Optimizing the purification of wild type and mutant recombinant phospholipase D: Approaches to developing a C. pseudotuberculosis vaccine

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Optimizing the purification of wild type and mutant recombinant phospholipase D: Approaches to developing a C. pseudotuberculosis vaccine

Honors Thesis

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Presented in Fulfillment of the Requirement For Honors in Biochemistry
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May 17, 2013
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Introduction

Disease

Pigeon fever, or dryland distemper, is a disease caused by the bacterium *Corynebacterium pseudotuberculosis*. As one of the “most common and economically important infectious diseases of horses,” it is very common in young adult horses and in warmer climates, becoming widespread in California, Arizona, and other states. Peak infection is during late summer and fall (Doher et al. 1998). Songer et al. found two main biovars of the bacteria: *equi* and *ovis*, by studying bacterial isolates from different animal species. While all the isolates contained phospholipase D, isolates in horses were able to reduce nitrates, unlike isolates from sheep and goats. This key difference is used to differentiate between the *ovis* and *equi* biovars of the bacteria.

![Figure 2: Horse with external abscess](http://www.wildhorsefoundation.org/pigeon%20fever.htm)

![Figure 2: Horse with multiple abscesses (red arrows)](http://elitequineks.com/wp-content/uploads/2012/11/Elite-Equine-Vet-Kansas-Pigeon-Fever.jpg)

Pigeon fever, named for characteristic pectoral swelling, refers to the disease manifestation in horses caused by the bacteria; in small ruminants, the disease is known as caseous lymphadenitis. Pigeon fever can manifest in one or more of three forms: external abscesses, internal infections, and ulcerative lymphangitis, with external being the most common form of the disease (Spier 2006).
Compared to the external form where horses commonly exhibit non-healing wounds, fever, and edema, internal abscesses are particularly difficult to diagnose because of non-specific symptoms such as fever and weight loss (Aleman, et al. 1996). It is not currently known what influences which form of the disease occurs. Incidence of disease does not correlate with age, breed, or sex of the horse (Spier 2006). Because diagnostic techniques and veterinary treatment are not completely effective, the disease is difficult to identify and treat. The bacteria are protected within the thick capsules of the abscesses, limiting the effectiveness of antibiotics (Dorella, et al. 2006). General strategies for addressing a breakout of the disease within a herd include limiting its spread by quarantining infected animals. Sanitation is essential by disposing of contaminated bedding, not using the same tools for healthy and infected horses, and ensuring that open wounds are kept clean and heal quickly (Thomas 2009).

Interestingly, there have been twelve reported cases where the disease has been contagious to human beings. Nine of these twelve have been in Australia, predominantly among farm workers or with open wounds that came in contact with infected animals (Peel, et al. 1996).

**Bacteria**

*Corynebacterium pseudotuberculosis* is a gram-positive facultative anaerobe bacterium that causes pigeon fever in horses and caseous lymphadenitis in ruminants such as cows and goats (Nieto, et al. 2009). The bacterium is a close relative to *C. diptheriae and C. ulcerans*, (Songer, Beckenbach, et al. 1988). First isolated from bovine samples in 1988, it exists in pleomorphic forms including filamentous rods and coccoids, but is non-motile, non-sporulating, and non-capsulated (Dorella, et al. 2006). The bacterium secretes multiple exotoxins, among which phospholipase D (PLD) is the major one and the most studied. These molecules are able to hydrolyze ester bonds in glycerophospholipids which damages cell membranes and contributes to spread of the disease (Spier, 2006).

Infection seems to happen by entry through wounds and skin abrasions in addition to through
mucus membranes and is believed to be spread by insect vectors including house flies and other diptera (Aleman, et al. 1996). Research on insects present around infected horses has shown that the bacteria are present in the insects, especially the house fly, horn fly, and stable fly. This is interesting considering that horn flies feed along the ventral midline of the horses, where most infections start (Thomas 2009). When the bacterium enters the organism, macrophages and neutrophils phagocytose the bacteria as a means of defense. However, it is able to survive within these cells and spread throughout the body within the blood and lymph. As the bacteria continue to replicate within the phagocytic cell, the host cell eventually dies, facilitating further pathogenesis (McNamara, Bradley and Songer 1994).

There are two main characteristics that make the bacteria particularly suited for infection: the cell wall’s high lipid content that helps the bacteria to survive inside macrophages and the bacteria’s secretion of exotoxins (Aleman, et al. 1996). The bacteria are susceptible to most antimicrobials including ampicillin, lincomycin, tetracycline, and others. However, researchers found that a few strains of bacteria are resistant to neomycin and all are resistant to streptomycin (Dorella, et al. 2006). In an effort made to reproduce natural conditions of infection, researchers grew the bacteria in a biofilm and found that the bacteria were highly resistant to all the drugs that were administered (Olson, et al. 2002).

**Phospholipase-D**

Phospholipase-D (PLD) is the main exotoxin produced by both the *C. pseudotuberculosis* and *C.

![Phospholipase-D](http://www.bioscience.org/1998/v3/d/roldan/fig1)

*Figure 3: Sites of Phospholipase Activity*

ulcerans species (Barksdale, et al. 1981). It was detected at early stages of research as a protein with a weight of about 31 kDa in Corynebacterium that was bound by sera antibodies of an infected sheep (Songer, Libby, et al. 1990) (Hodgson, Bird and Nisbet, Cloning, Nucleotide Sequence, and Expression in Escherichia coli of the Phospholipase D Gene from Corynebacterium pseudotuberculosis 1990). PLD forms dermonecrotic lesions and even death when introduced in different laboratory animals. The use of an antitoxin has been applied to stop the spread of the disease; however it is unable to prevent abscess formation (Dorella, et al. 2006). PLD acts by inhibiting neutrophil chemotaxis and degranulation of phagocytic cells and hydrolyzing phosphatidylcholine and sphingomyelin to increase vascular permeability (see figure 3), contributing to disease pathogenesis (Aleman, et al. 1996). Using protection studies, laboratories have shown that PLD is crucial for the spread of the bacteria within the host (McNamara, Cuevas and Songer, Toxic phospholipases D of Corynebacterium pseudotuberculosis, C. ulcerans and Arcanobacterium haemolyticum: cloning and sequence homology 1995).

To characterize the protein, multiple efforts have been made to clone and sequence the gene using Escherichia coli such as those done by Songer et al, McNamara et al, and Hodgson et al. Songer et al found that the two biovars of C. pseudotuberculosis differ not only in nitrate reduction activity or response to streptomycin, but also by restriction length polymorphisms in Southern blots (Songer, Libby, et al. 1990).

Immunology

The mammalian immune system must be understood in order to understand the aims of this research project. First, the immune response is divided into two subsets: the humoral and cell-mediated responses (figure 5). The humoral response primarily involves the formation of B lymphocytes which differentiate into plasma cells (that secrete antibodies) and memory B cells following exposure to a
specific antigen. Humoral immunity specifically involves the role of antibodies and complement proteins to mediate and direct the immune response.

On the other hand, the cell-mediated immune response deals with the role of cell to cell interactions including killing of infected cells by cytotoxic T-cells. Upon exposure to an antigen, T-cells differentiate into T_c (cytotoxic) cells and T_h (helper) cells. T_h cells are able to further differentiate into two subsets: T_{h1} and T_{h2} cells, both with different profiles of cytokine secretion (figure 4). Our lab has hypothesized that, generally, external abscesses are only able to form if the immune response is skewed towards a T_{h1} response, while the sometimes fatal internal abscesses are allowed by a T_{h2} skewed response. This theory is modeled after leprosy, a disease also caused by an intracellular pathogen. The two forms of leprosy: tuberculoid leprosy and lepromatous leprosy depend on the immune response of the host. A T_{h1} response results in tuberculoid leprosy, forming granulomas. On the other hand, T_{h2} response characterizes lepromatous leprosy, which leads to severe nerve and tissue damage (Kindt, Goldsby and Osborne 2007). By studying the cytokine responses of individual horses to an infection, we can attempt to determine if the various forms of pigeon fever are linked to the predominant T_h subset, important information for a diagnostic assay. The cell-mediated response activates cells to phagocytize foreign antigens, induces apoptosis in infected cells, and prompts cytokine secretion.

![Lymphocyte activation](Rang and Dale 2003)
Figure 5: Cell-mediated and humoral responses of the mammalian immune system (Kindt, Goldsby and Osborne 2007)

Research Project

We hope to study the immune response to *C. pseudotuberculosis* in order to develop diagnostic tools and vaccines. Currently, we measure the humoral immune response through the extensive use of ELISAs (Enzyme-Linked Immunosorbent Assays) which measure antibody levels to bacterial proteins in
sera from infected and uninfected horses (Kindt, Goldsby and Osborne 2007). Identifying if disease type is correlated to a certain T\(_h\) subset response or other factors would help develop a diagnostic assay. Furthermore, we hope in the long term to be able to promote the development of a commercial vaccine. Currently, there is a vaccine available for the sheep biovar of the bacteria, due to the danger the disease poses to sheep and other commercial farm animals. Released in 1983 in Australia and known as the Glanvac vaccine, it is multicomponent and contains an adjuvant (Peel, et al. 1996). This type of vaccine known as a toxoid vaccine, includes antigens of other bacteria and uses an inactivated version of PLD as one of its primary components (Dorella, et al. 2006). Eggleton et al studied whether the inclusion of antigens for multiple bacteria in the same vaccine has any impact on protective effects for caseous lymphadenitis. They found that when compared to mono-component vaccines using formalin-inactivated PLD, multi-diseases vaccines had the same level of effectiveness (Eggleton, et al. 1991). The commercially available and widely used multicomponent vaccine has shown protective effects for 70-90% of vaccinated sheep and goats (McNamara, Bradley and Songer 1994). The vaccine isn’t approved for use in horses. Because this formulation has proved to be highly effective in sheep, similar results should be expected from a horse variant as well (Pollock 2009). However, owners of companion animals will be less tolerant of side effects such as granulomas than owners of agricultural animals.

My previous research in the lab has involved studying both branches of the immune system: humoral (production of antibodies) and cell-mediated (cells having various functions such as killing infected cells). ELISAs that I previously carried out measured the level of antibodies to PLD in horse serum samples. T\(_h\) cells, or T-helper cells are one of the primary cell types involved in the immune system by prompting a response from both humoral as well as cell-mediated branches. Some subclasses of antibody can indicate a T\(_h\)1 or T\(_h\)2 response. Both the T\(_h\) cell types may have different responses depending on the form of pigeon fever occurring.
Research has shown that in the course of the disease, PLD-specific antibody responses have a protective effect in sheep and PLD-deficient or mutant PLD-producing *C. pseudotuberculosis* is unable to cause caseous lymphadenitis in sheep (Tachedjian, et al. 1995) (McNamara, Bradley and Songer 1994). As a result, my main project has focused on this protein and has involved purification of recombinant phospholipase-D, through growing bacteria that produce histidine-tagged wild type or mutant PLD, cell lysing via sonication, and purifying PLD from supernatant using a TALON column (Clontech Inc., Mountain View, CA). It has been important to optimize the yield of the purification process, in order to collect prep concentrations that exceed at least 500 µg/mL. The expression vector for producing wild type PLD in *E. coli* was made using the multiple cloning site of the *PTrcHis* plasmid and was a gift from Dr. Steve Billington from the University of Arizon at Tucson. When expressed, PLD is translated with a 5’ 6xHis tag, allowing us to use affinity chromatography to purify the protein.

