

## ABSTRACT

EZUMA-IGWE, UGONNA. Tackling The Problem of T-Cell Non-Hodgkin's Lymphomas By Targeting Cancer-specific Surface Antigens. (Under the direction of Paul Hess).

Peripheral T-cell lymphoma (PTCL) is a deadly cancer that responds poorly to chemotherapy. In humans with B-cell lymphoma, mAb-based immunotherapy targeting surface differentiation antigens (CD19, CD20) eradicates chemoresistant cells and improves outcomes, with loss of bystander normal B cells an accepted hazard of therapy. However, analogous total loss of T cells is not tolerable because of infectious disease risks, so alternate mAb targets are needed. Two promising candidates were found in PTCL biopsy specimens from dogs, our model system, by RNA-seq: PLAC<sub>1</sub> and CBY<sub>2</sub>, both present in 4 of 4 samples. Neither gene is expressed in normal non-reproductive tissues in humans, so risks of autoimmunity with immune targeting are probably low. We hypothesized that PLAC<sub>1</sub> and CBY<sub>2</sub> would have favorable profiles as PTCL targets: 1) high prevalence across patients; and 2) no expression in normal tissues, except placenta and testis.

To test this prediction, we tested canine normal tissue and PTCL samples. Briefly, we used reverse transcription-polymerase chain reaction (RT-PCR); placenta, for PLAC<sub>1</sub>, and testis, for CBY<sub>2</sub>, served as positive controls, while water constituted a negative control. We found that PLAC<sub>1</sub> was expressed in many lymphoma samples and

had limited expression in normal canine tissue. We observed that CBY2 was expressed in only a few lymphoma samples, with more expression in normal tissue than PLAC<sub>1</sub>. This data indicates that between antigens PLAC<sub>1</sub> and CYB2, PLAC<sub>1</sub> is the better candidate. Overall, this study showed that PLAC<sub>1</sub> has the potential to serve as a T-cell target, which is important because finding an antigen to target is the first step to creating a new antibody-based immunotherapy. Our next step will be to perform screening with commercially available anti-PLAC<sub>1</sub> antibodies to ensure that PTCLs make PLAC<sub>1</sub> protein, and if so, to determine the percentage of PLAC<sub>1</sub><sup>+</sup> cells in the population.

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Tackling The Problem of T-Cell Non-Hodgkin's Lymphoma By Targeting Cancer-specific Surface Antigens

By

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A thesis submitted to the Graduate Faculty of North  
Carolina State University in partial fulfillment of  
the requirements for the Degree of  
Master of Science

Comparative Biomedical Sciences

Raleigh, North Carolina

2023

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## **BIOGRAPHY**

Ugonna Ezuma-Igwe was born and raised in Durham, North Carolina, on June 10th, 2000. She originally hails from Arochukwu, Abia State, Nigeria. She received her Bachelor of Arts in Biological Science with a cross-discipline in Economics and a Minor in Africana Studies from North Carolina State University. As a Black woman in STEM, she was often the only Black person in her classes and work, if not the only Black woman. These experiences helped her decide to pursue her Master of Comparative Biomedical Sciences to work to dispose of health disparities and inequality in medicine. While the burden of disassembling institutional and internalized racism should not fall on the victims, Ugonna wants to advocate for underrepresented communities often pushed aside and left unaddressed. She has been accepted to the University of Georgia for their Pharmaceutical and Biomedical Sciences Ph.D. program and plans to continue her education there.

## ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Paul Hess, for his guidance and support on this project.

I would next like to thank my committee for all of their assistance and advice.

I want to extend my sincere thanks to Jennifer Holmes and Ching-Yen Lee, members of the Hess Lab, for their continuous helping hand.

I could not have undertaken this journey without my family and friend's love, prayers, and support—especially my grandparents, who are my biggest motivations.

Finally, I would like to express my deepest appreciation to God for providing me with this opportunity and granting me the strength and ability to complete this project.

