

**THE EXPLORATION OF ELISA DETECTION ASSAYS USING L1CAM
AS A BIOMARKER IN MALIGNANT CANCER**

by

Angelica F. Claxton

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

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AS A BIOMARKER IN MALIGNANT CANCER**

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Angelica F. Claxton

Approved: _____
Deni S. Galileo, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Robin W. Morgan, Ph.D.
Chair of the Department of the Biological Sciences

Approved: _____
George H. Watson, Ph.D.
Dean of the College of the Arts and Sciences

Approved: _____
James G. Richards, Ph.D.
Vice Provost for Graduate and Professional Education

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ABSTRACT

Metastasizing cells are generally the primary basis of malignancy in cancer. There is an array of signaling mechanisms that influences cancer cell migration and metastasis such as RTK (FGFR) and integrin signaling. Cancer cells manipulate signaling cascades such as these by multiple mechanisms, including the expression of proteins that otherwise would not be expressed, which results in aberrant behavior such as increased proliferation and motility. Abnormal proteins expressed on the cell surface of, or released by, migrating cancer cells can be considered biomarkers specifically to detect that metastasis is occurring. In this project, I have focused on the documented abnormally expressed cancer biomarker, L1CAM (L1; CD171). Several different cancer types such as breast, colon, myeloma, uterine, ovarian, pancreatic, and glioma have all been reported to have L1-positive tumors. Interestingly, clinical studies that have monitored survival outcomes of persons who have had L1-positive and L1-negative tumors, show that persons with L1-positive tumors have significantly lower survival rates compared to their counterparts. Therefore, the ability to detect L1 not only determines the presence of, or potential for, metastasis, but provides a better assessment of each cancer prognosis and the initial rigor of therapy to be administered. Toward this goal of better L1 detection, two commercial monoclonal antibodies, UJ127.11 and 5G3, were used in chromogenic capture and indirect poly-ornithine (p-

orn)-ELISAs to detect L1 in cell lysates/extracts, conditioned media, and both PBS and human serum spiked with known concentrations of L1. I found that in indirect (non-capture) p-orn-ELISAs, UJ127 detects L1 in PBS spiked with purified L1fc, and conditioned media from U118L1Le cell lines, but does not from 293TL1fc conditioned media or human serum spiked with purified L1fc. On the other hand, capture p-orn-ELISAs were capable of detecting L1 from all L1-containing samples with generally very low background. To optimize assay conditions, I tested both nitrocellulose and poly-ornithine for increased binding capacity, and poly-ornithine was selected as the best material to accomplish this. I also compared p-orn-coated ELISAs to the more commonly used high-pH buffer coated ELISAs, and found that p-orn-ELISAs were more sensitive for detecting L1. Using the p-orn-coated capture ELISAs, I was able to detect purified L1fc diluted in PBS as well as with human serum present, down to 0.4 ng/ml. This suggests that an improved L1 ELISA detection assay could be of benefit in a clinical setting to evaluate the potential degree of morbidity in cancer cases.

Chapter 1

INTRODUCTION

Approximately 14 million people are diagnosed with cancer every year [1]. Though there is no established cure for all cancer types, some cancers can be cured with treatment. Chemotherapy and radiation treatments available are often costly as well as debilitating for the patient. This is usually dependent on the stage at which the cancer is initially detected. Advanced staged cancers require more vigorous treatment with harsher side effects compared to earlier detected cases. If cancer can be detected early on, then early administration of treatment can increase the probability of cancer-free survival [2]. In addition to early cancer detection, the ability to determine the degree of malignancy can greatly influence the choice of treatment [3]. Though each cancer case is unique in its manifestations in individuals, there is evidence that aggressive cancer phenotypes express cell surface markers that can be indicative of poor outcomes[4] [5] [6] [7] [8].

L1CAM is a cell adhesion molecule that plays crucial roles in neurogenesis, axon outgrowth and fasciculation, and is necessary for correct cell migration during embryogenesis [9]. During post embryonic development, L1CAM is exclusively expressed in neurons and pre- and non-myelinating Schwann cells [10]. However,

there are numerous cancer types, such as uterine, ovarian, breast, and colorectal whose tumors have been positive for L1 expression [11] [12] [13] [14]. Consequently, the patients who possess the aforementioned cancers that are L1 positive, have a worse survival outcome compared to those patients whose cancers are L1 negative [11]. In addition to being abnormally expressed, L1CAM is cleaved by another membrane protein called ADAM10 [15]. This cleavage results in a soluble L1 ectodomain fragment that is able to interact with L1 homophilically, and initiate cell signaling via integrins and FGFRs heterophilically [16].

In my project, I used soluble L1 as a foundation for developing a simple yet efficient L1 detection system for analysis of L1 ectodomain in human serum via the enzyme-linked immunosorbent assay (ELISA) technique. In addition, I have uniquely altered the ELISA plate to increase the binding capacity by using polyornithine (p-orn), to coat the wells of these plates. The techniques described in this paper can not only be used to aid in the detection of L1 specifically, but can be applied to the use of ELISAs in general.

1.1 L1CAM: Structure and Function

L1CAM is a single-pass transmembrane protein that is 200-220 kDa in size when intact and 190-200 kDa when cleaved from the cell membrane. The exposed extracellular region of L1CAM is composed of six immunoglobulin or IgG-like and five fibronectin or FN-like domains (Figure 1). These repeats have specific binding partners that will be discussed in later chapters. These CAMS are on the surface of neurons and function to mediate certain cell adherences by interacting with specific proteins (including itself) on other cells, and to both guide and differentiate the cell during neuronal development [17]. L1 can interact homophilically via L1-to-L1

interactions, as well as heterophilically with integrins, and fibroblast growth factor receptors, or FGFRs [17, 18]. These abilities allow L1 to heavily influence neuron migration, extension, axon bundling, and the formation of synapses in the brain.

Cleavage occurs by the enzymatic activity of **a** **d**isintegrin **a**nd **m**etalloproteinase transmembrane protein or ADAM10, in conjunction with a gamma secretase, presenilin [19] [10]. Whether in its soluble form (cleaved) or intact in the cell membrane, the exposed extracellular portion of L1 is able to engage in numerous cell-to-cell interactions.

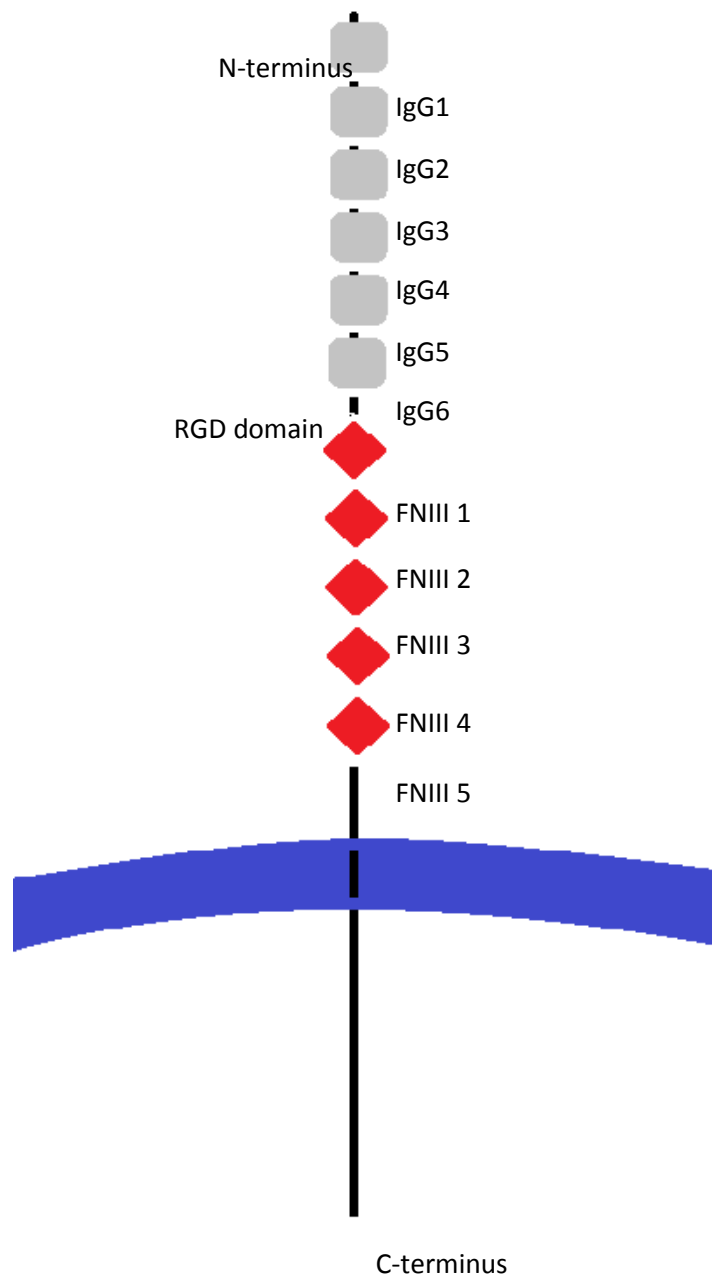


Figure 1 Simple Schematic of L1CAM Structure L1CAM is one-pass transmembrane protein with 6 immunoglobulin domains, and 5 fibronectin-like repeats. There is an RGD domain in the 6th Ig domain.

1.2 Metastasis

Metastasis often distinguishes malignant from benign tumors. However, in cancers confined to the nervous system, such as glioblastoma multiforme, the probability of metastasis is very low due to the blood brain barrier and compartmentalization of this system. Nevertheless, there have been reports of rare extracranial metastases in glioma cases [20] [21] [22] [23]. Almost 90 % of patients die from their cancer due to metastasis [24]. The simple definition metastasis is understood to be the change of a localized tumor mass migrating to another location in the body. See Table 1 [25] for common areas of metastasis. There is multi-step process involved in the metastasis cascade, including “dissemination from the primary tumor, extravasation, formation of micro-metastases and re-initiation of tumor cell proliferation” [26]. One major factor that can influence metastasis is the activity of the matrix metalloproteinases (MMPs) [27] [28].

Table 1 Cancer Types and Common Areas and Organs of Metastasis [25].

Cancer Type	Sites of Metastasis
Breast	Bone, Brain, Liver, Lung
Colorectal	Liver, Lung, Peritoneum
Renal	Adrenal gland, Bone, Brain, Liver, Lung
Lung	Adrenal gland, Bone, Brain, Liver, Opposite Lung
Melanoma	Bone, Brain, Liver, Lung, Skin/Muscle
Ovarian	Liver, Lung, Peritoneum
Pancreatic	Liver, Lung, Peritoneum
Prostate	Adrenal gland, Bone, Liver, Lung
Stomach	Liver, Lung, Peritoneum
Thyroid	Bone, Liver, Lung
Uterus	Bone, Liver, Lung, Peritoneum, Vagina

MMPs are zinc-dependent proteinase enzymes that change the extracellular environment of the cell [28]. These endopeptidases do so by having the ability to degrade the majority of the components that make up the extracellular matrix. Naturally, one could imagine that once the microenvironment is changed from a less confined to a “freer” state, the cell will be able to more readily move, and

subsequently migrate. A subclass of MMPs, the gelatinases (MMP-2 and MMP-9), have been reported to have a heavier impact in tumor progression and metastasis [12] [27] compared to other MMPs. Several studies have been undertaken by different groups to assess the role and regulation of gelatinases and MMPs overall in cancer. It turns out that MMPs are regulated by a wide array of molecules including integrins, cytokines, growth factors, and cell adhesion molecules [26] [29] [30]. In relation to my project, cell adhesion molecules and their role in regulating MMPs are particularly significant because they include L1CAM. Even though I do not focus on how L1CAM regulates MMPs, the over-expression of L1CAM in cancerous cells have been well elucidated and is discussed in the following sections. In addition, a variety of cancer types have been studied in regards to L1 expression and malignancy.

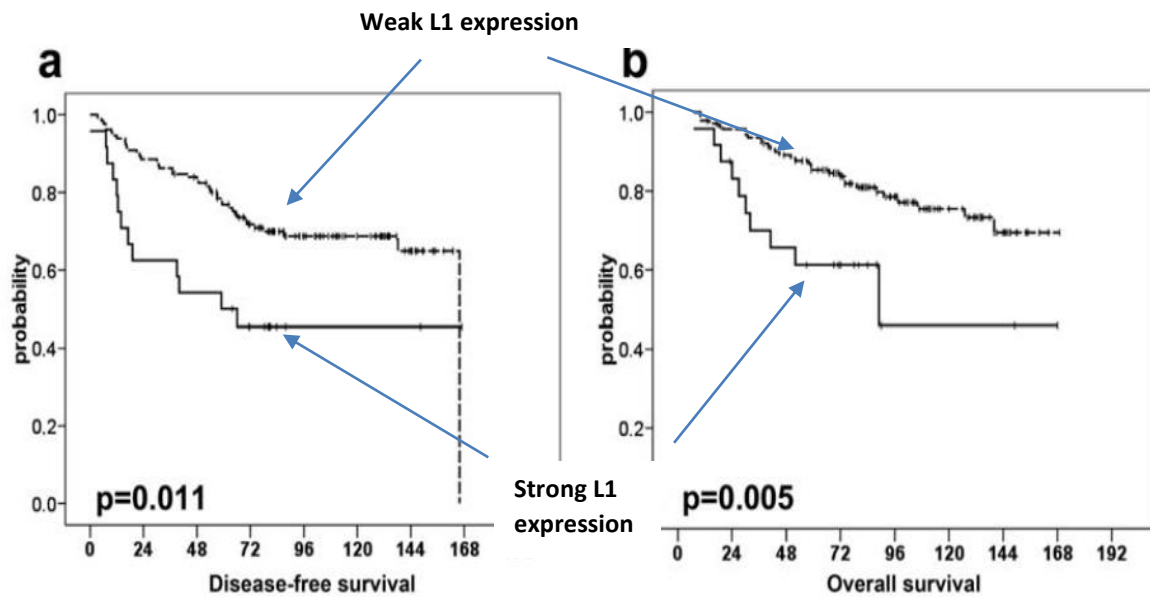


Figure 2 Survival rates and disease recurrence expectancy of breast cancer patients developed by Schroder et al. [31] Patients with weak L1 expression in their tumors had higher disease-free survival and overall survival compared to patients with higher L1 expression.

1.3 L1-Related Research in Breast Cancer

The ability for metastasis to occur is dependent on cancer cells' ability to detach from the extracellular matrix and each other. There are a variety of different junctions that are responsible for keeping cells adhered and closely bound, but obviously something changes that allows cells to become free and motile during metastasis. The Shtutman group explored this and found that L1 is able to disrupt e-cadherin adherens junctions in breast carcinoma cell lines MCF7 [32]. In addition, the

expression of full length L1, and not just portions of the extracellular domain of L1, increase motility in wound healing assays.

In a clinical scenario, generally, breast cancer can be treated in a straightforward manner if the tumor is in a localized form and remains in the breast. However, once metastasis occurs for instance, to the lungs, the percentage of patients who survive and remain cancer-free decrease significantly [33] [31]. In a study performed by Schroder et al. in 2009, breast cancer patients whose tumors had strong L1CAM expression had both lower survival and disease-free expectancies (Figure 2). These survival curves were generated, and found to have no influence from age of patient, stage of cancer, and presence of estrogen and progesterone hormone receptors. Other studies have found that the expression of L1CAM is distinctly preferentially positive in the highly aggressive triple negative breast cancer phenotype [14].

Similar studies done by our lab in the past have found positive correlations between stage of cancer and L1 expression in clinical samples, and high L1 expression in metastatic breast carcinoma cell lines [13]. Regardless of where the correlations lie, however, the fact remains that L1 is undeniably present if not increased during the point at which breast cancer progressively worsens. Even more convincingly, this L1 aberration is observed in other cancer types as well.

1.4 L1-Related Research in Prostate Cancer

Prostate cancer is the most common cancer related death in older male populations and second to lung cancer in the United States [34]. When metastasis occurs, bone is the most common site that cancer cells migrate toward, and 90 % of all patients who have died of prostate cancer have presented evidence of bone metastasis [35] [36]. Skeletal metastasis significantly compounds the morbidity of the patient

with addition of pain, fractures and compression of the spinal cord, bone-metastasis-evoked cranial neuropathy from base-of-skull syndromes, anemia, and infection [35] [37]. Sung et al. explored what would happen to highly aggressive and metastatic prostate cancer PC3 cell lines if L1CAM expression was hindered. It was found that using siRNA to knockdown L1 expression significantly decreases proliferation, motility/migration, and invasion *in vitro*. It was also noted that *in vivo* studies where mice models were injected with control PC3 cells and L1-knocked down PC3 cells, matrix metalloproteinases- MMP-2 and MMP-9, were suppressed with L1 knocked down. Most importantly, not only were tumors decreased in L1 positivity as well as size, but metastasis to bone had also been significantly decreased. These studies show that L1 expression is not just correlated with aggressive cancer subtypes, but is directly related to growth and migration of cancer cells.

1.5 L1-Related Research in Gastric and Colorectal Cancer

Colon cancer is the fourth most common cancer in the world and the third most common in the United States [38] . Evidence of L1 expression is also present in gastric cancer. Results of *in vitro* studies done to evaluate L1CAM in gastric cancer were found to be similar to results seen in both prostate and breast cancer studies. The Chen et al. group performed immunohistochemical staining on tumor biopsies from patients with gastric cancer were found to be positive for L1. They also found that L1 positive tumors correlated with poor prognosis [6]. The survival curves that were generated for these patients (see Figure 3) were very similar to breast cancer survival curves (see Figure 2), even across cancer stages.