I have been studying the various methods to inactivate PLD such as heat-denaturation, formalin-crosslinking, and genetic mutation with the intent to identify new components for developing a vaccine. A paper by Tachedjian et al (1995) noted that commercially available vaccines depended on formalin-inactivated PLD as the main antigen. They tested site-specific mutations at the PLD catalytic site and magnesium/calcium binding domain and found that one such mutation of site 20 (histidine), in the active site, to serine resulted in a 40% reduction in secretion, with minimal enzymatic activity (Tachedjian, et al. 1995). Previous research found that a mono-component sheep vaccine preparation using the mutant variety of PLD had only an effective disease protection rate of 44% compared to 95% for the multi-component formalin-inactivation preparation (Hodgson, Carter, et al. 1998). We hope that a vaccine with the mutation-inactivated PLD will be successful for horses as a more efficient and safe alternative to formalin-inactivation.
With the potential mutant PLD vaccine, we have started a mouse model using PLD to immunize mice. In addition to inactivated forms of PLD protein, we also used the mouse model to test a DNA vaccine, the mutant PLD sequence in a mammalian expression vector. Existing research has shown the potential of a DNA vaccine in sheep and we hope to replicate the results for the *equi* biovar (Chaplin, et al. 1999). The secretion of PLD by native immune cells would a natural antigen for eliciting an immune response and conferring protective effect through an increased cell-mediated response due to presentation on the multihistocompatibility complex (MHC). Using the services of an external company, *Nature Technology Corporation* (Lincoln, NE), we had a mutated version of the PLD gene inserted into a plasmid vector that gets expressed in mammalian cells. Once this or other vaccine components such as the mutant protein were injected into mice, we tracked the immune response using ELISA assays on sera collected at regular intervals. Currently we are carrying out a mouse model, with upcoming boosts and eventual bacterial challenge with live *C. pseudotuberculosis* this summer, to see if any of the various vaccines provide a protective effect.

**Materials and Methods**

**PLD Purification**

![Diagram of PLD purification process](Figure 6: Purification process)

PLD is harvested from DH5α *E. coli* transformed bacteria with a recombinant histidine-tagged PLD gene in multicloning site of the *pTrcHis* plasmid (a gift from Dr. Stephen Billington, University of Arizona, Tucson, AZ). We also used a company *Genscript* (Piscataway, NJ) to induce a site-specific mutation at site 20 of the protein; a histidine (CAC) was changed to serine (TCT). Our lab designed
sequence primers so that we could ensure that the full PLD sequence was included within the
multicloning site of pTrcHis. We also included two primers complementary to the pTrcHis sequence,
one on each side of the PLD insert so we could confirm that the insert was in the correct
orientation. Sequencing was completed by Genscript and we were sent mutant plasmid sequencing
information to ensure that the desired change was complete. As with the wild type PLD, the mutant
version of the protein is harvested from transformed DH5α E. coli.

We grew the transformed bacteria containing the wild type or mutant 6xHis tagged PTrcHis
(strand JG52274). The culture plates were then left overnight at 37°C. An isolated colony was then
picked and cultured in 3 mL of LB+AMP overnight. The culture was then added to
500 mL of LB+AMP for ~24 hours in the warm room. Following the time period, we
took a sample and read the OD_{600} of the culture to ensure it is above 0.6 absorbance.
If it is, we add 2.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce PLD
expression and leave the flask to shake at 37°C for three hours.

During the process of yield optimization, we changed the protocol. The following are the steps
with which our PLD was isolated and purified. Following culturing, the culture was then removed from
the warm room and transferred from the 2 L flask to two plastic centrifuge bottles. Cells were harvested
by centrifuging at 7000 xg for 15 minutes. The supernatant is then discarded. We then resuspended the pellet with 10 mL of binding buffer. Once the pellet in that centrifuge bottle was resuspended, we transferred the solution to the other centrifuge bottle and resuspended the pellet from the second plastic bottle. We transferred the resuspended pellets to a 50 mL conical tube and filled it with binding buffer until it reached 35 mL of volume. Cells were sonicated (50% duty cycle, output of six) three times at four minute intervals. Sonicated cells were transferred to two polycarbonate tubes, after we made sure each tube has the same volume with up to 0.2 g difference. The polycarbonate tubes were spun for 20 minutes at 15,000 x g at 4°C. Supernatant was collected in a 50 mL conical and we discarded the pellet. The PLD is in the supernatant.

PLD was isolated using a cobalt metal affinity column. Using a plastic flow column and TALON metal affinity resin seen in figure 8 (Clontech Inc., Mountain View, CA), we set up the chromatography. We washed the column five times with 8 mL of MES buffer and then once with 8 mL of binding buffer. We capped the column and added binding buffer, resuspending and transferring to 50 mL conical tube. It was spun at 700 xg for 1-2 minutes at 4°C. We then discarded the supernatant and repeated. The resin was shaken with the PLD supernatant gently for 20 minutes in the cold room at 4°C. We centrifuged again for 2 minutes at 700 xg and discard supernatant. We then added binding buffer to PLD and resin. It was shaken gently at 4°C for 10 minutes, then spun and the supernatant was discarded. Next step was

![Diagram](image)

Figure 8: Illustration of Clontech’s TALON™ Metal Affinity system.
A: BD TALON Cobalt affinity resin consisting of cobalt chelator ion bound to sepharose bead.
B: 6x histidine tagged PLD binds to the Co²⁺
Source: BD Talon™ Resin User Manual, Clontech (Mountain View, CA)
to add 8 mL of binding buffer and transfer back to column, discard flow through and repeat. We then soaked with 10 mM wash buffer for 8-10 minutes, discarding the flow through and repeating two more times. We added 2 mL of elution buffer (appendix) and let it settle for 8-10 minutes. We collected the PLD in 1 mL tube and stored resin at 4°C. This is the concentrated but not purified PLD.

The newer version of the protocol used for the last few PLD samples is described here. Following culturing, cells were harvested by centrifuging at 7000 xg for 15 minutes as before. We then resuspended the pellet with 10 mL of equilibrium buffer. Once the pellet was resuspended, we added equilibrium buffer to a total volume of 35 mL. Cells were sonicated three times at 50% intensity for 10 seconds, with 0.5 seconds on/off. Sample was cooled on ice for 30 seconds in between sonication cycles. Sonicated cells were spun for 20 minutes at 10,500 RPM in a Beckman JA-17 rotor at 4°C. Supernatant was collected in a 50 mL conical and we discarded the pellet. At this stage, the PLD is in the supernatant.

The next part involved extracting the PLD using a cobalt metal affinity column. Using a plastic flow column and TALON metal affinity resin, we set up the chromatography. We used 4 ml of resin slurry (2 ml bed volume). We centrifuged the slurry to pellet the resin and discarded the supernatant. Following this, we rinsed the resin twice with equilibrium buffer and spun to pellet once again. Following this, we added the clarified cell lysate to the resin. The mixture was then left on a vertical tube rotator for 20 minutes at 4°C. Using 700 xg centrifugation for 2 minutes, the components were then separated and the supernatant was discarded. We then rinsed the resin of unbound materials 2-3 times using successive equilibrium buffer washes and centrifugation of the same duration. The final resin/buffer slurry was then transferred to a Clontech™ (Mountain View, CA) gravity flow column and the resin was allowed to settle out of suspension. After a final rinse, we carried out one or two washes with equilibrium buffer with 10 mM of imidazole to remove weakly bound particles. Finally, for the first elution we used 2 mL of 150 mM elution buffer which we allowed to flow through. For the second
elution, we used a 100mM EDTA elution buffer which we allowed to soak the resin for 30 minutes. Flow through was collected.

The final step involves using the Slide-A-Lyzer dialysis cassette (ThermoScientific, Vernon Hills, IL). We immersed the cassette in dialysis buffer (1X phosphate-buffered saline at pH 7) for two minutes to moisten the membrane. After using a syringe to load in the PLD, we then spun the cassette in dialysis buffer at 4°C for 72 hours, replacing with new buffer every 24 hours. We then removed the cassette from the buffer. After dialysis, PLD was stored in a freezing vial in -20°C freezer. This was followed up with a bicinechnoninic (BCA) assay (Thermo Fisher Scientific, Rockford, IL) to determine protein concentration.

**Formalin-Inactivation**

For every 1 mL of concentrated PLD solution, we used 40 µL of 10% buffered formalin. The resulting solution was left for 72 hours at 37°C on a rocker. Following inactivation, formalin was dialyzed out using dialysis tubing or a dialysis cassette in a 1x PBS solution for 24 hours at 4°C. PBS solution was changed 3-5 times. During formalin-inactivation, PLD tends to precipitate out, so it’s important that any solid matter is re-suspended. Testing of final concentration is also done using a BCA assay.

**BCA Assay**

The bicinechnoninic acid assay (BCA assay) determines the total level of protein in a sample. The measurement is calculated by the degree of color change of the sample from green to purple. We used an array of standards including 2000, 1000, 500, 250, 125, 62.5, 31.25, and 0 µg/mL of bovine serum albumin. Using a 96-well plate, we added 25 µL of each BSA standard and unknown in triplicates. 200 µL of working reagent was added to each sample well, with a 50:1 ratio of substrates A and B from the ThermoFischerScientific (Rockford, IL) Pierce BCA Protein Assay kit. We agitated the plate for 30 seconds.
It was then incubated for 30 minutes at 37°C. Finally, we measured the OD$_{570}$ reading on a Biorad Benchmark Microplate Reader (*Hercules, CA*). Protein concentration was determined using a best fit polynomial curve for the standards.

**Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE)**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE, is a technique used to fractionate proteins by size. The protocol begins with opening a 4-15% poly-acrylamide gradient Tris-HCl gel. We prepared 800 mL of 1X running buffer (appendix) for the SDS-PAGE apparatus. The protein samples are heated in a 94°C heat block for about five minutes. We then loaded 15 µL of the kaleidoscope marker and 20 µL aliquots of the samples into wells. The gel was run at 200 volts for about 40 minutes, ensuring that the dye reaches the end of the gel. The gel was rinsed in ddH$_2$O. Then it was washed in ddH$_2$O three times for five minutes each, with shaking. We washed in Bio-Safe coomassie (*Bio-Rad Laboratories, Hercules, CA*) for one hour, with shaking. Lastly, the gels were washed with water for five minutes and then photographed.

**Western Blot**

To run a Western blot, we would run a duplicate to the SDS-PAGE gel being used for coomassie staining. Following completion of electrophoresis, we prepared the nitrocellulose membrane “sandwich” by layering wet membrane with Whatmann paper, sponge pads, and the gel to transfer. We ran the apparatus at 12 V overnight with a stir bar maintaining circulation. After completion, membrane was rinsed with ddH$_2$O and then immersed in blocking buffer (5% nonfat dry milk in TBST) for an hour. After a brief rinse with ddH$_2$O, we incubated the membrane for one hour in the primary antibody with binding buffer (appendix). After three washes with 150 mM tris-buffered saline 0.1% tween 20 (TBST), we added the secondary antibody diluted in blocking buffer and agitated on the shaker for one hour.
After two washes in TBST and one wash in just TBS (tris-buffered saline) for 15 minutes, we added the Opti-4CN (Bio-Rad, Hercules, CA) working reagent (substrate, diluent, and ddH2O) and incubated until lane darkness was adequate. After a brief rinse with ddH2O, we photographed and dried the membrane.

