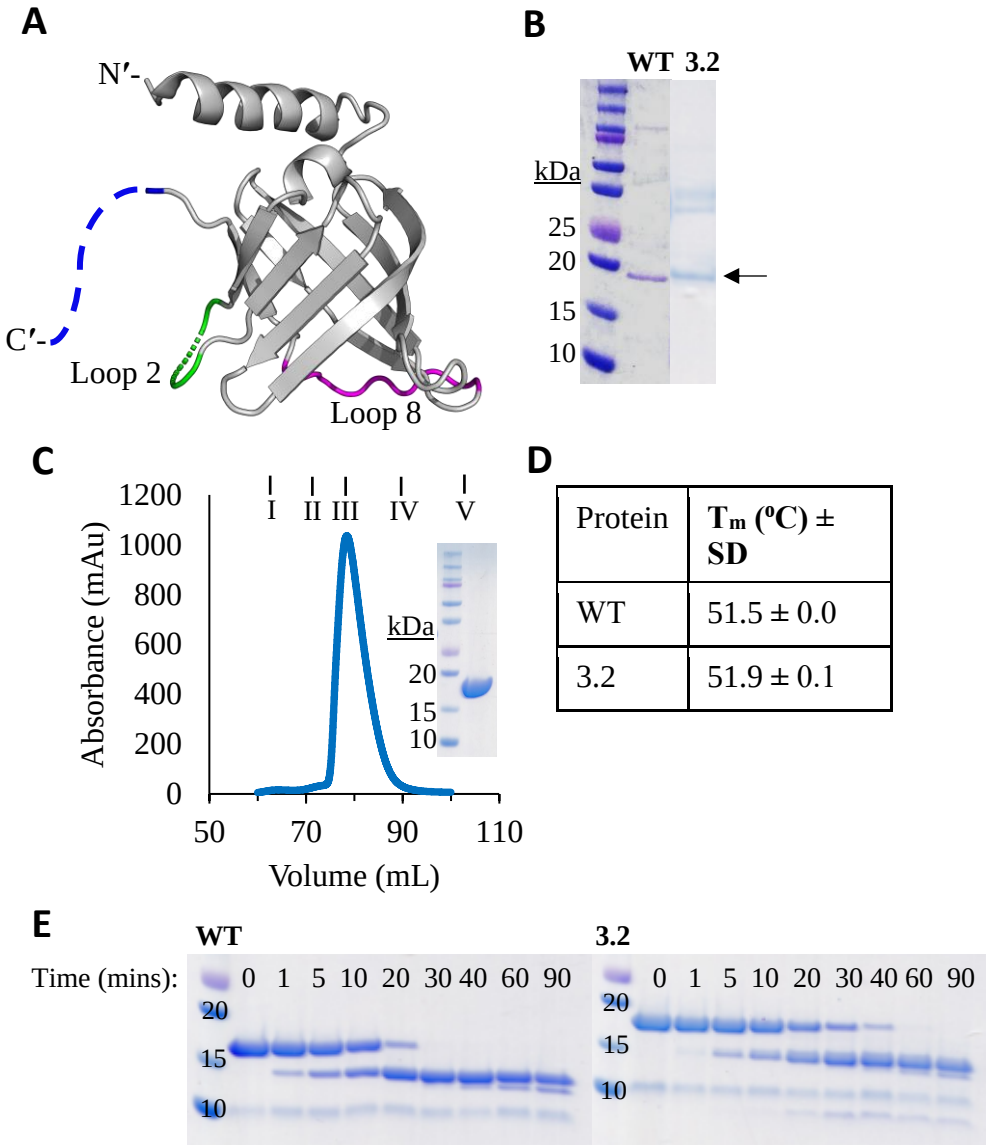
Figure 11. Purification data set for Tp0751 Construct 2.7 (Tp0751 ΔLoop 2 **ISPNS** → TprK Loop 2 **AKTIEATLH**, Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE). A)** Structural schematic showing targeted Tp0751 loops 2 (green) and 8 (magenta). **B)** Small-scale soluble expression of WT Tp0751 (99-237) and 2.7, showing that 2.7 has similar expression to WT Tp0751 (99-237). **C)** SEC trace and corresponding purity gel indicating successful purification of 2.7. Protein standards are listed as I-V above the chromatogram: I – conalbumin (75 kDa), II – ovalbumin (44 kDa), III – carbonic anhydrase (29 kDa), IV – ribonuclease (13.7 kDa), V – aprotinin (6.5 kDa). **D)** Melting temperature (T_m) for both WT Tp0751 (99-237) and 2.7,

showing that 2.7 is equivalent to WT Tp0751 (99-237). **E)** Proteolytic stability profiles of WT Tp0751 (99-237) and 2.7 following trypsin digestions at various time points, showing that 2.7 is equivalent to WT Tp0751 (99-237).

3.3.5.3 Construct 3.2 demonstrates the amenability of Tp0751 to triple epitope engraftments in Loop 2, Loop 8, and the C-terminus

Following the same iterative design used with Construct 2.7, the newly designed Construct 3.2 retains the same engraftments in Loop 2 and Loop 8 and additionally has a TprC epitope engrafted onto the C-terminus of Tp0751. As mentioned previously, the C-terminus of Tp0751 could not be fully modelled in the structure, consistent with inherent flexibility and greater potential to accommodate substitutions. This triple engraftment chimera followed the same workflow as Construct 1.21 and 2.7 to assess the stability profile of the newly designed protein.

As seen in the previously highlighted constructs, Construct 3.2 successfully met all criteria outlined in Figure 4. Small-scale expression trials display an expression band at the expected MW of 20.8 kDa, showing similar expression levels to WT Tp0751 (99-237) (Figure 12B). Following through large-scale purification and SEC, we see a strong monomeric and monodispersed peak on the SEC chromatogram and a clean band in the resulting purity gel, indicating proper protein folding and minimal protein degradation (Figure 12C). As seen with Construct 1.21, during large-scale purification, Construct 3.2 underwent a TEV cleavage to remove the N-terminal hexahistidine tag, resulting in a final MW of 18.1 kDa. This cleavage is responsible for the observed decrease in MW between the small-scale expression gel (Figure 12B) and the final purity gel (Figure 12C). Finally, the thermal shift and limited proteolysis data show Construct 3.2 displaying similar stability profiles to WT Tp0751 (99-237) (Figures 12D and F).



Protein	T_m (°C) ± SD
WT	51.5 ± 0.0
3.2	51.9 ± 0.1

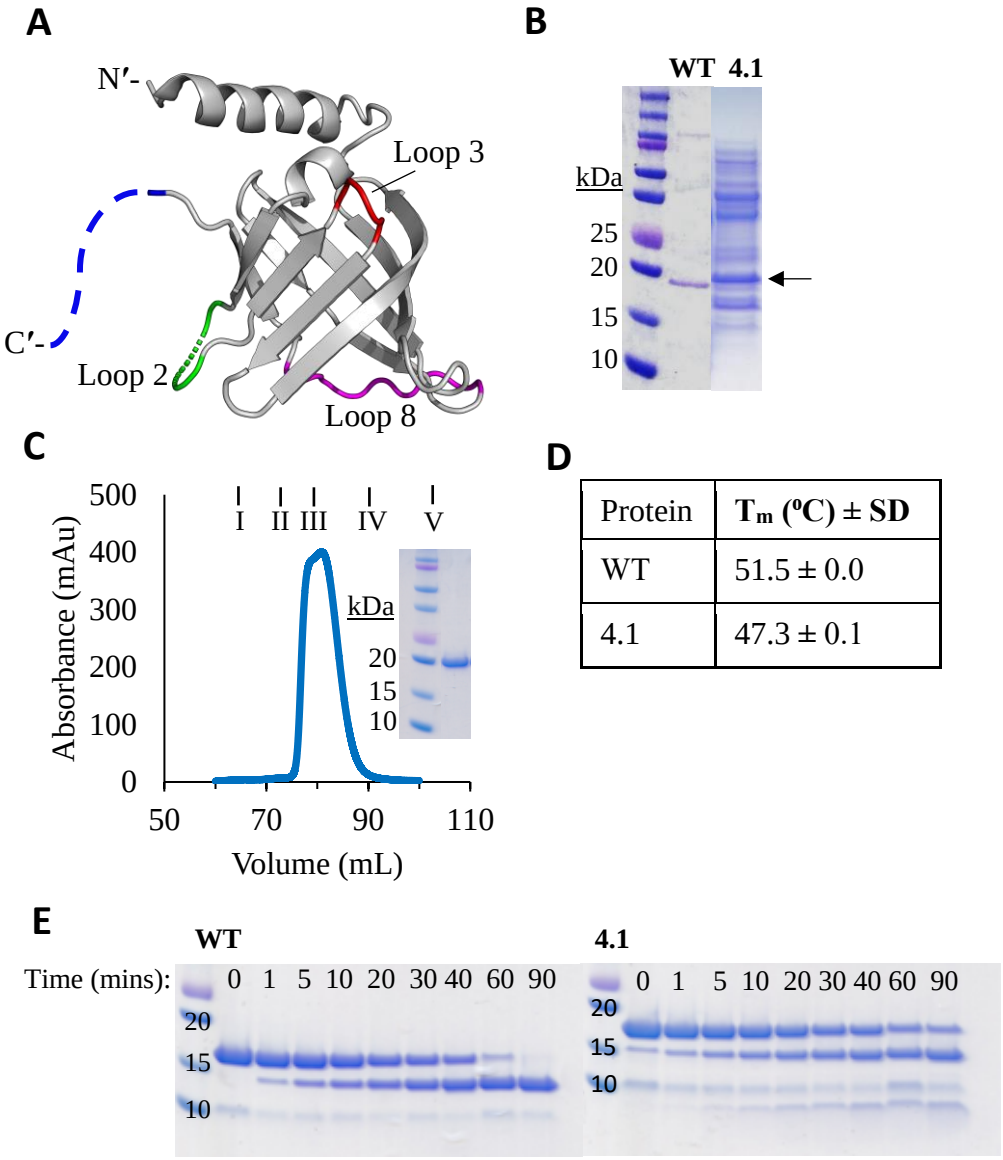
Figure 12. Purification data set for Tp0751 Construct 3.2 (Tp0751 ΔLoop 2 **ISPNS** → TprK Loop 2 **AKTIEATLH**, Tp0751 Expanded ΔLoop 8 **LKIGSTSM** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE**, Tp0751 ΔC-term **AGPASAPSP** → TprC Loop 6 **GKLVQNLPNIMMPPGIT). A)** Structural schematic showing targeted Tp0751 loops 2 (green) and 8 (magenta) and the C-term (blue). **B)** Small-scale soluble expression of WT Tp0751 (99-237) and 3.2, showing that 3.2 has similar expression to WT Tp0751 (99-237). **C)** SEC trace and corresponding purity gel indicating successful purification of 3.2. Protein standards are listed as I-V above the chromatogram: I – conalbumin (75 kDa), II – ovalbumin (44 kDa), III – carbonic anhydrase (29 kDa), IV – ribonuclease (13.7 kDa),

V – aprotinin (6.5 kDa). **D)** Melting temperature (T_m) for both WT Tp0751 (99-237) and 3.2, showing that 3.2 is equivalent to WT Tp0751 (99-237). **E)** Proteolytic stability profiles of WT Tp0751 (99-237) and 3.2 following trypsin digestions at various time points, showing that 3.2 is equivalent to WT Tp0751 (99-237).

3.3.5.4 Construct 4.1 demonstrates the amenability of Tp0751 to quadruple epitope engraftments in Loop 2, Loop 3, Loop 8, and the C-terminus

Rather than iterating on the previously highlighted constructs as seen with Construct 2.7 and 3.2, this Generation 4 chimera resulted from iterative design using the epitope engraftments used in Constructs 1.6, 1.9, and 1.18. Structural analysis of the TprK-derived epitopes onto the TprK predicted structural model revealed a lack of loop localization for one predicted epitope, TprK loop 2. Because we want to focus our engineering efforts using epitopes predicted to be surface-exposed loops, the loop 2 engraftment (TprK loop 2) used in Constructs 2.7 and 3.7 was replaced during Generation 4 design. By revisiting other successful Generation 1 chimeras, we were able to engraft four epitopes within the Tp0751 scaffold successfully.

Throughout the protein assessment, Construct 4.1 met all success criteria outlined in Figure 4. Small-scale expression trials display an expression band at the expected MW of 21.0 kDa, displaying expression levels similar to WT Tp0751 (99-237) (Figure 13B). Following through large-scale purification and SEC, we see a strong monomeric and monodispersed peak on the SEC chromatogram and a clean band in the resulting purity gel, which indicates proper protein folding and minimal protein degradation (Figure 13C). Thermal shift data shows Construct 4.1 displaying a T_m close to WT Tp0751 (99-237), and limited proteolysis suggests a similar proteolytic stability profile to WT Tp0751 (99-237) (Figures 13D and F).



Protein	T_m (°C) ± SD
WT	51.5 ± 0.0
4.1	47.3 ± 0.1

Figure 13. Purification data set for Tp0751 Construct 4.1 (Tp0751 Expanded ΔLoop 2 **ISPNSGD** → TprK Loop 3 Truncation 1 **DYKSKGDKPVYE**, Tp0751 Expanded ΔLoop 3 **REHA** → TprK New Loop 2 **ESNGGAK**, Tp0751 Expanded ΔLoop 8 **RLKIGSTSM** → TprC Loop 3 **RAYSEKDTRYA**, Tp0751 ΔC-term **AGPASAPSP** → TprC Loop 6 **GKLVQNLPNIMMPPGIT). A)** Structural schematic showing targeted Tp0751 loops 8, 2 and 3 and the C-term (dark grey). **B)** Small-scale soluble expression of WT Tp0751 (99-237) and 4.1, showing that 4.1 has similar expression to WT Tp0751 (99-237). **C)** SEC trace and corresponding purity gel indicating successful purification of 4.1. Protein standards are listed as I-V above the chromatogram: I – conalbumin (75 kDa), II –

ovalbumin (44 kDa), III – carbonic anhydrase (29 kDa), IV – ribonuclease (13.7 kDa), V – aprotinin (6.5 kDa). **D)** Melting temperature (T_m) for both WT Tp0751 (99-237) and 4.1, showing that 4.1 is equivalent to WT Tp0751 (99-237). **E)** Proteolytic stability profiles of WT Tp0751 (99-237) and 4.1 following trypsin digestions at various time points, showing that 4.1 is equivalent to WT Tp0751 (99-237).