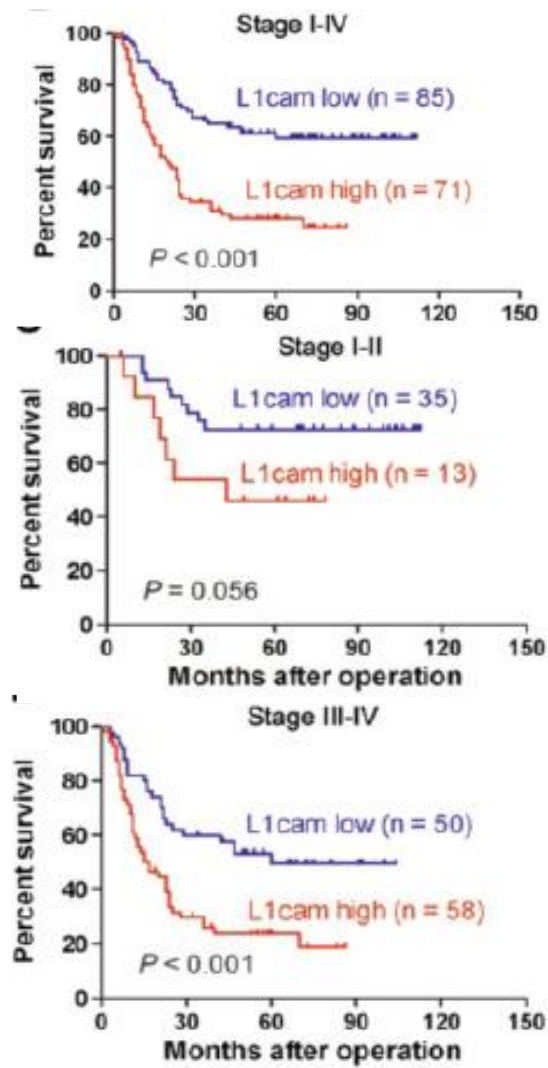


Figure 3 Chen et al. survival curves of gastric cancer patients with low and high levels of L1 in tumors [6]

1.6 L1-Related Research in Ovarian and Uterine Cancer and Detection of L1 in Serum

Ovarian and uterine cancers are the leading cause of death in gynecological malignancies. Endometrial-type uterine cancer is low risk and generally positive outcomes are predicted for patients. However, a subpopulation of women die from this disease. L1 one was assessed in the Fogel et al. (2003) study [11] to determine whether or not there was a correlation between this aggressive type of cancer and L1 expression. Patients with epithelial ovarian and endometrial carcinomas were investigated and their tissue from tumors surgically removed within the past ten years were stained for L1 using immunohistochemistry. At the same time, these patients were monitored over several months post-surgery to evaluate survival. Blood from individuals whose tissues that were determined to be L1 positive was collected. Blood was also collected from patients with malignant breast, colon, skin, and prostate cancers. Immunoprecipitation as well as capture ELISAs were used as methods to detect L1 in patient serum. UJ127 coupled to sepharose beads were used in the immunoprecipitation experiments, and 5G3 was used as a coating antibody while UJ127 was used as the detecting antibody in the ELISAs. Tumor samples were homogenized to create cell lysates and were analyzed via western blot.

Immunohistochemical staining revealed that L1 was not diffuse throughout the tumor, but mainly was expressed at the forefront of the advancing edge of ovarian and uterine tumors. Staining failed to reveal L1 in tumors of the other cancer types. Unlike the results of gastric cancer where L1 was present across cancer stages, L1 was only detected in later stages in the Fogel et al. study of ovarian and uterine cancers for western blot analysis and capture ELISA (See Figure 4) [11].

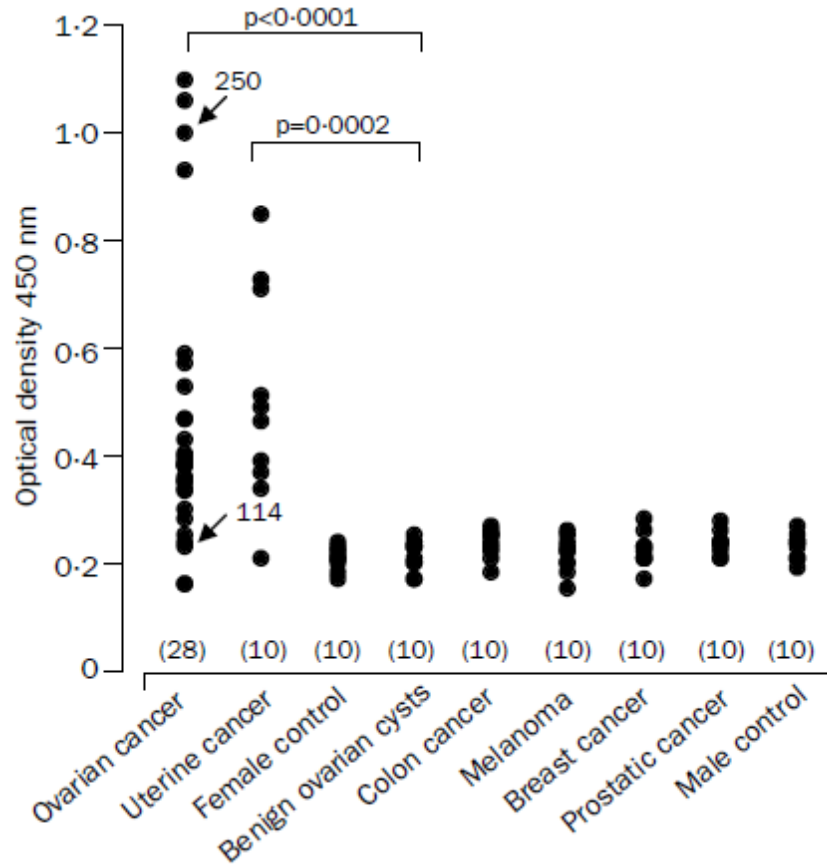
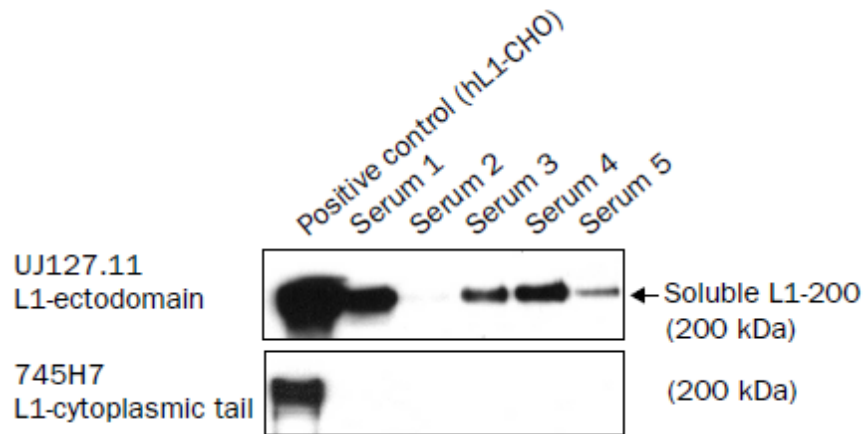


Figure 4 Immunoprecipitation blot and ELISA graphs from Fogel et al. [11]. Using UJ127 primary antibody, soluble L1 was detected in serum from patients with ovarian and uterine cancer. Immunohistochemical staining of ovarian and uterine tumors from patients showed L1 detection but not in the other cancer types.

1.7 L1-Related Research in Glioblastoma Multiforme

Glioblastoma multiforme is the major cancer type that we focus on in our lab. It is the most common of all brain tumors affecting adults, and the most aggressive form, grade IV, is most prevalent in humans [39] [40]. Glioblastoma tumors can arise from astrocytes, which are star-shaped glial cells with long projections, or neural progenitor and stem cells [41]. Astrocytes are glue-like (glial), and make up the supportive tissue of the brain. Surgery remains the most common component of treatment regimens, however, due to the invasive nature of the glioblastoma cells, it is impossible to remove all of them, and surgery is used mainly to relieve pressure. Almost invariably, the tumors recur and the patient dies in about a year [39].

L1 has been documented to have a variety of active roles in this cancer type as well. In 2011, our lab found that L1CAM expression is correlated with FAK activation. In one of many experiments, soluble L1 ectodomain was added to L1-attenuated glioma cells and not only rescued cell motility, but also stimulated cell migration in a human glioma cell line that expressed L1-binding integrins but lacked endogenous L1 expression. This shows that L1 has a key role in motility, and suggests that L1 is able activate FAK through binding with integrins [18].

In addition to interacting with FAK, in 2013 our lab had also shown that L1 can bind FGFRs and stimulate cell motility and cell proliferation in high grade glioma cell lines. Mohanan et al has shown that FGFRs are overexpressed regardless of the grade of glioma. Knocking down L1 and inhibiting any interaction with FGFRs and specific L1-FGFR interaction had significant decreasing effects on cell motility [16].

Other researchers have found that attenuating L1 in glioblastoma stem cells, a highly tumorigenic subpopulation of the glioblastoma cells that drive the tumor, results in other molecular aberrations. The Cheng et al. group found that knocking

down L1 reduced DNA damage checkpoint activation and subsequent repair. This results in increased sensitivity to radiation. L1CAM is thought to regulate NBS1, a vital part of the MRN complex needed to activate the ATM kinase, which is needed to phosphorylate a number of important proteins that initiate activation of the DNA damage checkpoint [42]. Similarly, Held-Feint et al. performed studies that suggest L1 also offers glioma cells chemoresistance. First, they found that inhibition of TGF- β 1 signaling reduced L1 expression, whereas stimulation increased expression. Glioma cells expressing L1 were more resistant to temozolomide than cell lines with attenuated L1 [43].

1.8 L1CAM ELISA Kit

DRG International is a medical diagnostics and equipment manufacturer. It is one company that has developed and marketed an L1CAM ELISA detection system, and I will compare this company's system with the work done in my project. Their colorimetric ELISA is based on the principle of the sandwich, or capture, ELISA. The microtiter plates in the kit are pre-coated with a monoclonal antibody (unspecified) to L1CAM. There is no blocking buffer included in the kit, but this could be due to wells being pre-blocked, then packaged. The kit offers suggestions on how specimens to be tested should be collected and prepared: Blood samples should be collected, allowed to clot, and then centrifuged for serum isolation. The kit strongly recommends the inclusion of a standard curve with each assay procedure as a positive control. The standard curve and samples are incubated for 1 hour. Interestingly, the kit's protocol recommends that the primary detection antibody, or "Enzyme Conjugate", be dispensed into the well immediately after the samples are added, and are incubated together for the hour. A streptavidin-peroxidase solution, or "Enzyme Complex" is

added afterward and left to incubate for 30 minutes. These incubation times are shorter than the more common 60 minute incubation, and may be done to expedite the time in which the assay takes. These changes are strikingly different compared to the protocol of my project described in later sections. One similarity between the kit and my ELISA is the developing substrate used. TMB or tetramethylbenzidine is a chromogenic substrate that yields a blue-green color when mixed with ethyl acetate and reacts with peroxidase. This reaction is stopped with the addition of an acid, which is commonly sulfuric acid. The result is a gold-yellow color that is read at 450 nm with a microtiter plate reader. Figure 5 shows a table provided in the DRG kit, which shows protein standards 0-5 and their concentrations along with corresponding OD readings [44]. Although this is provided as an example, it is recommended to develop one's own concentration to OD ratio.

Standard (ng/mL)		Optical Units (450 nm)
Standard 0	(0)	0.06
Standard 1	(8.75)	0.12
Standard 2	(35)	0.46
Standard 3	(70)	0.83
Standard 4	(140)	1.52
Standard 5	(280)	2.66

Figure 5 Protein concentrations and corresponding OD readings provided in the DRG L1 detection ELISA kit An example of an array of protein standards were read and the OD readings for each standard is listed.

1.9 Types of ELISAs

To ensure proper understanding of the different ELISA protocols used here, I have included this section to introduce the different types of these assays that exist, and why they are generally used. In my project, I have utilized two ELISA types: Indirect ELISA and Capture ELISA.

1.9.1 Direct ELISA

The direct ELISA is used to detect the presence and quantity of a purified antigen in each microtiter well. The sample containing the antigen (usually a purified protein) is added to the wells and incubated to allow attachment to the solid phase (plate). A blocking buffer is then added to reduce nonspecific binding of the antibody to the plate. The antibody is added and left to detect the antigen for the incubation period. The unique aspect about this assay is that it utilizes only one antibody that is labeled with an enzyme such as peroxidase or alkaline phosphatase for detection. This method can be done fairly quickly due to the fact only one antibody is used, but this can be a drawback as well due to cost obtaining enzyme linked primary antibodies, and the effect of labeling on the immunosensitivity of the antibody.

1.9.2 Indirect ELISA

An indirect ELISA is somewhat similar to the direct ELISA, but it differs with the addition of an extra antibody called a secondary antibody. The antigen is adsorbed to the solid phase, and blocked. An unlabeled primary antibody is added, incubated, and then a labeled secondary antibody is added. As the name suggests, the antigen is detected by primary antibodies, but the signal from the secondary antibody is what is read and also *indirectly* detects the antigen. The signal comes from the label which could be alkaline phosphatase or biotin. If biotin is used, an additional incubation with

streptavidin is required. This method takes a longer period of time compared to the direct ELISA, but it is more sensitive, cost effective, and allows for more cross use between biological procedures.

1.9.3 Capture ELISA

Capture ELISAs are also known as sandwich ELISAs. This is because the antigen is essentially “sandwiched” between two antibodies in this assay. The first step is to coat or adsorb an antibody that is specific to the antigen to the well. The sample containing the antigen is then added to the wells and left to incubate so that the adsorbed antibody can “capture” the antigen. This is especially important if the antigen is in low concentration in a complex biological mixture, such as blood serum or a cell lysate. The blocking step is next, and afterward another antibody is added for incubation. This is another primary antibody that is required to detect the antigen, but it has a different isotype than the capturing primary antibody and must bind to a different region of the molecule to be detected. A labeled secondary antibody is then added, and this secondary should be specific for the isotype of the second primary with no cross-reactivity to the capture antibody isotype. Common labels used in chromogenic ELISAs are alkaline phosphatase or biotin. If biotin is used, an additional incubation step with enzyme-linked streptavidin is required.

1.9.4 Competitive ELISA

The competitive ELISA is a very unique type of assay that is unlike any of the others previously described. The principle of the competitive ELISA is the opposite of the other ELISA types, however the same quantitative information can be obtained. The first step in this assay is to coat wells with an antibody and block, such as in a

capture ELISA. Next, the sample and a designated amount of purified labeled antigen is mixed together, then added to the wells for incubation. The developing substrate is then added for detection. The idea behind this method is that the labeled purified antigen is “competing” with the antigen in the sample on the plate. The less competing antigen in the sample, the stronger the signal, and vice versa. This inverse relationship can be applicable in other competitive ELISA setups as well, such as incubating the antigen with antibody first and then adding it to pre-coated wells if labeled antigen is not available or determining the specificity of different antibodies. Two advantages to using this method are the high specificity (though not higher than capture) that is offered, and the flexibility of tailoring the assay based on what is already in stock.

1.9.5 ELISAs in this Project

My project consisted of using both indirect and capture ELISAs to detect known amounts of purified L1fc from PBS and human serum. I used polyornithine to increase the binding capacity compared to high pH buffer and polystyrene. The theory behind our indirect ELISA, is that the antigen (L1fc) bound to the polyornithine coated on the bottom of the well. Blocking with an albumin-milk buffer blocked open sites on the polyornithine. Primary antibody (UJ127) bound the antigen, and a biotin-conjugated secondary antibody bound to the primary. Lastly, peroxidase-conjugated streptavidin, a bacterial protein with high affinity for biotin, was added and then the plates were developed with TMB.

Similarly, the capture ELISA worked with the same concept. However, instead of the antigen, the first primary antibody (5G3) bound to the polyornithine. The open sites were blocked, and antigen was added to bind to the antibody. After this, another primary antibody (UJ127) was added to bind the antigen again, but at a different

domain. Secondary antibody and streptavidin followed, and then wells were developed with TMB. A schematic of this is shown later in Section 3.7.

Chapter 2

MATERIALS AND METHODS

2.1 Cell Lines and Culture

HEK 293T cells were transfected with an L1fc construct prior to the beginning of this project. These cells were used to make L1fc conditioned media using serum free media, Pro293A-CDM, for adherent cells, or Sigma Aldrich serum free media, (product# 14571C) supplemented with penicillin-streptomycin, and 2 mM L-glutamine for 2-3 days. Normal cell culture was done with Sigma Aldrich DMEM (product# D5796) supplemented with penicillin-streptomycin, 2 mM L-glutamine, and 10 % FBS at 37° C and 4% CO₂.

2.1.1 293T-flhL1

293T cells were transfected with full length human L1 (flhL1) provided by TransOMIC Technologies (item# TCH1103) in *E. coli*. The plasmid was grown and purified using 5 Prime Maxi prep kit (Ref# 2300120). 15 ug of plasmid was added to 500 ul of sterile 0.25 M CaCl₂. 500 ul of 2X BBS buffer was slowly added to this mixture, and left to incubate at room temperature for 20 min. After incubation, the mixture calcium phosphate precipitate was added dropwise to a 60 mm plate of 293T

cells 60-70% confluent. The media was changed to 24 hr later, and puromycin selected 2 days later at 2 ug/ml.