**Bacterial Transformation**

The bacteria were transformed to contain the plasmid with ampicillin resistance and the PLD insert. To start off, the 50 µL of competent cells was incubated on ice for 30 minutes after adding 5 µL of the ligation mix product (appendix). Then, the cells were incubated in ice for 30 minutes. The cells were then heat-shocked at 42°C for 20 seconds to increase permeability. The tube was placed on ice for 2 minutes following which 950 µL of super optimal broth with catabolite repression (SOC medium) was added to facilitate adaptation of the bacteria to glucose as its energy source. The tube was then incubated at 37°C with shaking for an hour. The two LB agar + ampicillin (LBA) plates were labeled “100 µL” and “900 µL.” The tube was flicked to mix the cells. The plates were spread with 100 µL of the cells. Then, the remaining bacteria were concentrated by transferring bacteria to a microfuge tube and spinning bacteria down for about 5 seconds in the microfuge. Then, the bacterial pellet was resuspended by finger flicking and then gently pipetted. These were then plated onto the plate labeled “900 µL.” The plates sat at room temperature for few minutes to air dry and we then put into the warm room upside-down overnight.

Once the plates were removed from the refrigerator, four culture tubes were set up with 1.8mL/tube of LB-AMP. By using the wire loop, a white colony was picked and placed into each tube. Two of the four colonies came from the “100 µL” plates, and other two came from “900 µL” plates. The tubes were placed in the 37°C shaker for 12-16 hours. The LB + AMP with the bacteria was transferred to four separate 1.5mL Eppendorf tubes and stored at 4°C.
Mini-preps

Minipreps were carried out with the QIAprep Spin Miniprep Kit (Qiagen, Venlo, Netherlands). The culture tube was centrifuged at 2200 x g for 2 minutes to pellet the cells. The supernatant was discarded. The cell pellet was resuspended in 250 µL of Qiagen buffer P1. Then, 250 µL Qiagen buffer P2 was added and the solution was mixed gently by inverting the tube 5 to 6 times. Then, 350 µL of Qiagen buffer N3 was added and mixed by inverting the tube again 5-6 times. The tube was placed in the microfuge and spun at 16,000x g for 10 minutes to pellet the cell debris. The supernatant then was transferred into a Qiaprep column.

The column was placed in a 1.5 mL collection tube and centrifuged at 10,000x g for a minute. Flow through was then discarded. We then added 750 µL of Qiagen buffer PE to the column and it was centrifuged at 10,000x g for one minute, following which the flow through was discarded once again. The column was then centrifuged to remove liquids. Lastly, 50 µL of Qiagen buffer EB (10mM Tris, pH 8.5) was added to the top of the column and spun at 10,000x g in a microfuge for one minute.

Restriction Enzyme Digests

The restriction enzyme digests of the mutated PLD DNA were set up: 7.0 µL of sterile H₂O, 2 µL of 10X EcoRI restriction buffer, 10 µL of mutant PLD product, and 1 µL of EcoRI restriction enzyme were added into the “EcoRI digest” tube. For the double digest, we used PvuI as the second restriction enzyme. The digest tube was flicked and spun briefly in the microfuge and placed in the 37°C incubator. The digest was incubated overnight for approximately 16 hours. Then, the digest was stored at -20°C.

Agarose Gel Electrophoresis

After preparing the mutant PLD restriction digests, we prepared the gel for electrophoresis of the DNA samples. We added 8 µL (20% of reaction volume) of 10X loading buffer. We added ethidium
bromide solution (0.5 mg/mL) to each sample for a final concentration of 0.5 µg/mL, as well as 1.0 g of electrophoresis grade agarose to a 100 mL volume of electrophoresis buffer to make 1% agarose gel. The agarose was melted in a bottle with the cap loosely on in a microwave oven and swirled to ensure even mixing. We then sealed the gel-casting platform with tape. We poured in the melted agarose and inserted the gel comb, making sure that no bubbles are trapped underneath the combs and all bubbles on the surface of the agarose were removed before the gel sets.

After the gel had hardened, we started the electrophoresis. We removed the tape from the open ends of the gel platform and withdrew the gel comb, taking care not to tear the sample wells. We placed the gel-casting platform containing the set gel in the electrophoresis tank. We added sufficient electrophoresis buffer to cover the gel to a depth of about 1 mm. We prepared the DNA sample by adding the appropriate amount of loading buffer to a volume of 20 µL. We loaded each well with 20 µL of sample. We included the appropriate DNA molecular weight markers to label our gel at the DNA sizes we were expecting. We then run the gel at 100 V for one hour and photographed it.

**Results**

**PLD Purification**

The majority of data involves our efforts to optimize the purification process for PLD. Initially, we managed to achieve a concentration of 378.1 and 201.5 µg/mL respectively in our first two purifications. Figure 10 shows the SDS-PAGE that we ran using samples from different stages of the purification in order to analyze our collection of PLD. For all figures, mM notations refer to imidazole concentration of the buffer solution. We can see that during the washing process, we are removing impurities along with some PLD seen at 32.1 kDa. This can be seen in lane 8, where a significant band of PLD is visible. A similar, but decreasing pattern of band darkness can be seen for lanes 10 and 11 which are the subsequent washes. For PLD prep 1 (lane 12) we can see some protein contaminants with
heavier molecular weight. For PLD prep 2 (lane 13), we can also see a contaminant, but one with a molecular weight only slightly larger than PLD.

![SDS-Page Gel](image)

**Figure 10**: SDS-Page Gel A: 1- Marker; 2- Culture supernatant; 3- Flow through with binding buffer; 4- Flow through with BB; 6- 2nd 10mM imidazole wash; 7- 3rd 10 mM wash; 8- 1st 300 mM wash; Gel B: 9- marker; 10- 2nd 300 mM wash; 11- 3rd 300 mM wash; 12- UPNY PLD 1; 13- UPNY PLD 2

<table>
<thead>
<tr>
<th>PLD Sample</th>
<th>Concentration (µg/ml)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPNY 3a</td>
<td>32.7</td>
<td>Old culture wash 1</td>
</tr>
<tr>
<td>UPNY 3b</td>
<td>41.7</td>
<td>Old culture wash 2</td>
</tr>
<tr>
<td>UPNY 4a</td>
<td>680.8</td>
<td>New culture wash 1</td>
</tr>
<tr>
<td>UPNY 4b</td>
<td>381.2</td>
<td>New culture wash 2</td>
</tr>
<tr>
<td>UPNY 5a</td>
<td>408.6</td>
<td>Two BCAs</td>
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<td></td>
<td></td>
<td>423.38</td>
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<tr>
<td>UPNY 5b</td>
<td>234.4</td>
<td>Two BCAs</td>
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<tr>
<td></td>
<td></td>
<td>210.1</td>
</tr>
<tr>
<td>UPNY 5c</td>
<td>329.2</td>
<td>Not dialyzed</td>
</tr>
<tr>
<td>UPNY 5d</td>
<td>531.8</td>
<td>Not dialyzed</td>
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</table>

*Table 1: Spring 2012 PLD Yields, BCA refers to Bicinchoninic Assay use to measure protein concentration. For UPNY 5a and 5b, two BCAs were carried out, with slight variation in result.*
Over the course of the spring semester 2012, we conducted several rounds of purification. The various sample concentrations can be seen in Table 1. Previously, we had been conducting only one elution at 300 mM imidazole for each purification. However, for sample 3 onwards we used a 500 mM imidazole elution buffer and 2 rounds of elution. The number following ‘UPNY’ indicates the elution prep number and the letter indicates the elution. For each purification, we conducted two elutions per sample with a soak time of 15 minutes (compared to the previous 6-8 minutes). For UPNY 3, we had to temporarily halt the procedure during the large culture step, before adding IPTG. As a result, we had to store the large tissue culture in the cold room and so the cells probably died.

When we started the next purification we purified the PLD from the bacteria stored in the cold room (UPNY 3), in addition to the new PLD culture (UPNY 4). The old culture gave a very low PLD yield as expected, but with the new sample, we were quite successful with a concentration of 680.8 μg/mL using 500 mM imidazole in the first 2 mL elution. We attempted a second elution with the same imidazole concentration and got a relatively high value of 381.2 μg/mL for our 2 mL collected. This indicated to us the necessity of continuing using two elutions to collect the maximum amount of PLD possible. We proceeded to carry out a third purification (UPNY 5). However, mistakenly, 3.5 times the normal elution volume was used. For PLD preparation 5, elutions a through d refer to four different fractions that were collected. Despite our excessive elution volume, 7 mL, we still collected a concentration over 400 μg/mL.

Figure 11 shows the SDS-PAGE results from our next purification, UPNY PLD 3. As we had learned from the BCA, the PLD content was quite low. In lane 7 and 8, the PLD bands at ~37 kDa (PLD is 31,500) are very faint. Additionally, in the first elution, there was a second band located lower than the PLD band. Lanes 4-6, which were the washes, also contain small traces of PLD, particularly lane 5. However, these amounts are low. In Figure 12, one can see the gel run with samples from PLD 4, the new culture. The PLD bands are more pronounced in lanes 7 and 8 at the 32.1 kDa mark. However, there
appears to be some impurity or other protein appearing in lane 8 much higher than the PLD band. The lanes containing our washes do not have visible PLD bands, indicating that we did not lose a significant amount of protein during the isolation process.

Figure 13 shows the gel used to run samples from UPNY PLD 5 as well as samples of autoclaved PLD from last semester. Unlike before, this and succeeding gels were run under reducing conditions (loading dye contained β-mercaptoethanol), which may have contributed to the higher resolution. PLD
5a and b had high concentrations of PLD which can be seen in the very prominent bands in lanes 5 and 6 at ~32,000 Da. PLD 5b and c had smaller amounts of PLD at expected. However, we noticed a lot of unwanted contaminants in the sample both above and below the PLD bands in lanes 5 and 6. Much of these were washed out in the three washes on lanes 2-4, however some remains. We could also see levels of PLD (higher than the c and d elutions) in wash 1 and 2 especially. Finally, lanes 9 and 10 contained the autoclaved PLD from samples UPNY PLD 1 and 2. As can be seen, the autoclaving process completely degraded the protein, leaving small bands distributed from 31,000 Da and smaller, ruling out the possibility that this could serve as an alternative form of inactivation.

**Mutant Transformation**

Once we received the plasmid encoding the His-tagged PLD with His20 mutation from *Genscript*, we transformed it into DH5-α cells and selected 10 colonies using ampicillin. To test the transformation, we used restriction analysis with *EcoRI* and *PvuI* on plasmid DNA obtained from transformed bacteria. Figures 14 and 15 show the results of our digests when run on an agarose gel. We used a combination of double enzyme digests (DD) in wells 9 and 10 on the first gel and wells 3 through 10 on the second gel, and three pUC19 transformation controls. We used single enzyme digests to test just mutant line 1 (M1). We were expecting that the mutant double digest would show bands at 3531, 950, and 751 bp.
EcoRI did not seem to work, possibly due to age of the enzyme. For the double digests, on the control lanes three bands can be seen representing the 3 cuts made by the enzymes which were expected at 3531, 950, and 751 bp. On the second gel (figure 15), mutants 4, 5, 7, and 8 have very little DNA appearing in bands. The only lanes with prominent bands that matched expected sizes were 3 and 6.