3.4 Discussion

A successful syphilis vaccine would help to overcome many of the current challenges involved in global syphilis reduction and elimination, such as limited access to healthcare as well as limited clinical diagnostic tools. A protective vaccine candidate would decrease infection incidence by preventing infection rather than relying solely on diagnosis and treatment of active infections. Previous efforts to develop a protective vaccine have been hindered due to the limited knowledge of protective antigens, as well as the limited number of surface-exposed antigenic targets. One of the key challenges with vaccine development is the limited amount of structurally characterized *T. pallidum* proteins to support engineering. Many of these antigens, specifically surface-exposed antigens, adopt transmembrane β-barrel structures that are recalcitrant to recombinant protein production. Our approach to solving this problem involves using Tp0751, a *T. pallidum* adhesin protein, as the base scaffold on which to build a protective single recombinant vaccine candidate. Tp0751 is a lipocalin-like protein comprised of an 8-stranded β-barrel connected by a network of flexible loop regions, which we hypothesize are amenable to substitution with epitopes from various immunogenic *T. pallidum* OMPs such as TprC and TprK. By decorating this scaffold in immunogenic epitopes derived from other functionally important surface-exposed *T. pallidum* proteins, our goal is to retain the immunogenicity of these large OMPs (TprK and TprC) while expressing their epitopes in a stable and soluble scaffold that protects against dissemination.

3.4.1 Proof-of-concept engineering shows that loop 2 and loop 8 are prime candidates for epitope engraftment

We began by interrogating the engineering potential of Tp0751 by replacing the native flexible loop regions with the loops of structurally similar lipocalins NmfHbp and

NHBA. Small-scale expression trials were used as a guide to determine the expression and solubility of each newly designed construct. These trials yielded successful results, where each construct was expressed in a soluble form, even if at low concentrations for the loop 3 constructs (Construct 0.5 and 0.6). During large-scale purification, as foreseen by the loop 3 construct small-scale expression trials, Construct 0.5 could not be purified. This inability to be purified was likely due to the comparatively small insertion site, indicating that the loop 3 engineering strategy may not allow for construct stability. Conversely, all other loop 2, 6, and 8 constructs were successfully purified, indicated by the ease of concentration and a successful single SEC peak.

Following purification, we conducted thermal shift analyses to assess the thermal stability of each protein, showing that loops 2, 6, and 8 all maintained similar stability to WT Tp0751 (99-237) (Figure 8). Construct 0.6, despite showing strong expression in small scales and stability during purification, showed a significant reduction in thermal stability, indicating that the loop 3 engineering strategy used was not able to maintain protein stability. Similarly, our loop 6 constructs, while not exhibiting a large T_m decrease (>10% WT Tm), did show a slight decrease in T_m of 6.5%. This slight decrease in Tm does indicate that there may be issues with engineering this loop in the future, for example, when engrafting in an epitope from a structurally dissimilar protein. In the following section, we revisit loop 3, focusing on expanding the WT Tp0751 (99-237) residues removed for epitope insertion. The constructs engineered using loops 2 and 8 showed great promise in the thermal shift assays. Nearly all 6 of these constructs maintained a similar T_m to WT Tp0751 (99-237), indicating that these modifications had close to no effect on overall construct stability. However, we see a slight decrease in T_m for Construct 0.3, suggesting that the engrafted sequences play a role in the resulting protein stability. This decrease in stability is likely the result of a combination of the larger size of this epitope (10 amino acids) compared to the other Loop 2 construct epitopes (7 and 8 amino acids), as well as the biophysical properties of the engrafted residues. The epitope used in Construct 0.3 has many large bulky residues, such as tyrosine, asparagine, glutamine, glutamic acid, and lysine, all of which may not be compatible with the flexible
nature required for protein stability.

These results strongly support the hypothesis that the loops of Tp0751 are amenable to substitution using the loops of structurally similar proteins. Loops 2 and 8 show great promise as epitope engraftment sites, and our findings demonstrate that the WT Tp0751 (99-237) residues in these loops are not required for overall construct stability and can be replaced with peptides of similar size.

3.4.2 Tp0751 is amenable to engraftment in four distinct locations, loops 2, 3, 8, and the C-terminus

To utilize the Tp0751 scaffold to its full potential, I aimed to engraft four epitopes into loops 2, 3, 8 and the C-terminus. By replacing as many non-functionally relevant locations on the Tp0751 scaffold as possible in TprC and TprK epitopes, I aimed to create a single protein vaccine candidate capable of inducing an immune response against three separate *T. pallidum* proteins. This approach relies on iterative design processes, whereby data from previously engineered constructs was used to improve the scaffold for future design generations. This process involved testing a plethora of epitopes in various engraftment locations on the Tp0751 scaffold to determine which combination of epitopes resulted in the most stable chimeric protein. To do this efficiently, I refined a workflow (Figure 4) that begins with the lower resolution moderate throughput small-scale expression trials, followed by the higher resolution assessments to assess protein folding and stability, such as SEC, thermal shift, limited proteolysis, and mass spectrometry. These analyses allow us to prioritize successful chimeras for future iterations.

Each candidate highlighted in the results section represents successful protein candidates from each generation of engineering, exemplifying the success of our iterative design workflow. By working with one loop engraftment at a time, I could mitigate the addition of multiple variables into the scaffold, allowing me to determine whether each individual engraftment hindered chimera stability. Through this iterative design process, I have shown that Tp0751 is amenable with up to four separate epitope engraftments in loops 2, 3, 8, and the C-terminus.

This protein scaffold is not limited to only TprC and TprK epitope engraftments but to any protective epitopes that may be uncovered in future research. As the field continues developing and discovering vaccine candidates, Tp0751 can continue to be used

as a stable and soluble scaffold on which to express their immunogenic epitopes. This is especially useful when considering that most OMPs are integral membrane-spanning proteins, meaning they cannot be recombinantly produced in a properly folded and soluble state. Many of the most suitable epitopes are displayed in the surface-exposed loops of these OMPs. By engrafting these epitopes into the loops of Tp0751, their native structures are accurately recapitulated, improving the efficacy and immunogenicity of the resulting chimeras. Thus, a critical feature of the Tp0751 scaffold is its ability to not only be decorated in antigens but also to present these epitopes in their native loop conformations.

In these experiments, we have established that Tp0751 is a tractable scaffold on which to build a recombinant vaccine. We have shown that Tp0751 is amenable to up to four separate epitope engraftments, solidifying the success of our engineering strategy.

Chapter 4: An engineered Tp0751-based chimeric protein exhibits partial protection in an animal model

Contributions: L. Thompson performed all chimeric engineering and design work. L. Thompson performed all protein purification and analysis work, including preparing protein for protection experiments. A. Gomez performed all *in vivo* work relating to protection trials with assistance from A. Haimour, A. Geppert, E. Schovanek, and S. Waugh. A. Gomez performed all data analysis relating to protection experiments.

Chapter 4 will cover the preparation of our top Generation 3 vaccine candidate to be used in protection experiments, as well as a summary of the protection experiment results. This protection experiment began in February 2022, and the final data collection was completed in January 2023. Though in Chapter 3 I outline chimeras that have been engineered beyond Generation 3, to complete data collection and analysis in time for this thesis to be written, the protection experiments had to begin in 2022.

4.1 Introduction

An effective syphilis vaccine must inhibit the invasive nature of the pathogen during dissemination and must protect against the spread of infection by limiting lesion formation and ulceration. Identifying protective surface-exposed *T. pallidum* antigens has historically been difficult, partly due to their low density in the outer membrane (114, 115, 164, 165). Despite this, there have been great advancements made to identify several predicted OMPs such as TprC, TprK, and Tp0751 that, when used as recombinant vaccine candidates, have been shown to significantly attenuate lesion formation, ulceration, and dissemination (133, 140, 143).

Rabbits provide an ideal model for evaluating vaccine candidates, as they are readily susceptible to *T. pallidum* infection and display all of the classical primary disease manifestations seen in humans, such as lesion formation and CNS invasion (13, 166–168). Rabbits also display similar immune responses to those seen in humans, such as a Th-1 skewed immune response and CD4+ and CD8+ lymphocyte infiltration (13, 166, 167). The outbred model of the rabbit ensures genetic diversity similar to that of humans, allowing for the generalizability of responses.

To prepare a recombinant protein vaccine candidate for immunization, safety must be ensured by both sterile filtering and endotoxin assessment. Endotoxins are heat-stable lipopolysaccharides found in the outer membrane of Gram-negative bacteria. These pyrogenic endotoxins can induce severe physiological reactions, including activation of monocytes and macrophages, a defensive inflammatory response against bacteria (169–171). When Gram-negative bacteria, such as *E. coli*, are used for recombinant protein production, endotoxin is an inherent contaminant (172). To mitigate any potential side effects derived from endotoxin contamination, vaccine candidates must undergo endotoxin level testing to ensure that the immunization solutions are below the FDA threshold of 5 endotoxin units (EUs)/kg (173).

In the studies reported here, we describe the assessment of two top vaccine candidates, the methods used to prepare them for use in rabbit protection trials, and a summary of the protection trial results. Here we show that the top vaccine candidate remains stable and soluble following purification, and that the immunization regimen outlined here significantly attenuates lesion formation, lesion ulceration, and dissemination. This study demonstrates the promise of the Tp0751 scaffold for use as a recombinant vaccine platform and demonstrates the success of the current top chimeric candidate.

4.2 Materials and Methods

See Chapter 2: Materials and Methods for all general methods used throughout the remainder of this chapter.

4.3 Results

4.3.1 Identifying the lead chimeric candidate to be used in protection experiments

Following Generation 3 engineering, two key chimeric protein candidates emerged as top candidates for protection trials, Construct 3.1 and 3.2. Both proteins were nearly identical in their engineering, with each having the same epitope engraftments located in loops 2, 8, and the C-terminus. The key difference between Construct 3.1 and 3.2 was in the engineering performed on the C-terminus. In Construct 3.1, the epitope was engrafted directly onto the native C-terminus without removing or substituting residues. In contrast,

Construct 3.2 had the 9 terminal residues removed (**AGPASAPSP**) from the C-terminus prior to engraftment. This engineering strategy resulted in Construct 3.1 having a slightly longer C-terminal tail than Construct 3.2, though the engrafted epitope remained the same. Both proteins were assessed using the workflow outlined in Figure 4 to differentiate the optimal candidate for protection trials. See Table 3 for full construct descriptions.

Both proteins were purified successfully without major precipitation or aggregation throughout purification, indicating high protein stability. Both proteins purified as monomeric and monodispersed peaks (Figure A2.), indicating monomeric protein folding, and the resulting purity gels showed a strong single band, indicating acceptable protein purity following purification (Figure A4.). Thermal shift analysis demonstrated that both constructs had similar levels of thermal stability, with Construct 3.1 and 3.2 showing Tms of 50.7ºC ± 0.0ºC and 51.9ºC ± 0.1ºC, respectively. Limited proteolysis revealed that both proteins have similar proteolytic stability, with the major band (~18 kDa) disappearing at 60 minutes (Figure 14).

As a final measure of protein stability, mass spectrometry was conducted. The results revealed a large mass difference of -1407.698 Da from the expected mass in Construct 3.1 (Table 4). Conversely, Construct 3.2 was measured to be the exact theoretical MW, indicating increased protein stability compared to Construct 3.1 (Table 4). The decreased stability in Construct 3.1, as measured by the large difference in expected mass, indicated that this construct was unfit for protection trials. This reason, combined with the measured stability in Construct 3.2, informed our decision to use Construct 3.2 in future protection trials.

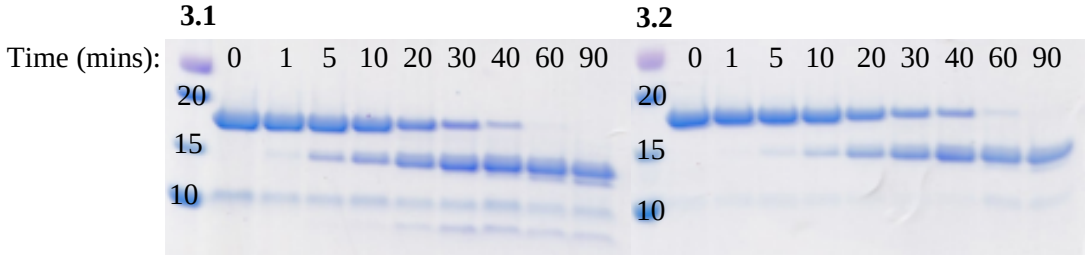


Figure 14. Limited proteolysis of Constructs 3.1 and 3.2 display similar levels of proteolytic stability. Trypsin was added to a mixture of 0.4 mg/mL protein in SEC buffer to a final concentration of 0.004 mg/mL and incubated at room temperature. 30 µL

aliquots were removed at 0, 1, 5, 10, 20, 30, 40, 60, and 90 minutes and inactivated with 0.3 µL of Millipore Protease Inhibitor Cocktail Set III, EDTA-Free (Cat. ID: 539134-1SET). Each timepoint was then separated on a 12% Bolt 12% Bis-Tris Plus gel at 200 V for 30 minutes for visualization.

Table 4. Mass spectrometry data for Construct 3.1 and 3.2, showing theoretical and experimental molecular weights.

Protein	Theoretical MW (Da)	Experimental MW (Da)	Mass Difference from Theoretical (Da)
Construct 3.1	18839.68	17431.982	-1407.698
Construct 3.2	18104.32	18104.334	-0.014

4.3.2 Preparation of Construct 3.2 for immunization

4.3.2.1 Filter sterilization

To sterilize the protein solution for immunization, the solution was filtered through a Millex ® GV 0.22µM Hydrophilic Durapore (PVDF) Membrane. To ensure that no significant amount of protein was lost through this filtering process, protein concentrations were measured before and after filtering. The final concentration before sterile filtration was 1.25 mg/mL, and after sterile filtration was 1.18 mg/mL, indicating minimal protein loss.

4.3.2.2 Endotoxin levels are within safety limits for immunization

To ensure the endotoxin levels within the protein solution were below the FDA tolerance limit (≤5.0 EU/kg for non-intrathecal drugs), a chromogenic limulus amebocyte lysate (LAL) endotoxin test kit was used. Using the average weight of each rabbit (3.5 kg) and the immunization dose of 0.125 mg of protein, the maximum acceptable amount of endotoxin within the protein solution would be 140 EU/mg of protein. Using a standard curve (Figure A5), Construct 3.2 solution's endotoxin levels were 5.8 EU/mg, well under the FDA threshold for safety.

4.3.2.3 Freeze-thaw experiments reveal no loss of protein stability

After purification and filter sterilization, the protein solution was aliquoted and flash-frozen in liquid nitrogen before storage at -80ºC. To ensure the protein remained stable after freezing, samples of unfrozen and frozen-thawed protein were assessed by thermal shift. No significant reduction in T_m was observed, with a T_m of 51.9ºC ± 0.1ºC for unfrozen and 52.0ºC ± 0.1ºC for those frozen and thawed.