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# **Chapter 1: The Promise Of A Cure For Non-Hodgkin's Lymphoma (NHL) Through Immunotherapy.**

## **1.1. T cells and B cells**

Lymphocytes are white blood cells that play an integral role in the immune system especially in recognizing and targeting pathogenic microorganisms and eliciting a response. There are two main types of lymphocytes: T cells and B cells. The T in T cells is derived from their primary production in the thymus. The immature precursors of T lymphocytes originate in the bone marrow and then migrate to the thymus where they complete their development. There are several types of T cells such as Memory, Helper and Cytotoxic. The function of these T cells is dependent on the type. For example, CD4<sup>+</sup> T cells, a helper T cell, function to elicit cell-mediated immunity in order to evoke immune responses. Another type of T cells is CD8<sup>+</sup> T cells, a cytotoxic T cell, that enlist the help of helpers T cells, such as CD4<sup>+</sup>, to induce apoptosis, a form of targeted cell death, when confronted with infected or foreign cells. [41]

While the B in B cells was derived from the Bursa of Fabricius in birds, a lymphoid organ in young chickens where lymphocytes mature. In humans, B cells originate in the bone marrow.

## **1.2. Non-Hodgkin Lymphomas**

Non-Hodgkin Lymphomas (NHL) is one of the two types of malignant cancers affecting the lymphatic system, the other being Hodgkin's disease. [1] NHL is an all-inclusive name for various subtypes of malignant neoplasms arising from B cells, T cells, or natural killer cells found in lymphoid tissues. [2] The prevalence of NHL globally has established it as the most common hematological malignancy. [3] Categorizations ranging from stage, type, and symptoms to familial history, age group, and sex all play a part in determining treatment. [2,3] The common forms of treatment are chemotherapy, radiotherapy, immunotherapy, and stem cell transplant. In 2018, 2.6%, approximately 248,700, of all deaths globally from cancer were accredited to NHL. [3]

## **1.3. T-cell Lymphomas**

NHL's have two main categories: B-cell (B-lymphocyte) and T-cell (T-lymphocyte). T-cell lymphomas are less common than B-cell lymphomas. High-grade peripheral T-cell lymphoma (PTCL) is a routinely aggressive form of lymphoma that develops from T-cells and natural killer (NK) cells. [4] Less than 10% of non-Hodgkin lymphomas are classified as PTCL. [5] PTCL has universally poor outcomes in terms of patient health and survival rate. This is due to its aggressive nature characterized by the ability to form, grow, and spread quickly. In terms of an optimal strategy for PTCL

treatment, there is currently no consensus. Still, a common form of treatment is cyclophosphamide, doxorubicin hydrochloride, vincristine (Oncovin), and prednisone (CHOP) though its efficacy is limited. [12]

### **1.3.1 Treatment efficacy**

Attempts to further improve CHOP's efficacy on PTCL have proven futile, specifically when used in addition with Romidepsin, a histone deacetylase inhibitor, and antineoplastic agent. [13] Romidepsin's proven effectiveness as a treatment for cutaneous T-cell lymphoma (CTCL), and PTCL individually led scientists to test if its addition to CHOP would further improve CHOPs use as a therapy. Investigations concluded that it did not improve the progression-free survival rate, response rates, or overall survival. [12]

Overall, 60-80% of adults with NHL can go into complete remission, but unfortunately, 20-40% will eventually relapse. [11] When PTCL is treated with strategies similar to those for aggressive B-cell lymphoma, though, the strategies are not as effective. Those treated this way experienced early relapse and had an overall survival rate of fewer than two years. [6]