Using the resulting cell colonies from the first transformation we carried out two purifications, PLD 6 and 7 with mutant clones 3 and 5 from figure 15 respectively. But, as can be seen in table 2, PLD yields were low. Even though the previous transformation appeared to be successful according to plasmid DNA digests, we decided to re-do the transformation in the hopes of increasing yields. The results on the gel can be seen in figure 16 and 17. The warped nature of the bands is evident. The uncut controls in lanes 2 and 3 did not move for the most part as can be seen from the DNA that remains in the well. However, there are faint bands extending across, midway on the gel, till lane 6 at around 751 bp. Lane 7, 8, and 9 all exhibited 2 consistent bands as a result from PVUI’s two cutting sites on the plasmid, as we expected. The second gel, figure 17, shows the double digests. Results are mostly uniform across the gel. In particular, lanes 3, 5, 7, and 8 had the best results, showing our three

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conc. (µg/ml)</th>
<th>Notes</th>
</tr>
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<tbody>
<tr>
<td>UPNY 6a</td>
<td>36.2</td>
<td>Ave. of 2 BCAs</td>
</tr>
<tr>
<td>UPNY 6b</td>
<td>23.9</td>
<td>Ave. of 2 BCAs</td>
</tr>
<tr>
<td>UPNY 7</td>
<td>36.5</td>
<td></td>
</tr>
<tr>
<td>UPNY 8a</td>
<td>16.39</td>
<td></td>
</tr>
<tr>
<td>UPNY 8b</td>
<td>41.41</td>
<td></td>
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</table>

Table 2: First Purification of Mutant PLD

Figure 16: Gel 1 of digests on 2nd transformation attempt
Lane 1- 1kb ladder; 2- C1 uncut, 3- M1 uncut; 4- M1 EcoRI; 5- M3 EcoRI; 6- M8 EcoRI; 7- M1 PvuI; 8- M3 PvuI; 9- M8 PvuI; 10- 100 bp ladder

Figure 17: Gel 2 of digests on 2nd transformation; Lane 1- 1kb ladder; 2- C1 double digest, 3- M1 DD; 4- M2 DD; 5- M3 DD; 6- M4 DD; 7- M5 PvuI; 8- M6 DD; 9- M8 DD; 10- 100 bp ladder
expected bands at 3531, 950, and 751 bp.

Using these newly transformed bacteria (mutant clone 7), we carried out another PLD purification. We hypothesized that a possible reason for the lack of significant PLD yield was changed solubility of the PLD due to the site specific mutation. As such, we included an additional solubilization step to check if the mutated PLD was insoluble in the cell lysate during our purification procedure. The result was UPNY PLD 8a and 8b. In table 2, the PLD concentration is even lower than that of 6 and 7, both of which were already negligible.

Troubleshooting of Mutant and Wildtype Purification

Multiple purifications of both mutant and wild type PLD were done in my efforts to maximize the yield. Initially, preps 6a through 14b (figure 18 and 19), as can be seen from the graph below, protein concentrations were below 150 µg/mL. However, with changes to purification technique, later batches of mutant PLD eventually crossed above 150 µg/mL (prep 14c) and reached almost 200 µg/mL
(prep 15b). However, this was still a ways from our minimum of 500 µg/mL. The two tables, figures 18 and 19, illustrate the various yields for protein purifications, both mutant and wild type over the course of the summer. The tables show the type of elution used to collect the PLD.

Our primary goal recently has been to purify mutant PLD. As such, we carried out analysis to identify which culture condition promotes the most mutant PLD synthesis. One of our first troubleshooting tests was based on the fact that our cell pellets seemed somewhat small. We thought that perhaps the cells were not growing well. Separate from our purification attempts, we cultured the bacteria at different temperatures and induction times with IPTG to compare PLD levels. The results can be seen in figure 20 and 21, which were the SDS-PAGE of culture lysates and the Western blot probed for PLD using hybridoma supernatant positive for anti-PLD antibodies. Each band contains samples from a condition of culture temperature, induction time, and samples using the old and new sonicator. PLD appears right near the 32.1 kDa band on the marker. It seems that 37 degrees with 3 hours of induction is the faintest. Using the blot, we can compare the darkness of the PLD bands to identify which culture condition had the most protein with volume kept constant. The legend shows the contents of each lane, applying to both figures.

Alternatively, we hypothesized that the site specific mutation could have altered the folding of the protein and that this might have affected the solubility of the protein in the binding buffer. We
created a solubility testing assay to check the amount of PLD in various cell culture components. The flowchart below exhibits our efforts. Each section was solubilized with a specific technique and then run on a gel to compare amounts of PLD. A Western blot allowed us to better visualize the comparative amounts of only PLD in figure 23. Lanes are labeled by their corresponding number in the solubilization flow chart.

Following the solubilization tests, we hypothesized that another explanation for low yield could be if the His-tag of the mutated PLD bound in a different way to the Co²⁺ of the TALON resin. We usually eluted with 500 mM imidazole solution, which we considered to be very strong. However, if the mutated PLD’s affinity was higher than we might have needed stronger elution regents. In conversation with the company, we decided to try a pH 4 and 5 elution in addition to 100 mM and 200 mM EDTA to strip the resin. Figure 25 shows the comparative effects when we use different elution reagents. PLD 14
involved pH 5 elution, but the absence of proteins in the band proves that it was unsuccessful. On the other hand, 15 c and d contained an imidazole wash and had more PLD visible in the Western blot.

The next method of testing to ensure that there were no obvious errors with our protocol: were we losing PLD when we were washing? Did the PLD inadequately bind to the resin in the beginning? While this step was included in the protocol for each purification round, in prep 15 we carefully observed the results. By running various samples on a gel (Figure 26), we were able to compare the approximate protein concentration. The washes show that only small amounts of PLD were present (lanes 9d and 12b). Comparing the cell lysate (B) lane in both the first and second gel to the clarified supernatant (S), we can see how much PLD was transferred into the column with the resin. 15d and e also show the importance using EDTA as an alternative (figure 25). It was highly effective at 100 mM and produced larger yields of PLD. Figure 27 shows the progression of purification over the course of the summer on mutant PLD purification.
The final change was reducing the intensity and duration of sonication to lyse the cells. Furthermore, through research done by Dr. Karen Molinder, the concentration of imidazole used to elute PLD from the column was changed to better reflect protocols suggested by the manufacturer. The results were very high yields of 546.7 µg/mL and 1516.3 µg/mL for the 150 mM imidazole and 100 mM EDTA respectively (carried out by Dr. Molinder). The SDS-PAGE in figure 28 shows the relative protein
presence indicative of the high concentration. Each sample (the imidazole and EDTA elution) was run at 1.5 µg and 3 µg quantities respectively in lanes 7/8 and 9/10.

We continued to use the new protocol for wild type PLD purification to see how high the yields could be made. Figure 29 shows the results of purification for PLD 19. Yields were very high. PLD sample 19a (eluted in 150 mM imidazole) was 233.93 µg/mL and PLD 19b (eluted in 150 mM EDTA) was 925.13 µg/mL, a record high. As can be seen in figure 29, the concentrated PLD samples in lanes 8 and 9 are significantly darker than the others. However, we can see a significant amount of protein contaminants present in all the lanes, darkest in the concentrated PLD rather than being successively fainter. The difference in darkness of the PLD bands between the cell lysate (L) before incubation with resin and the first equilibrium buffer rinse lane (B) shows that the majority of PLD did bind to the TALON resin. Furthermore, all of the rinses (B) seem to have washed away unwanted contaminants but not excessive amounts of PLD. However, it is important to note that PLD samples were loaded at a standard volume without calculating and ensuring that a uniform amount of PLD was loaded; as a result, direct comparison of band darkness is misleading.

Figure 29: SDS-PAGE of Mutant PLD with new protocol and sonication conditions. M: Marker; L: Cell lysate; B: Rinse with equilibrium buffer; W: wash with 10mM imidazole in equilibrium buffer, 19a: PLD eluted in 150 mM imidazole, 19b: PLD eluted in 150mM EDTA, +: positive PLD control
Another round of purification was attempted, this time with the addition of an additional wash step with 10 mM imidazole equilibrium buffer to determine if contaminants could be better removed. The concentration for PLD 20a (eluted in 150 mM imidazole) and 20b (EDTA) were 396.22 \( \mu g/mL \) and 841.38 \( \mu g/mL \) respectively. This was interesting considering that the imidazole-eluted elution rose by 100 \( \mu g/mL \) from purification 19. However, the EDTA-elution decreased by about 100 \( \mu g/mL \). SDS-PAGE was not done for this prep.

**Discussion**

The results of the protein purifications have helped us determine whether our protocol changes have been effective. From our analysis of SDS-PAGE gels in the summer of 2011, we made changes to the protocol. Earlier purifications had been done with 50 mM imidazole washes and we found that those removed some of the PLD. As a result, we decreased the imidazole wash concentration to 10 mM to prevent loss of PLD. In comparing techniques, we noticed a difference in the SDS-PAGE of the flow through for both washes shown in figure 10. The SDS Page from our second purification attempt is also shown in figure 10. As expected, in lanes 2 and 3 we can see significant impurity in the sample that is then removed during the initial wash steps. In the lanes 8-10, with the 300 mM elution, we can see the presence of a band indicating PLD, showing that our purification was successful.

When we purified that sample UPNY 2 (lane 13 of figure 10), we could see a much stronger band of pure PLD compared to PLD 1. However, there is also a larger protein that wasn’t present in earlier stages of the purification. We are unsure what that could be because it did not appear earlier in the washes. It might be some protein that bound strongly to the resin due to presence of histidine and was eluted off the column under these conditions. Compared to our first sample of PLD (lane 12, figure 10), UPNY 2 seems to be purer than UPNY 1. There are multiple bands of contaminants in our first sample
that were not completely removed by the purification process. While we were able to collect fairly pure PLD in these first two preps, our concentration was low. We hypothesized that these early low yields could be due to a malfunctioning sonicator. If it was not working correctly, the *E. coli* would not have been completely lysed, therefore when we discarded the pellet, much of the PLD would have been trapped in the unlysed cells and debris. As such, we decided to dedicate the next steps of the project to optimizing the yield.

During the spring of 2012, we spent time altering purification techniques. With our efforts to purify wild type PLD, we received greater concentrations due to changes in our elution strength. We increased the concentration of our imidazole elution buffer to 500 mM from the previous 300 mM. Figure 11 shows the SDS-PAGE purification 3. Comparing the difference in PLD band intensity in lane 2, the cell lysate, with lane 3, the discarded supernatant after binding between PLD and the resin, it is clear that the resin bound most of the PLD in the sample, indicated by the faint band at 31.5 kDa in lane 3. We also increased the soaking time for the elution to 15-30 minutes, allowing us to maximize the collection of PLD 4 (figure 12) and 5 (figure 13) which can be seen in table 1. It was hypothesized that some PLD may be left bound to the resin after we finish elution. We realized that when we eluted PLD off the resin twice, we could collect more PLD. Lane 6 of figure 13 shows the results of the second elution, still a significantly high concentration (234.4 µg/mL). We realized that we should continue using two elutions to collect all the PLD possible. With PLD 5, 3.5 times the elution volume was accidentally added. Despite more dilution, the relatively high concentration of PLD, 423 (UPNY 5a) and 234 µg/mL (UPNY 5b) respectively, suggests a high protein concentration had we eluted in only 2 mL of imidazole solution.