4.3.3 Immunization with Construct 3.2 induces partial protection in a rabbit model

4.3.3.1 Immunization with Construct 3.2 significantly attenuates lesion development and *T. pallidum* burden at the initial site of infection

For 22 days following challenge, rabbit challenge sites were monitored for the development of lesions, or chancres, that represent the first clinical sign of infection. Monitoring lesion development provides an indicator of vaccine efficacy. Construct 3.2 immunized animals had significantly attenuated lesion volume (Figure 15A) and ulceration (Figure 15B) compared to unimmunized control rabbits. qPCR analysis of day 19 lesion aspirates also revealed that immunized rabbits had significantly lower *T. pallidum* burden at the local infection site than unimmunized control rabbits (Figure 15C).

4.3.3.2 Immunization with Construct 3.2 induces high antibody titers against Tp0751, TprC and TprK

Antigen-specific antibody titers were determined by ELISA assay using Construct 3.2, WT Tp0751 (99-237), and peptides corresponding to Construct 3.2 loop engraftments (TprK loop 3, TprC loop 6). Immunization induced a high antibody titer against both full-length Construct 3.2, WT Tp0751 (Figure 16A), and against both tested epitopes (Figure 16B).

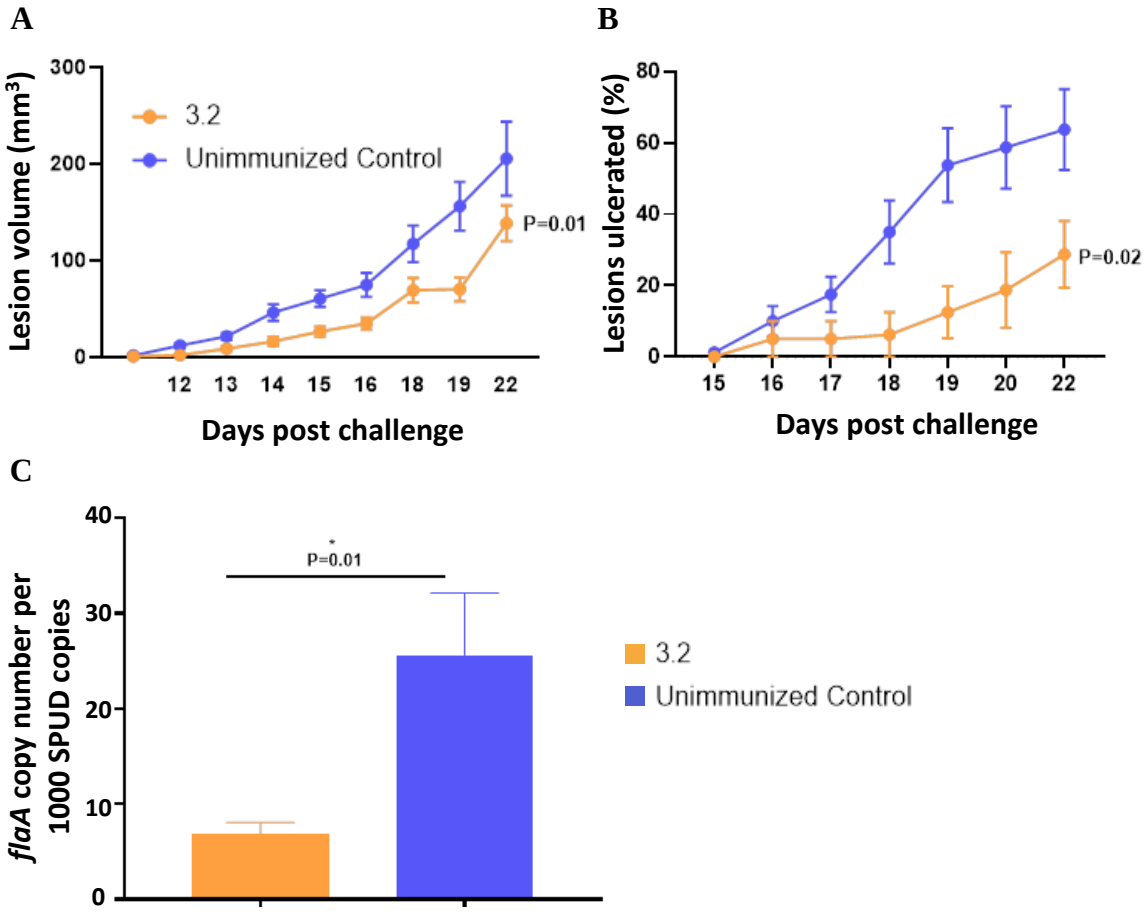


Figure 15. Immunization with chimera Construct 3.2 significantly attenuates both lesion volume, ulceration, and *T. pallidum* burden at the local infection site (immunized rabbits, n=8, unimmunized control rabbits, n=8). **A)** Lesion volume in the chimera immunized rabbits was smaller compared to the unimmunized control rabbits (P=0.01). **B)** Proportion of lesions ulcerating was also significantly reduced in the chimera-immunized animals, compared to the unimmunized animals (P=0.02). Data shown are mean +/- SEM. Significance was assessed using two-way ANOVA. **C)** The total number of *T. pallidum* in pooled lesion aspirates [day 19 PC] was assessed via qPCR. Local sites of chimera-immunized animals had significantly lower total treponemal burden on day 19 PC (P=0.01). Data presented as mean +/- SEM and significance was assessed using an unpaired t-test.

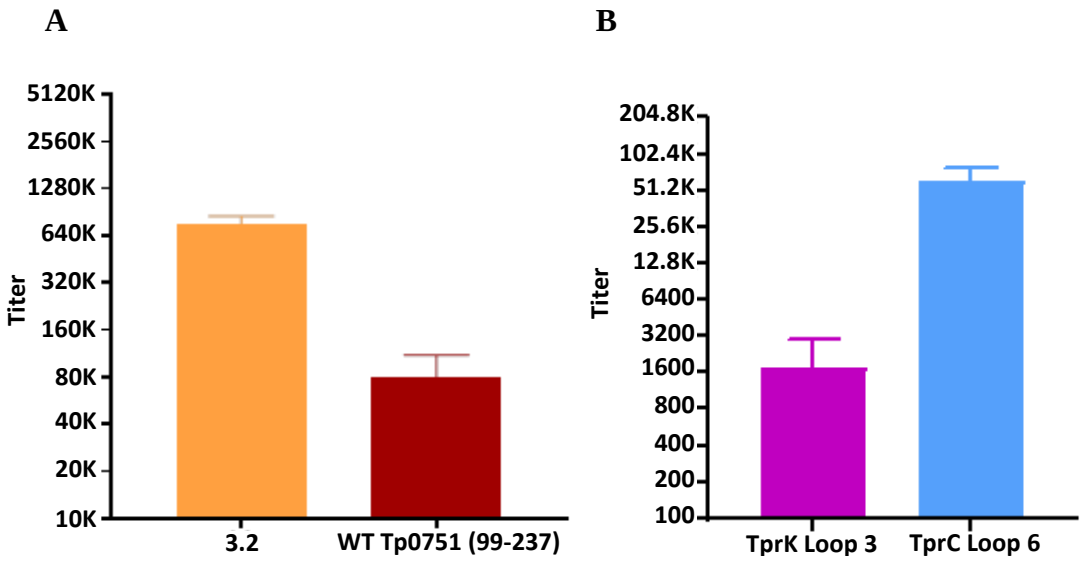


Figure 16. Immunization with Construct 3.2 induced high antibody titers against Tp0751, TprC, and TprK. A) Immunization with Construct 3.2 induced high titers against both full-length Construct 3.2 and WT Tp0751 (99-237). **B)** Immunization with Construct 3.2 induced high titers against TprK loop 3 and TprC loop 6 epitopes.

4.3.3.3 Immunization with Construct 3.2 inhibits *T. pallidum* dissemination

The rabbit infectivity test (RIT) was used to measure dissemination, where time to orchitis development was used as a metric to measure the number of viable treponemes in the transferred popliteal lymph nodes. A longer time to orchitis development indicates a lower treponemal burden in the lymph nodes, corresponding to a decrease in dissemination. Lymph node transfer was conducted on day 22 PC, and rabbits were monitored daily for 180 days. Of eight rabbits receiving lymph nodes from immunized rabbits, two showed no sign of orchitis development. The other six rabbits exhibited a three-day delay in orchitis development compared to rabbits receiving lymph nodes from unimmunized control rabbits (Figure 17). Using the standard curve described in Lukehart, S.A., et al. 2022 (163), the observed delay in orchitis development was calculated to be an approximate 85% reduction in dissemination to the popliteal lymph nodes in the chimera-immunized animals.

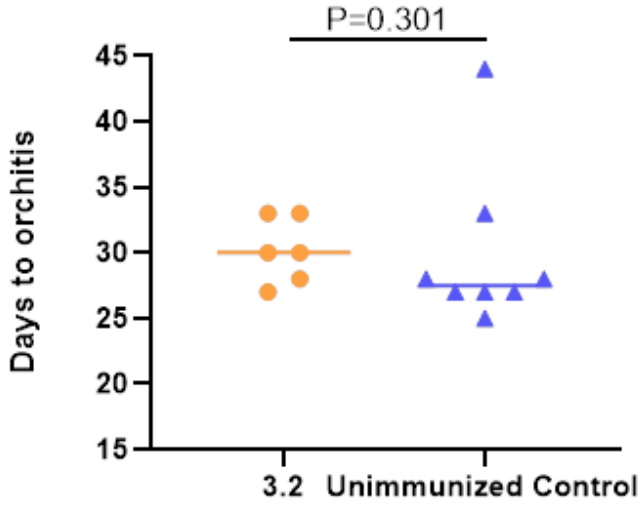


Figure 17. Inhibition of dissemination in the immunized animals was observed using the RIT. On day 22 PC, popliteal lymph nodes were extracted from the challenged rabbits and were injected into the testes of naïve rabbits to assess dissemination. The rabbits were then monitored for 180 days, where 8/8 of the animals in the unimmunized control group developed orchitis and 6/8 of the animals in the chimera immunized group developed orchitis. However, the median days to orchitis for the immunized group was three days later than the unimmunized control group. Comparisons were conducted by Mann-Whitney test. The bar observed in each group represents the median.

4.4 Discussion

The ultimate goal of a prophylactic syphilis vaccine candidate is to prevent infection by any *T. pallidum* strain through the prevention of lesion formation, dissemination, and treponemal persistence. Many candidates have been tested for protective capacity in animal models including BamA (174, 175), TprK (127, 139, 140), TprI (176), TprC (133), TprF (133), Gpd (177), TmpB (178), TpN15 (179), TpN47 (175), Tp0155 (175), Tp0483 (175), Tp0956 (175), 4D (180), Tp0751 (143) and endoflagella (181). However, none of these studies demonstrated complete protection of both dissemination and lesion formation. There is only one report, in a rabbit model, where complete protection was achieved. This vaccine regimen required 60 intravenous doses of

γ-irradiated *T. pallidum* over 37 weeks, making this regimen not viable for human administration. However, it does provide proof-of-concept for a protective syphilis vaccine (182). An effective vaccine must protect against both lesion formation and dissemination, to inhibit transmission and protect against late-stage disease development, respectively. Our chimeric Tp0751-based vaccine candidate was engineered with both goals in mind. By using Tp0751, a candidate to protect against dissemination, and TprC and TprK, candidates to protect against lesion formation, we were able to successfully engineer a single vaccine candidate capable of recapitulating the collective immune responses raised against each separate protein.

4.4.1 Construct 3.2 is the lead chimeric candidate to be used in protection trials

Prior to protection trials, two key chimeric proteins presented themselves as top candidates, Construct 3.1 and 3.2. Each of these proteins were nearly identical, with both proteins having the same epitope engraftments in the same locations, with the key difference being the engineering performed on the C-terminus. In Construct 3.1, the C-terminal epitope was engrafted directly onto the native C-terminus, and in Construct 3.2, the 9 terminal C-terminus residues were removed prior to epitope engraftment. Using the success criteria listed in Figure 4, each protein was assessed for stability to differentiate the best candidate for immunizations. Throughout purification, thermal stability, and proteolytic stability assessment, both proteins showed similar levels of stability with no discernable difference in stability or purity between the two. As a final measure of protein stability, both proteins were assessed by mass spectrometry, revealing that Construct 3.1 was ~1408 Da smaller than expected. Conversely, Construct 3.2 showed no significant decrease in MW, indicating that the C-terminal engineering in Construct 3.2 was likely more stable than in Construct 3.1. By retaining the 9 terminal residues in Construct 3.1, the engrafted epitope may protrude further from the core protein, decreasing stability and perhaps increasing accessibility for enzyme-based cleavage. This finding made it clear that Construct 3.2 was the optimal candidate to move forward with into protection trials.

4.4.2 Immunization with Construct 3.2 elicited partial protection in a rabbit model

The protection trial data showed that chimera-immunized rabbits demonstrated partial protection against *T. pallidum* infection, including decreased lesion volume and ulceration, decreased *T. pallidum* burden at lesion sites, and inhibition of dissemination to popliteal lymph nodes. Additionally, ELISA assays show that immunization induced a high antibody titer against WT Tp0751 (99-237), as well as against the engrafted TprC and TprK epitopes. These high titers indicate both that the WT Tp0751 scaffold is still able to be recognized by the immune system and that the engrafted Tpr epitopes are also recognized. It should be noted that due to the expense and complexity of these protection experiments, we were unable to directly compare these results to the protection achieved by each individual antigen (Tp0751, TprC, and TprK). This limitation makes it difficult to assess the contribution of each individual antigen within the chimera to overall protection. Despite this, we can compare the protection profile of our chimera to that of the tri-antigen cocktail in Lukehart *et al.,* 2022 (163), where both the UW and UVic experiments closely follow the immunization and challenge regimen conducted in our experiments. In Lukehart *et al,* 2022, researchers studied the protection profile of a tri-antigen cocktail consisting of Tp0751 (24-237), refolded N-terminal TprK, and refolded N-term Subfamily I (TprC), all of which have epitopes presented within our Construct 3.2. In these experiments, both UW and UVic showed that immunization with this tri-antigen cocktail was significantly able to inhibit lesion volume and ulceration, showed decreased *T. pallidum* burden at lesion sites, and inhibited dissemination of treponemes to popliteal lymph nodes, demonstrating the reproducibility of the results (163). In our experiments, immunization with Construct 3.2 was able to recapitulate similar levels of protection to those seen with the UW and UVic tri-antigen cocktail immunizations, such as decreased lesion volume and ulceration, decreased *T. pallidum* lesion burden, and inhibition of dissemination to popliteal lymph nodes. These findings indicate that we were able to successfully recapitulate the immune response raised against three separate antigenic targets using one single recombinant chimeric protein.