2.1.2 Sp2/0

Sp2/0 myeloma cells used for the fusion in the antibody development were obtained from Dr. William Cain. They were maintained and grown with IMDM (Corning Cellgro, Iscove's DMEM, ref# 10-016-CV) with 10 % FBS, 2 mM L-glutamine, and penicillin streptomycin at 37° C and 4 % CO₂.

2.1.3 U-118-L1LE

U-118-L1LE cell lines were prepared prior to the beginning of this project by transfection of U-118 MG astrocytoma-glioblastoma cells (Grade IV) with a truncated L1CAM construct. This construct only allows for the L1 ectodomain to be expressed.

2.1.4 Conditioned Media

Serum-free media (Sigma Aldrich EX-CELL 293 Serum-free medium for HEK 293 cells product# 14571C; Lonza Pro293A-CDM catalog# BE12-764Q) was supplemented with L-glutamine and penicillin-streptomycin and used to collect all conditioned media. Cells were washed with PBS then 10 mL of serum free media was added to dish (10 cm dish) for 2-3 days. The media was then removed from cells and centrifuged at 3760 RPM for 10 minutes to pellet cellular debris. The supernatant was decanted and filtered through a 0.45 um filter. After filtration, the conditioned media was concentrated using a 100,000 molecular weight cutoff unit (Millipore Amicon Ultra ref# UFC910024 lot# R3CA89259).

2.2 Antibodies and Reagents

To detect L1 in both western blot and ELISA experiments, a mouse monoclonal anti-L1 antibody (UJ127.11) (Santa Cruz Biotechnology sc53386 lot# G1310) was used as a primary antibody. In western blot experiments UJ127.11 was used at a concentration of 1:10,000 or 20 ug/mL in 10% milk/TBST. For secondary antibody, peroxidase conjugated goat anti mouse (Jackson ImmunoResearch code# 115-035-003, lot# 109786) was used at 1:20,000. The developing chemiluminescent substrate (Lumigen ECL Ultra, Lumigen TMA-6, cat# TMA-100) was used at a 1:1 ratio for a total volume between 400-600 uL. The incubation time for the substrate to develop the blot was approximately 2 minutes.

Another primary antibody used to bind L1 in capture ELISAs (5G3) was used in the amount of 1 ug per well. This antibody was obtained from two companies: Santa Cruz Biotechnology NCAM-L1 (5G3) 200 ug/mL: cat# sc33686, and BD Biosciences Purified Mouse Anti-human neurite cell adhesion molecule L1 0.1 mg cat# 554273.

The secondary antibodies used in alkaline phosphatase ELISA experiments were goat anti-mouse phosphatase labeled (ReserveAP Phosphatase labeled, cat# 4751-1806, lot# 091177), and used at 1:5,000. The developing substrate used for ELISA experiments with alkaline phosphatase secondary is p-nitrophenyl phosphate or PNPP. To make substrate buffer A of developing substrate, see Table 1. Substrate B (Sigma Aldrich, Phosphatase Substrate tablets p-nitrophenyl phosphate, cat# 104-105, lot# 118H6128) was dissolved in substrate A at 2 mg/mL, vortexed, then immediately used to develop ELISA samples.

For ELISAs developed with tetramethylbenzidine (TMB), goat anti-mouse biotinylated secondary (Jackson ImmunoResearch code# 205-065-208) was found to

be best used between 1:40,000 -1:55,000. 100 uL of secondary antibody solution, diluted in PBS, was added to each well and left to incubate for 1 hr at room temperature. Streptavidin (Jackson ImmunoResearch code# 016-000-084) was found to be best used 1:265,000, but was used at dilutions between 1:250,000-1:300,000 in the project. TMB colorimetric substrate (Thermo Scientific prod# 34021 lot# PC197906) was mixed 1:1, added to wells and allowed to develop for at least 5 minutes. The reaction was stopped with 2 M sulfuric acid after the desired color was reached, which usually took 5-6 minutes.

Table 2 Substrate Buffer A Components for Developing an Alkaline Phosphatase ELISA

Substrate buffer A	
Diethanolamine	48.5mL
NaN ₃	0.1g
MgCl-6H ₂ O	50mg
H ₂ O	to 400mL
HCl/NaOH	to pH 9.8
H ₂ O	To 500mL

2.3 Attempts at Antibody Development

Human L1fc (R&D Systems L1fc chimera, 100 ug, catalog# 777-NC-100) was reconstituted in 500 uL sterilized PBS (20 ug/100 uL). The adjuvant system used (Sigma Aldrich, product # S6322) was reconstituted in 1 mL of sterile saline. One female balb/c mouse was immunized with 25 ug of antigen (L1fc) mixed 1:1 with adjuvant. The remainder was stored at 4° C until the next injection. The mouse was immunized with approximately half of the antigen/adjuvant solution intraperitoneally and half subcutaneously (Figure 6). The mouse was boosted with another 25 ug of L1fc 3 weeks after the initial injection. One week later, retro-orbital test bleeds were performed to obtain a blood sample. The blood was allowed to clot for 30 minutes at 37° C, and centrifuged at 13,000 RPM for 5 minutes. The serum was carefully removed with a pipet and used for titer analysis via ELISA. The serum was stored at 4° C between uses. After 10 days, the mouse received a final intravenous tail injection of 20 ug of antigen (no adjuvant). Three days later the mouse was sacrificed by asphyxiation with CO₂, and the spleen was harvested for fusion with sp2/0 myeloma cells.

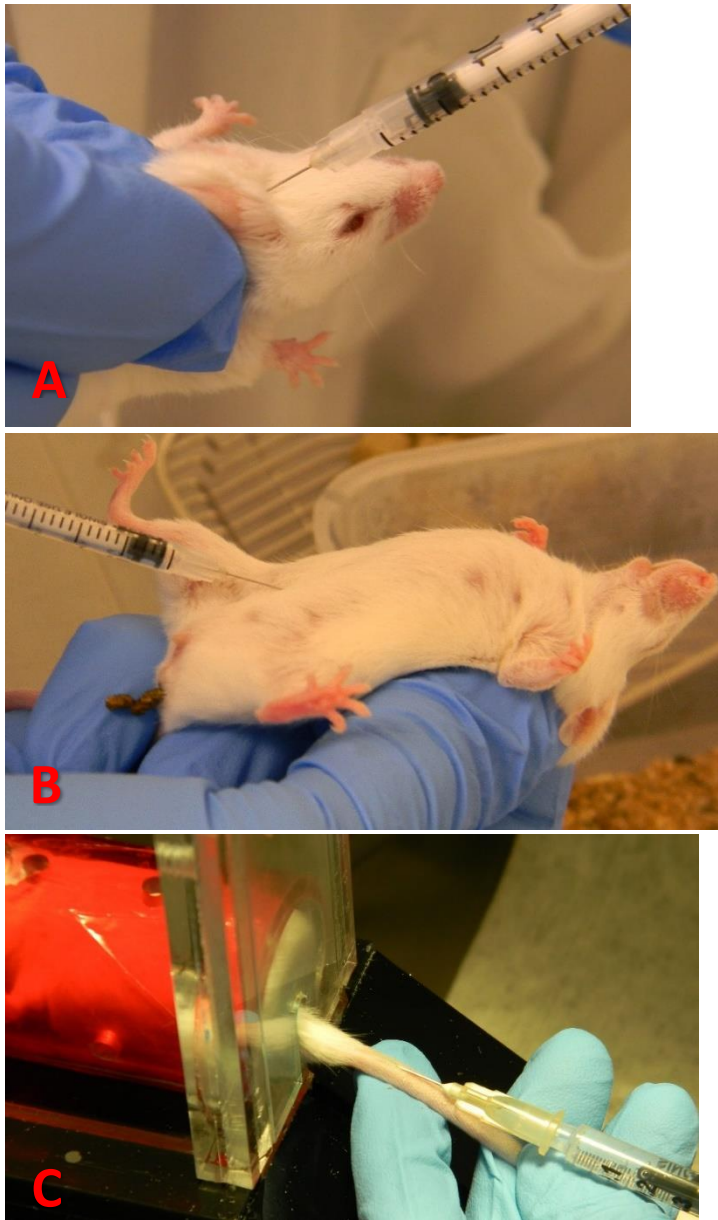


Figure 6 Subcutaneous (A), intraperitoneal (B), and tail vein (C) are injection sites on balb/c mouse for L1fc immunization.

Immediately after sacrificing, the mouse was soaked with 70 % ethanol. The pelt was cut and removed to expose the inner abdomen. With sterile instruments, the spleen was removed and transferred to 60 mm Petri dish with 5mL of plain IMDM. Using a 25 gauge syringe, the spleen was perfused in multiple sites with the same IMDM. The spleen was then transferred to sterile sieve and placed directly over a Petri dish. A sterile tool was used to crush the spleen into the grid of the sieve so that the splenocytes (B cells) are in the dish. The cells were transferred to a 50 mL conical tube. The sieve was washed with another 5 mL of IMDM and transferred to the tube as well. Splenocytes were spun for 5 minutes at 500 x G. After centrifugation, the media was decanted and the tube was firmly tapped to disrupt the pellet. These cells were then re-suspended in 5 mL of cold NH₄Cl and incubated on ice for 10 minutes. During incubation, the myeloma cells were transferred from flasks to a 50 mL conical tube. The cells were washed twice with 45 mL of IMDM and re-suspended in 2 mL of IMDM. Ten more mL of cold complete IMDM (+ FBS + L-glut + pen-strep) was added to the splenocytes, and immediately centrifuged at 500 x G for 10 minutes. The supernatant was decanted and the pellet re-suspended in 10 mL of IMDM. This 10 mL was drawn up into a 10 mL serological pipet and held horizontally for 20 seconds to allow cellular debris to settle. Both myelomas and splenocytes were counted. Approximately 1.4×10^7 splenocytes were mixed with 1.7×10^7 myeloma cells in a 13 x 100 mm round bottom tube. The cells were washed with 5 mL of IMDM and pelleted. The pellet was loosened by tapping the tube on the benchtop/hood floor. One mL of 50 % polyethylene glycol (1:1 PEG:IMDM) was added to the tube slowly over the course of one minute. The tube was centrifuged immediately afterwards for 3 minutes at 500 x G. One mL of IMDM was added to the tube slowly over a one

minute period, and incubated at room temperature for 1 minute. This was repeated 2 more times. After the last incubation, 5 mL of IMDM was added to the tube slowly in a minute's time, incubated for an additional 2 minutes and transferred to a 50 mL conical tube. The round bottom tube was rinsed with 10 mL of IMDM and then slowly, for 1 minute, added to the 50 mL conical. After another minute, 20 mL of complete medium was added and the tube was centrifuged for 5 mins. The pellet was then washed with 20 mL of complete medium. Cells were re-suspended in HAT (Sigma Aldrich, HT media supplement 50X, cat# H-0137 lot# 112K8927) supplemented medium, poured into a reservoir and transferred to 96-well plates, and then finally put into incubator at 37° C and 5% CO₂.

2.4 Making and Performing Indirect ELISA with Nitrocellulose Coating

To enhance the binding capacity of ELISA plates, 0.22 um nitrocellulose was dissolved in a selection of solvents to coat the wells of 96 well polystyrene dishes. Solvents tested to dissolve the nitrocellulose included DMSO, ethanol, methanol, and acetone. According to Stobbs and Schagen, 1986 [45], 20% DMSO in ethanol was adequate to dissolve nitrocellulose to recondition and re-use ELISA plates. 0.75 %, 1 %, and 2 % nitrocellulose was made in 1:1 methanol to acetone. 0.5 %, 0.75%, and 1 % nitrocellulose was made in 20 % DMSO in ethanol. Enough of the nitrocellulose solution to cover the surface of the bottom of the well (100 uL) was deposited into the wells of a 96-well plate and left to dry until all the solvent has evaporated or overnight at room temperature (≈4hrs). When depositing nitrocellulose, it was important that the pipet tip was placed in the bottom corner of the well, then the pipet plunger was depressed to release the solution. Wide orifice tips are best used for this procedure. Otherwise, the end result would be nitrocellulose lost to the sides of the walls of the

well. Clean tips, plates, and area for drying should be used for coating, otherwise, the risk of adding debris to the coat is a strong possibility. It is important to note that trying to speed up the drying nitrocellulose coat by adding a fan, affects the drying of the coat. It is best to coat the wells and allow the plates to dry slowly overnight in a hood or benchtop.

After drying, 100 μ L of concentrated L1fc or L1LE-conditioned media was deposited into wells and incubated at 4 $^{\circ}$ C overnight. Due to concern of phosphotyrosine cross reaction from BSA fraction V blocking buffer or IgG cross reaction from normal human serum, the wells were blocked with 0.5 % or 40 ng/mL albumin, 5 % non-fat dry milk in PBS [46]. For informative purposes, the albumin came from bovine standard ampules (PierceTM Bovine Serum Albumin cat #23209). Essentially, one ampule per 50 ml of 5% milk-PBS was used as blocking buffer. We also explored using 5 % normal goat serum as blocking buffer because of its complexity, but I would not recommend using serum as a blocking agent due to nonspecific signal from secondary antibody. Blocking was done at room temperature for 1 hr on a rocker. Wells were rinsed with PBS and primary antibody was added. UJ127 was diluted at 1:200 (stock: 200 μ g/ml) in PBS and 100 μ L was added to wells and left to incubate at room temperature for 1 hr. Wells were washed 2-3 times with PBS-Tween 20 (0.05%). Alkaline phosphatase secondary was made up at 1:500 in PBS and added to wells after washing, and left to incubate for 1 hr at room temperature. After incubation, wells were washed and immediately developed with p-nitrophenyl phosphate (see section 2.2 Antibodies and Reagents) at 490 nm.

2.5 Making and Performing Indirect ELISA with Polyornithine Coating

Polyornithine was another material that was considered for increasing the binding capacity of the ELISA plates. Due to its hydrophobicity, virgin polystyrene dishes were not suitable for coating with polyornithine (Figure 7). Instead, I opted to use 96 well tissue culture dishes (Costar 3595 tissue culture treated non pyrogenic dish). 100 microliters of 200 ug/mL poly-L-ornithine (molecular weight 30,000-70,000 g/mol) was deposited into wells of a 96 well plate and left to dry overnight at 37° C in a small bacterial plate incubator. Multiple plates in the incubator slowed down the evaporation time, therefore, the drying process could be done more efficiently by adding a dish of dessicant (anhydrous calcium sulfate or silica gel) in the incubator along with plates to be dried. Nevertheless, on average it required at least 10 hrs to completely dehydrate 100 uL from the wells in the tabletop incubator that was used. Polyornithine was tested in diluents, water and PBS. The result of polyornithine diluted in PBS was a thin dry layer of salt precipitated polyornithine on the bottom of the surface each well that could be distinguished from polyornithine diluted in water (Figure 8). Antigens such as lysates, L1fc, or conditioned media were diluted in PBS for serial dilutions in a plain polystyrene plate and then transferred to each well and left to incubate at 37° C overnight. Wells were blocked with 0.5 % albumin, 5 % non-fat dry milk in PBS for 1 hr at room temperature. UJ127 was diluted at 1:1,000 (stock: 200 ug/ml) in PBS and 100 uL was added to wells and left to incubate at room temperature for 1 hr. Wells were washed 3 times with PBS-tween. Goat anti-mouse biotinylated secondary (Jackson ImmunoResearch code# 205-065-208) was found to be best used between 1:40,000 -1:55,000. 100 uL of secondary antibody solution, diluted in PBS, was added to each well and left to incubate for 1 hr at room temperature. Streptavidin (Jackson ImmunoResearch code# 016-000-084) was found to be best used 1:265,000, but was

used at dilutions between 1:250,000-1:300,000 in the project. TMB colorimetric substrate (Thermo Scientific prod# 34021 lot# PC197906) was mixed 1:1, added to wells and allowed to develop for at least 5 minutes. The reaction was stopped with 2 M sulfuric acid after the desired color was reached, which usually took 5-6 minutes.