As mentioned earlier, figures 11-13 show the results of our SDS-PAGE on our wild type PLD samples. In Figure 11, it is clear that there is a significant presence of PLD in both our ‘a’ and ‘b’ samples, indicated by a prominent band at ~31.5 kDa. A second band appears in lane 7; however we are unsure
what it represents. Since that gel contains PLD 3, the old culture, it is possible that the band is made of some proteins secreted by the older or dying cells. Lanes 4, 5, and 6, our washes, all contain slight traces of PLD, indicating that we lost small amounts of PLD with our wash.

Figure 12 shows the samples from PLD 4. Unlike figure 11, we did not lose any PLD with our washes. There is a very prominent PLD band in lane 7, indicative of a higher concentration. The second elution, 4b, also shows PLD, however it has a large amounts of proteins. We are unsure what the additional bands are. We also cannot explain why they were unseen on the gel throughout the washes and the first elution, only to appear at the end possibly because they bound tightly to the column. The final gel, figure 13, shows the results of wild type PLD 5. Intense PLD bands in wash lanes 2-4 show that we were losing a large amount of PLD earlier along with many other impurities. In lanes 5 and 6, there are very intense PLD bands, reflective of the measured high concentration. However, there are other protein bands as well, representing unknown contaminants. The final lanes, 9 and 10, were our autoclaved PLD samples from a previous prep. The autoclave inactivation was clearly effective as the proteins were virtually destroyed, degraded to into a vast collection of protein fragments. The complete breakdown suggests that it would be unsuitable as a vaccination since the protein was degraded.

To use the mutated plasmid to express PLD, we carried out a transformation and restriction digest. The results in figures 14 and 15 show our double digest controls (gel 1, lanes 3 and 4), confirming that our transformation worked, with three clear bands indicated the total three cuts by PvuI and EcoRI. However, lane 5 seems to have faint bands perhaps a result of a loading error. On gel 2, lanes 3 and 6 show the best digests with three clear bands. As a result, we used mutant clone 3 (lane 3) for our purification. Our concentration for mutant PLD 6 was incredibly low; despite following the full protocol, we achieved a low yield of 36.2 μg/mL on the first elution. We re-purified with the same mutant line to ensure that we had not made errors the first time. Mutant UPNY 7 was 36.5 μg/mL, still very low. Our
restriction digest confirmed that the transformation had successfully taken place. However, to ensure that we had not made a mistake, we repeated the transformation with the mutant PLD expression plasmid.

The results can be seen in figures 16 and 17. We were unsure why the gels were so warped. One explanation was that the TAE buffer we used to run the gel was quite old. This might have caused solute precipitation within the buffer and obstructed even flow of the current. Fortunately the gel was readable. Compared to the first digest, the bands were more consistent; perhaps a result of newer enzymes. Our uncut DNA in lanes 4, 5, and 6 of gel 1 were as expected. In lanes 7, 8, and 9 there are only two bands, indicative of the two cuts made by PVUI. Gel 2 had all of our mutant double digests. We found that all of the cultures contained our plasmid. This was not surprising since theoretically the cells shouldn’t have survived in ampicillin culture without the plasmid inserted correctly; the construct contained the ampicillin resistant gene. We found that mutant clones 1, 3, 5, and 8 had the strongest bands in the double digests, as we expected at 3531, 950, and 751 bp respectively. As a result we chose mutants clones 1 and 3 respectively, for our next two purifications.

After consultation with the company that manufacturers the TALON resin, Clontech (Mountain View, CA), we concluded that the low yields might be due to a solubility problem of the mutated protein. As a result, we added an additional component to the purification protocol. Rather than throw away the pellet after sonication and centrifugation, we solubilized proteins from the insoluble pellet component, hoping to find some PLD in this layer. We then purified the various components using the TALON column. UPNY 8 a, b, c, and d were the results of these efforts. There were some mistakes when carrying out the new protocol. A centrifugation was missed midway and, in spite of our efforts to correct it, our yield was very low: 16.39 μg/mL and 41.41 μg/mL for our first and second elutions respectively. We hypothesized that the inclusion of cell debris had lowered the TALON resin’s affinity for PLD through
non-specific binding. As a result, we attempted a ninth purification on the same protocol as well, this time ensuring that all centrifugation steps were done correctly, so that cell debris was removed before addition of the beads. However, this once again little to no PLD was obtained.

The next testing assay we carried out was to identify the optimum culture conditions for the *E. coli* to express the mutant PLD. Figures 20 and 21 show the gel and Western blot from the experiment. First of all, it becomes apparent that at each discrete temperature condition, the 5 hour induction allows for maximal PLD expression (by comparing the darkness and size of the band in the blot). We also used the assay to test the efficiency of our new sonicator. Viewing lanes 2 and 3 on the gel, one can see that the new sonicator released more proteins, as can be seen from the darker collection of bands. Usually, our *E. coli* is cultured in the 37 degree warm room. However, we noticed that the 38 degree conditions seemed to provide maximum protein expression. This is something that would need to be explored further to identify if a higher temperature would be optimal for growth. For the purposes of experimentation, we decided that the 37 degree culture times were sufficient for our purification.

Since preps 8 and 9 did not work, we conducted a complete solubility assay to identify which cell fraction the PLD was in, as can be seen in figures 23 and 24. The flow-chart for the various components is in figure 22. We were surprised to see that PLD remained in every sample, indicating that PLD gets trapped in the cell pellet as well. We found that when we used urea to solubilize the pellet (lane 7), we were able to isolate PLD. Using SDS, a denaturing agent, was not necessary since it did not produce that much more PLD (lane 8) than urea (lane 7). We noticed that the lane containing a sample grown at 37 degrees with 3 hours of induction was faint, but we suspect that that could have been caused by experimental error during loading of the sample. From examining the Western blot and comparing the relative quantities of PLD in each component, we concluded that there was enough PLD in the clarified cell lysate (lanes 4 and 6) for us to use our regular purification protocol.
The biggest changes made were to our elution process. Previously we had used 500 mM imidazole solution to displace the PLD from the Co$^{2+}$ ions in the TALON resin. We suspected that due to the mutation perhaps the conformation had changed and the PLD bound very strongly to the resin, so only smaller amounts were eluting off. In conversation with Clontech, we were advised to try acidic binding buffer to elute. Figure 25 shows the alternate elution reagents that we used. 15a, 14a, and 14b were the acidic elutions of pH 4 and pH 5. However, as can be seen in the lanes, there is no significant protein content, let alone PLD. Hence we concluded that the acidic elution buffer was ineffective.

Next we tried 100 mM EDTA, as can be seen in 15 d and e. Both contained detectable protein in the 32.1 kDa region, where we expected PLD. This was an amount that was significantly high, especially considering that two elutions with imidazole had already taken place meaning that all the PLD had not been eluted from the column. We concluded therefore that EDTA seems to be the best eluting agent that we tried. By using EDTA earlier in the process, before the use of an imidazole elution, we can maximize the concentration. As a chelating agent, EDTA irreversibly strips the cobalt off the sepharose backbone of the TALON beads. Like imidazole, EDTA can be removed from the PLD using dialysis in PBS. However, since the Co$^{2+}$ was removed from the resin, the TALON resin is no longer usable and must be replaced. Once we started using EDTA, our yield increased tremendously.

Our final troubleshooting step involved basic action to ensure that we were not losing PLD due to lack of binding to resin or during washing with low-concentration imidazole. Figure 26 shows our routine gels to test the flow through that we collected during the purification process. As can be seen from the wash (W) lanes, numerous impurities are being washed off from the column. However, compared to the amount of PLD in the final eluted sample, only small amounts of PLD are being washed away. Therefore, we were able to conclude that we were not losing PLD during the wash process.
To determine whether or not our PLD was binding to the resin, in the second gel of figure 26, we can compare the protein content of the lysate (L) band before incubation with the resin to the binding buffer (B) band, representing the supernatant after incubation. The band in the buffer (B) lane shows the amount of protein that was retained in the resin. As can be seen, the difference is noticeable, particularly in the 32.1 kDa region of the gel, where the PLD is located. We were able to conclude that the PLD was indeed binding to the resin. This ruled out the possibility that protein losses were occurring even before the column purification started. As a result, we have concluded that our main problem with expressing and purifying mutant PLD was with our elution methods. Our yields increased dramatically between spring 2012 and the end of summer, as can be seen in figure 27.

During the fall of 2012, research was done by Dr. Karen Molinder to determine how our protocol differed from that of the various manufacturers’ instructions and that of other labs. Dr. Molinder determined that many research groups and manufacturers had used alternate reagents and sonication times in order to purify. The protocol was changed, cutting sonication time drastically. Rather than using 500 mM imidazole to elute, we started using a first elution of 150 mM imidazole, followed by 100 mM EDTA. In the first prep with the new protocol, there was a significant rise in yield of mutant PLD: 546.7 µg/mL for the imidazole elution (KM 1a) and 1516.3 µg/mL for the EDTA elution (KM 1b) compared to previous preps. We hypothesize that the excessive sonication of the previous protocol was having a deleterious effect on the PLD. Despite the fact that the process was done on ice, the PLD solution would rise significantly in temperature. It is highly likely that some of the protein was being denatured or altered in a way so that it would not bind to the Co^{2+} in the resin.

Figure 28 shows an SDS-PAGE comparing different components, with the PLD samples equalized by amount loaded. Leading up to lane 6, we can observe how a series of washes progressively removed impurities. The washes with imidazole (W) removed protein from the column that had not been washed
off in the previous samples. We further observed that the use of a preliminary imidazole elution can help to remove many of the impurities that remain in the sample even after multiple 10mM imidazole washes. Comparing lanes 8 and 10, we can see that the EDTA-eluted PLD is far more pure than the imidazole-eluted PLD. These results were a break through and allowed us to continue an effective protocol.

Figure 29 was our next attempt at purification for wild type PLD. We were similarly successful in this attempt, with a yield of 233.93 µg/mL for imidazole-eluted and 925.13 µg/mL for EDTA-eluted PLD. As can be seen, there are significant contaminant bands in many of the lanes. We can confirm that most of the PLD was indeed bound to the resin when we compare the lysate prior to resin incubation (L) with the discarded buffer supernatant post incubation (B). There were also many impurities that were removed during our buffer washes and during our low-concentration imidazole wash. However, in our samples 19a and 19b there is still a high amount of contaminants. In this gel, volumes of loaded samples were not selected to ensure an equal amount of protein, so the relative bands cannot be compared to ascertain relative sample concentration. Regardless, we can objectively see the high amount of PLD as well as contaminant proteins.

To address this problem, we decided to add an additional 10 mM imidazole wash to the protocol to reduce the presence of extraneous protein matter. The result, wild type PLD 20, had similarly high yields, with a 396.2 µg/mL for imidazole-eluted and 841.4 for EDTA-eluted. Interestingly, the yield for our EDTA-elution dropped by 100 µg/mL while the yield for imidazole rose. We were expecting yields to drop slightly as a result of the PLD lost during the additional wash, however that wasn’t the case. Due to our improvement in purification technique, we were able to collect significant amount of both wild type and mutant PLD.
To apply our results on PLD purification and inactivation, the lab has expanded vaccine
development. Using the help of the Biology 330: Immunology class, the laboratory initiated a mouse
model for the different PLD vaccines. The first vaccine component tested was formalin-inactivated PLD,
the most common component of the commercial sheep vaccine. Since the formalin crosslinked the PLD,
the protein was inactive and could be injected into the mice without being toxic. However, the formalin
itself is highly toxic and, if inadequately dialyzed out, could have deleterious effects on the mice. Large
amounts of PLD are lost during the formalin-inactivation process due to precipitation and “clumping”. As
such, we hoped to test alternate forms of inactivated PLD.