Chapter 5: Conclusions and Future Directions

5.1 Conclusions

An effective syphilis vaccine would help to overcome many of the challenges involved in global syphilis reduction, such as limited access to healthcare and limited clinical diagnostic tools. A prophylactic approach would decrease infection incidence altogether, rather than relying solely on diagnosis and treatment of active infections. A protective vaccine must target the highly invasive nature of *T. pallidum*, including both the primary lesions that facilitate transmission, as well as its propensity for dissemination throughout the bloodstream. Previous attempts to uncover protective vaccine candidates have had limited success, with no candidate showing complete protection of both lesion formation and dissemination (97, 127, 133, 139, 140, 174–181, 183). Tp0751 has been a key target for vaccine development, as it is one of the only proteins known to be implicated in treponemal dissemination (143). In addition to the evidence of its presence on the bacterial surface, it has also been shown to bind directly to multiple components of the host vasculature, including collagen, laminin, fibronectin and fibrinogen (77–82, 142). Despite its success as a vaccine candidate in protecting against dissemination, it is insufficient as a single protein candidate to prevent both lesion formation and reinfection (143). The inability of a single protein candidate to confer complete protection against syphilis infection exemplifies that a multi-component approach to vaccine design must be taken. The difficulty with a multi-component vaccine approach is that many currently characterized surface-exposed proteins adopt transmembrane β-barrel structures. These large transmembrane proteins are largely unable to be purified recombinantly due to their hydrophobic nature, making them unable to be used in classical multi-component vaccine approaches.

By leveraging our structural knowledge of Tp0751, we engineered a multi-component vaccine using a single protein that is stable, easily produced, and targets both lesion formation and dissemination. In this thesis, I have outlined the development of a highly modular vaccine platform by using Tp0751 as a scaffold on which to build a multi-component vaccine. By decorating this scaffold in immunogenic epitopes derived from other functionally important surface-exposed *T. pallidum* proteins, our goal is to retain the

immunogenicity of these large OMPs, TprC and TprK, while expressing their epitopes in a stable and soluble scaffold that provides protection against dissemination.

5.1.1 Tp0751 is a tractable scaffold on which to build a recombinant vaccine

In Chapter 2 of this thesis, initial proof-of-concept experiments were conducted to assess the engineering potential of Tp0751, where we replaced the native flexible loops regions of Tp0751 with the loops of structurally similar lipocalins NmfHbp and NHBA. From these experiments, we were able to show that two top loop candidates, loops 2 and 8, showed great promise as epitope engraftment sites. The resulting loop 2 and loop 8 constructs were both stable and soluble, indicating that the native Tp0751 residues were not required for structural stability and can be replaced with peptides of similar size.

By using the data acquired during proof-of-concept experiments, as well as by focusing on re-assessing engineering strategies used therein, we aimed to engraft up to four separate TprC- and TprK-derived epitope engraftments into the flexible loop and termini regions of the Tp0751 scaffold. By refining and following the engineering workflow and success criteria outlined in Figure 4, we successfully showed that Tp0751 is stable and soluble with up to four separate epitope engraftments, highlighted in Construct 4.1. The success of these experiments demonstrates the potential for Tp0751 to be used as a stable and soluble scaffold on which to express epitopes from various other *T. pallidum* proteins.

5.1.2 A Tp0751-based chimeric protein offers partial protection in an animal model

In addition to the engineering success, in Chapter 4 of this thesis, we have shown that Construct 3.2, a lead Generation 3 chimeric candidate, is both recognized in an animal model as shown through antibody titers and able to elicit a similar protective response to its constituent proteins, Tp0751, TprC, and TprK. These studies demonstrate that Tp0751 is a tractable scaffold on which to build a multi-component protein vaccine. To my knowledge, this is the first instance of a multi-component syphilis vaccine within a single recombinant protein. This vaccine platform provides a sophisticated method to express protein epitopes that would otherwise be incompatible with large-scale manufacturing processes due to the difficulty of maintaining solubility and proper protein folding of the full-length proteins. Though our chimera did not elicit complete protection in protection

trials, it elicited similar levels of protection to previous immunization experiments involving separately produced Tp0751, TprC and TprK proteins (163). In addition, immunization with our engineered chimera was able to inhibit *T. pallidum* dissemination at an efficacy similar to that observed in previous experiments, indicating that our engineering strategies did not affect the immunogenicity of Tp0751 (143, 163). This body of work, combined with future investigations that further apply to the technology described here, will provide an innovative solution to the many roadblocks involved in syphilis vaccine development.

5.2 Future directions

5.2.1 Future designs

In future design work, research should focus on expanding and uncovering new regions within Tp0751 for epitope engraftment. Multiple potential engraftment regions have yet to be tested for amenability, such as the N-terminus, loop 1, loop 5, and loop 7. Though loop 5 and loop 7 are markedly small loops (only two amino acids each), with the proper engineering strategy alongside the engraftment of relatively small epitopes (~six-ten amino acids), there is great potential for the success of these engraftment locations.

In addition to the TprC- and TprK-epitope chimeras outlined in this thesis, the Tp0751 scaffold offers an innovative solution to all future syphilis vaccine development. Since opsonic targets are crucial for proper immune clearance of treponemes, outer membrane proteins will continue to be key targets for vaccine development. As new outer membrane vaccine targets are discovered and validated within the field, Tp0751 will continue offering a stable and soluble scaffold on which to express their immunogenic epitopes.

5.2.2 Vaccine deployment strategies

The Tp0751 vaccine platform will enable not only multiple modes of delivery, but also the ability to be multimerized prior to delivery. This multimerization can be homologous to increase avidity, or heterologous to encompass a broader range of immune responses. Heterologous constructs can be created where each Tp0751 construct contains different epitope engraftments that are then multimerized into dimers, trimers, or more

using a multimerization linker region to enhance immunogenicity while maintaining stability and solubility.

Alternatively, homologous or heterologous chimera constructs can be expressed and displayed on outer membrane vesicles (OMVs) or contained within liposomes, either with or without a human-track adjuvant, to allow exposure of a multimerized vaccine construct to the immune system in a conformationally conserved manner. OMVs resemble their bacterial antigenic surface and inherently contain components that stimulate both humoral and cell-mediated immune responses (184). By embedding homologous or heterologous chimeric constructs within an OMV, it could allow for enhanced immunogenicity and protection within a host.

Finally, mRNA vaccines can be created with the Tp0751 vaccine scaffold sequence, thus taking advantage of the inherent adjuvant capacity, multivalency potential, and high immunogenicity associated with this mode of delivery. In addition, mRNA vaccines have the potential for rapid, inexpensive, and scalable manufacturing compared to recombinant protein vaccines, all important aspects when considering a globally available vaccine.

Appendix

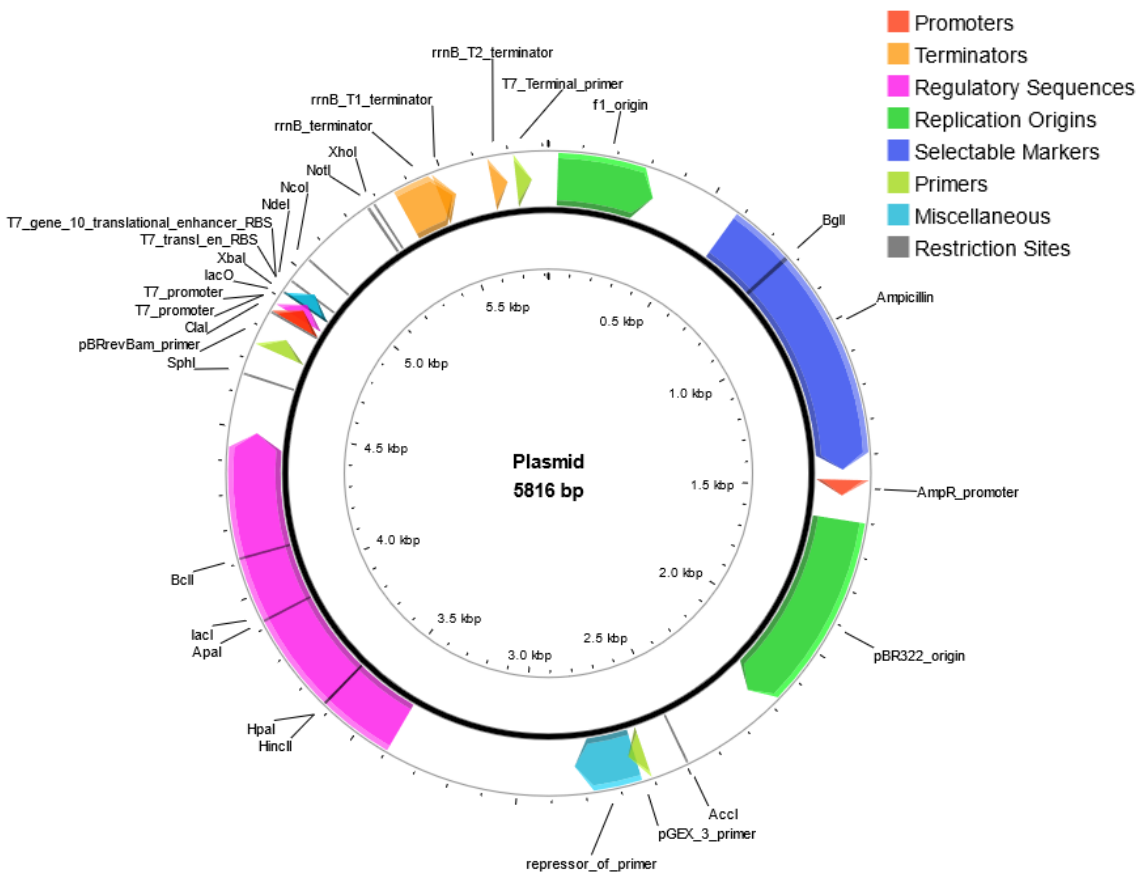


Figure A1. pET hisTEV N'term full-length vector map.

Table A1. pET hisTEV N'term full-length vector sequence.

#	Vector Sequence
1	TTGGCGAATG GGACGCGCCC TGTAGCGGCG CATTAAGCGC GGCGGGTGTG GTGGTTACGC
61	GCAGCGTGAC CGCTACACTT GCCAGCGCCC TAGCGCCCGC TCCTTTCGCT TTCTTCCCTT
121	CCTTTCTCGC CACGTTCGCC GGCTTTCCCC GTCAAGCTCT AAATCGGGGG CTCCCTTTAG
181	GGTTCCGATT TAGTGCTTTA CGGCACCTCG ACCCCAAAAA ACTTGATTAG GGTGATGGTT
241	CACGTAGTGG GCCATCGCCC TGATAGACGG TTTTTCGCCC TTTGACGTTG GAGTCCACGT
301	TCTTTAATAG TGGACTCTTG TTCCAAACTG GAACAACACT CAACCCTATC TCGGTCTATT
361	CTTTTGATTT ATAAGGGATT TTGCCGATTT CGGCCTATTG GTTAAAAAAT GAGCTGATTT
421	AACAAAAATT TAACGCGAAT TTTAACAAAA TATTAACGTT TACAATTTCT GGCGGCACGA
481	TGGCATGAGA TTATCAAAAA GGATCTTCAC CTAGATCCTT TTAAATTAAA AATGAAGTTT
541	TAAATCAATC TAAAGTATAT ATGAGTAAAC TTGGTCTGAC AGTTACCAAT GCTTAATCAG
601	TGAGGCACCT ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT GACTCCCCGT
661	CGTGTAGATA ACTACGATAC GGGAGGGCTT ACCATCTGGC CCCAGTGCTG CAATGATACC
721	GCGAGACCCA CGCTCACCGG CTCCAGATTT ATCAGCAATA AACCAGCCAG CCGGAAGGGC
781	CGAGCGCAGA AGTGGTCCTG CAACTTTATC CGCCTCCATC CAGTCTATTA ATTGTTGCCG
841	GGAAGCTAGA GTAAGTAGTT CGCCAGTTAA TAGTTTGCGC AACGTTGTTG CCATTGCTAC