2.6 Performing Capture ELISA with Polyornithine Coating

Polyornithine-coated plates were made as described in the previous section. 5G3 (Santa Cruz Biotechnology NCAM-L1 (5G3) 200 ug/mL: cat# sc33686, and BD Biosciences Purified Mouse Anti-human neurite cell adhesion molecule L1 0.1 mg cat# 554273), a commercially available primary antibody was selected as the capturing antibody. It detects L1 at a different binding domain (N-terminal end) than UJ127 (C-terminal end of ectodomain). 1 ug of 5G3 per well (1 ug/100 uL) was diluted in PBS and allowed to dehydrate onto the polyornithine overnight. Though not realized in the beginning, one washing step prior to blocking significantly increased the sensitivity of the assay, as will be shown later in the RESULTS. Blocking was done at room temperature using 100 uL of 5% milk-5% albumin in PBS for 2 hrs. Wells were then washed 3 times with PBS-Tween 20. The sample containing the antigen was then added to each well and incubated at room temperature for at least 2 hrs. When preparing the serial dilutions for the antigen, I made a stock of 1 ug/mL of L1fc diluted in human serum or PBS. Serial dilutions were made in a plain polystyrene plate with PBS as the diluent at all times. So, in the samples of serial dilutions of L1fc diluted in human serum, the serum got diluted as well. After serial dilutions were made, they were transferred to p-orn-coated plate. After incubation, the wells were washed 3 times to remove unbound antigen. The detecting primary antibody, UJ127, was diluted at 1:1000 (0.2 ug/ml) in PBS, dispensed into wells (100 uL), and set for a

1 hr incubation at room temperature. The wells were washed 3 times with PBS-Tween 20 after this step. Biotin-conjugated secondary antibody was made up at 1:50,000 (stock: 1.4 mg/mL), dispensed into wells (100 uL), incubated at room temperature for 1 hr. The wells were washed 3 times with PBS-tween. Streptavidin-peroxidase was diluted in PBS at 1:265,000 (stock: 1 mg/mL), dispensed into wells (100 uL), and then incubated at room temperature for 1 hr. The wells were then washed 3 times with PBS-Tween. Developing substrate TMB as mixed 1:1 and added to each well and allowed to develop for at least 5 minutes. The reaction was stopped with 2M sulfuric acid and read at 450 nm on a Bio-Rad model 450 plate reader. To determine the appropriate dilution ratios for secondary antibody and streptavidin, a criss-cross ELISA was performed with no antigen, milk-albumin blocking, and serial 3-fold dilutions of secondary antibody and streptavidin incubated for 1 hr with intermittent washes of PBST. Prior to this analysis, strong background signal was a problem. Figure 9 shows the results of the assay. The dilution ratio 1:243,000 for streptavidin was still a little too high. I performed assays at 1:300,000, and this was too dilute and the main consequence of this was the extended time it took for the signal to show during development with TMB. The average time for signal to show using the established dilution ratio is 5 mins, but the more dilute dilution ratios took 20-25 mins. I also tried 1:250,000, and this was still too concentrated as high background still resulted. With trial and error, I established that secondary antibody could be used between 1:50,000 and 1:60,000 and streptavidin at 1:265,000 with no noticeable nonspecific signal.

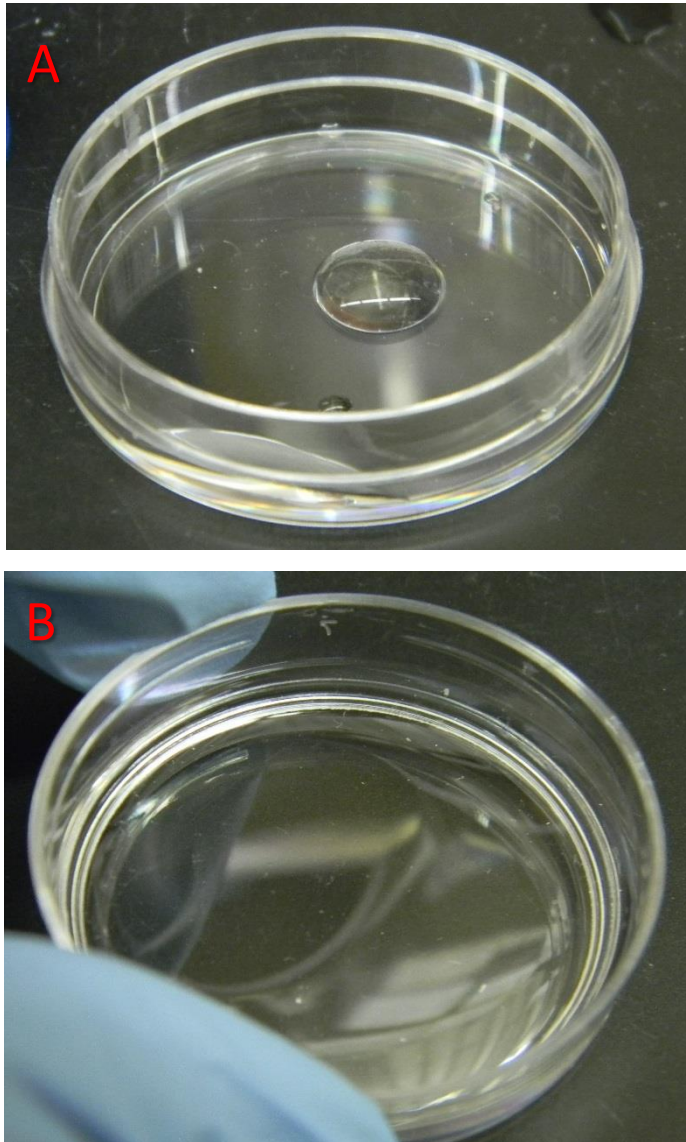


Figure 7 Polyornithine on virgin polystyrene vs. polyornithine on tissue culture dish. The hydrophobicity of polystyrene repels the polyornithine coat (A). The hydrophilic surface of the tissue culture plate displays a better interaction (B).

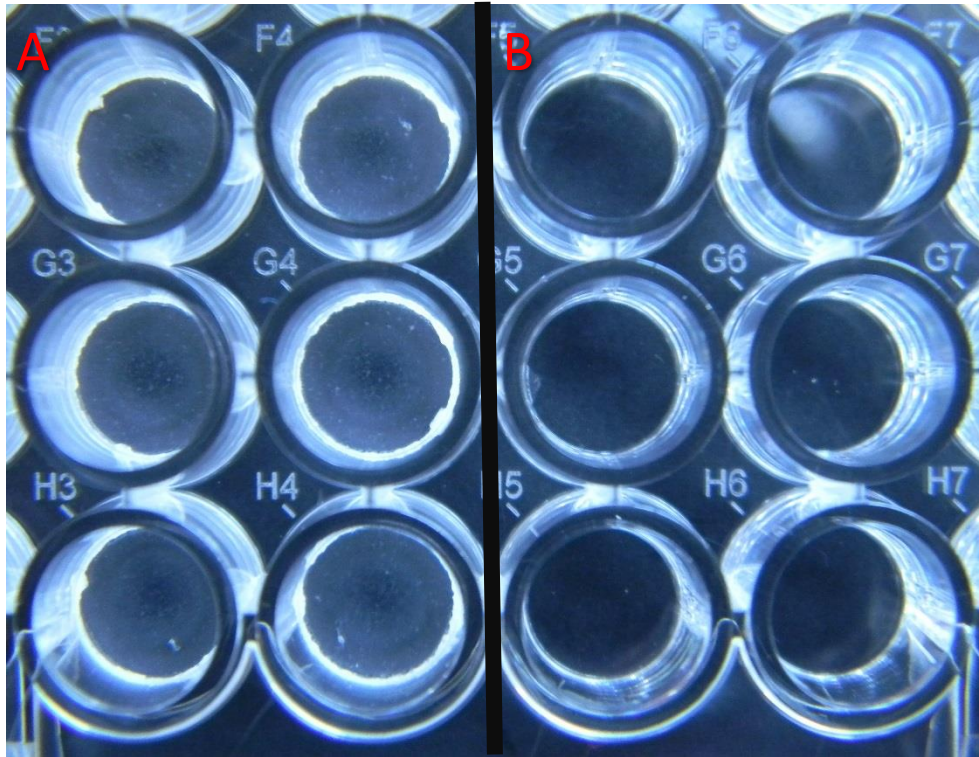


Figure 8 Polyornithine diluted in PBS vs. polyornithine diluted in water. Polyornithine dilute in PBS yields a salt precipitation at the bottom of the well (A). PBS only did not yield this effect. Polyornithine diluted in water, results in a clear unnoticeable coating.

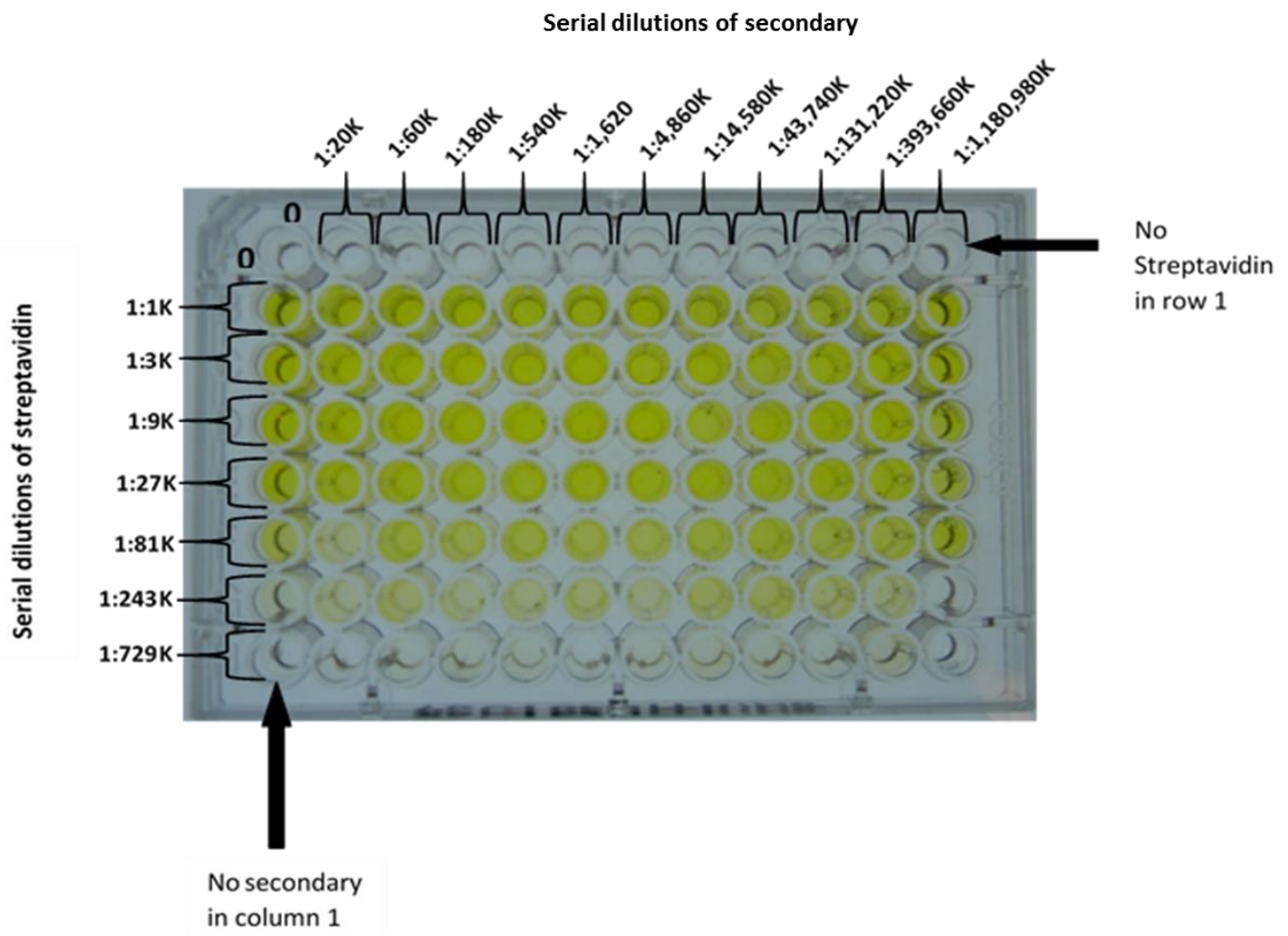


Figure 9 Criss-Cross ELISA to determine appropriate dilution ratios This ELISA was performed with no antigen, milk-albumin blocking, and 3-fold serial dilutions of secondary antibody across and streptavidin down the plate.

2.7 Attempts of L1fc and flhL1 Purification

Because I needed to use the same antigen that I immunized the mouse with in my ELISA experiments, I attempted to purify L1fc from 293T-L1fc cell lines and full length L1CAM from 293T-flhL1 cell lines using 2 methods. The first method I tried was using Millipore's Amicon (cat# ACR5000PA lot# Q2092465) Pro Purification kit to purify L1fc from 293T-L1fc 50-fold concentrated conditioned media. I performed this method according to the protocol included in the kit. The next protein purification method I used was an immunoprecipitation procedure to purify flhL1. Cell lysates were made from a ≈ 90 % confluent 10 cm plate of 293T-flhL1 cells using the lysis buffer described below in Table 3.

Table 3 Lysis Buffer Components for flhL1 Purification

<u>Lysis Buffer</u>
150 mM NaCl
50 mM Tris-HCl pH 7.4
1 % Nonidet P-40
0.25 % Sodium deoxycholate
1 mM EDTA
15 mM KCl
Protease inhibitor cocktail (use fresh)

Plates were washed with PBS and 1 mL of cold lysis buffer + protease inhibitor (Roche complete mini EDTA-free cat# 04693159001) was added to each plate and placed on ice for 10 mins. After incubation, a cell scraper was used to help lift remaining cells still attached to the plate so that they were suspended in the buffer. Cells were transferred to a tube for a 20-30 sec sonication. Cell lysates were then centrifuged at high speed (14,000 RPM) to pellet cellular debris. The supernatant was decanted into a fresh tube and stored at -20° C or at 4° C if the lysate was to be used within 24 hrs.

For immunoprecipitation, cell lysates were incubated with 2-5 ug of UJ127 for 2 hrs at 4° C on a rotator/rocker. After incubation, beads were added to the lysate and left to incubate overnight at 4° C. Both protein A sepharose (Sigma Aldrich cat# P-3391 lot# 19F-0447) and protein G agarose beads (Roche Diagnostics ref# 11719416001) were tested in their abilities to capture antibody in this experiment. Beads were pelleted by centrifugation at 2500 RPM for 10 seconds. The supernatant was discarded, but a portion was saved for western blot analysis. The beads were washed 3 times with PBS. After beads were pelleted and supernatant was discarded for the last time, 40 uL of 2X sample buffer was added and beads were boiled for 2 mins.

Chapter 3

RESULTS

3.1 Antibody Development

After fusion of splenocytes to sp2/O cells and HAT selection, we failed to see hybridoma colonies. We performed an immunization for a second time with a slight increase in the amount of antigen for the final tail vein injection, and were still unsuccessful in creating hybridomas. Retro-orbital test bleeds were performed on our mouse during the immunization process to ensure that an immune response was being generated. Not only was it important to see that there was a presence of an immune response, but also what the immune response was against. Because the mouse was immunized with L1fc, antibodies can be raised against the L1 portion of the molecule as well as the fc portion of the molecule. I coated polystyrene plates with L1fc and human IgG at 1 ug/mL diluted in bicarbonate coating buffer pH 9.5. The plate was left to incubate at 4° C overnight and blocked with either 2 % BSA-PBS or 10 % dry milk-PBS for 1.5-2 hrs. Mouse serum obtained from retro orbital bleeds of both immunized and un-immunized mice were used as primary antibody and serially diluted across the plate, and incubated for 1 hr on rocker at room temperature. Alkaline phosphatase secondary antibody was used at 1:1000 (0.2 ug/ml) across the entire plate. Figure 10

shows the resulting developed plate, where immunized mouse serum elicits a reaction against both L1-fc and purified IgG, but control mouse serum does not. Thus, the mouse did at least raise antibodies against the fc portion of L1-fc.

In addition to the polyclonal antibodies working in an ELISA format, the serum also worked in a western blot procedure (Figure 10). L1fc conditioned media and concentrated L1fc conditioned media was run on a 4-20 % SDS PAGE gel and transferred to PVDF membrane (30 V overnight, cold). The blot was then stained with Ponceau stain and cut into strips. Each strip was blocked in 10 % milk-TBST and probed for 1 hr with anti-serum, control serum, or UJ127 at 1:10,000 (0.02 ug/ml). After primary antibody incubation, the strips were washed for 10 mins with TBST. Next, HRP-labeled secondary was added to the strips at 1:20,000 and incubated for 1 hr, followed by a 10 min wash with TBST and then chemiluminescent detection. The strips were developed and L1fc bands were observed (Figure 11). Thus, the antiserum also worked in western blots to identify L1-fc. However, neither assay nor western blot was done using a source of L1 without the attached fc, so the extent of antibodies in the antiserum against L1 vs. fc was not determined.