The mutant variety of PLD protein was the second vaccine component to be tested. The
genetically inactivated recombinant protein was purified and used to vaccinate the mice. Since the
protein is almost identical to the wild type variety except for the active site (residue 20), we expected
that it would have similar antigenic properties to regular PLD. The purification of mutant PLD is far easier
and more efficient than the formalin-inactivation of wild type PLD. Furthermore, there is no worry of
formalin-toxicity to the mice.

The lab’s final mouse model vaccine component was a DNA vaccine designed for expression in
mammalian cells. Using services provided by Nature Technology (Lincoln, NE), Dr. Molinder selected the
NTC8682 vector that allows for expression of secreted protein in mammalian cells. The proteins are
targeted for secretion by the cell through use of TPA (tissue plasminogen activator) signal peptide. The
proprietary selection system by Nature Technology does not need antibiotics but, rather, sucrose in the
growth medium. The vectors express an RNA anti-sense strand called RNA-OUT that represses
expression of counter-selectable marker SacB. SacB encodes for the production of levansucrase. This is
toxic when combined with sucrose, so plasmid selection is carried out with the addition of the sugar to
the growth medium (Nature Technology 2011).
The Immunology class immunized four groups of mice (A through D), each consisting of four mice that were immunized with one of the three different vaccine components tested. Group A was immunized with formalin-inactivated PLD. Mice in group B as well as C1 and C2 were immunized with mutant PLD. Finally, C3, C4, and D-group mice were immunized with the DNA vaccine. Mice were bled regularly and sera were collected for testing by ELISA. Samples were then tested for IgG antibody response to both wild type and mutant PLD using an ELISA. Figures 30 and 31 show the initial results from this trial, one graph showing the mouse anti-wild type PLD antibody response and the other showing the mouse anti-mutant PLD antibody response. ELISAs were carried out by Margaret Winterkorn and I analyzed the data. The displayed values are a ratio of the measured optical density to the positive and negative controls used on the plate.

![Graph](image)

**Figure 30:** Mouse IgG response to wild-type PLD divided by immunization group and graphed according to the number of days from immunization that sample was drawn. Group averages displayed along with standard deviation bars.

Unfortunately, the sera for bleed dates after 22 days from formalin-inactivated PLD immunized mice were finished and serum from two out of the four mice was unaccounted for. For this group, averages were calculated from the two available samples. Figures 30 and 31 show the averaged murine
antibody response to both wild type and mutant PLD based on immunization administered. As can be seen in figure 30, formalin-inactivated PLD and DNA vaccine mice did not exhibit strong immune responses to wild type PLD. However, the mice immunized with the mutant PLD reacted very strongly to wild type PLD. In figure 31, we can see that the mutant PLD mice exhibit a similar robust antibody response to mutant PLD, as we would expect. The formalin-inactivated response has quite a sharp rise at day 22. Finally, the DNA vaccine-immunized mice start to show a rising response following 41 days from immunization. Large standard deviations for the mutant PLD mice’s response to both wild type and mutant PLD indicate inconsistency between the reactions of the six mice. In particular for the last bleed dates, three mice had very strong responses while three had very weak responses. This accounts for the larger standard deviation bars.

The data collected from this mouse trial showed a lot of variation in response. However, when averaged and displayed along with standard deviation bars, we can follow the primary response to the vaccinations. For the response to wild type PLD, the mutant seemed to react the strongest. This was

![Anti-Mutant PLD Response](image)

Figure 31: Mouse IgG response to mutant PLD divided by immunization group and graphed according to the number of days from immunization that sample was drawn. Group averages displayed along with standard deviation bars.
very heartening, indicating that the site-specific mutation of the PLD in the vaccine still resulted in the production of antibodies that recognize wild type PLD. This presents a large amount of potential for vaccine development. The mutant PLD vaccine could be recognized by the immune system the same way as wild type PLD, without the toxicity associated with active PLD or formalin-inactivation.

Unfortunately, there were few sera samples available to study the formalin-inactivated vaccine group. However, that response has already been well-documented in mice and has been studied widely due to its role in the sheep vaccine. We do not see a significant DNA vaccine response, as we had expected this. However, the manufacturers suggest 3-5 boosts for maximum effectiveness. Since we had not boosted to that extent, it is possible that, with further boosts, a response can be detected.

Work with the mouse model will be continued once we have set up our Bio Safety Level-2 facilities. Along with boosts with all the various vaccine components, the next step will be to challenge the mice with live bacteria. We will be able to measure the murine immune response and identify if any of the vaccines confer a protective effect against the bacteria. Of particular interest is the mutant PLD protein due to its antigenic recognition as both wild-type and mutant PLD. It has a lot of potential as an alternative to formalin-inactivated PLD due to its inactivity and non-toxicity. Similarly, the DNA vaccine has potential as a “Trojan-horse” of sorts. The host cells will produce mutant PLD within the body itself, serving as a sustained source of antigen, to elicit a response and, hopefully, provide protection against *Corynebacterium pseudotuberculosis*. 
Bibliography


Appendix

Basic Reagents

Tryptic Soy Broth
30g Tryptic Soy Broth powder (Sigma Aldrich, St. Louis, MO)
1L ddH20
Autoclaved
10x Running Buffer
30g Tris-Base
144g of glycine
10g SDS
1L ddH20
pH 8.3
Store @ room temperature
Dilute to 1x before using

10x Transfer Buffer
288.4 g glycine
60.5 g Tris-Base
2L ddH20
1x Transfer Buffer
100mL 10x Transfer Buffer
200mL Methanol
700mL H2O
pH 8.0-8.5

PBS-Tween 20
1ml Tween 20
Combine with 2mL PBS
LB Medium
10g Tryptone
5g Yeast Extract
10g NaCl
Dissolve above contents in 1L ddH20
Adjust pH to 7.0 and autoclave to sterilize

IPTG (500mM stock)
0.357g IPTG
Dissolve in 3mL water

1M Tris stock
60.5 grams Tris base in 400 mL ddH20
pH 8.0 w/ concentrated HCl
Bring to 500 mL with ddH20

Binding Buffer: 20 mM Tris HCl, 100 mM NaCl
5.84g NaCl
20mL 1M Tris stock
900mL ddH20
pH 8.0 (adjust)
Add ddH20 for a final volume of 1L

**MES Buffer**
(formula weight is 195.24)
3.9 grams MES
5.84g NaCl
Dissolve in 800mL ddH20
pH 5.0 (HCl or NaOH)
Adjust to 1L with ddH20

**Imidazole**
1) 8mL binding buffer + 10mM imidazole
2) 10mL binding buffer + 150 mM imidazole
10% Buffered Formalin
100ml 37% formaldehyde
900ml ddH20
12.0g Na2HPO4 (anhydrous)
3.0g KH2PO4 (anhydrous)
Nonbuffered ethanol w/ 0.1% sodium azide (NaN3)
Previously made by Hector

**New Recombinant PLD Purification Protocol: Non-denaturing Conditions**

1M NaH$_2$PO$_4$ (200 ml): MW = 120 g/mol
24 g in total volume of 200 ml
Note: Will precipitate at room temp. Warm until dissolved.

1M Na$_2$HPO$_4$ (200 ml): MW = 142 g/mol
28.4 g in total volume of 200 ml

100 mM sodium phosphate buffer pH 7.0 (1 L) -see Maniatis Vol. 3; Appendix B.21
42.3 ml of 1M NaH$_2$PO$_4$
57.7 ml of 1M Na$_2$HPO$_4$
bring total volume to 1L. pH to 7.0

**1X Equilibrium Buffer**
for 500 ml:
50 mM sodium phosphate buffer
300 mM NaCl
pH to 7.0

150 mM imidazole in 1x elution buffer
pH to 7.0

**Imidazole elution buffer (150 mM imidazole)**
for 100 ml:
1.02 g imidazole
100 mM EDTA elution buffer pH 8.0:
For 100 ml: use 3.72 g EDTA (MW = 372.2 g/mol)

Start pHing toward 8.0: EDTA will not fully go into solution until nearing this range.

II. Sample Preparation

1. Harvest cell culture by centrifugation at 1000 – 3000 g (3500 – 5500 RPM for Beckman JA-10 rotor) for 15 min at 4C. Remove supernatant.
2. Resuspend the cell pellet by vortexing in chilled 1X Equilibration/Wash buffer. For cultures < 100 ml, use 2 ml buffer for every 25 ml of original culture; for cultures > 1L, resuspend the pellet in 2% of the original culture volume; for 900-1000 ml culture, use 35 ml buffer.
3. Sonicate on ice. Run three cycles of program 4 (10 sec at 50%; 0.5 sec on; 0.5 sec off. Keep for 30 sec on ice in between cycles).
4. Transfer lysate to two 50 ml Nalgene specialty centrifuge tubes: Make sure tube masses are within 0.2 g. Centrifuge at 10,000 – 12,000 g (~ 10,500 RPM for Beckman JA-17 rotor) for 20 min at 4C.
5. Carefully transfer supernatant to clean tube without disturbing pellet. Save small sample for later SDS-PAGE.

III. Column Purification

1. Thoroughly resuspend the BD Talon resin, then transfer 4 ml slurry to a sterile 50 ml conical. 4 ml slurry will = 2 ml bed volume of packed resin
2. Centrifuge at 700 g for 2 min at 4C to pellet resin. Discard supernatant.
3. Wash resin twice with 10x bed volume of equilibration/wash buffer
   a. Add 10 bed volumes of 1X equilibration/wash buffer and mix briefly
   b. Centrifuge at 700 g for 2 min at 4C to pellet resin. Discard supernatant.
   c. Repeat steps a. and b.
4. Add the clarified lysate from section II to the resin in the conical. Rotate conical in the cold room on a vertical tube rotator for 20 min.
5. Centrifuge at 700 g for 5 min at 4C to pellet resin. Remove as much supernatant as possible without disturbing the resin pellet.
6. Wash resin twice with 10x bed volume of equilibration/wash buffer
   a. Add 10 – 20 bed volumes of 1X equilibration/wash buffer. Agitate the suspension gently. Rotate conical on a vertical tube rotator for 10 min.
   b. Centrifuge at 700 g for 5 min at 4C to pellet resin. Discard supernatant.
   c. Repeat steps a. and b.
7. Add 1 bed volume of 1X equilibration/wash buffer to the resin and resuspend by vortexing
8. Transfer the resin to a 2 ml gravity flow column with end cap in place. Allow the resin to settle out of suspension at 4C.
9. Remove end cap and (at 4C) allow buffer to drain into waste container below until it reaches the top of the resin bed, making sure no air bubbles are trapped in the resin bed.
10. Add 5x bed volumes of 1X equilibration/wash buffer to the column to wash. Drain at 4C.
11. Optional: If necessary, wash column with 1X equilibration/wash buffer including 5 - 10 mM imidazole.
12. Elutions: Prepare tube for collection. Perform at 4C
   a. Optional imidazole elution: Add 1 bed volume of 150 mM imidazole elution buffer. Collect.
b. **EDTA elution:** Make sure column end cap is in place. Add 1 bed volume of 100 mM EDTA elution buffer. Let resin soak for 30 min at 4C. Position collection tube, uncap, and collect.