901	AGGCATCGTG GTGTCACGCT CGTCGTTTGG TATGGCTTCA TTCAGCTCCG GTTCCCAACG
961	ATCAAGGCGA GTTACATGAT CCCCCATGTT GTGCAAAAAA GCGGTTAGCT CCTTCGGTCC
1021	TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTTATCA CTCATGGTTA TGGCAGCACT
1081	GCATAATTCT CTTACTGTCA TGCCATCCGT AAGATGCTTT TCTGTGACTG GTGAGTACTC
1141	AACCAAGTCA TTCTGAGAAT AGTGTATGCG GCGACCGAGT TGCTCTTGCC CGGCGTCAAT
1201	ACGGGATAAT ACCGCGCCAC ATAGCAGAAC TTTAAAAGTG CTCATCATTG GAAAACGTTC
1261	TTCGGGGCGA AAACTCTCAA GGATCTTACC GCTGTTGAGA TCCAGTTCGA TGTAACCCAC
1321	TCGTGCACCC AACTGATCTT CAGCATCTTT TACTTTCACC AGCGTTTCTG GGTGAGCAAA
1381	AACAGGAAGG CAAAATGCCG CAAAAAAGGG AATAAGGGCG ACACGGAAAT GTTGAATACT
1441	CATACTCTTC CTTTTTCAAT CATGATTGAA GCATTTATCA GGGTTATTGT CTCATGAGCG
1501	GATACATATT TGAATGTATT TAGAAAAATA AACAAATAGG TCATGACCAA AATCCCTTAA
1561	CGTGAGTTTT CGTTCCACTG AGCGTCAGAC CCCGTAGAAA AGATCAAAGG ATCTTCTTGA
1621	GATCCTTTTT TTCTGCGCGT AATCTGCTGC TTGCAAACAA AAAAACCACC GCTACCAGCG
1681	GTGGTTTGTT TGCCGGATCA AGAGCTACCA ACTCTTTTTC CGAAGGTAAC TGGCTTCAGC
1741	AGAGCGCAGA TACCAAATAC TGTCCTTCTA GTGTAGCCGT AGTTAGGCCA CCACTTCAAG
1801	AACTCTGTAG CACCGCCTAC ATACCTCGCT CTGCTAATCC TGTTACCAGT GGCTGCTGCC
1861	AGTGGCGATA AGTCGTGTCT TACCGGGTTG GACTCAAGAC GATAGTTACC GGATAAGGCG
1921	CAGCGGTCGG GCTGAACGGG GGGTTCGTGC ACACAGCCCA GCTTGGAGCG AACGACCTAC
1981	ACCGAACTGA GATACCTACA GCGTGAGCTA TGAGAAAGCG CCACGCTTCC CGAAGGGAGA
2041	AAGGCGGACA GGTATCCGGT AAGCGGCAGG GTCGGAACAG GAGAGCGCAC GAGGGAGCTT
2101	CCAGGGGGAA ACGCCTGGTA TCTTTATAGT CCTGTCGGGT TTCGCCACCT CTGACTTGAG
2161	CGTCGATTTT TGTGATGCTC GTCAGGGGGG CGGAGCCTAT GGAAAAACGC CAGCAACGCG
2221	GCCTTTTTAC GGTTCCTGGC CTTTTGCTGG CCTTTTGCTC ACATGTTCTT TCCTGCGTTA
2281	TCCCCTGATT CTGTGGATAA CCGTATTACC GCCTTTGAGT GAGCTGATAC CGCTCGCCGC
2341	AGCCGAACGA CCGAGCGCAG CGAGTCAGTG AGCGAGGAAG CGGAAGAGCG CCTGATGCGG
2401	TATTTTCTCC TTACGCATCT GTGCGGTATT TCACACCGCA TATATGGTGC ACTCTCAGTA
2461	CAATCTGCTC TGATGCCGCA TAGTTAAGCC AGTATACACT CCGCTATCGC TACGTGACTG
2521	GGTCATGGCT GCGCCCCGAC ACCCGCCAAC ACCCGCTGAC GCGCCCTGAC GGGCTTGTCT
2581	GCTCCCGGCA TCCGCTTACA GACAAGCTGT GACCGTCTCC GGGAGCTGCA TGTGTCAGAG
2641	GTTTTCACCG TCATCACCGA AACGCGCGAG GCAGCTGCGG TAAAGCTCAT CAGCGTGGTC
2701	GTGAAGCGAT TCACAGATGT CTGCCTGTTC ATCCGCGTCC AGCTCGTTGA GTTTCTCCAG
2761	AAGCGTTAAT GTCTGGCTTC TGATAAAGCG GGCCATGTTA AGGGCGGTTT TTTCCTGTTT
2821	GGTCACTGAT GCCTCCGTGT AAGGGGGATT TCTGTTCATG GGGGTAATGA TACCGATGAA
2881	ACGAGAGAGG ATGCTCACGA TACGGGTTAC TGATGATGAA CATGCCCGGT TACTGGAACG
2941	TTGTGAGGGT AAACAACTGG CGGTATGGAT GCGGCGGGAC CAGAGAAAAA TCACTCAGGG
3001	TCAATGCCAG CGCTTCGTTA ATACAGATGT AGGTGTTCCA CAGGGTAGCC AGCAGCATCC
3061	TGCGATGCAG ATCCGGAACA TAATGGTGCA GGGCGCTGAC TTCCGCGTTT CCAGACTTTA
3121	CGAAACACGG AAACCGAAGA CCATTCATGT TGTTGCTCAG GTCGCAGACG TTTTGCAGCA
3181	GCAGTCGCTT CACGTTCGCT CGCGTATCGG TGATTCATTC TGCTAACCAG TAAGGCAACC
3241	CCGCCAGCCT AGCCGGGTCC TCAACGACAG GAGCACGATC ATGCTAGTCA TGCCCCGCGC
3301	CCACCGGAAG GAGCTGACTG GGTTGAAGGC TCTCAAGGGC ATCGGTCGAG ATCCCGGTGC
3361	CTAATGAGTG AGCTAACTTA CATTAATTGC GTTGCGCTCA CTGCCCGCTT TCCAGTCGGG
3421	AAACCTGTCG TGCCAGCTGC ATTAATGAAT CGGCCAACGC GCGGGGAGAG GCGGTTTGCG
3481	TATTGGGCGC CAGGGTGGTT TTTCTTTTCA CCAGTGAGAC GGGCAACAGC TGATTGCCCT
3541	TCACCGCCTG GCCCTGAGAG AGTTGCAGCA AGCGGTCCAC GCTGGTTTGC CCCAGCAGGC
3601	GAAAATCCTG TTTGATGGTG GTTAACGGCG GGATATAACA TGAGCTGTCT TCGGTATCGT
3661	CGTATCCCAC TACCGAGATG TCCGCACCAA CGCGCAGCCC GGACTCGGTA ATGGCGCGCA
3721	TTGCGCCCAG CGCCATCTGA TCGTTGGCAA CCAGCATCGC AGTGGGAACG ATGCCCTCAT
3781	TCAGCATTTG CATGGTTTGT TGAAAACCGG ACATGGCACT CCAGTCGCCT TCCCGTTCCG
3841	CTATCGGCTG AATTTGATTG CGAGTGAGAT ATTTATGCCA GCCAGCCAGA CGCAGACGCG
3901	CCGAGACAGA ACTTAATGGG CCCGCTAACA GCGCGATTTG CTGGTGACCC AATGCGACCA
3961	GATGCTCCAC GCCCAGTCGC GTACCGTCTT CATGGGAGAA AATAATACTG TTGATGGGTG
4021	TCTGGTCAGA GACATCAAGA AATAACGCCG GAACATTAGT GCAGGCAGCT TCCACAGCAA
4081	TGGCATCCTG GTCATCCAGC GGATAGTTAA TGATCAGCCC ACTGACGCGT TGCGCGAGAA
4141	GATTGTGCAC CGCCGCTTTA CAGGCTTCGA CGCCGCTTCG TTCTACCATC GACACCACCA
4201	CGCTGGCACC CAGTTGATCG GCGCGAGATT TAATCGCCGC GACAATTTGC GACGGCGCGT
4261	GCAGGGCCAG ACTGGAGGTG GCAACGCCAA TCAGCAACGA CTGTTTGCCC GCCAGTTGTT

4321	GTGCCACGCG GTTGGGAATG TAATTCAGCT CCGCCATCGC CGCTTCCACT TTTTCCCGCG
4381	TTTTCGCAGA AACGTGGCTG GCCTGGTTCA CCACGCGGGA AACGGTCTGA TAAGAGACAC
4441	CGGCATACTC TGCGACATCG TATAACGTTA CTGGTTTCAC ATTCACCACC CTGAATTGAC
4501	TCTCTTCCGG GCGCTATCAT GCCATACCGC GAAAGGTTTT GCGCCATTCG ATGGTGTCCG
4561	GGATCTCGAC GCTCTCCCTT ATGCGACTCC TGCATTAGGA AGCAGCCCAG TAGTAGGTTG
4621	AGGCCGTTGA GCACCGCCGC CGCAAGGAAT GGTGCATGCA AGGAGATGGC GCCCAACAGT
4681	CCCCCGGCCA CGGGGCCTGC CACCATACCC ACGCCGAAAC AAGCGCTCAT GAGCCCGAAG
4741	TGGCGAGCCC GATCTTCCCC ATCGGTGATG TCGGCGATAT AGGCGCCAGC AACCGCACCT
4801	GTGGCGCCGG TGATGCCGGC CACGATGCGT CCGGCGTAGA GGATCGAGAT CGATCTCGAT
4861	CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA TTCCCCTCTA
4921	GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATACATAT GGGCTCCACT AGTGGTAGCG
4981	GCCACCATCA CCATCACCAT TCTTCTGGTC GTGAAAACCT GTACTTCCAG GGCTCCATGG
5041	CTAGCTAACC AGTGCGATGT AAGCTAATGA CTGAGCGCTA ATATGCATCG CTTGAGCCCG
5101	AGAATAAACT CACGAATAAG GGAGAGAGGC TACGGAATCC ACATAAAAGA TAGATTCTTG
5161	ATTGTAGGGA TAACTGGCAC GGTTCCAACA GACCCTGGGT GTCCTTCGAC CAGAACCTGG
5221	ATTACCAAAT CGGTTACATC TGCTCTGGAG TCTTCGGTGA CGCGGCCGCC TAAGTAACTA
5281	AACTCGAGCA CCACCACCAC CACCACTAAT GTTAATTAAG TTGGGCGTTC CTAGGCTGAT
5341	AAAACAGAAT TTGCCTGGCG GCAGTAGCGC GGTGGTCCCA CCTGACCCCA TGCCGAACTC
5401	AGAAGTGAAA CGCCGTAGCG CCGATGGTAG TGTGGGGTCT CCCCATGCGA GAGTAGGGAA
5461	CTGCCAGGCA TCAAATAAAA CGAAAGGCTC AGTCGAAAGA CTGGGCCTTT CGTTTTATCT
5521	GTTGTTTGTC GGTGAACGCT CTCCTGAGTA GGACAAATCC GCCGGGAGCG GATTTGAACG
5581	TTGCGAAGCA ACGGCCCGGA GGGTGGCGGG CAGGACGCCC GCCATAAACT GCCAGGCATC
5641	AAATTAAGCA GAAGGCCATC CTGACGGATG GCCTTTTTGC GTTTCTACAA ACTCTTTTGT
5701	TTATTTTTCT AAATACATTC AAATATGTAT CCGCTGAGCA ATAACTAGCA TAACCCCTTG
5761	GGGCCTCTAA ACGGGTCTTG AGGGGTTTTT TGCTGAAAGG AGGAACTATA TCCGGA

Table A2. Tp0751 (99-237) mutant chimera designations, construct sequences, and sequence IDs.

Construct ID	Peptide Sequence
0.1	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTE**GDDLHMG**GDIHPLL FFDREHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRRRLEVTF ESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP
0.2	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTE**KTVNGIRH**GDIHPL LFFDREHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRRRLEVT FESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP
0.3	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTE**VLYNQAEKGS**GDIH PLLFFDREHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRRRLE VTFESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP
0.4	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTEISPNSGDIHPLLFFDR **GDDLHMG**AEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRRRLE VTFESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP
0.5	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTEISPNSGDIHPLLFFDR **VLYNQAEKGS**AEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRR RLEVTFESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP

0.6	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTEISPNSGDIHPLLFFDR EHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTA**GDDLHMG**SIRRRL EVTFESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP
0.7	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTEISPNSGDIHPLLFFDR EHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTA**KTVNGIRH**SIRRRL EVTFESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP
0.8	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTEISPNSGDIHPLLFFDR EHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTA**VLYNQAEKGS**SIRR RLEVTFESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP
0.9	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTEISPNSGDIHPLLFFDR EHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRRRLEVTFESHE VIHVRAVEDVAR**GDDLHMG**MWDGQYTRYHAGPASAPSP
0.10	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTEISPNSGDIHPLLFFDR EHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRRRLEVTFESHE VIHVRAVEDVAR**KTVNGIRH**MWDGQYTRYHAGPASAPSP
0.11	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTEISPNSGDIHPLLFFDR EHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRRRLEVTFESHE VIHVRAVEDVAR**VLYNQAEKGS**MWDGQYTRYHAGPASAPSP
1.1	VQTAMRIALWNRATHGEQGALQHLLAGLWIQT**RAYSEKDTRYA**HPLL FFDREHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRRRLEVTF ESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP
1.2	VQTAMRIALWNRATHGEQGALQHLLAGLWIQT**GKLVQNLPNIMMPP GIT**HPLLFFDREHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIR RRLEVTFESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP
1.3	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTE**AKTIEATLH**GDIHPL LFFDREHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRRRLEVT FESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP
1.4	VQTAMRIALWNRATHGEQGALQHLLAGLWIQT**AKTIEATLH**HPLLFFD REHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRRRLEVTFESH EVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP
1.5	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTE**ESNGGAK**GDIHPLL FFDREHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRRRLEVTF ESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP
1.6	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTE**DYKSKGDKPVYE**IH PLLFFDREHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRRRLE VTFESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP
1.7	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTE**LQENSNVVIEKNV**I HPLLFFDREHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRRRL EVTFESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP
1.8	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTEISPNSGDIHPLLFFD**D NNQPFDLTV**EITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRRRLE VTFESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP

1.9	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTEISPNSGDIHPLLFFD**E SNGGAK**EITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRRRLEVTF ESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP
1.10	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTEISPNSGDIHPLLFFD**L QENSNVVIEKNV**EITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRR RLEVTFESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP
1.11	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTEISPNSGDIHPLLFFDR EHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRN**DNNQPFDLTV**IRRRLE VTFESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP
1.12	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTEISPNSGDIHPLLFFDR EHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRN**RAYSEKDTRYA**IRRRL EVTFESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP
1.13	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTEISPNSGDIHPLLFFDR EHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRN**ESNGGAK**IRRRLEVTF ESHEVIHVRAVEDVARLKIGSTSMWDGQYTRYHAGPASAPSP
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2.1	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTE**RAYSEKDTRYA**GDI HPLLFFDREHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRRRL EVTFESHEVIHVRAVEDVAR**DYKSKGDKPVYE**WDGQYTRYHAGPASA PSP
2.2	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTE**GKLVQNLPNIMMP PGIT**GDIHPLLFFDREHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTA ISSIRRRLEVTFESHEVIHVRAVEDVAR**DYKSKGDKPVYE**WDGQYTRY HAGPASAPSP
2.3	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTE**GTTNRFNIINAAGN LLN**GDIHPLLFFDREHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAI SSIRRRLEVTFESHEVIHVRAVEDVAR**DYKSKGDKPVYE**WDGQYTRYH AGPASAPSP
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2.8	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTE**GKLQENSNVV**GDIH PLLFFDREHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRRRLE VTFESHEVIHVRAVEDVAR**DYKSKGDKPVYE**WDGQYTRYHAGPASAP SP