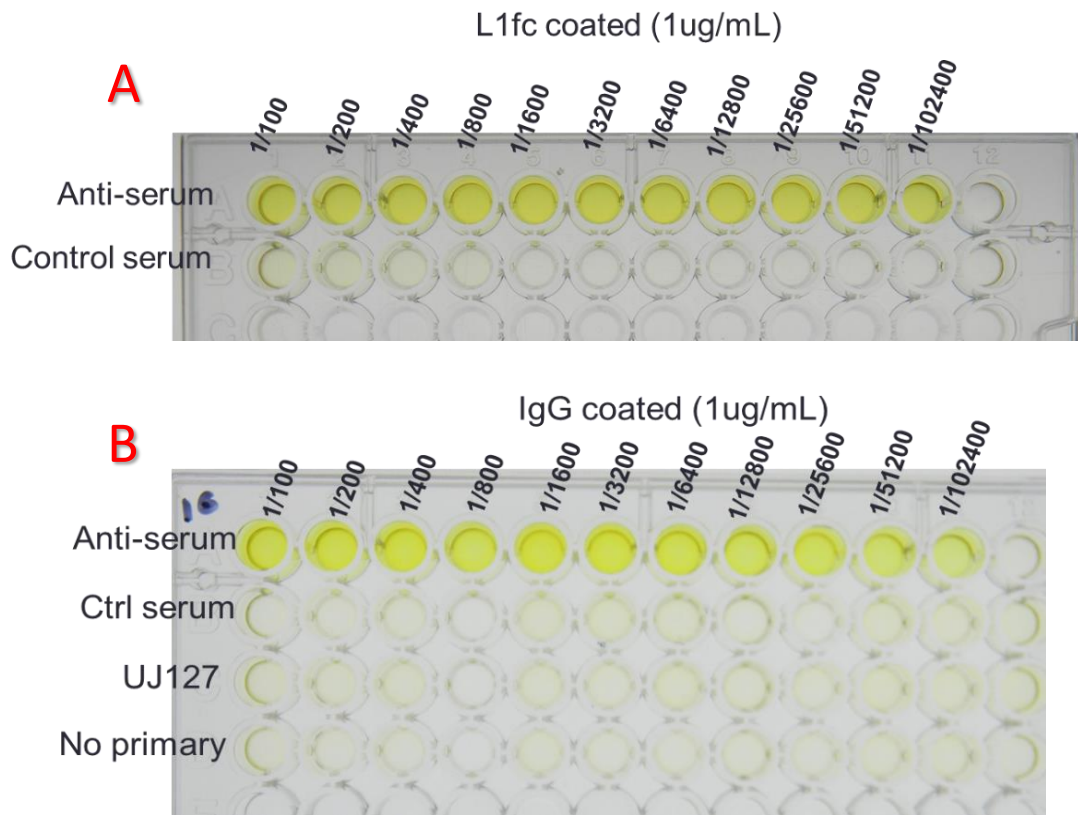


Figure 10 Dr. Cain's protocol for alkaline phosphatase ELISA with mouse antisera 10A shows the results when 2-fold dilutions of 1 ug/mL of L1fc was coated onto 96-well polystyrene plates using a high pH buffer. Primary antibody (mouse antisera) and alkaline phosphatase secondary were used at 1:1000. Control serum was used as a negative control. 10B shows the results of 2-fold dilutions of 1 ug/mL IgG. Control serum, antiserum, UJ127, and no primary controls were implemented in this assay. Both plates were developed with alkaline specific substrate in SECTION 2.2

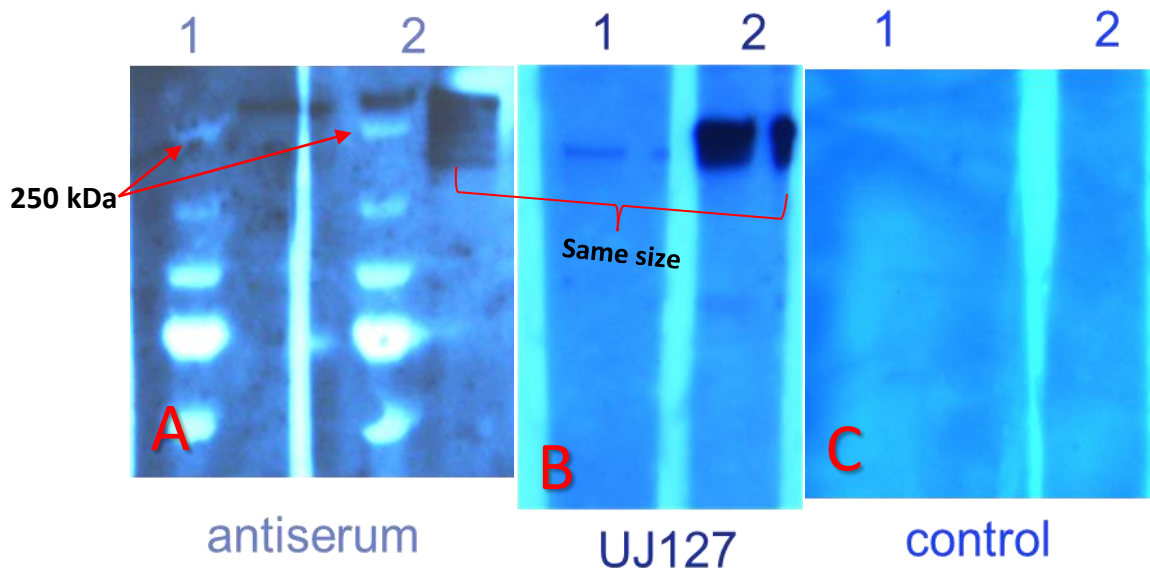


Figure 11 Mouse antiserum works as a primary antibody in western blots Regular (1) and concentrated (2) 293T-L1fc conditioned media was ran through 4-20 % SDS PAGE and transferred to PVDF membrane. The membrane was cut into strips and then probed with L1fc mouse antiserum (11A), UJ127 (11B), and control serum (11C).

3.2 Nitrocellulose-Coated Plates

A 1:1 mixture of methanol to acetone was shown to be the best solvent to dissolve nitrocellulose. Figure 12 shows nitrocellulose dissolved in 100 % acetone and 100 % methanol. It is clear that acetone dissolves nitrocellulose more uniformly, and methanol yields a colloidal-like cloudy mixture.

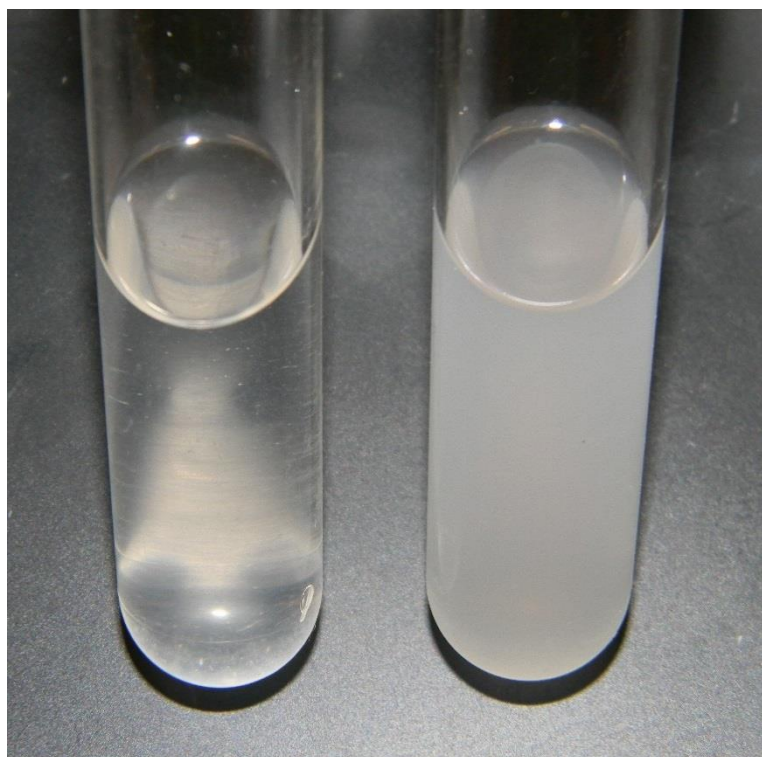


Figure 12 Nitrocellulose dissolved in 100 % acetone (left) and 100 % methanol (right)

It also took a significantly longer time for methanol to dissolve nitrocellulose compared to acetone (Table 4). Dissolving nitrocellulose in flasks with screw caps wrapped with parafilm were ideal for making large volumes of liquid nitrocellulose. These flasks can be placed in a rotating incubator at 37° C overnight. This enables proper dissolving with agitation and heat, but very minimal loss of solvent evaporation and residual nitrocellulose fragments. Table 4 and Figure 13 describes observations of the resulting layers of dissolved nitrocellulose deposited into wells of a 24 well plate.

Table 4 Observations of Dissolved Nitrocellulose in meOH and etOH Post-drying

	100 % Methanol	100% Acetone	1:1 Methanol to Acetone	3:1 Methanol to Acetone
Time to dissolve	35-40mins w/ frequent agitation	Within seconds	Within 2-3 mins w/ stirring	Within 5-7 mins w/ stirring
Appearance in tube after dissolving	Very cloudy; colloidal texture	Clear; Completely homogenous	Clear; homogenous	Translucent; homogenous
Appearance after drying in dish	Solid white layer formed at bottom	n/a	Solid white layer	Cloudy and glue-like, yet dry
Layer formation 1mL added to 15.62 mm well	Major crumpling and peeling of layer	n/a	Large net-like holes remained hovering over the surface of the bottom of well	Continuous and even coating in middle of well, but puffiness and lifting at perimeter of well
Layer formation 750uL added to	Net-like holes (smaller than 3:1 @	n/a	Large net-like holes (bigger than 100% meoH at 75ul)	Layer has formed but has completely lifted from bottom of well

15.62 mm well	1mL) remained hovering over the surface of the bottom of well			remained hovering over the surface of the bottom of well	
Layer formation 500uL added to a 15.62 mm well	Even solid surface in appearance but puffy at perimeter		n/a	Even solid surface in appearance but puffy at perimeter	Failed to reach perimeter of well; nitro. was lost at sides of well
Layer formation 350uL added to a 15.62 mm well	Even solid surface in appearance, but layer thinned near the center		n/a	Even solid surface in appearance, but layer thinned near the center	Failed to reach perimeter of well; nitro. was lost at sides of well
Layer formation 250uL added	Papery and thin, even more so in the center of well		n/a	Papery and thin, even more so in the center of well	n/a
Second coating in dish	n/a	n/a	Did not help, only thickened where nitrocellulose had already dried	n/a	

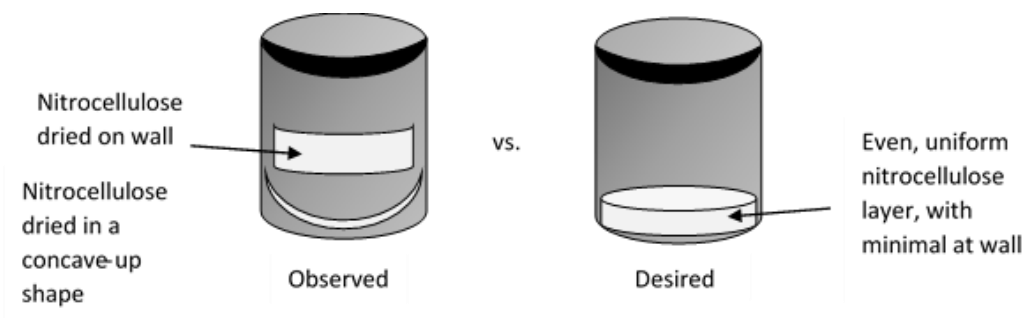


Figure 13 Schematic of the visual of dried nitrocellulose layer Observed dried layers of nitrocellulose in a well of a 96-well plate. Desired layers should be a uniformed, flat layer settled at the bottom of the well. Instead, layers that were observed were not layers at all, and nitrocellulose had dried on the wall and in a bowl shape at the bottom of the well.

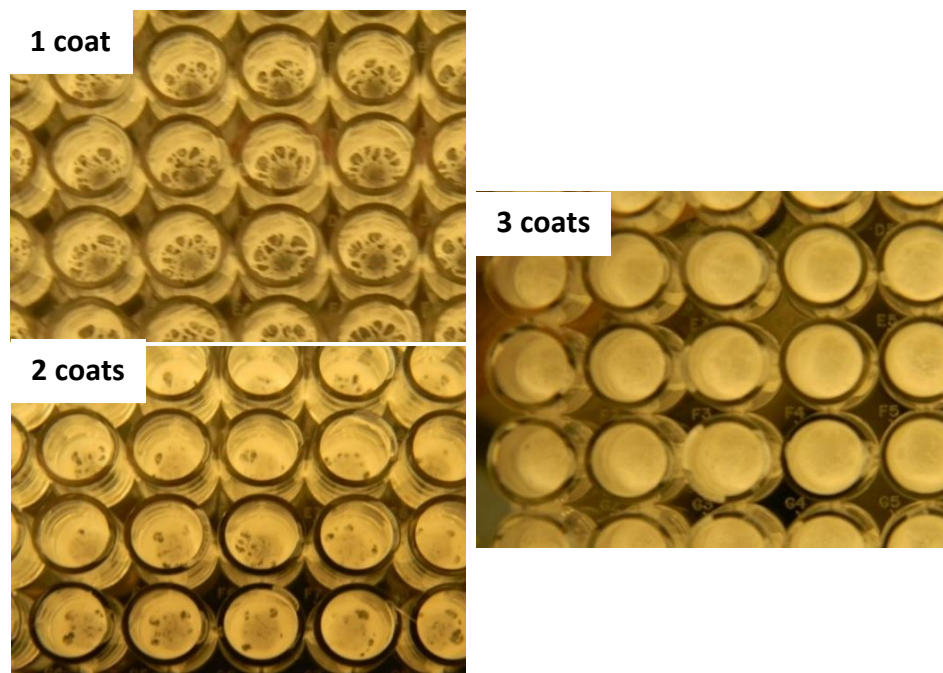


Figure 14 Three coatings of 1 % nitrocellulose (1:1 MeOH to acetone)

The different percentages of nitrocellulose resulted in different coatings in polystyrene plates. 0.5 %, 0.75 %, 1 % did not dry evenly with one coating, but this was corrected with additional coatings (Figure 14). The downside to coating with multiple layers is a large amount of time needed to dry each layer (≈ 2.5 hrs to dry a 100 uL coat).

Increasing the concentration of nitrocellulose to 2 % makes one coating sufficient.

Figure 15 shows one layer coatings of 2 %, 1 %, and 0.75 % nitrocellulose in a polystyrene plate.



Figure 15 One coating of 0.75 %, 1 % and 2 % nitrocellulose 2 % nitrocellulose yields a solid uniformed coating compared to 1 % and 0.75 % coatings

Nitrocellulose dissolved in 20 % DMSO in ethanol was not used in any ELISA experiments. The coating that resulted in Figure 16 seemed as if it would be ineffective, and it took an additional hr for the nitrocellulose to dissolve in this solvent compared to 1:1 methanol.

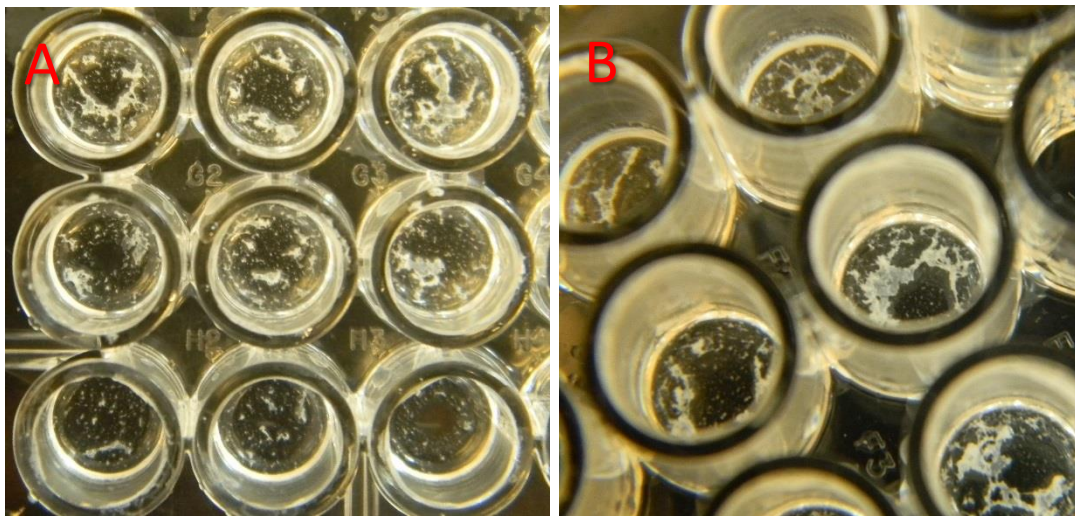


Figure 16 One coating of nitrocellulose dissolved in 20 % DMSO in etOH from Stobbs et. al [45]

3.3 Purification Attempts of L1fc and flhL1

I attempted to purify L1fc from 293T-L1fc cells using the Amicon Pro Affinity concentration kit with a protein A beads (ref # ACS510012, lot # R3BA54490). I

performed purification according to the protocol using 1 mL of L1fc conditioned media with the buffer exchanged to PBS, and 400 uL of protein A slurry. Using a nanodrop reader to test the amount of purified protein we obtained resulted in a zero reading. Numerous solutions were used to “blank” the instrument such as PBS, serum free media used to make the conditioned media, the elution buffer used to elute the purified protein, as well as the wash buffer. All of these resulted in readings of zero of the purified protein. However, a western blot performed over a year later showed a 200 kDa band for L1fc (Figure 17). It is possible that using a nanodrop reader to detect the amount of protein we purified is not a sensitive enough method. At the same time, the amount of protein we have purified could be so low that the instrument could not detect any L1fc.

I also attempted to purify full length L1 from 293T-flhL1 using the procedure described in the METHODS section. Using western blot, I probed the end product with UJ127 for a full length L1 band, however, I saw no band or a very faint band. It seems like the majority of L1 was lost during the washing steps (data not shown).

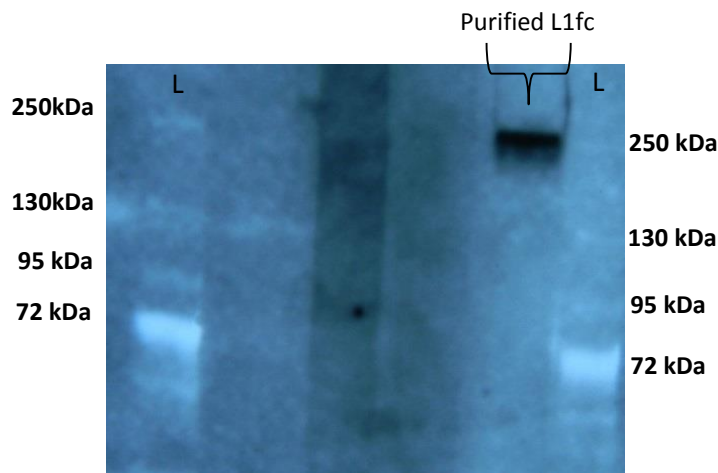


Figure 17 Western Blot of Purified L1fc from 293TL1fc conditioned media L1fc was purified from 293TL1fc conditioned media, and stored at 4 C for approximately 1 year. This western blot was performed as a last effort to see if L1 was present because there were no readings using a nanodrop instrument. A band close to the 250kDa band size of the ladder was observed, presumably L1, showing that the nanodrop was not sensitive enough to detect purified L1fc.