IV. Dialysis: Load G2 Dialysis cassette with needle and syringe

1. **Hydrate membrane**
   a. Remove cassette from packaging. Handle cassette by its frame only!! Do not touch the membrane with ungloved hands!
   b. Immerse cassette in dialysis buffer (1X PBS) for 2 min to hydrate. It may be necessary to hold the cassette under the surface for the hydration step as the air inside the cassette may cause it to float sideways
   c. Remove cassette from buffer. Gently tap on a paper towel to remove excess. Turn cassette upside down and tap again. Do not blot membrane!

2. **Add sample:** Maximum volume for 3 ml cassettes is 1 ml.
   a. Use 18 or 21 gauge 1 – 1½” needle. Fill the syringe with sample, leaving a small amount of air in the syringe
   b. With base of cassette on benchtop, SLOWLY penetrate the gasket through one of the syringe ports. Push needle in **just enough** to see the open end is in the cavity.
   c. Slowly inject about half the quantity of sample in the syringe. Then withdraw some air from the cassette by gently pulling back on the syringe plunger. Then inject remaining sample.
   d. Withdraw some air from the cassette by pulling back on the syringe plunger, to compress the membrane windows around the sample. Stop before windows touch needle.
   e. Remove needle from cassette while retaining air in the syringe. Mark which port you used on the cassette.

3. **Dialyze**
   a. For the volume of dialysis buffer, use at least 300 times the sample volume. Float cassette vertically in the dialysis buffer.
   b. Stir gently with stir bar – make sure you do not create a vortex that pulls the cassette down in contact with the stir bar.
   c. Dialyze 2 hr; change buffer. Dialyze 2 hr; change buffer. Dialyze overnight
(4) Recover sample: use the unused syringe port
   a. Suck some air into the syringe. Penetrate gasket and push needle in until you can just see open end. Inject air into cassette to inflate and separate the membrane windows. With needle in place, turn cassette so needle is on the bottom. Allow sample to collect near port and withdraw the sample with syringe.

**His-PLD Purification (Old protocol)**

**Grow and Sonicate E. coli Bacteria**

1. Streak *E. coli* bacteria (strand JG52274) containing the 6xHis tag on an LB+AMP plate. The bacteria is in the -80°C freezer in the box labeled “Immunology.”

2. Put the plate upside down in the 37°C warm room overnight, or until ready to pick.

3. Before picking, make sure you have 500 ml of LB. Right before picking, add 1 ml of 50 mg/ml of AMP to 500 ml of LB and swirl. The AMP is currently in the freezer in a 15 ml conical tube at a concentration of 50 mg/ml. The final concentration of the LB will be 100 μg/ml.

4. When ready to use the *E. coli*, pick an isolated colony and transfer gently into a bacteria culture tube filled with 3 ml of LB+AMP. Make sure to sterilize the loop before each use by dipping in ethanol and then flaming.

5. Wrap the plate with Parafilm and store in the cold room.

6. Incubate the small culture in the 37°C warm room with shaking for 12 hours, or until cloudy.

7. Pour the small culture into ~500 ml of LB+AMP (100 μg/mL) in a 2 L flask. Put the large culture in the 37°C warm room with shaking for 24 hours.

8. Add 3 ml of 500 mM IPTG (0.357 g of the IPTG into 3 ml of ddH₂O). Put the large culture back in the 37°C warm room with shaking for 5 hours.

9. Remove the large culture from the warm room. Pour the culture from the 2 L flask into two plastic centrifuge bottles. Try to get each bottle to have the same volume (~250 ml).

10. Harvest the cells by centrifuging at 7000 rpm for 15 minutes. Use the centrifuge located in the room with the -80°C freezer.

11. Discard the supernatant. Resuspend one of the pellets with 10 ml of binding buffer.

12. Once the pellet has been resuspended, transfer the cell solution to the other centrifuge bottle. Resuspend until the second pellet dissolves.

13. Transfer the cell solution to a 50 ml conical tube. Fill up to 35 ml with binding buffer.

14. Sonication should be done with 1:1 ratio of pulse time and an output amplitude of 80-100%. To clean the sonicator, sonicate ~40 ml of ddH₂O for two minutes. Gently dry off the tip with a Kim wipe.

15. Sonicate the 35 ml of cell solution for four minutes. Clean the sonicator after each use with ddH₂O for two minutes. Repeat two more times (three times total).

16. Transfer the sonicated cells to two polycarbonate tubes. Make sure each tube has the same volume with less than 0.2 g difference. Use a scale to determine the mass.

17. Spin the polycarbonate tubes using the centrifuge in -80°C for 20 minutes at 15,000 x g (angular velocity between 10,000 – 10,500 rpm) at 4°C.

18. Collect supernatant in a 50 ml conical and discard the pellet. The PLD is in your supernatant. Keep the PLD supernatant on ice, but do not freeze!

**B. Purifying PLD with a Metal Affinity Column**
1. Get a column from the cold room and a TALON® Superflow™ Metal Affinity Resin from the fridge. 
   
   If the resin is not new, follow steps 2-9:

2. Set up the column on a metal stand with a clamp. Put a medium-sized beaker underneath the column to collect waste. Let the sodium azide solution flow through. Wash the column three times with 8 ml of MES buffer.

3. Wash the resin once with 8 ml of binding buffer. Cap the column and add binding buffer, resuspending and transfer to 50 mL conical tube.

4. Spin in the TC room at 1800 rpm for 1 minute at 4°C. Discard supernatant.

5. Add another 8 ml of binding buffer and repeat step 4.

6. Add the PLD supernatant and shake gently for 20 minutes in the cold room. Make sure the shaker is set up in the cold room. Repeat step 4.

7. Add 8 ml binding buffer to PLD + resin. Shake gently at 4°C for 10 minutes. Repeat step 4.

8. Add 8 ml of binding buffer and transfer back to the column. Discard flow through.


10. Soak with 8 ml of binding buffer and transfer back to the column. Discard flow through. Repeat two more times (three times total).

11. Cap column. Add 2 ml of 300 mM elution buffer and soak for 15 minutes. Collect PLD flow through in a 2 ml freezing vial.

12. Rinse the resin with 8 ml of MES buffer. Cap the column and fill with sodium azide solution. Store resin at 4°C.

13. Continue PLD purification (recommended), but the PLD can be frozen at this point in the -20°C freezer.

Formalin-Inactivation

MATERIAL:
10% buffered formalin:
10ml 37% formaldehyde
90ml distilled water

1.20g Na₂HPO₄ (anhydrous)
0.30g KH₂PO₄ (anhydrous)
5 ml 37% formaldehyde
45 ml distilled water
0.60 g Na₂HPO₄ (anhydrous)
0.15 g KH₂PO₄ (anhydrous)

METHOD:

1. For every 1ml of toxin solution (concentration 1-5mg/ml), add 40μl of 10% buffered formalin (final concentration of 0.4%)
2. Incubate at 37°C for 72 hours with gentle agitation (on the rocker).
3. Dialyze the toxoid against 3 changes of PBS (at least 2 liters, 4 liters is better) overnight at 4°C. We use 14,000 molecular weight cutoff dialysis tubing. If multiple samples are being dialyzed together, do 4 to 5 changes of PBS.
4. Test by a protein assay such as BCA. Much of the protein may precipitate out, so be sure to resuspend well and note whether you observe an obvious precipitate.

### BCA Assay

#### Standards

<table>
<thead>
<tr>
<th>Vial</th>
<th>Volume of Diluent (1X PBS)</th>
<th>Volume of BSA</th>
<th>Final BSA Concentration</th>
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</thead>
<tbody>
<tr>
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<td>200 µL</td>
<td>2000 µg/ml</td>
</tr>
<tr>
<td>B</td>
<td>100 µL</td>
<td>100 µL</td>
<td>1000 µg/ml</td>
</tr>
<tr>
<td>C</td>
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<td>100 µL of B</td>
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<td>H</td>
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<td>0 µL</td>
<td>0 µg/ml</td>
</tr>
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</table>

#### Working Reagent (WR)

\[(\text{# of Standards} + \text{# of unknowns}) \times (\text{# of replicates}) \times (\text{volume of WR added per sample}) = \text{Total volume of WR needed}\]

**WR Recipe**

50 parts of BCA Reagent A with 1 part Reagent B (50:1). Mix until a clear green WR develops

**Protocol**

1. Add 25 µL of each standard and unknown to the wells. I tested each sample in triplicates (So make sure you have at least 75 µL of each standard and unknown.
2. Add 200 µL of WR to each sample well.
3. Mixed plate for ~30 seconds
(4) Cover plate and incubate for 30 mins @ 37°C
(5) Cool plate to RT
(6) Measure \( \text{OD}_{570} \)

**Plate Reader Settings**

\[ M = 4:570 \text{ (single)} \]

Mixing = ON (30 seconds)

Incubation = ON (37°C)

---

**Western Blot**

Notes:

1. Use premade gel (ie BioRad 4-15% gradient TGX Ready Gels)
2. Start heating block to 94°C while you are setting up apparatus

1. Remove green tape along the bottom of the gel
2. Put gel on gel stand, shorter plate facing in and making a snug fit just below the gasket pads (see figure 1)
3. Put other gel or dam on opposite side of apparatus in the same manner

**If using older Biochem Department apparatus:**

4. The clamp apparatus flaps should be open. Holding gels to stand, insert the gel stand into the top of the clamp apparatus. Be sure to push down firmly as you slide it into place so it is all the way at the bottom of the clamp apparatus
5. While pushing downward, clamp the gel into place by closing the flaps on the gel clamp.
6. Lower the clamp apparatus into the electrophoresis tank.

**If using Tetra-cell:**

4. Make sure to put the gel stand with banana plugs in the proper tank slot so that the lid to the tank will fit onto the electrodes.
5. Gel stand has “butterfly” clamps that fold up to lock the plates in place. May need to start raising them slightly before plates will sit perfectly.
6. Lower clamped gel stand into electrophoresis tank
7. Fill the top chamber in between the plates with running buffer (buffer level must be higher than the top of the wells). Fill the bottom of the chamber past where the clamp apparatus wire with running buffer.
8. Remove comb
9. With a transfer pipet, flush the wells with running buffer.
Samples:

1. Add β-mercaptoethanol to the appropriate amount of 1X loading dye: 5% (50 ul β-mercaptoethanol to 950 ul dye.
2. Put cap holders on tube lids if desired. Boil samples for 3-5 minutes. Markers we use do not need to be boiled.
3. Quick spin samples
4. Load 10 ul of marker per lane and up to 25 ul of each sample per lane.

Running gel

1. Put top on electrophoresis tank matching the red lead to the red electrode and the black lead to the black electrode.
2. Run at 100V for 1 – 1.5 hrs. Protein will run toward the positive electrode

After gel has run:

1. Turn off power supply and remove electrophoresis tank lid
2. Pour off buffer from upper chamber
3. Remove the clamp apparatus. Open the flaps
4. Pull the gel stand up out of the clamp apparatus. Be sure to keep a finger holding each gel so they don’t fall off stand
5. Take gels off the gel stand.
6. Insert the BioRad opening lever between the plates at one of the arrows marked on the gel plates and gently pry plates appart. Repeat at each of the other three arrows (one in each corner)
7. Now that the plates have been loosened, use the lever to fully pry them apart, so that the gel stays on one of the plates.