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3.2	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTE**AKTIEATLH**GDIHPL LFFDREHAEITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIRRRLEVT FESHEVIHVRAVEDVAR**DYKSKGDKPVYE**WDGQYTRYH**GKLVQNLP NIMMPPGIT**
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4.1	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTE**DYKSKGDKPVYE**IH PLLFFD**ESNGGAK**EITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSIR RRLEVTFESHEVIHVRAVEDVA**RAYSEKDTRYA**WDGQYTRYH**GKLV QNLPNIMMPPGIT**
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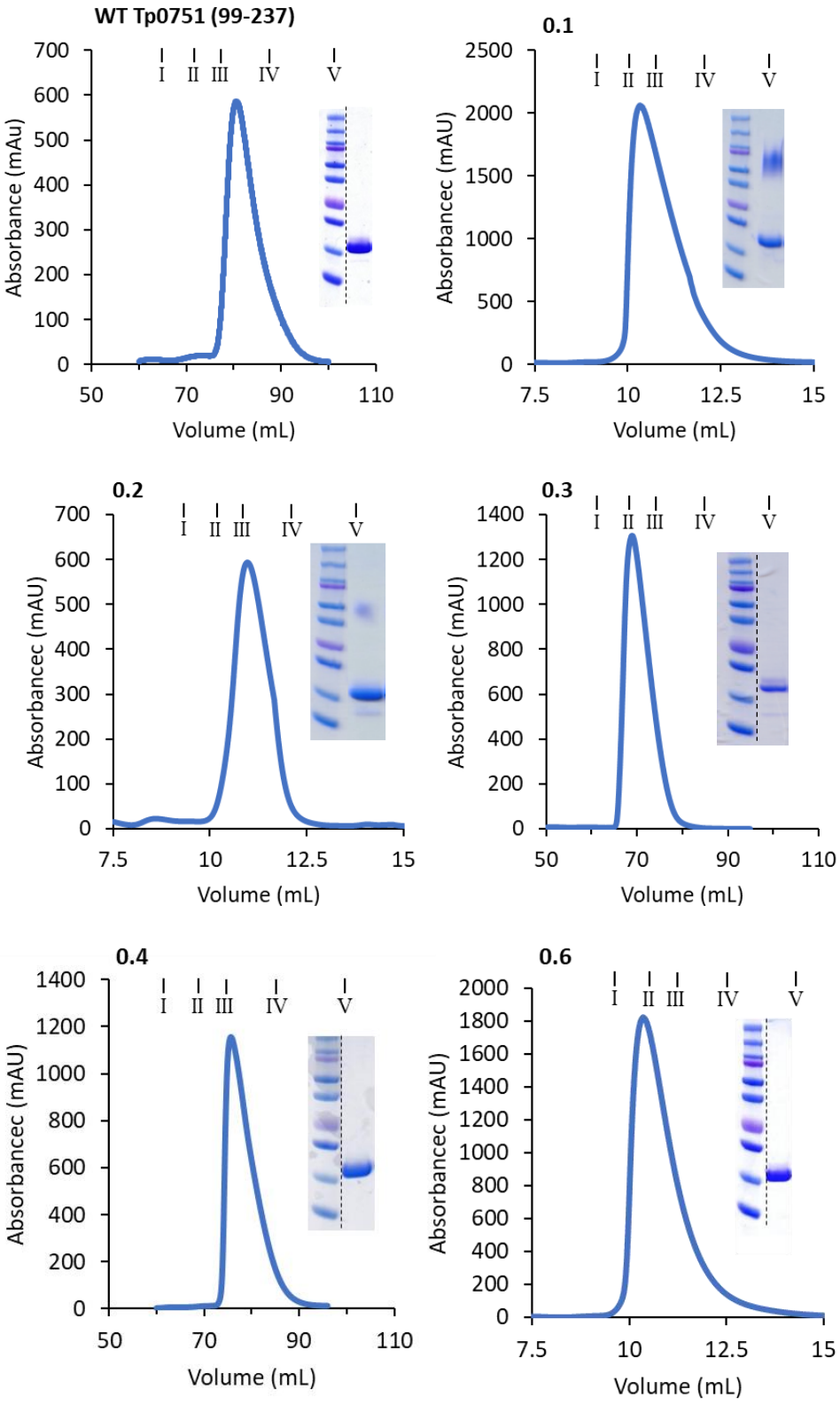
4.3	VQTAMRIALWNRATHGEQGALQHLLAGLWIQTE**LQENSNVVIEKNV**I HPLLFFD**ESNGGAK**EITFSRASVQEIFLVDSAHTHRKTVSFLTRNTAISSI RRRLEVTFESHEVIHVRAVEDVA**DYKSKGDKPVYE**WDGQYTRYH**GK LVQNLPNIMMPPGIT**


WT Tp0751 (99-237)
Absorbance (mAu)
Volume (mL)
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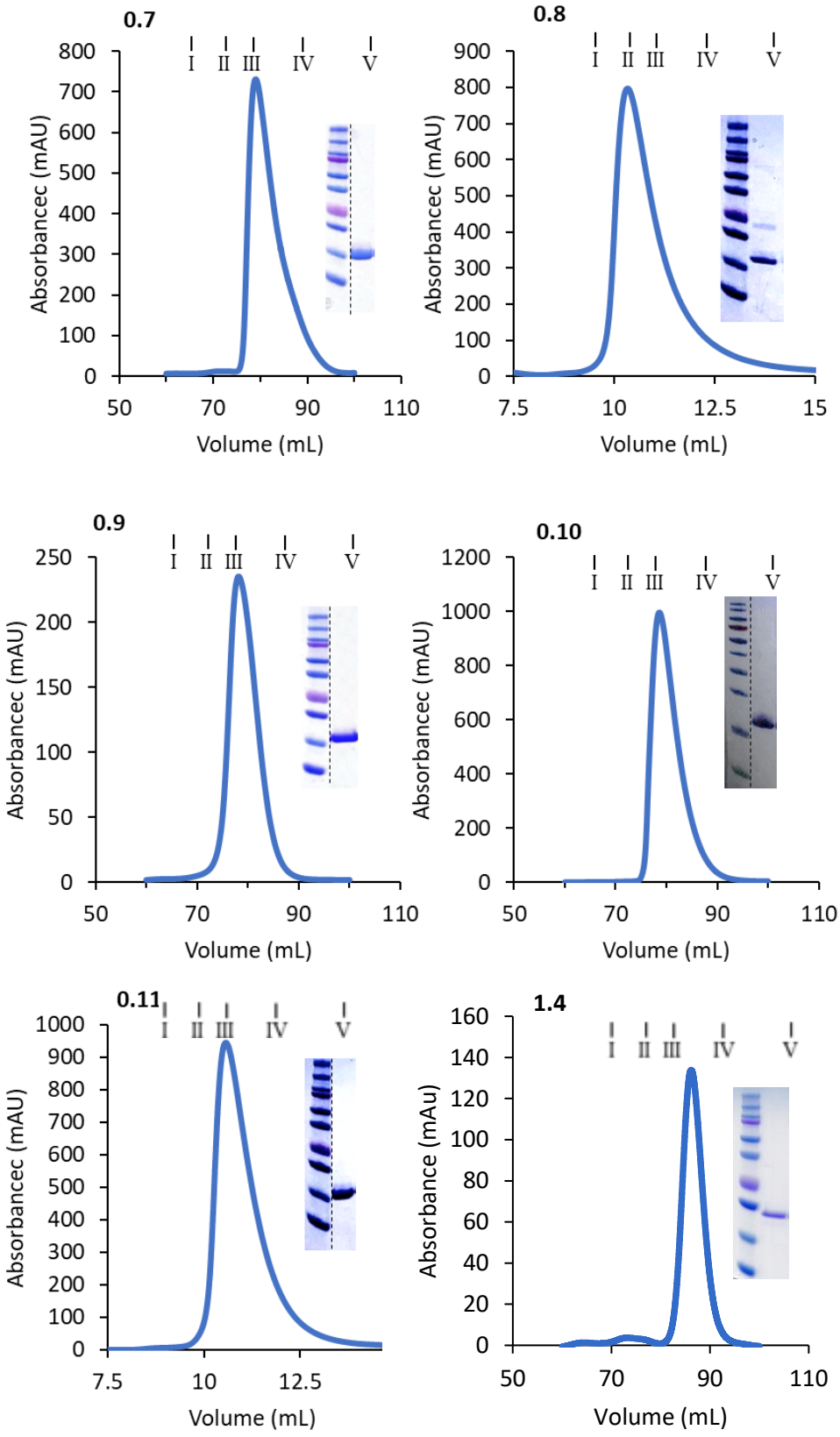
0.1
Absorbancec (mAU)
0.2
0.3
0.4
0.6


0.7
I II III IV V
Absorbancec (mAU)
Volume (mL)
0.8
I II III IV V
Absorbancec (mAU)
Volume (mL)
0.9
I II III IV V
Absorbancec (mAU)
Volume (mL)
0.10
I II III IV V
Absorbancec (mAU)
Volume (mL)
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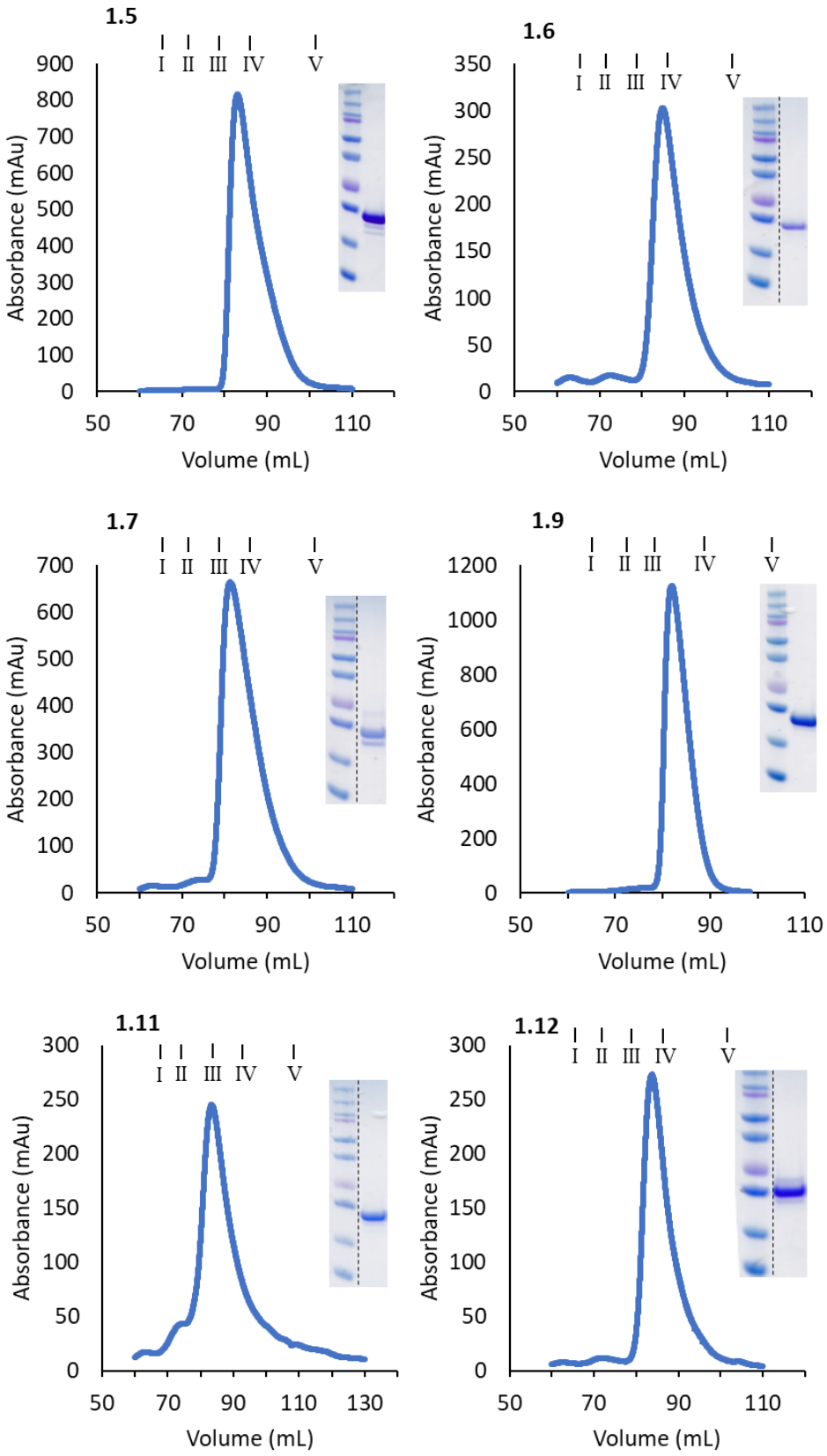
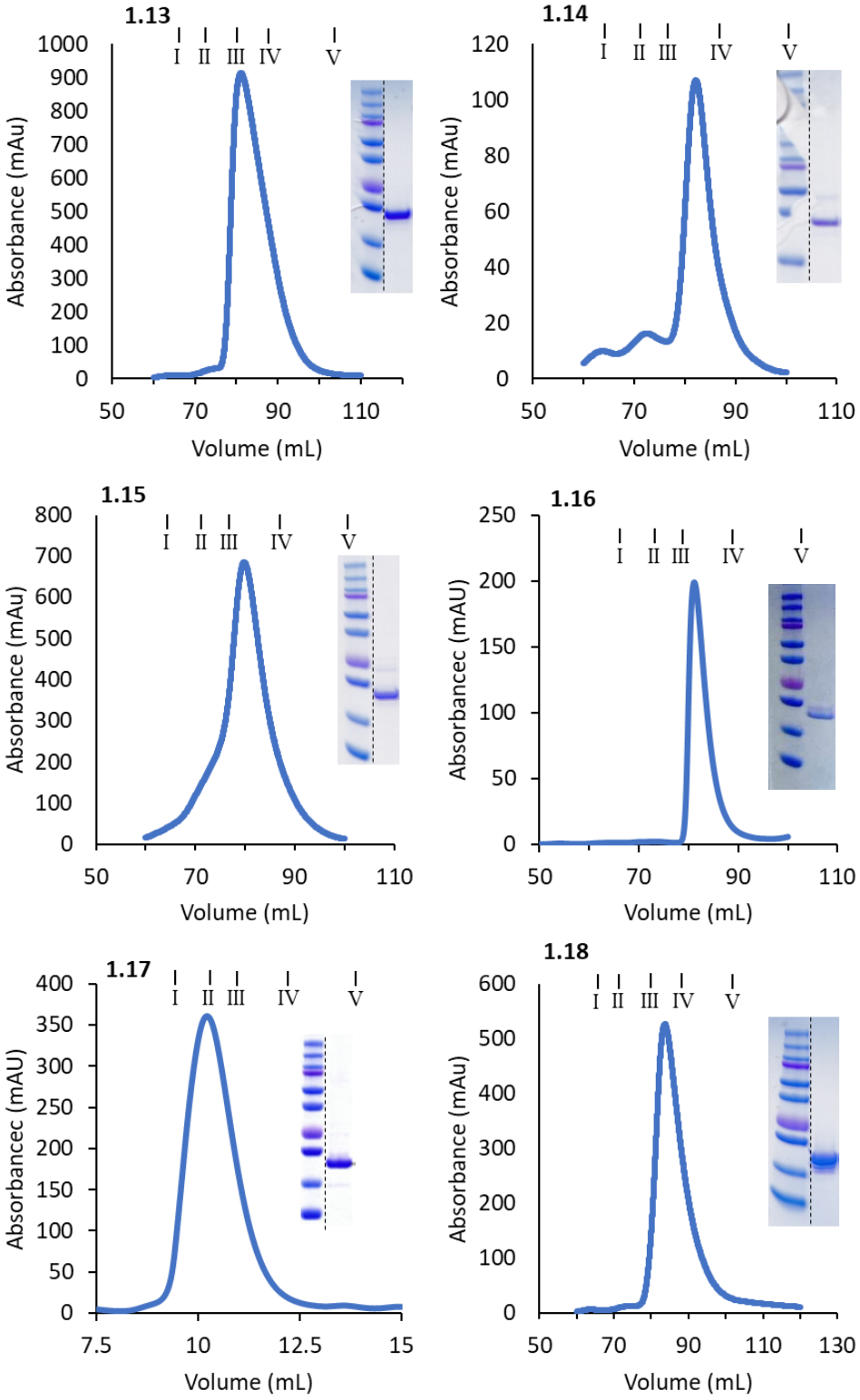
Absorbancec (mAU)
Volume (mL)
1.4
I II III IV V
Absorbance (mAu)
Volume (mL)