3.4 Nitrocellulose- vs. Polyornithine- vs. Buffer-coated ELISAs

Using nitrocellulose to increase the binding capacity of ELISA plates did not result in reproducible results. In a simple assay of 2-fold dilutions of purified L1-fc antigen across the plate, where primary and secondary antibodies were kept constant across the plate, there was no color gradient as expected. The signal appeared random across wells as if the binding capacity varied per well (data not shown). In other experiments where parallel ELISAs were performed to compare the three techniques (nitrocellulose-coated, polyornithine-coated, and buffer-coated plates), nitrocellulose again was shown to be unsuitable material for use in the ELISA. Figure 18 shows comparative ELISAs using a 1:1000 dilution of primary and 1:1000 dilution of

alkaline phosphatase-conjugated secondary antibody. This revealed extensive nonspecific binding of secondary antibody in the nitrocellulose-coated plate resulting in false positives. Buffer-coated plates showed no signal, even after 20 min of time to develop. The polyornithine-coated plate however, did develop a gradient of color (Figure 18A). Thus, nitrocellulose led to non-specific binding, the coating buffer resulted in no detection, and polyornithine gave an expected gradient of staining. These experiments led me to move forward with polyornithine-coated plates for the ELISA, and not with the nitrocellulose coated or buffer-coated plates.

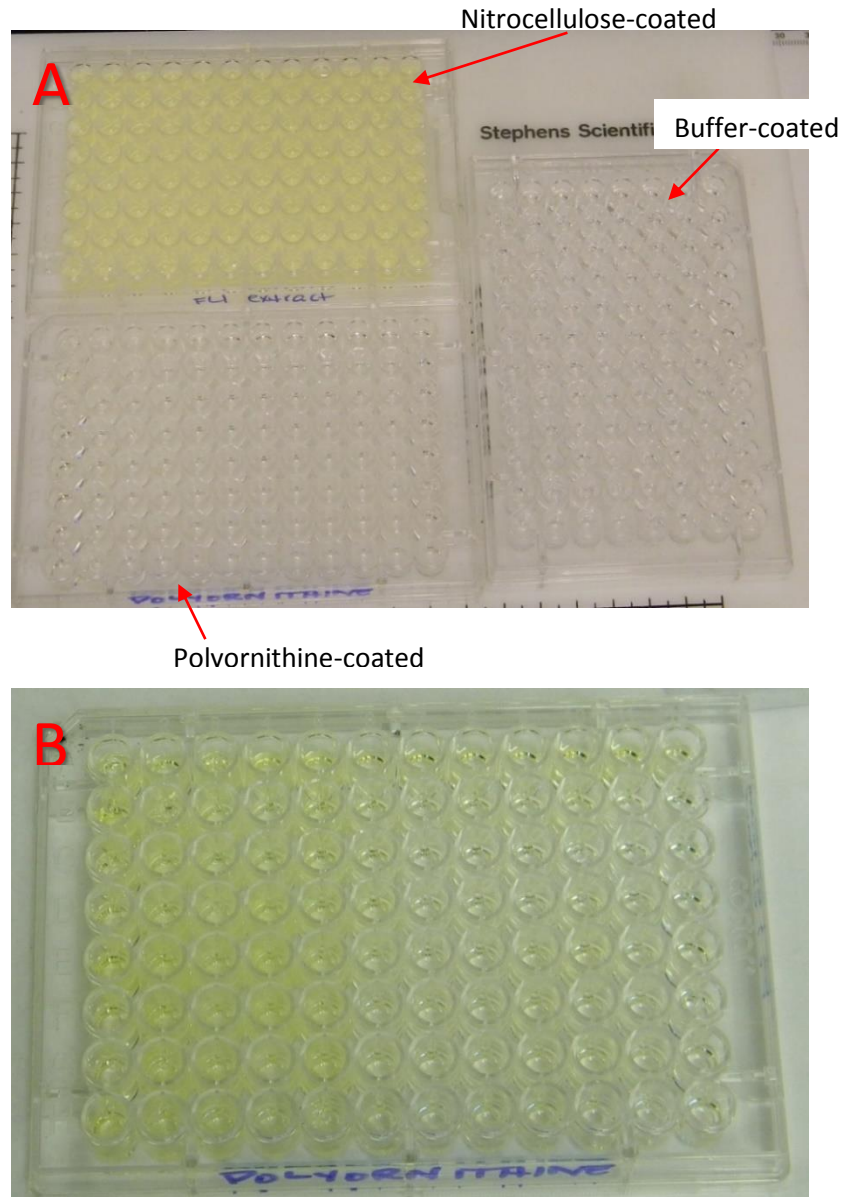


Figure 18 ELISA developed with alkaline phosphatase 2-fold dilutions of 293TflhL1 lysates were coated on p-orn plates, high pH buffer coated plates, and nitrocellulose coated plates. P-orn-plates worked better in producing a signal gradient as expected, with little nonspecific signal. Immediately after adding developing substrate nitrocellulose exhibited extensive nonspecific signal (A). After 5 mins the p-orn ELISA showed the expected gradient (B), but the buffer-coated plate showed no signal (not shown).

3.5 Cold vs Warm Antigen Incubations for Indirect Non-Capture ELISAs

Protocols for ELISAs commonly recommend overnight incubations of antigen in high pH buffer at 4° C. Because the focus of this project is to develop an assay that can detect very low quantities of antigen, there was concern that the cold incubation method would result in reduced antigen binding and would not be sensitive enough. To circumvent this, my ELISAs were fashioned similar to dot blot procedures where the sample is left to dry on the membrane [47]. Allowing the sample to dry on the membrane, and in the case of ELISAs in the well, the antigen is being concentrated and loss of antigen is minimal due to evaporation of water. In the previous section, I already have shown that polyornithine works much better than coating buffer to attach proteins. However, to determine if warm incubation is more effective than cold incubation, indirect ELISAs were performed identically with the only difference being incubation temperatures of the antigen. Blocking and concentration of antibodies were kept the same for each. Figure 19 shows the results of each plate.

Visually, it can be determined from the color differences alone that the dehydrated plate fulfills the expected trend, whereas the 4° C incubated plate is random in signal with a great deal of non specific binding (e.g., row F-lysis buffer control). This indirect ELISA experiment was done with the two plates prepared in parallel, with the only difference being the incubation temperature of the antigen. Although lower incubation temperatures may prevent degradation of the targeted protein, it apparently does not help in the solid-phase binding process. Between samples, it appears the L1fc-conditioned media does not contain enough L1fc to be

detected, or there are too many other components in the sample that blocks antibody-antigen interaction.

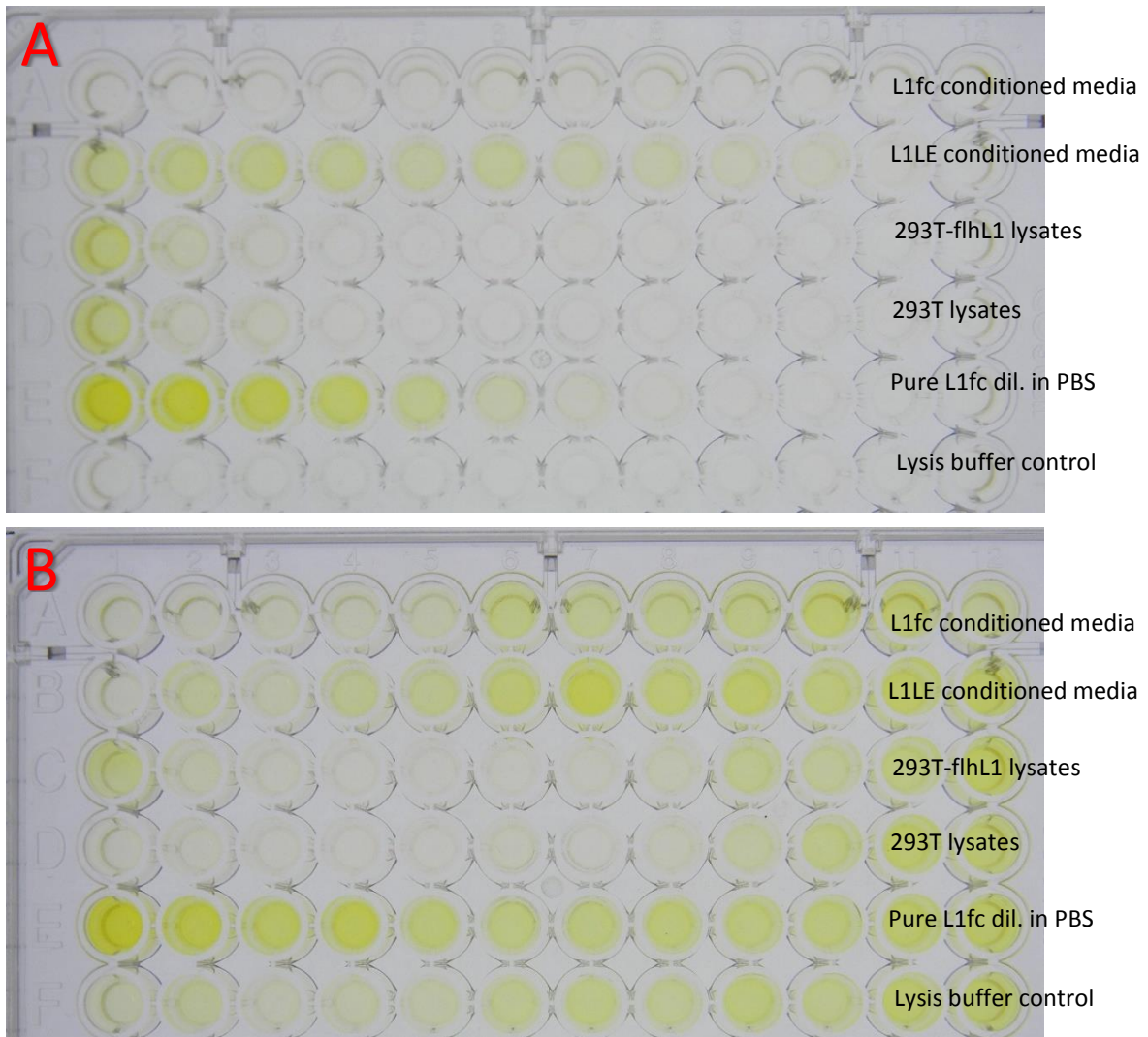


Figure 19 Antigen Incubation at 37° C vs 4° C for Indirect p-orn ELISA Panel A shows p-orn indirect ELISA with the indicated antigens incubated, and dehydrated at 37° C. Plates were blocked with milk-albumin buffer for at least 2 hrs. UJ127 was used at 1:1000, and plates were incubated for 1 hr at room temperature. Plates were washed and goat anti-mouse secondary was used at 1:50,000 and incubated at room temperature for 1 hr. Plates were washed and streptavidin was used at 1:265,000, and incubated for 1 hr. Plates were washed and developed with TMB. Panel B is the same procedure, except that the antigen was incubated at 4° C.

Conditioned media from the U118-L1LE cell line (row B) appeared to have enough detectable L1, but there may be components that need to be diluted out so that L1 can bind the antibody. 293T lysates (row D) and flhL1 lysates (row C) look very similar, though the flhL1 lysates should show stronger immunoreactivity. This could be due to non-specific binding to proteins in both cell lysates and/or not enough L1 being produced by the flhL1 transfected cell line. Lysis buffer was included as a negative control, and incubated as “antigen” along with the other samples. This ensured that the blocking step was sufficient to inhibit non-specific signal. Purified L1fc diluted in PBS showed signal that was expected, and OD values for all samples are shown in the following section. Though not included, several indirect non-capture ELISAs were conducted to determine, as well as compare, the signal of purified L1fc diluted in normal human serum. However, each of these ELISAs resulted in no detection signal, even at the most concentrated well (1 ug/mL) of L1fc diluted in human serum, presumably due to the high concentration of serum proteins binding to the polyornithine. This suggested that an alternative ELISA method such as a capture ELISA was needed to detect L1 from human serum.

3.6 The Limit of Detection for Indirect ELISA

Figure 20 shows the graphical representation of Figure 19A. I was able to confidently detect L1 down to less than 7.8 ng/mL in PBS. If one looks carefully at the zoomed in graph of Figure 20B, the curve of the concentration at 3.9 ng/mL is above baseline. Nevertheless, we wanted to improve sensitivity even further, as well as

attempt to gain values for L1 diluted human serum. Though these results show that the standard ELISA can be altered using this method when testing samples with very little purified antigen, I ultimately want to see the sensitivity of L1 in human serum, which is essentially the goal of this project.

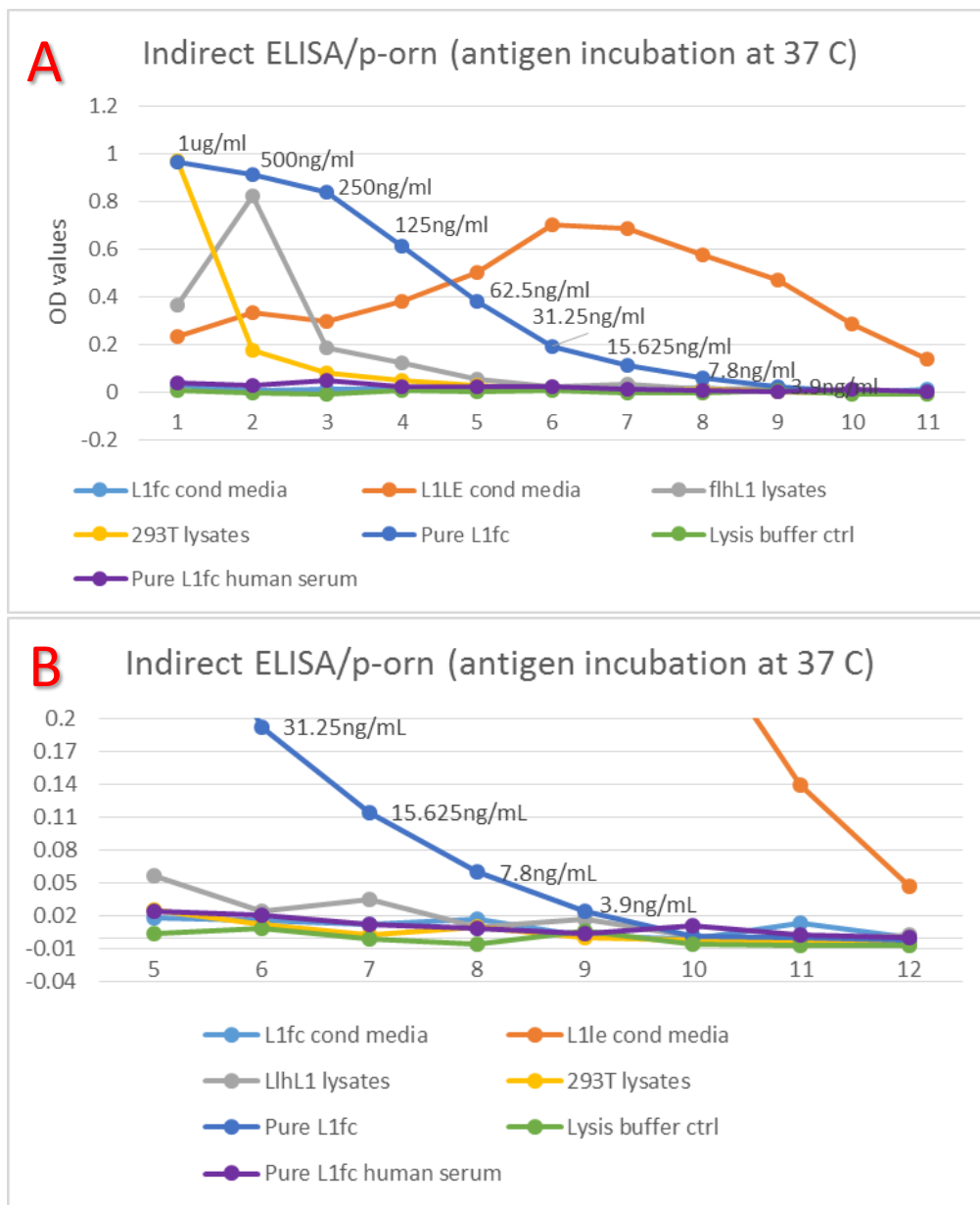


Figure 20 Graph of ELISAs in Figure 19A Graph of OD output at 450 nm generated in Microsoft Excel. Lowest limit of detection for this ELISA is less than 7.8 ng/mL of pure L1fc diluted in PBS.