### **1.3.2 Models for treatment**

PTCL is rare in humans, and the less common form of lymphoma diagnosed in dog as well [7] Though there are many differences between humans and dogs, it has been established that canine PTCL is molecularly similar to human PTCL. Both share similarities in their activation of the PI3 kinase pathways, loss of the gene PTEN, and production of the tumor suppressor CDKN2. [8] Similarly to humans, when dogs with PTCL are given treatment similar to those done on B-cell lymphomas, effects are subtle. The overall survival rate is only ~6–9 months. [9] These similarities have made dogs an attractive model for PTCL cancer research in regard to further understanding its occurrence in humans. [10]

### **1.4. Immunotherapy**

Immunotherapy is one form of treatment against NHL. These therapies help equip the immune system with the tools to fight the spread of lymphoma and effectively administer an antitumor immune response. There is a range of immunotherapies – varying in their form of administration, target approach, and intended mechanism of action. Examples include antibody-based (Ab) immunotherapy, cell-based therapies, and immune checkpoint inhibitors. [17]

There has been extensive interest in monoclonal antibodies (mAbs) since the first one was generated in 1975 by César Milstein and Georges Köhler, two researchers funded by the National Cancer Institute (NCI) [18]. As of June 2022, the US Food and Drug Administration (FDA) has approved 122 therapeutic mAbs, many specifically for humans, to treat various diseases and conditions, including cancer. [38] Their specificity and flexibility have made the mAbs market a predominant focus in the global pharmaceutical market. [18, 19] Interest in the mAbs antitumor effect has pushed scientists to work to further develop and maximize their efficacy.

Rituximab is an anti-CD20 monoclonal antibody approved by the US FDA in 1997 for treating B-cell NHL. Rituximab has been incorporated as the standard form of treatment (CHOP) for B-cell lymphomas, as it has the ability to prolong disease progression and extend overall survival. [14] CD20 is a non-glycosylated protein expressed on the surface of pre-B cells and mature peripheral B lymphocytes. Though CD20's specific role in B cells is unclear, data suggests it may regulate the development and maturation of B cells through the cell cycle, apoptosis, and calcium regulation. [16]

CD20 was one of the first successful immunotherapy molecular targets. [15] In order for rituximab to target CD20, it uses multiple effector mechanisms, including complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity

(ADCC), antibody-dependent phagocytosis (ADP), and induction of cell death pathways such as apoptosis.

ADCC and ADP are induced when CD20 molecules begin reorganizing, which is triggered by rituximab binding to the tumor cells. In turn, this activity activates the classical pathway of the complement system. [14] This initial reaction of rituximab binding triggers ADCC and apoptosis. In response, ADCC is a defense protocol leading to release of cytokines, chemokines, and mediators that kill target cells. Apoptosis is initiated by the crosslinking of CD20 molecules. [14] The combination of these mechanisms enables Rituximab to kill its targeted cancer cells.

### **1.5. Harnessing T-cells**

Rituximab works by enlisting effector cells such as macrophages (MØ) and NK cells to kill targets. Antibodies can also harness T cells to kill targets. The concept of utilizing T cells for immunotherapy, specifically cancer therapy, has been promisingly studied due to T cells' function in cell-mediated immunity and ability to produce prolonged cytotoxic, antigen-specific, effector, and immune memory responses. Also, the presence of T-cell receptors (TCRs) on the T cell's surface, unlike other lymphocytes, has stimulated intrigue into how T cells can effectively promote and aid

tumor eradication. [21] Two tested techniques that have been utilized are Bi-specific T-cell Engagers (BiTEs) and Chimeric Antigen Receptors (CAR Ts). [20]

### **1.5.1 Bi-specific T-cell Engagers (BiTEs)**

BiTEs are fusion proteins that are a category of bispecific antibodies (BsAbs) that target a tumor antigen and an immune-related molecule. BsAbs have two binding . BiTEs are created when two varying single-chain variable fragments (scFv) are joined together by a peptide linker. BiTEs are unique in their ability to link to TCRs. BiTEs are designed to specifically target T cell-specific molecule CD3 and a tumor-specific antigen concurrently to induce T cell activation. The simultaneous interaction of the T cell-specific molecule and the tumor-specific antigen is essential for BiTE to activate T cells. This interaction leads to the formation of an immunological synapse commissioning T cell activation. Without this interaction, meaning both anti-CD-3 and anti-tumor antigen expressing concomitantly, BiTE's cytotoxic mechanism will not be initiated. [21]