1.5
I II III IV V
Absorbance (mAu)
Volume (mL)
1.6
I II III IV V
Absorbance (mAu)
Volume (mL)
1.7
I II III IV V
Absorbance (mAu)
Volume (mL)
1.9
I II III IV V
Absorbance (mAu)
Volume (mL)
1.11
I II III IV V
Absorbance (mAu)
Volume (mL)
1.12
I II III IV V
Absorbance (mAu)
Volume (mL)


1.13
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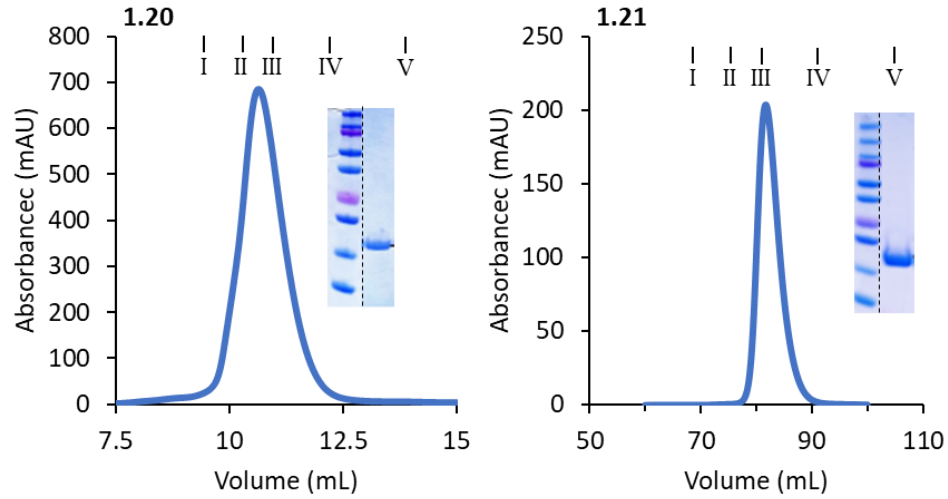
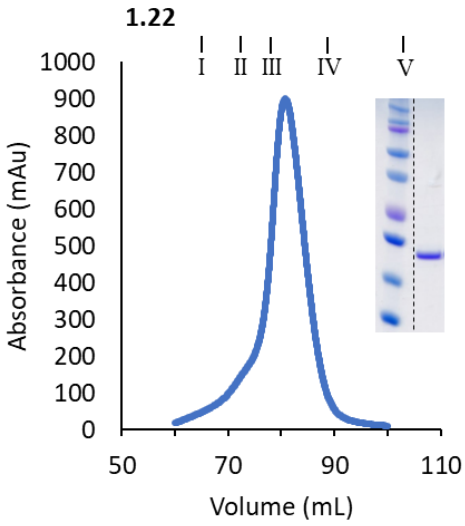
Absorbance (mAu)
Volume (mL)
1.14
I II III IV V
Absorbance (mAu)
Volume (mL)
1.15
I II III IV V
Absorbance (mAu)
Volume (mL)
1.16
I II III IV V
Absorbancec (mAU)
Volume (mL)
1.17
I II III IV V
Absorbancec (mAU)
Volume (mL)
1.18
I II III IV V
Absorbance (mAu)
Volume (mL)


1.20
I II III IV V
Absorbancec (mAU)
800
700
600
500
400
300
200
100
0
7.5
10
12.5
15
Volume (mL)

1.21
I II III IV V
Absorbancec (mAU)
250
200
150
100
50
0
50
70
90
110
Volume (mL)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I II III IV V
Absorbance (mAu)
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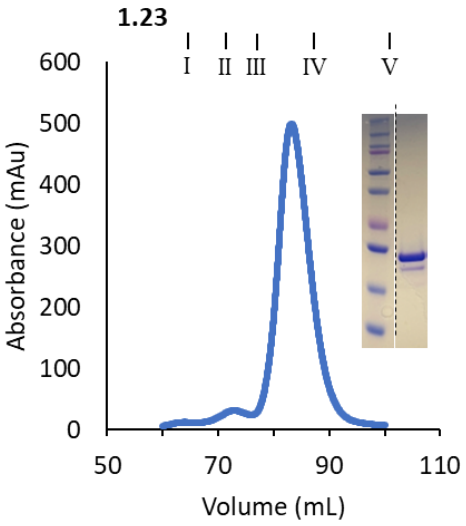
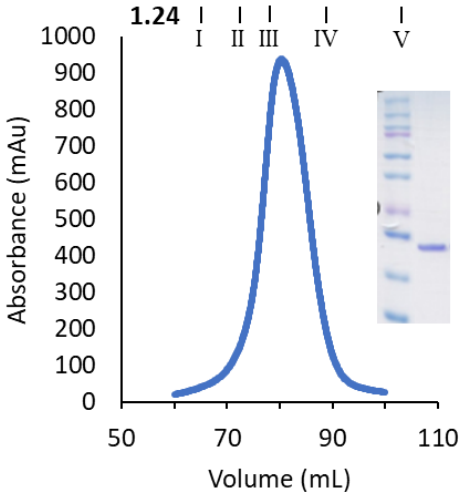
900
800
700
600
500
400
300
200
100
0
50
70
90
110
Volume (mL)


1.23
I II III IV V
Absorbance (mAu)
600
500
400
300
200
100
0
50
70
90
110
Volume (mL)


1.24
I II III IV V
Absorbance (mAu)
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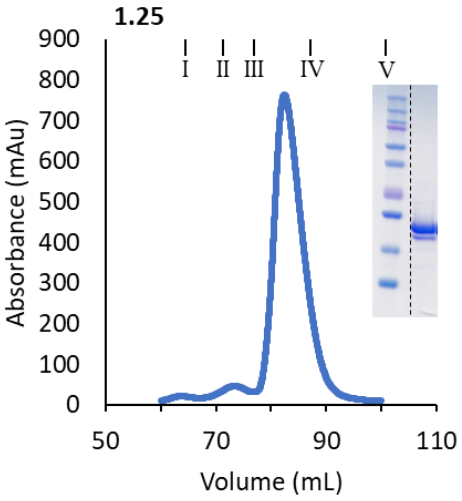
900
800
700
600
500
400
300
200
100
0
50
70
90
110
Volume (mL)


1.25
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Absorbance (mAu)
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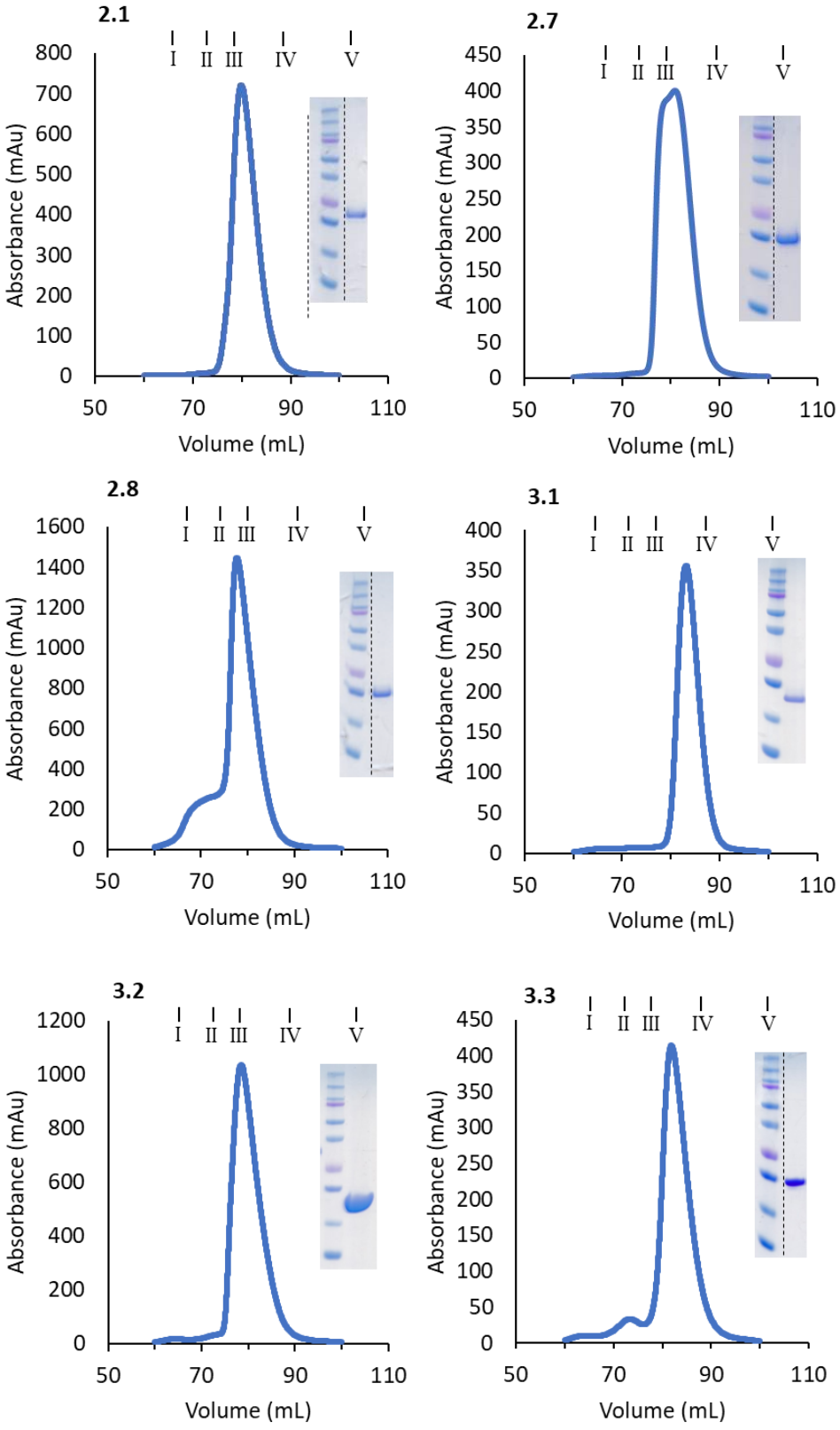
800
700
600
500
400
300
200
100
0
50
70
90
110
Volume (mL)


2.1
I II III IV V
Absorbance (mAu)
Volume (mL)
2.7
I II III IV V
Absorbance (mAu)
Volume (mL)
2.8
I II III IV V
Absorbance (mAu)
Volume (mL)
3.1
I II III IV V
Absorbance (mAu)
Volume (mL)
3.2
I II III IV V
Absorbance (mAu)
Volume (mL)
3.3
I II III IV V
Absorbance (mAu)
Volume (mL)

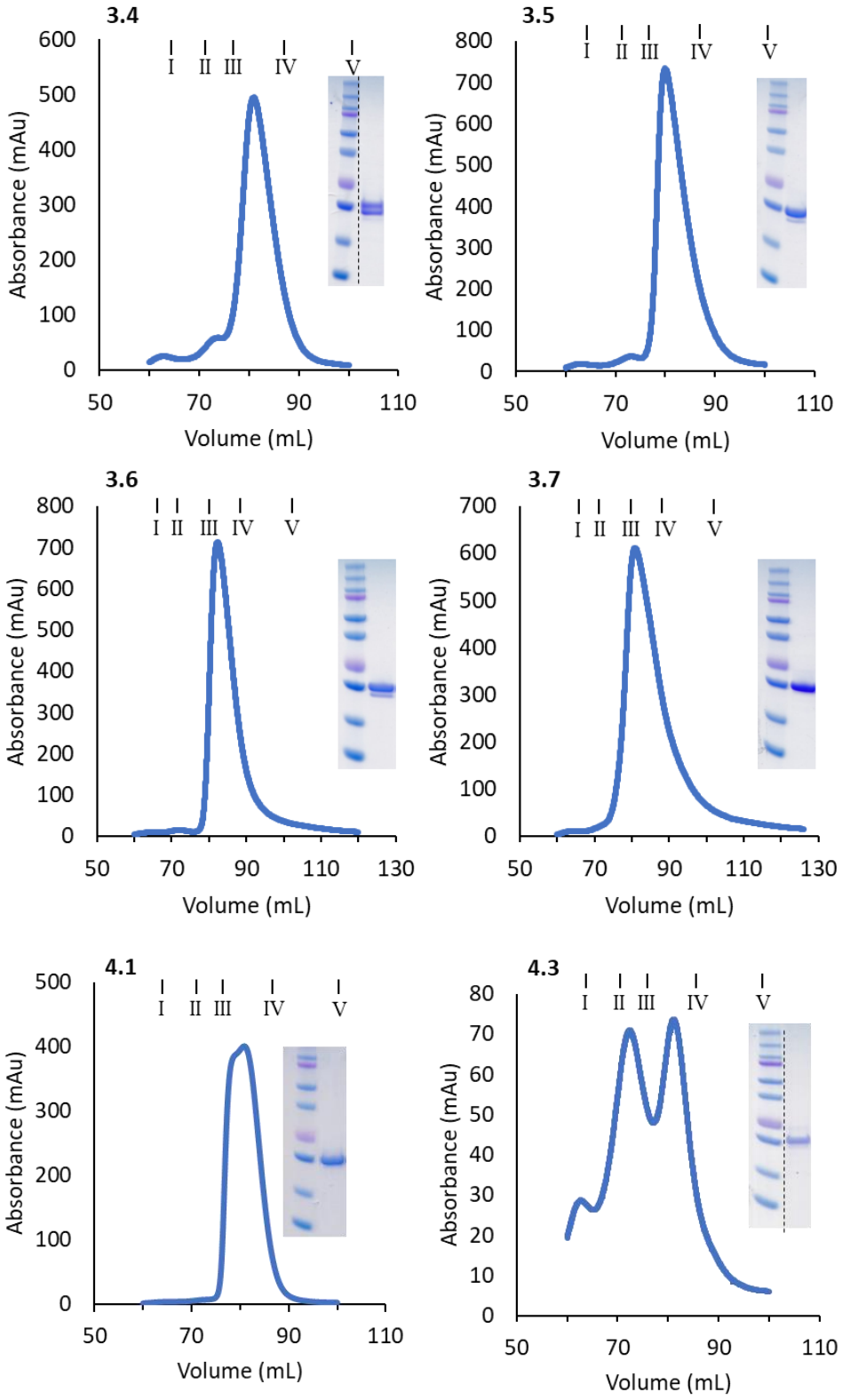

3.4
I II III IV V
Absorbance (mAu)
Volume (mL)
3.5
I II III IV V
Absorbance (mAu)
Volume (mL)
3.6
I II III IV V
Absorbance (mAu)
Volume (mL)
3.7
I II III IV V
Absorbance (mAu)
Volume (mL)
4.1
I II III IV V
Absorbance (mAu)
Volume (mL)
4.3
I II III IV V
Absorbance (mAu)
Volume (mL)

Figure A2. Size exclusion chromatograms of all successfully purified Tp0751 (99-237) chimeras. Purity gels of each construct are shown on 4-12% acrylamide SDS-PAGE gels alongside Bio-Rad Dual Color Precision Plus Protein Standards molecular weight ladder. Protein standards listed above the chromatogram are as follows: (I) conalbumin (75 kDa), (II) ovalbumin (44 kDa), (III) carbonic anhydrase (29kDa), (IV) ribonuclease A (13.7kDa), (V) aprotinin (6.5kDa).