3.7 Cold vs Warm Antibody Incubations in Capture ELISA

There was some hesitation proceeding with this experiment because we did not know how capture antibodies, in this case 5G3, would hold up during the dehydration process. It is generally believed that antibodies should be kept at low temperatures as often as possible, ranging from -20°C to 4°C , to preserve the immunologic properties. This may be true in terms of storage, but for immediate experiments it may not be needed. I tested this idea in my capture ELISA experiments and found that antibodies can still retain their binding properties after multiple lyophilization-like events. Similar to the procedure previously described in the preceding section, I performed 2 assays in parallel with the only differences being the incubation temperature of the coating antibody. Designated rows of each plate were coated with 1 μg of 5G3 anti-L1 monoclonal antibody per well. One plate was covered and left to incubate at 4°C overnight, while the other was left uncovered in the 37°C incubator overnight. In the same plate of each condition, indirect ELISAs were done in order to observe a comparison between capture and indirect of the same samples under the same conditions. It is crucial to note that after antibody incubation, blocking must be done before adding antigen. Many of these assays were done with the blocking step done after antigen incubation and no signal resulted in the end. This was later discovered to be a critical error that can alter immunoreactions thereafter. Because I was using two different anti-L1 monoclonal antibodies for the capture ELISA, I had to acquire a subtype specific biotinylated secondary antibody to react specifically with UJ127 detection primary antibody, and not with 5G3. Figure 21 shows an illustration of the assay's concept. Figure 22 shows the results of these assays. Figures 23 and 24 show the graphical representation of these results.

First, these results show that the capture ELISA procedure is needed to detect L1 from the complex biological fluid of human serum. The differences between warm and cold incubations can be seen immediately simply by inspecting the plates. There is a striking difference in signal intensity between each plate, however the overall trend of samples appear to be similar.

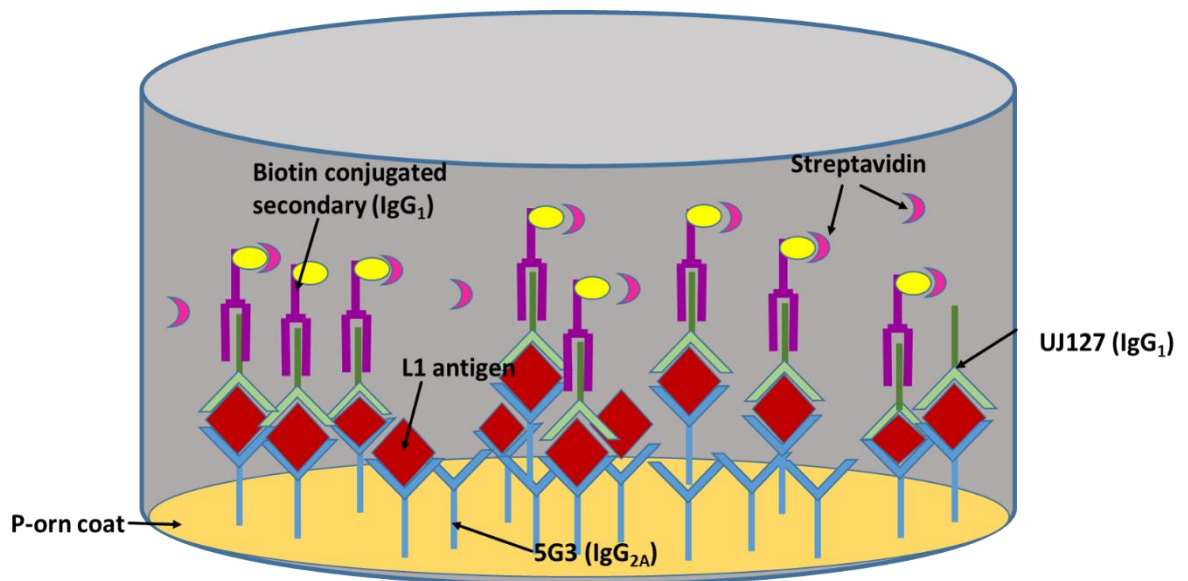


Figure 21 Schematic of capture ELISA procedure Illustration of the theory of capture ELISA.

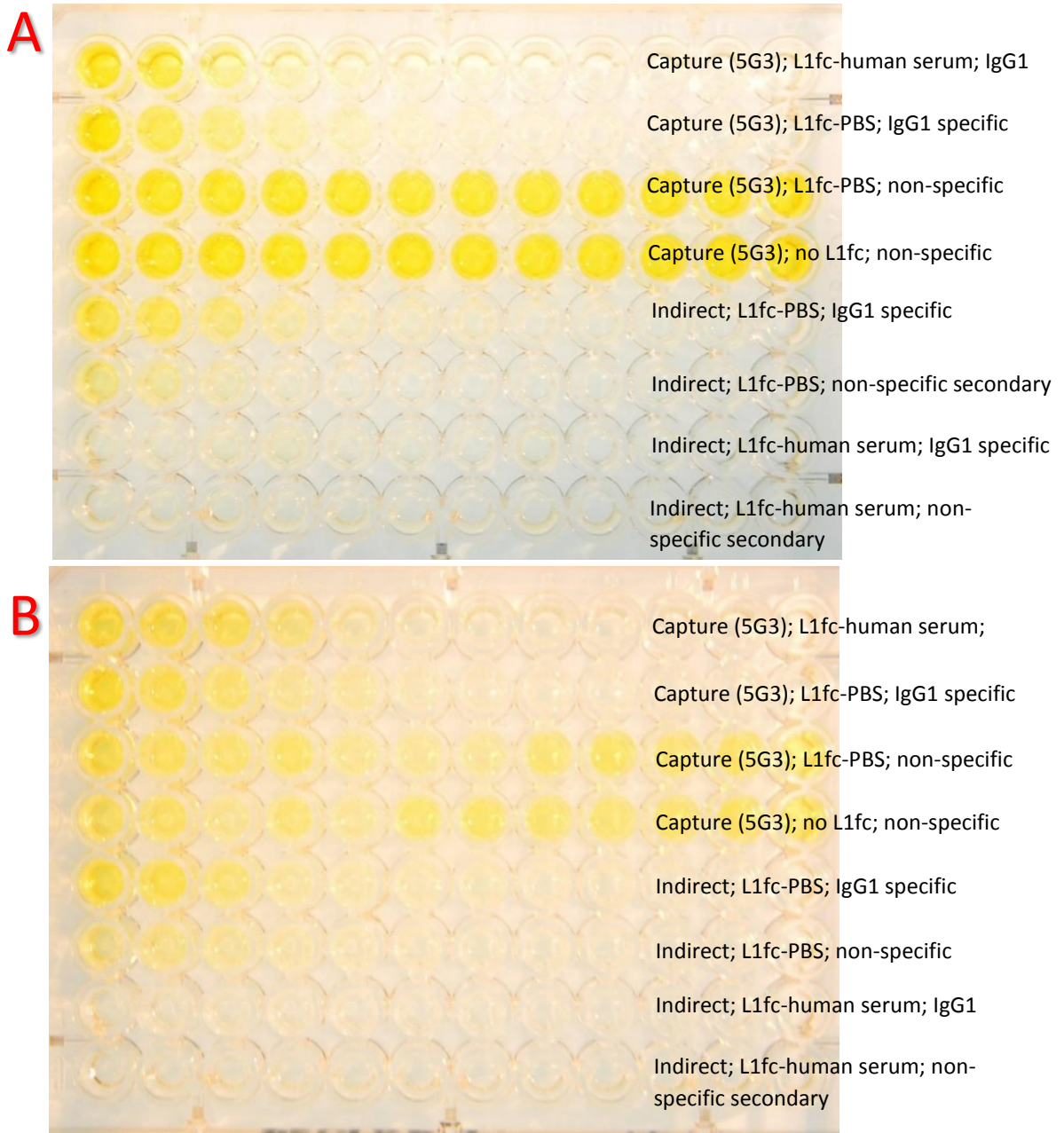


Figure 22 P-orn Capture and Indirect ELISA at 37° C and 4° C Panel A shows capture antibody, 5G3, (row A-D) incubated and dehydrated at 37°C. Antigen for indirect ELISA (row E-H) were also incubated at 37°C. Capturing of antigen was 2 hrs at room temperature. Blocking was done for 2 hrs at room temperature. Detecting primary antibody (1:1000), secondary antibody IgG₁-specific (1:50,000), and streptavidin (1:265,000) were incubated for 1 hr respectively. Panel B is the same except for 5G3 incubation at 4°C.

3.8 The Limit of Detection for Capture ELISA

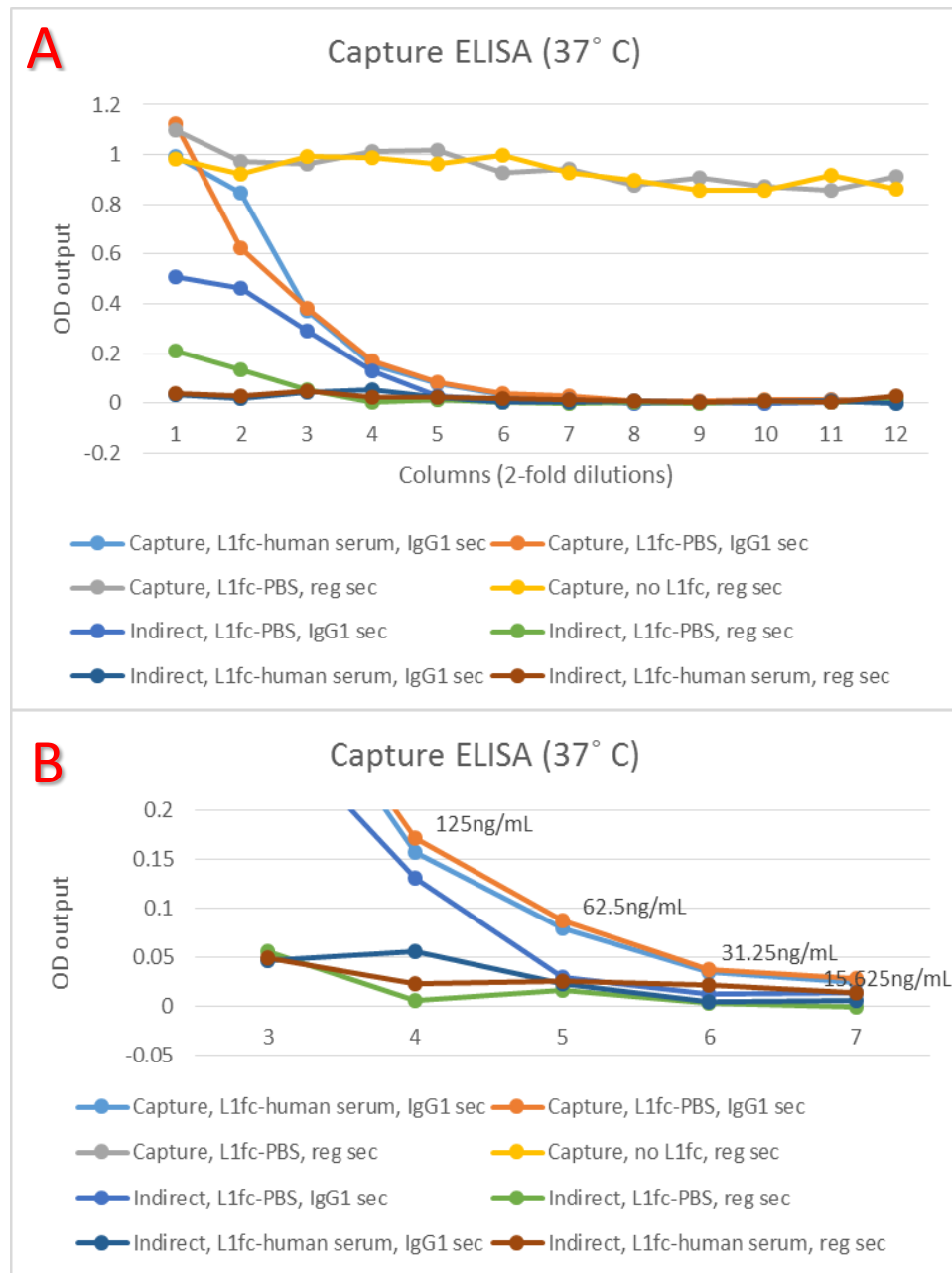


Figure 23 Graph of ELISA of Figure 22A Graph of OD output at 450 nm generated in Microsoft Excel. Lowest limit of detection for this ELISA is less than 62.5 ng/mL of both L1fc diluted in PBS and human serum. A-H in Panel A correspond with rows in Figure 22A. Panel B represents a zoom in of the higher dilutions of L1fc.

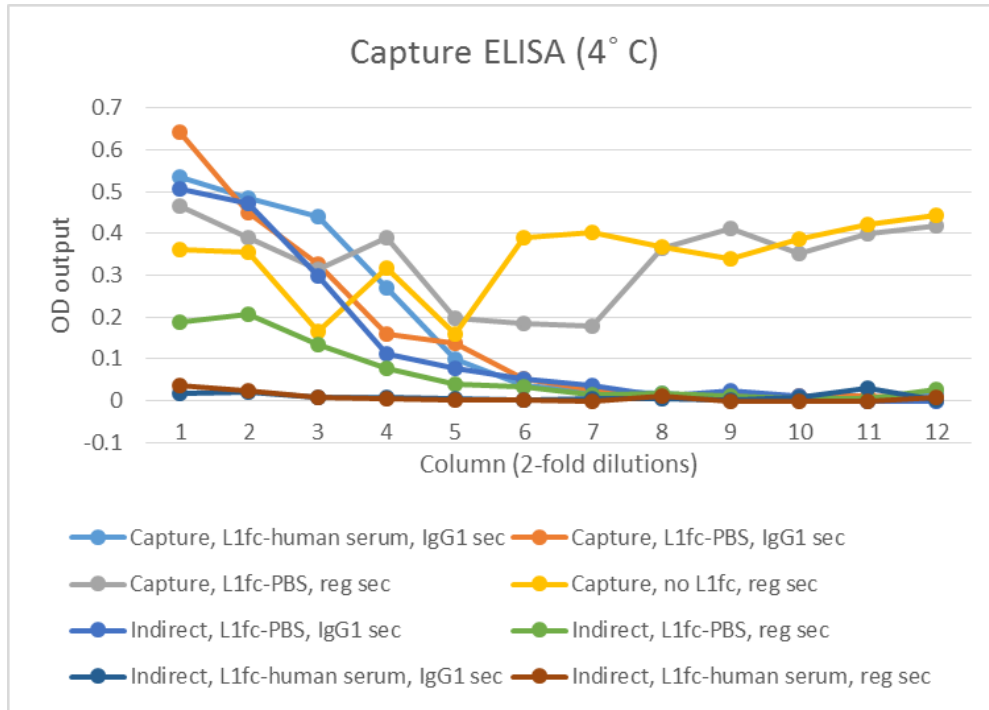


Figure 24 Graph of ELISA in Figure 22B Graph of OD output generated in Microsoft Excel.

It should be noted that the trend between purified L1fc diluted in human serum is very similar to the trend of purified L1fc diluted in PBS. These results indicate that there are no competing substances in human serum that inhibits antibody-L1 interaction. I wanted to get results showing this ELISA assay was at least as sensitive as the DRG L1-CAM ELISA kit, whose limit of detection is 2.74 ng/mL. However, this was not the case.

Giving my protocol a closer look, we realized that additional washes may be needed to increase sensitivity. I only used washes between primary and secondary steps. Thus, it was possible that antigen and antibody might have been complexing in

solution, and washed out resulting in low binding to the bound L1 and, therefore, low signal. I implemented 1 wash with PBS-tween after antibody dehydration to remove unbound antibody, blocking, then 3 washes, antigen incubation, and then an additional 3 washes to remove unbound antigen. The remaining steps of the protocol remained the same. These subtle changes made a significant difference in the sensitivity of detection, which now was 487.5 pg/ml for L1 diluted in either PBS or human serum. Figure 25 shows the results of this assay. Additional washes after 5G3 dehydration, blocking and antigen incubation may improve sensitivity. We tried this and the results are pictured below in Figure 25. Thus, the most sensitive conditions for this capture ELISA to detect L1 is included in the Appendix.

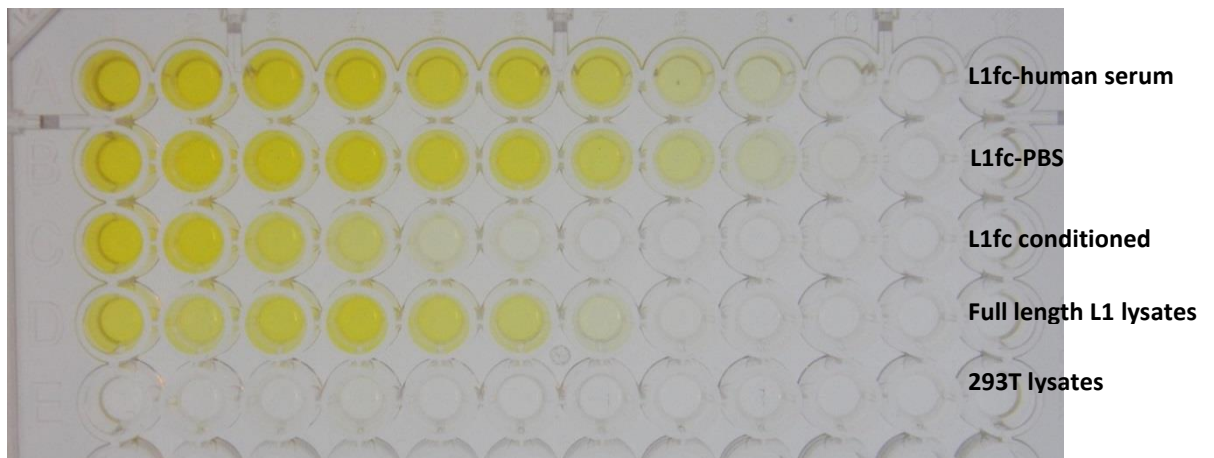


Figure 25 P-orn Capture ELISA with revised protocol Capture antibody, 5G3, was incubated and dehydrated at 37°C. Capturing of indicated antigen was done for 2 hrs after blocking. Primary, secondary, and streptavidin were incubated for 1 hr respectively. See the appendix for the complete protocol.