BiTEs have been used for the treatment of B-Cell lymphomas. The only BiTE with US FDA and European Medicines Agency (EMA) approval is the BiTE, Blinatumomab, which targets the B-cell surface differentiation antigen CD19. CD-19 is a transmembrane glycoprotein belonging to the immunoglobulin superfamily of proteins,

and because of expression on most B-cell phases, is an excellent target for immunotherapy. This BiTE redirects T cells to kill CD19-positive B-cell lymphomas. CD3 crosslinking triggers T-cell proliferation, killing the B-cell lymphomas through membrane perforation and programmed cell death. [22]

After a clinical study on patients with Philadelphia-negative chromosome relapsed/ refractory B-cell acute lymphoblastic leukemia, a rapidly progressing blood and bone marrow cancer, presented a full remission rate of 43%, the FDA authorized BiTE Blinatumomab in 2014. Since its initial approval, BiTE Blinatumomab use has expanded and is now approved for treating B-Cell lymphomas. [28] When studies clinically tested BiTE blinatumomab on patients with Non-Hodgkin lymphoma, it was administered as a continuous intravenous infusion over a period of weeks and then followed by a treatment-free period to avert T-cell exhaustion. [22] A constant disadvantage of BiTE blinatumomab is the need for continuous intravenous infusion which limits its use as a long-term and ongoing treatment. BiTE blinatumomab's small molecular weight and rapid clearance from circulation is an advantage, as smaller proteins migrate faster.

### **1.5.2 Chimeric Antigen Receptors (CAR T's)**

Another strategy, called chimeric antigen receptor (CAR) T cell or CAR T, genetically alters the programming of T cells of the cancer patients by re-directing them to target specific tumor-associated antigens. T cells are collected from a patient through apheresis. Those cells are genetically re-engineered to have CARs on their surface in order to recognize, target, and bind specific antigens on the surface of tumors through the fusing of a tumor-specific scFv that contains the immunoreceptor tyrosine-based activation motif (ITAM) of the TCR complex CD3 zeta chain. The T cells are then transferred back to the patient with the intent for them to go forth and multiply within the patient's body, actively building a defense against cancer growth. The binding of the tumor antigen activates the ITAM of the CAR T cell and subsequently kills the tumor cells. CAR Ts are unique in their approach as cells are specifically tailored for each patient. CAR Ts are able to eradicate tumor cells quickly and successfully as they are unhindered by target cells' antigen processing and MHC restrictions. This process enables CAR T to have a considerably short treatment time while allowing for greater success at long-term remission. [23]

CAR T cells have also been used for the treatment of B-Cell lymphomas. Currently, the only CAR T-cell therapies approved by the US FDA and the EMA to treat B-cell malignancies are Tisagenlecleucel and Axicabtagene Ciloleucel. [22] In 2017, Tisagenlecleucel became the first ever CAR T-cell therapy to receive FDA approval,

originally as a treatment for a form of acute lymphoblastic leukemia. A year later it was approved as a treatment for Non-Hodgkin Lymphoma. [29] In 2017, Axicabtagene Ciloleucel became the first FDA-Approved CAR T-Cell Therapy for Relapsed/Refractory Large B-Cell Lymphoma. [30]