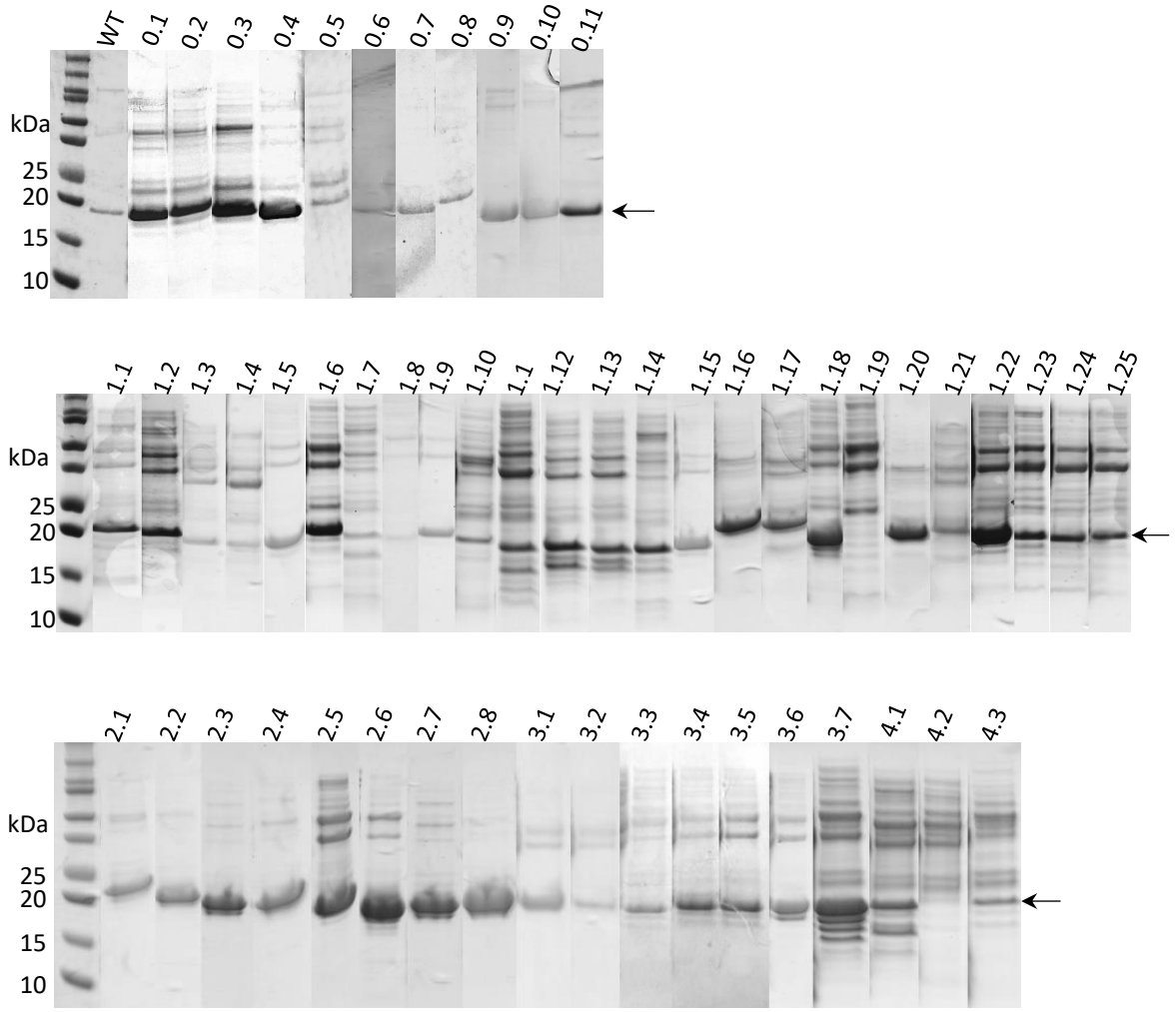


Figure A3. Small-scale expression trials of Tp0751 chimeras. Samples taken from multiple stages of purification (insoluble pellet, lysis supernatant, purified sample) from *E. coli* cultures were evaluated on a 12% Bolt Bis-Tris Plus gel at 200 V for 30 minutes, and expression was assessed by the presence or absence of a strong band at the expected molecular weight (designated by arrows). Only nickel-enriched samples are shown above. Chimeric constructs that showed expression equivalent to wild type Tp0751 (99-237) and that could be enriched by nickel pulldown indicated correctly folded protein and were advanced to large-scale expression trials.

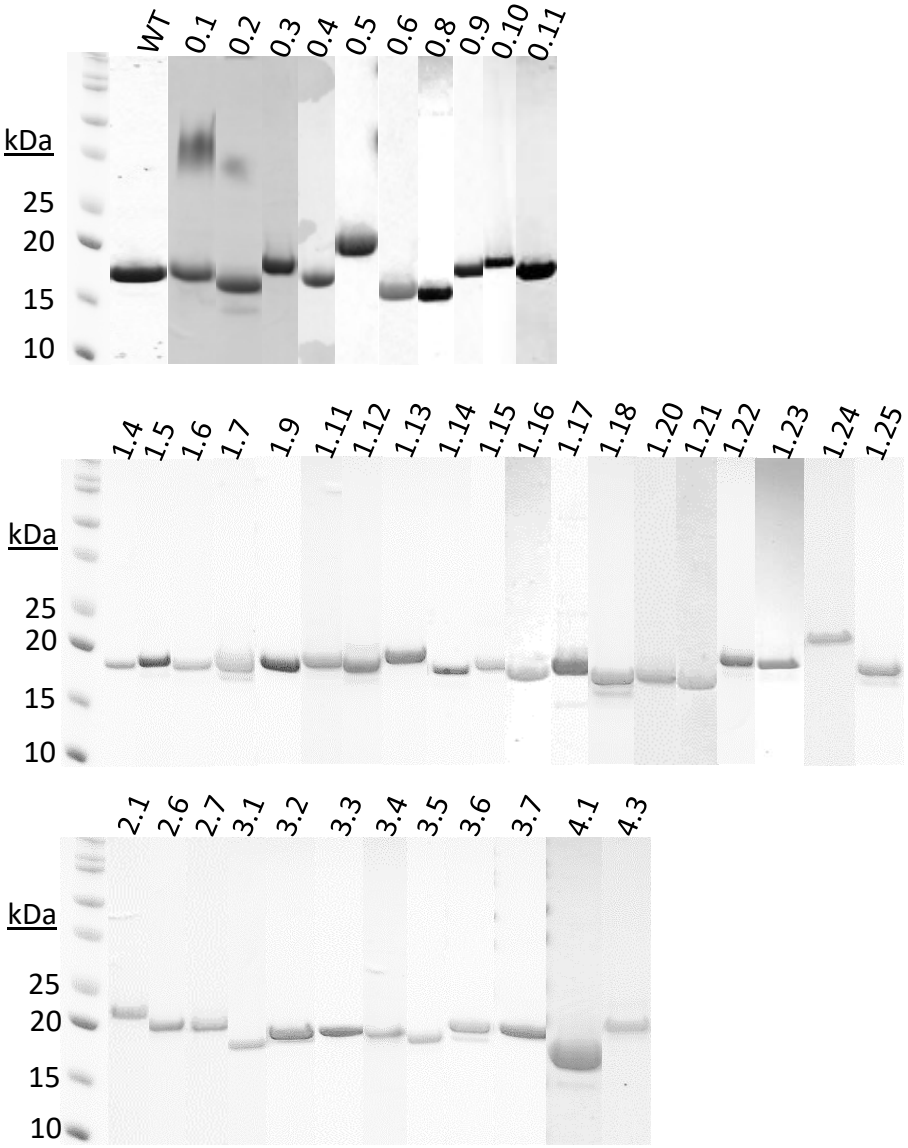


Figure A4. Purity gels from large-scale Tp0751 chimera preparations following nickel affinity and SEC purification. Each chimera displayed here was purified from large-scale *E. coli* cultures with minimal protein aggregation or precipitation and was successfully on either an ENrich™ SEC 70 or a Superdex™ 75 column in 20 mM HEPES pH 8.0, 150 mM NaCl + 1% glycerol. Purified proteins were then visualized on a 12% Bolt Bis-Tris Plus gel at 200 V for 30 minutes. Note: loading volumes were normalized to expression to allow for direct comparison to WT Tp0751 (99-237).

Table A3. Melting temperature (Tm) results for each chimerically engineered protein purified from large-scale *E. coli* preps. For T_m analysis, each protein was prepared in a reaction mixture to a final concentration of 0.125 mg/mL protein and 5X SYPRO Orange hydrophobic binding dye. From there, samples were run in triplicate on a CFX Opus 96 Real-Time PCR machine for 60 minutes on a continuous temperature ramp (1%) from 25 to 99 ºC.

Construct Design	**Purification successful?**	**T_m ± std dev (°C) (°C)**
WT	Yes	51.5 ± 0.0
0.1	Yes	52.3 ± 0.0
0.2	Yes	52.9 ± 0.2
0.3	Yes	50.2 ± 0.1
0.4	Yes	42.9 ± 0.3
0.5	No	N/A
0.6	Yes	49.9 ± 0.1
0.7	Yes	49.9 ± 0.0
0.8	Yes	48.0 ± 0.2
0.9	Yes	52.5 ± 0.0
0.10	Yes	52.2 ± 0.2
0.11	Yes	52.9 ± 0.1
1.1	No	N/A
1.2	No	N/A
1.3	N/A	N/A
1.4	Yes	51.7 ± 0.1
1.5	Yes	53.0 ± 0.1
1.6	Yes	51.1 ± 0.1
1.7	Yes	50.3 ± 0.1
1.8	No	N/A
1.9	Yes	49.8 ± 0.0
1.10	No	N/A
1.11	Yes	45.7 ± 0.2
1.12	Yes	51.9 ± 0.1
1.13	Yes	52.6 ± 0.0
1.14	Yes	49.9 ± 0.1
1.15	Yes	53.4 ± 0.1
1.16	Yes	48.3 ± 0.0
1.17	Yes	47.7 ± 0.0
1.18	Yes	49.8 ± 0.0
1.19	N/A	N/A
1.20	Yes	49.6 ± 0.0
1.21	Yes	51.3 ± 0.1

1.22	Yes	52.2 ± 0.1
1.23	Yes	50.3 ± 0.1
1.24	Yes	51.5 ± 0.0
1.25	Yes	50.3 ± 0.1
2.1	Yes	52.5 ± 0.2
2.2	No	N/A
2.3	No	N/A
2.4	No	N/A
2.5	No	N/A
2.6	No	N/A
2.7	Yes	50.2 ± 0.1
2.8	Yes	52.2 ± 0.1
3.1	Yes	50.7 ± 0.0
3.2	Yes	51.9 ± 0.1
3.3	Yes	52.4 ± 0.0
3.4	Yes	52.1 ± 0.1
3.5	Yes	51.4 ± 0.0
3.6	Yes	54.5 ± 0.1
3.7	Yes	53.5 + 0.5
4.1	Yes	47.3 ± 0.1
4.2	No	N/A
4.3	Yes	48.2 ± 0.5

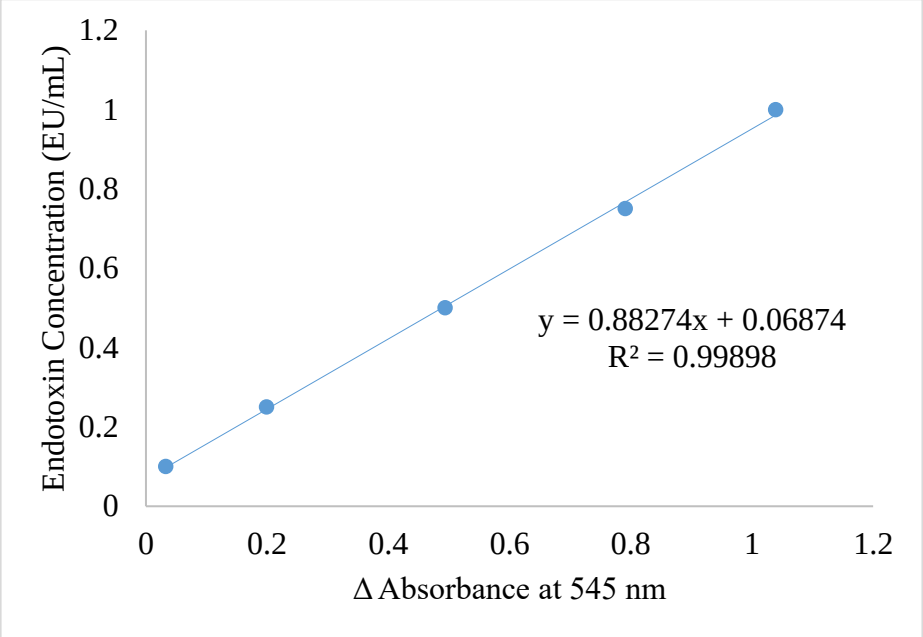


Figure A5. Standard curve of endotoxin standards ranging from 0.1 EU/mL to 1 EU/mL. Absorbance was read at 545 nm.

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