3.9 Limit of Detection of Revised Capture ELISA

The lower limit of detection decreased to 467.5 pg/mL according to the OD output. The additional washes implemented in the revised protocol improved the sensitivity and detection of the capture ELISA. Figure 26 shows the graph of this assay.

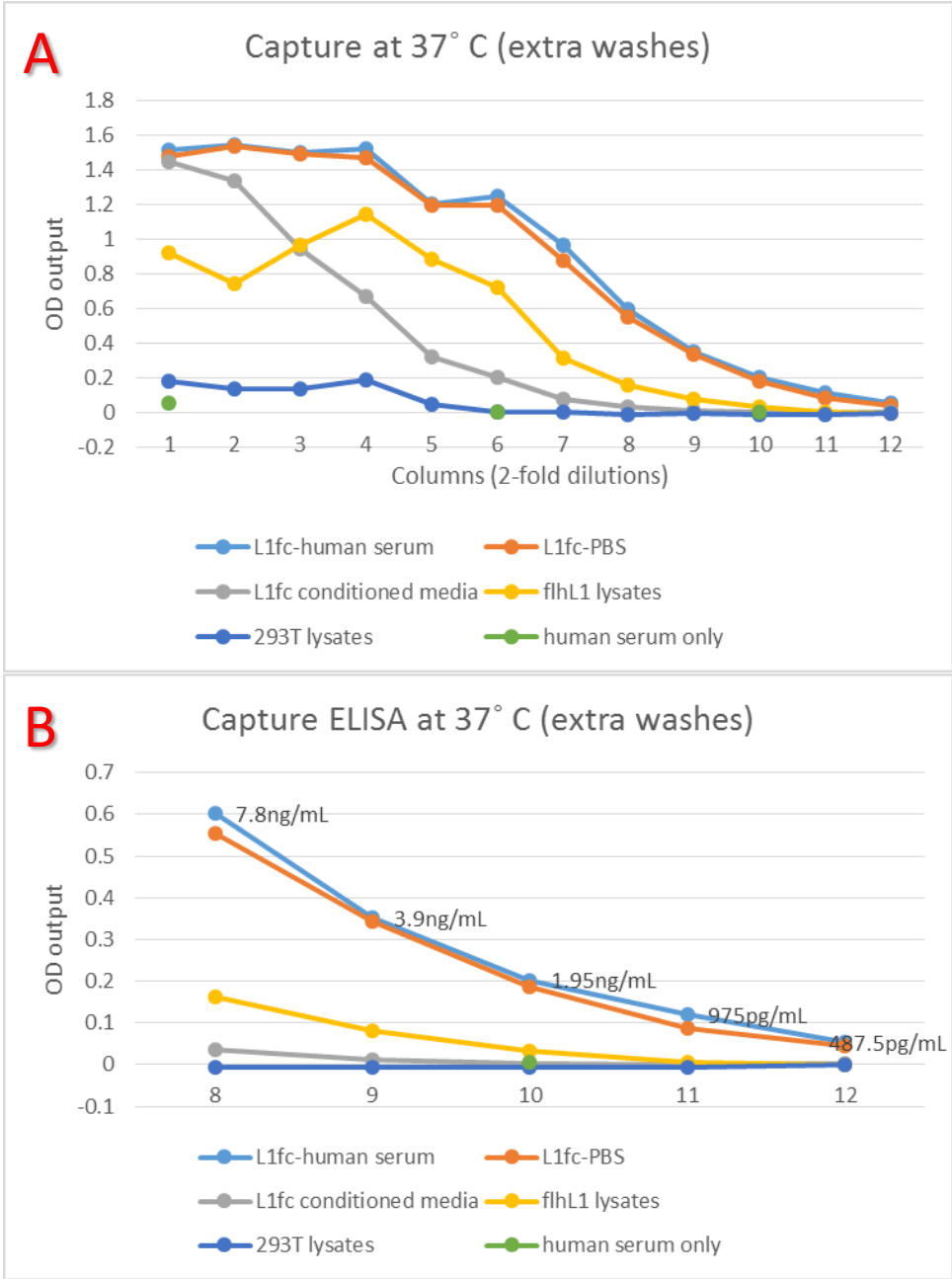


Figure 26 Graph of ELISA with revised protocol for capture p-orn ELISA Graph of OD output generated in Microsoft Excel.

Chapter 4

DISCUSSION AND FUTURE DIRECTIONS

Detecting cancer biomarkers via ELISA is a common practice. Figure 27 shows a table from a review paper [48] showing a list different cancer biomarkers and their respective diagnostic techniques. Studies presented in the introduction (Chapter 1) show the significance and prominence of L1 in cancer studies. However, it is still not being screened for as a cancer biomarker. Although the listed cancer biomarkers can give an indication as to whether there may be cancer present in a patient, they do not also correlate with the aggression and malignancy of the supposed cancer as L1 does. It may be possible that potential biomarkers such as L1, are not implemented into regular screening because a substantial amount is not released into the blood stream, and current ELISA techniques may not be sensitive enough. The dehydrated ELISA technique described in this thesis may help to improve on the sensitivity. Many ELISA improvement tips and techniques available online state that temperature is a crucial factor to be taken into consideration when performing the ELISA [49] [50] [51]. I found this to be true, but not in the same sense as these sources do. One source states that, “Lower temperature can decrease OD values,” and “Higher temperatures can increase OD values” [49]. On the surface, these statements can give the impression of a false positives resulting if ELISAs are performed at higher temperatures. According to my ELISA comparisons at 4°C and 37°C, I did have higher

OD readings in the ELISAs incubated at 37°C compared to 4°C, but I am arguing that this is due to greater availability of antigen-antibody binding due to the purposeful evaporation process.

Table. Cancer biomarkers for diagnosis and prognosis of the disease			
Biomarker	Tumour	Application	Sample type/ Method of detection
<i>Cancer antigen (biomolecules) based biomarkers:</i>			
Prostate specific antigen (PSA)	Prostate cancer	Diagnostic and prognostic	Serum/ Immunoassay
Alpha-fetoprotein (AFP)	Hepatocellular carcinomas (HCC)	Diagnostic and prognostic	Serum/ Immunoassay
Cancer antigen 125 (CA125)	Ovarian cancers Fallopian tube cancer	Diagnostic and prognostic	Serum/ Immunoassay
Cancer antigen 15-3 (CA15-3)	Breast cancer	Diagnostic and prognostic	Serum/ ELISA, Lymph node/ IHC, Bone marrow/ IHC
Cancer antigen 19-9 (CA 19-9)	Pancreatic cancer Bladder cancer	Diagnostic and prognostic	Serum/ ELISA Urine/ ELISA
BRCA-1, BRCA-2	Breast cancer	Diagnostic	Tumour samples/ RT-PCR
Carcinoembryonic antigen (CEA)	Colorectal cancer	Diagnostic and prognostic	Serum/ ELISA
Human chorionic gonadotrophin (hCG)	Germ cell tumours (ovarian and testicular)	Diagnostic	Serum/ ELISA
Thyroglobulin (Tg)	Papillary and follicular thyroid cancer	Diagnostic and prognostic	Serum/ ELISA or IHC with TPO Ab
Heat shock proteins (HSPs) Hsp27; Hsp70	Gastric, prostate carcinoma, osteosarcomas, uterine, cervical, and bladder carcinoma	Diagnostic and prognostic	Serum/ ELISA
TGFβ	Malignant tumours	Diagnostic and prognostic	Serum / ELISA
<i>Metabolic biomarker:</i>			
Glucose metabolism	All cancers, general	Diagnostic, prognostic and therapeutic	Imaging/ FDG-PET scan
<i>Genetic biomarkers:</i>			
Genetic translocations viz. Philadelphia chromosome, Bcl2 and other gene translocation fusion products	AML, ALL, CML, MDS and Burkitt's lymphoma	Diagnostic	Bone marrow or peripheral blood/ FISH
APC gene	Adenocarcinoma, squamous cell carcinoma of the stomach, pancreas, thyroid and ovary	Diagnostic and prognostic	Blood, Tumour sample/ RFLP of chromosome 5q21-22, Methylation status of APC gene
<i>Cells as biomarker:</i>			
Circulating tumour cells (CTCs)	Metastatic breast cancer, etc.	Diagnostic and prognostic	Blood/ Immunocytometry
Cancer stem cells (CSCs)	AML, melanoma, brain tumour, breast cancer, prostate cancer	Diagnostic, prognostic and therapeutic	Tumour sample/ Immunocytometry

Figure 27 List of cancer biomarkers from Bhatt et al. [48]

Published work that has compared temperature incubation of serum in relationship to sensitivity in ELISAs, western blots, and particle agglutination, has shown no difference in results in these diagnostic techniques [52].

Degradation of protein is also of concern when incubating at higher temperatures. Salt concentration, pH, and heat are all factors that can alter the structure and integrity of proteins. Ionic concentration and pH were not factors in my project as buffers and pH were in normal and neutral range. But the fact that heat can affect proteins is part of the reason why cold incubation temperatures are often employed, particularly regarding antibodies. But does this necessarily need to be as concerning in the ELISA as is implied? The short answer is, it depends on the protein. Certain proteins, depending on their properties natural function can withstand higher temperatures, whereas others function optimally at lower temperatures. According to a thermal stability study performed by Vermeer and Norde, denaturation of IgG does not occur before 61 °C [53]. This confirms that my 37 °C incubations do not negatively affect 5G3, and binding site is intact.

Incubation time is another aspect that has been highlighted as a contributing factor in ELISA results. Strict adherence to incubation time is strongly recommended for accurate and reproducible results in ELISAs. I found this to be also true, but again, the entirety of the statement has to be considered. For example, one may read a protocol for an ELISA, and the incubation time for each step is 30 minutes. One might think that for every time an ELISA is performed, the incubation time must be precisely 30 minutes. However, it is possible that the incubation time can be extended to an hour, and similar, if not better results can occur. The strict adherence to incubation time lies *between* ELISA experiments, especially of the same samples being tested.

There is a great likelihood to have significant differences between ELISA experiments performed with varying incubation times. I can attest to this from ELISAs performed but not included in this thesis.

Detecting L1fc via indirect non-capture ELISA was successful in the samples of U118-L1LE conditioned media and samples where purified antigen was diluted in PBS. It was not successful with samples of 293T-L1fc conditioned media, 293T-flhL1 lysates, or purified L1fc diluted in human serum. Failure to detect L1 from the conditioned media could be due to the cell lines needing re-selection with puromycin. It is possible that the L1fc manufacturing cells are a smaller subpopulation from which the conditioned media was made. It also a possibility that that cells are fully selected, but have lost the L1fc region in the vector, but the puromycin cassette remained intact. These hypotheses could also be applied to the failure to detect flhL1. But in addition, the lysates contained many more proteins than did the conditioned media and PBS-L1fc, which also would bind to the polyornithine and block L1 binding. Unfortunately, just as the concentration of L1fc within each well would increase during evaporation at 37°C, the additional proteins in the lysates increase as well. This is the same problem with L1fc diluted in human serum. The enormous amount of proteins block the antigen and antigen binding sites, even after several dilutions. I recommend that indirect non-capture ELISAs be used only for purified proteins, conditioned media (serum-free), and low-protein-density samples, such as saliva and urine with the protocol described in the APPENDIX B.

Capture ELISAs work well to detect antigen with many other proteins in solution. By saturating the polyornithine with 5G3, there are very few areas where proteins from human serum can bind (but blocking was still necessary after 5G3).

However, the 5G3 antibody is specific enough to “pick out” L1fc from the very protein-dense serum or cell lysate solutions. It is important to rinse one time after 5G3 incubation to remove excess antibody. Otherwise, antigen can complex in solution and be washed out, resulting in signal lower than the maximum. It is also crucial to block before adding antigen. The APPENDIX C contains the complete protocol.

In the future, chemiluminescent ELISAs should be implemented to determine if this mode of detection further increases sensitivity. I expect that the range of the limit of detection will increase significantly with the switch from chromogenic to chemiluminescent detection. Solid white polystyrene plates should be used for these assays, which are known to amplify the chemiluminescent signal and limit cross signals between wells. Even though the interaction between polyornithine and unmodified polystyrene exhibit a repelling effect, it is possible that once the dehydration process occurs that the polyornithine will adhere to the plate. Other polyaminoacids such as polylysine and polyarginine are other materials that can be explored to enhance the binding capacity of the plate, but this is unlikely to yield results different from polyornithine, since they are similar positively charged amino acids.

Nitrocellulose-coated plates have potential to be revisited with the protocol developed for p-orn ELISAs. Looking back, it is possible that those ELISAs did not work because their incubation temperatures were at 4° C, and the appropriate dilution ratios had not been established yet. In addition, this type of ELISA should only be applied to chemiluminescent ELISAs. It is very likely that the coating will disrupt absorbance measurements of the plate reader.

The Fogel et al paper has demonstrated that it is possible for L1 to be detected in human serum in ovarian and uterine cancer patients. They stated that they were not able to detect L1 in other malignant cancer types, however, it was unclear if those cancers were L1 positive. In addition, Figure 4 shows the plot of their OD readings for their ELISAs where it appears that they have a background of approximately 0.18. This is not reflective of my ELISAs, where I corrected for background in my calculations and still observed an average background reading of 0.01. For this reason, I believe that the ELISA methodology used in the Fogel paper was not sensitive enough to show the presence of L1 in the other cancer types (provided they were L1 positive). This paper may have suggested that certain cancer types shed more L1 than others, but certainly not that L1 is unable to be detected in cancers aside from uterine and ovarian. My method is unique in two ways and may be able to correct the high background problem. My utilization of polyornithine to increase the binding capacity of the plates may enable more protein to adhere to the plate compared to a plain polystyrene dish. Then, the purposeful evaporation allows maximal sample/antibody incubation. With my method, it is expected that L1 will be able to be detected in the serum of a variety of cancer patients who have L1 positive cancers.

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Appendix A

MAKING POLYORNITHINE COATED PLATES (TISSUE CULTURE PLATES)

Dilute polyornithine in water at 200 ug/mL

For 96-well plates, 100 uL is sufficient to coat the wells

For 24-well plates, 500 uL is sufficient to coat the wells

Place plate in 37°C incubator overnight for 10-16 hrs until dried

Appendix B

P-ORN INDIRECT (NON-CAPTURE) ELISA PROTOCOL (96 WELL PLATE)

Make up desired serial dilutions of antigen/sample in a clean separate polystyrene plate

Transfer antigen/sample to p-orn-coated plate

Incubate plate at 37°C overnight for 10-16 hrs

Block plate for at least 2 hrs

Blocking buffer consists of 1 albumin ampule (2 mg) per 50 mL of 5 % milk- PBS

Rinse plate **one** time with PBS-Tween

Make up primary antibody at 1:1000 in PBS (**not** PBS-Tween)

Deposit 100 uL of primary antibody and incubate at room temperature for 1 hr

Wash plate 3 times with PBS-Tween

Make up secondary antibody (between 1:40,000-1:55,000 for TMB or 1:1000 for alkaline phosphatase)

Deposit 100 uL secondary antibody and incubate at room temperature for 1 hr

Wash plate 3 times with PBS-Tween

Make up streptavidin (if using TMB) at 1:265,000

Deposit 100 uL, and incubate for 1 hr

Wash plate 3 times with PBS-Tween

Develop for at least 5 mins.

Read at 450 nm

Appendix C

P-ORN CAPTURE ELISA PROTOCOL (96 WELL PLATE)

Dilute capturing primary antibody in PBS at 1 ug per 100 uL

Deposit a total of 1 ug per well in a p-orn plate

Incubate plate overnight at 37° C for 10-16 hrs

Rinse plate **one** time with PBS-Tween

Block for at least 2 hrs

Blocking buffer consists of 1 albumin ampule per 50 mL of 5 % milk-
PBS

Rinse plate **one** time with PBS-Tween

If needed, make up desired dilutions of antigen/sample in a clean separate polystyrene plate and transfer 100 uL of sample to the p-orn plate.

Incubate antigen/sample at room temperature for 2 hrs

Rinse plate **three** times with PBS-Tween

Make up detecting primary antibody at 1:1000 in PBS (**not** PBS-Tween)

Deposit 100 uL of primary antibody and incubate at room temperature for 1 hr

Wash plate 3 times with PBS-Tween

Make up secondary antibody (between 1:40,000-1:55,000 for TMB or 1:1000 for alkaline phosphatase)

Deposit 100 uL secondary antibody and incubate at room temperature for 1 hr

Wash plate 3 times with PBS-Tween

Make up streptavidin (if using TMB) at 1:265,000

Deposit 100 uL, and incubate for 1 hr

Wash plate 3 times with PBS-Tween

Develop for at least 5 mins.

Read at 450 nm

Appendix D

CELL LINES AT A GLANCE

293T	Transfectable cell line; derived from human embryonic kidney cells
293T-flhL1	293T cells transfected with a full length human L1 construct via calcium phosphate transfection; puromycin selected
293T-L1fc	293T cell transfected with a L1fc recombinant protein construct; puromycin selected; GFP
Sp2/0	Mouse myeloma cell line
U118	Human malignant glioma cell line
U118-L1LE	U118 cells infected with a lentiviral vector expressing the L1 ectodomain only; puromycin selected