For Tisagenlecleucel, T cells collected from a patient are engineered to link to the CD3, TCR and CD137 and recognize and bind to CD19. [26] While for Axicabtagene Ciloleuce, T cells collected from a patient are engineered to link to the CD3 TCR and CD28 and recognize and bind to CD19. [27] Again, CD19 is the target because most B-cell lymphomas express the antigen, reflecting its expression on the surface on non-cancerous B-cells throughout most phases of development. It is important to note that “the majority of B cell malignancies express CD19 at normal to high levels (80% of ALL, 88% of B cell lymphomas and 100% of B cell leukemias).” [39] Further, CD19 is not expressed on non-B cells, limiting off-target effect. Once the anti-CD19 CAR T cell recognizes its target, the production and release of cytokines is triggered by T-cell activation, which leads to tumor cell lysis. There are numerous factors that have the ability to affect this process, such T-cell exhaustion, but the benefit of CAR-T cell therapy is its ability to counteract this independent of the major histocompatibility complex (MHC), as they are able to override MHC class restrictions. T-cell response is improved and fueled further with the introduction of CAR T-cell Tisagenlecleucel and Axicabtagene Ciloleucel to the body. [26]

CAR T-cell therapies' most common adverse effects are Cytokine Release Syndrome (CRS), neurotoxicity, hypogammaglobulinemia, and prolonged cytopenias. CRS is an inflammatory response induced when (CAR) T cells bind to its antigen and subsequently activate bystander immune and non-immune cells causing hyperactivation and excessive cytokine release. [31] Neurotoxicity is typically associated with CRS, mainly seen in patients with concurrent or preceding CRS. Neurotoxicity is a functional or structural change in the nervous system caused by an outside agent that can lead to symptoms such as delirium, tremor, transient focal weakness, ataxia, and more (Symptoms specific to CAR-T cell-induced neurotoxicity). [32] Hypogammaglobulinemia is triggered by CD19 CAR T cells' ability to target malignant CD19-expressing B cells and destroy normal B cells resulting in low immunoglobulin levels. CAR T cells can have long-term and ongoing responses to tumors in the body, potentially leading to long-term hypogammaglobulinemia. Signs of low IgG levels can be detected as early as nine weeks after CAR T-cell infusion making it essential for those treated with CAR T-cell therapy to have access to effective IgG replacement options. [33] Like hypogammaglobulinemia, prolonged cytopenias can manifest early after CAR T-cell therapy treatment. Prolonged cytopenia is a dangerous decrease in healthy blood cells for an extended period of time. After CAR T-cell infusion, patients commonly experienced neutropenia, anemia, and thrombocytopenia. [34] These symptoms, CRS, neurotoxicity, hypogammaglobulinemia, and prolonged cytopenias, often work in

tandem with one another. Managing these symptoms is essential to attenuate the severity of these adverse effects on the success of CAR-T-cell therapies.

### **1.5.3 Major CAR Ts and BiTEs Similarities**

Both use scFv antibodies to target antigens expressed on the surface of tumor cells to facilitate T-cell response. Also, BiTEs and CAR T cell therapies are quite costly. The estimated per-patient average cost of CAR T cell treatment prep is \$350,000, and the total CAR T cell costs are \$1 to 1.5 million. 1-2 cycles of BiTEs treatment costs about \$72 000. [22] Similarly, both treatments facilitate tumor cell death independent of MHC and redirect T-cell cytotoxicity to tumor cells. [22,23]

### **1.5.4 Major CAR Ts and BiTEs Differences:**

CAR Ts provides a long-term and ongoing response to tumors, while BiTEs are unable to have the same effect without continuous administration. [22,23] BiTEs components are available “off-the-shelf”, whereas CAR T cells must be customized to each patient. [22,23] CAR Ts uses active targeting through their continuous interaction with tumor-associated antigens allowing long-term response, while BiTEs use passive targeting due to their biodistributions’ dependence on multiple factors such as diffusion rates.[24]