For Coomassie staining a gel:

1. Put plate with gel into a container of H2O. Float gel off the plate
2. Wash gel 3 times in ddH2O for 5 min each with shaking.
3. Stain gel in Bio-Safe Coomassie Stain for at least 1 hr with shaking
4. Wash gel one time in ddH2O for 5 min, then in ddH2O for at least 1 hr.
5. Photograph stained gel

For Western Blotting a gel

1. Soak gel for 30 min in room temp transfer buffer to equilibrate (so gel doesn’t shrink during transfer)
2. Prepare nitrocellulose membrane: wet slowly in distilled water (with one edge at a 45° angle, slowly dip corner into water and slowly lower the membrane in until membrane is completely wet. Then equilibrate for 10-15 min in transfer buffer
3. Put transfer cassette in large pyrex pan. Black side of open cassette should be flat against the bottom of the pan. Assemble blot sandwich on top of black side.
4. Put soaked pad on the black side. Pour enough transfer buffer into the pyrex pan to keep pad moist but keep liquid level below the top of the pad (so subsequent layers don’t float!)
5. Assemble the rest of sandwich. Make sure everything is soaked with transfer buffer. Whatmann paper and membrane should be cut to the size of the gel. Keep everything wet with buffer and roll with pipet or conical to remove any air bubbles each time you add a layer!

Order:

a. Soaked sponge
b. 1 piece Whatmann paper. Use this piece of Whatmann paper to slide under the gel to move it to cassette
c. Gel
d. membrane
e. 1 piece Whatmann paper
f. Soaked sponge

6. Close sandwich tightly. Insert into transfer apparatus with black side of cassette against black side of apparatus. Add transfer buffer to apparatus until the cassette is completely covered.

7. Transfer at 20 V for 12 hours (or 12V O/N) in the cold room on top of stir plate. Have stir bar in apparatus below the transfer cassette (stir bar may not be able to turn in a full circle but will keep buffer moving). Should be small stream of bubbles visible on the black side of the apparatus (may have to turn up voltage temporarily to see the bubbles).

8. Make blocking buffer (so you are sure milk is fully dissolved by the next day).

9. After transfer is complete, open transfer cassette in pyrex pan.
   a. Mark wells on membrane with pencil. Cut corner to orient if needed.
   b. Rinse membrane in ddH2O

10. Block membrane in blocking buffer for 1 hour at room temperature (or 0/N at 4C) with rocking.

11. Add primary antibody diluted in blocking buffer (usually sera dilution is 1:1500). Incubate for 1 hour with rocking.

12. Wash membrane in wash buffer (TBST). Wash 3-4 times for 10-15 minutes each.

13. Change tray if desired. Add secondary antibody (usually anti-IgG) diluted in blocking buffer (usually 1:1500 dilution). Incubate for 1 hour at room temperature with rocking.

14. Wash 2 times in TBST for 10-15 minutes each.

15. Wash once in TBS (no tween! Tween inhibits the Opti 4-CN reaction) for 10-15 min.

16. Add the Opti-4CN Substrate to Opti 4-CN diluent (per 10 ml: 1mL of Opti-4CN Diluent + 9 mL ddH2O + 0.2 mL of Opti-4CN Substrate). Mix Well. Pour onto membrane. Incubate with gentle agitation until desired signal level is reached.

17. Rinse w/ dd H2O and photograph

**5X SDS Electrophoresis buffer**

15.1 g Tris base (final concentration in 5x = 0.125 M)
72.0 g glycine (final concentration in 5x = 0.96 M)
5.0 g SDS (final concentration in 5x = 0.5%)
H2O to 1000 ml
Adjust pH to 8.3 only after diluting to 1x
Store at 4C (for up to a month)
10X Transfer Buffer (1L)
30.25 g Tris base
144.2 g glycine
ddH2O to 1000 ml
pH will be around 8.3 – 8.4
Note: pH reads at ~8.7 at first. Takes a few ml of concentrated HCl (~ 6.5 ml) to pH to 8.4

1X Transfer Buffer (4 L)
400 ml 10X Transfer Buffer
800 ml methanol
ddH2O to 4 L

Tris-Buffered Saline (TBS): 1L
12.1 g Tris base (100 mM)
800 ml ddH2O
pH to 7.5 with HCl
Note: adjust pH at the temperature that you plan to use the solution.
Takes a few ml (~6.75 ml) of concentrated HCl to pH to 7.5
Then add...
8.8 g NaCl (150 mM or ~0.9%)
ddH2O to 1000 ml

Wash buffer (TBST: Tris-Buffered Saline + Tween)
TBS + 0.1% Tween 20
Can be stored several months at 4C

Blocking Buffer
5% nonfat dry milk in TBST

QIAprep Miniprep for E. coli Plasmid Isolation – Protocol
1. Grow E. coli colonies on an LB agar + AMP plate overnight at 37°C.
2. Once colonies look healthy, pick a single colony from a freshly-streaked selective plate and inoculate a culture in 5mL LB medium containing AMP. Incubate 12-16 hours at 37°C with vigorous shaking.
3. Aliquot bacteria into 2- or 1- mL microcentrifuge tubes. Harvest the bacteria cell by centrifuging at 13,000 rpm for 3 min at RT.
4. Resuspend the bacteria pellet in 250 uL Buffer P1 and transfer to a microcentrifuge tube.
5. Add 250 uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times.
6. Add 350 uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
7. Centrifuge for 10 min at 13,000 rpm in a microcentrifuge.
8. Apply the supernatant from Step 7 to the QIAprep spin column by decanting or pipeting.
9. Centrifuge for 30-60 sec and discard the flow-through.
10. Wash the QIAprep spin column by adding 0.5 mL Buffer PB and centrifuging for 30-60 sec. Discard the flow-through.
11. Wash QIAprep spin column by adding 0.75 mL Buffer PE and centrifuging for 30-60 sec. Discard flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.
12. To elute DNA, place the QIAprep column in a clean 1.5 mL microcentrifuge tube. Add 50 uL Buffer EB or water to the center of each QIAprep spin column, let stand 1 min, and centrifuge for 1 min.
13. Store in -20°C.

**Restriction Mapping** *(Current Protocols in MoBio, Vol 1, 3.2.1)*

Definition: To construct a map of specific sites where restriction endonucleases cleave DNA

Purpose: To generate a restriction map, aid in nucleotide sequence analysis, subclone, and/or confirm the identity of an isolated plasmid

How: The DNA is cleaved at or near specific nucleotide sequences with restriction endonucleases, and determination of the sizes of the resulting DNA fragments is done via agarose or acrylamide gel electrophoresis.

Parameters: Can be done with a variety of restriction enzymes individually or in combo

- Cloned DNA segments up to 20kb in length
- Use restriction enzymes that cleave the DNA relatively infrequently

Materials
- DNA sample in TE buffer on ice
- Restriction endonucleases *(EcoR1, Pvu1)*
  - *EcoR1*: G/AATTC, 37°C rxn, 65°C inact
  - *Pvu1*: CGAT/CG, 37°C rxn, 100°C inact
- 10x restriction endonuclease buffers *(EcoR1 and Pvu1 use the same buffer)*
- 10x loading buffer

1. Pipette the following into a microcentrifuge tube **IN THE FOLLOWING ORDER:**

<table>
<thead>
<tr>
<th></th>
<th>20uL reaction</th>
<th>50uL reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH2O</td>
<td>8.0 uL</td>
<td>33.0 uL</td>
</tr>
<tr>
<td>10X restriction buffer</td>
<td>2.0 uL</td>
<td>5.0 uL</td>
</tr>
<tr>
<td>DNA (127ug/mL)</td>
<td>8.0 uL</td>
<td>8.0 uL</td>
</tr>
<tr>
<td>Restriction endonuclease</td>
<td>2.0 uL</td>
<td>4.0 uL</td>
</tr>
</tbody>
</table>

**NOTE:** The volume of restriction endonuclease added should be less than 1/10 the volume of the final reaction mixture.

2. After adding the restriction endonuclease, incubate the mixture overnight at 37°C.

**NOTE:** Since the reaction temperatures of the restriction endonucleases are the same, they can be added simultaneously and incubated at 37°C.
3. Immediately before loading a gel, stop the reaction by adding 4μL or 10μL (20% of rxn volume) of 10X loading buffer.

**Agarose Gel Electrophoresis**

Purpose: To separate, identify, and purify 0.5-25 kb DNA fragments

How: Prepare an agarose gel w/ concentration appropriate for size of DNA fragments to be separated; run gel; and stain gel or illuminate with UV light if ethidium bromide is incorporated

**Materials**
- Electrophoresis buffer (0.5X TBE or 1X TAE)
- Ethidium bromide solution
- Electrophoresis-grade agarose
- 10X loading buffer (provided by Hector)
- DNA molecular weight markers
- 55°C water bath
- Horizontal gel electrophoresis apparatus
- Gel casting platform
- Gel combs
- DC power supply

**Preparing the gel**

WEAR GLOVES. ETHIDIUM BROMIDE IS A CARCINOGEN!

1. Prepare 600mL of electrophoresis buffer to fill the electrophoresis tank. Add 600μL of stock ethidium bromide solution (0.5mg/mL) for a final concentration of 0.5µg/mL.
   
   **NOTE:** Make sure the concentration of ethidium bromide in the gel is identical to the concentration in the buffer.

2. Add 1.0g of electrophoresis-grade agarose to a 100mL volume of electrophoresis buffer for constructing a 1% agarose gel.
   
   **NOTE:** A 1% agarose gel has an effective range of resolution of linear DNA fragments listed as 10 to 0.5 kb. Gels typically contain 0.8-1.5% agarose.

3. Melt the agarose in a bottle with the cap loosely on in a microwave oven and swirl to ensure even mixing. You can also autoclave it in an Erlenmeyer flask with a foil lid taped on. Only fill the flask half-full.

4. Seal the gel-casting platform with tape. Once the agarose has cooled so that you can touch your hand to the bottom of the bottle or flask (very warm, not hot), add 100μL of stock ethidium bromide solution. Pour in the melted agarose and insert the gel comb, making sure that no bubbles are trapped underneath the comb and all bubbles on the surface of the agarose are removed before the gel sets.

**Loading and running the gel**

5. After the gel has hardened, remove the tape from the open ends of the gel platform and withdraw the gel comb, taking care not to tear the sample wells.
NOTE: Low percentage gels and gels made from low gelling/melting temperature agarose should be cooled in 4°C to gain extra rigidity and prevent tearing.

6. Place the gel-casting platform containing the set gel in the electrophoresis tank. Add sufficient electrophoresis buffer to cover the gel to a depth of about 1 mm. Make sure no air pockets are trapped within the wells.

7. Prepare the DNA sample by adding the appropriate amount of 10X loading buffer—20% of reaction volume. Load each well with 20uL sample. Include the appropriate DNA molecular weight markers.

8. Run the gel at 100V for ~1.8 hours. Make sure the leads are attached such that the DNA will migrate toward the positive lead (red). Usually you will run the gel until the bromophenol blue dye is 2/3 of the way down the gel and the xylene cyanol is 1/3 of the way. 
*Note: If you are using pre-made loading buffer, it might have different dyes, so check the directions.*

9. Turn off the power supply when the fragments have migrated sufficiently. Photograph the gel.