## 1.6. Differentiation Antigens

Many therapies used to treat lymphomas focus on target-based treatments—for example, Rituximab and anti-CD19 CAR T target differentiation antigens, CD20 and CD19. “Differentiation antigens represent normal proteins which are expressed as a consequence of a specific function of the target tissue” and are typically only seen at particular phases of differentiation. [35] Targeting differentiation antigens has adverse effects as they are not selective to malignant tumors. The targeted differentiation antigens are also displayed on benign cells, causing an attack on healthy and unhealthy cells. There are extra hazards when targeting T cells with this approach, as there is the possibility of prompting autoimmune disorders such as severe skin rashes, vitiligo lesions, and transient colitis. [35] The effects of targeting differentiation antigens are seen in patients administered with Alemtuzumab, an anti-CD52 monoclonal antibody, specifically depleting T and B lymphocytes, approved to treat multiple sclerosis (MS) and Chronic Lymphocytic Leukemia (CLL). While Alemtuzumab is tailored to illnesses other than NHL, the effects speak to extra hazards associated with targeting differentiation antigens, specifically T cells. A study tested the effects of Alemtuzumab on patients with MS and established the correlation between the number of therapy cycles and T and B cells. As the treatment cycles increased, the number of circulating T

and B cells dramatically reduced. [36] T-cell depletion actively increases the risk of infection and recurrence of illness. [37]

### **1.7. Research Objectives**

Our project focuses on exploring potential new targets for CAR T-cells. As stated previously, CAR T-cells alter the programming of T lymphocytes by instructing them to target specific tumor-associated antigens on the cell surface. Our goal is to find PTCL-specific surface antigens not expressed on normal T cells or other tissues, as it is crucial to target tumor-specific antigens or antigens with limited/no expression in normal tissues. [35] We investigated two promising antigens, PLAC<sub>1</sub> and CYB<sub>2</sub>. These antigens were selected due to their expression in four PTCL samples by RNAseq. We hypothesized that PLAC<sub>1</sub> and CYB<sub>2</sub> would have favorable profiles as PTCL targets: 1) high prevalence across patients; and 2) no expression in normal tissues, except placenta and testis. In the next chapter, we will further discuss whether PLAC<sub>1</sub> and CYB<sub>2</sub> turned out to be new targets for CAR T-cells and the implications of whether or not the two antigens should be further investigated.

### **1.8. List of Abbreviations**

Antibody-based	Ab
Antibody-dependent Cellular Cytotoxicity	ADCC

Antibody-dependent Phagocytosis	ADP
Bi-specific T-cell Engagers	BiTEs
Bi-specific Antibodies	BsAbs
Chimeric Antigen Receptors	CAR T
Complement-dependent Cytotoxicity	CDC
Cyclophosphamide, doxorubicin hydrochloride, vincristine (Oncovin), and prednisone	CHOP
Chronic Lymphocytic Leukemia	CLL
Cytokine Release Syndrome	CRS
Cutaneous T-cell lymphoma	CTCL
European Medicines Agency	EMA
Fragment of Antigen Binding	Fab
Food and Drug Administration	FDA
Immunoreceptor Tyrosine-based Activation Motif	ITAM
Monoclonal Antibodies	mABs
Major Histocompatibility Complex	MHC
Macrophages	MØ
Multiple Sclerosis	MS
National Cancer Institute	NCI
Non-Hodgkin Lymphomas	NHL

Natural Killer	NK
Peripheral T-cell Lymphoma	PTCL
Single-chain Variable Fragments	scFv
T-cell Receptors	TCRS

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## **Chapter 2: Investigating Shared Cancer-specific Surface Antigens in Canine Peripheral T-cell Lymphoma**

### **2.1 Introduction**

T-cell lymphomas have many subtypes with varying levels of aggression. The Peripheral T-cell lymphoma (PTCL) subtype is highly aggressive, causing medical prognosis to be quite bleak as poor health and low survival rates are standard. Especially in comparison with survival rates for B-cell lymphoma, which has a 5-year survival rate of 30%-40% for its most prevalent subtypes. [2] Canine PTCL is the second most common form of lymphoma diagnosed in dogs, while in humans, it is considered a relatively rare disease. [3] When diagnosed, the first form of treatment for all PTCL is cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) though its efficacy is limited, and the prognosis is still poor. [2] There is an overall understanding that CHOP is the first line of defense, but it is not necessarily the most effective. Scientists have actively worked to improve the efficacy of CHOP through the addition of other drugs to it, such as Romidepsin and Etoposide, but efforts have proven futile. [7, 8]

Though there are many differences between humans and dogs, it has been established that canine PTCL is molecularly similar to human PTCL. [1] Dogs and humans share similarities in their activation of the PI3 kinase pathways, loss of the gene PTEN, and production of the tumor suppressor CDKN2. [1] Also, when humans

and dogs with PTCL are given treatment similar to those for B-cell lymphomas, the results and effects are not substantial.[5] These similarities have made dogs an attractive model for the human form of PTCL as we actively work to understand its occurrence in humans further. [6]

While antibody-based (Abd) immunotherapies have shown success in eliminating chemoresistant cells when tested on B-cell Non-Hodgkin's Lymphoma (NHL), there are special hazards with differentiation antigens when targeting T cells. An issue brought forth by using differentiation antigens to target T cells is that they are not specific to malignant cells. The lack of specificity to T cell lymphomas means the targeted differentiation antigens can be displayed on normal T cells. The attack on healthy and unhealthy cells brings forth extra hazards as there is the possibility causing profound immunodeficiency, similar to Severe Combined Immunodeficiency Disease (SCID). SCID patients lack proper T lymphocyte and B lymphocyte function, leading them not to be able to exhibit T-cell dependent antibody responses and cell-mediated immune responses. [40] Without these responses, individuals with SCID are susceptible to all kinds of infections as their bodies cannot counteract the introduction of bacteria.

It is important to acknowledge that there are different types of antigens, all with varying properties that affect their efficacy for CAR T cells. While there are broad category terms, such as tumor-specific antigens, and tumor-associated antigens, there are more specific categories: differentiation antigens, viral antigens, cancer germline antigens, overexpressed antigens, and antigens affected by mutated genes. Understanding each of these variations makes it more evident why differentiation antigens are ideal for Ab $\alpha$  immunotherapies. For example, a mutated antigen occurs when a neoantigen is formed from an individual's mutated genes. These antigens are unique to each individual's cancer and genes. This specificity is undesired for Ab $\alpha$  immunotherapies as CAR T cells and other Ab $\alpha$  therapies should target proteins shared by cancer patients, not by specific individuals. [14] When looking for ideal antigen types, we want ones that are [9, 20]:

- 1) De-silenced in lymphomas
- 2) Not expressed on normal T cells
- 3) Prevalent in PTCL's
- 4) Shared by cancer patients

Using RNA Sequencing (RNA-Seq), researchers in our laboratory curated a list of potential candidates for a new T-cell target. Antigen name recognition, human gene expression databases, and a review of the cancer literature were used to narrow down

the list of candidates from tens of thousands of transcripts to a few hundred candidates for further analysis. When analyzing, plasma membrane surface proteins were solely considered. Of all human protein-coding genes, only 11% encode proteins that are localized to the plasma membrane. [16] With all these considered, the list was ultimately narrowed down to two genes, PLAC1 and CBY2.

The first candidate is placenta-specific protein 1 (PLAC1), a one-exon gene activated in several malignant tissues, but its expression in tissues is limited to placenta. It was discovered by sequencing near the hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene on the human X-chromosome and was found to be highly conserved to placental mammals. PLAC1 does not have one singular function, as there is evidence that supports that PLAC1 has the ability to affect multiple tumor cell responses and is essential to placental function. [11] A study suggested that PLAC1 is likely not a receptor on its own but an accessory receptor or a receptor-activity-modulating protein (RAMP). While it is unclear which it is, the idea that it has receptor-like functions indicates why PLAC1 has the potential to be helpful for immunotherapy. [10]

The first evidence of PLAC1 expression in a cancer cell was in 2006, and there has been continuous evidence that there is a correlation between the amount of PLAC1

and tumor metastasis. [11] The expression of PLAC1 has been established in multiple cancer cell types, including but not limited to non-small cell lung cancer (NSCLC), hepatocellular carcinoma, breast cancer, colorectal cancer, ovarian cancer, gastric cancer, uterine cancer, and prostate cancer. [11] Since PLAC1's expression is mainly localized to tumor cells; this has made it an attractive candidate for Ab-based immunotherapies. However, it is unclear why so many cancer cells express this gene since it is switched off in most normal tissues. An antibody target is desired to be expressed solely on cancer cells to limit the attack on healthy and unhealthy cells. This double attack on healthy and unhealthy cells is a hazard that comes with using differentiation antigens when targeting T cells. [9] The interest in PLAC1 as a potential target is also linked to its believed role in promoting cancer progression and cell proliferation. By understanding PLAC1's role, position, and effects, there is potential for it to be used as a target for immunotherapy to inhibit the aggressive spread of tumor cells.

The other candidate, Chibby Family Member 2 (CBY2), is a two-exon protein-coding gene with its expression primarily restricted to testis tissues. CBY2 is involved in spermatogenesis, expressed solely during the elongation stage of spermatids. Also, there is a notable absence of expression in mature spermatozoa spermatids. [13] CBY2 enables identical proteins to bind to each other activity. According to the Human

Protein Atlas, CBY2 was expressed and enhanced in multiple cancer cell lines, i.e., urothelial, esophageal, and head and neck cancer. [12] CBY2's multiple expressions of activation in tumor cells and a lack of expression in normal cell tissue have made it an attractive potential candidate for Ab immunotherapies.

In this experimental study, we investigated if either PLAC1 or CBY2 could be a promising CAR T cell targets for dogs. This was done through the use of RNA-Seq and reverse transcription-polymerase chain reaction (RT-PCR) to:

- 1) Determine the prevalence of expression of both genes in canine T-cell lymphomas.
- 2) Determine if both genes are expressed in normal canine tissues, and if so, which ones.
- 3) Determine if they are expressed on the surface of the Canine Histiocytic Sarcoma (HS) cell line DH82 and the T-cell lymphoma line OSW.

The results of these experiments illustrated that PLAC1, but not CBY2, is the better candidate due to the high prevalence in canine PTCL's and limited expression in normal canine tissue.

## **2.2. Materials and Methods**

### **2.2.1 Canine Tissue, Lymphomas & Cell Lines Acquisition and Storage**

Canine PTCL samples were a gift from Dr. Steven Suter. Normal canine tissues were collected from NCSU-CVM Laboratory Animal Resources-owned healthy young adult Beagles and hound-mix dogs that were euthanized for reasons unrelated to our study. Normal testis tissue was obtained following routine elective castration of a healthy young adult mixed-breed dog at NCSU. All tumor and tissue samples were stored individually in RNAlater and at  $-20^{\circ}\text{C}$  until time to prepare cDNA.

The canine HS cell line DH82 and the T-cell lymphoma line OSW were purchased from the American Type Culture Collection (ATCC) and stored in liquid nitrogen until ready to be used.

### **2.2.2 RNA Isolation and cDNA Creation**

RNA was isolated from tissue and lymphoma samples using the Direct-zol RNA MiniPrep Kit, all manufacturer's instructions were followed. RNA was then DNase treated using the TURBO DNA-free<sup>TM</sup> Kit (ThermoFisher Scientific), then the DNase-free RNA was placed in a PCR Thermal Cycler (Eppendorf Mastercycler Nexus) for one hour at  $32^{\circ}\text{C}$ . RNA was inactivated for 5 minutes at room temperature. According to the manufacturer's instructions, the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, California) was used to create cDNA. Two mixtures were created: a reverse

transcriptase (RT) mixture and No RT mixture. No RT was created by omitting RT while using the iScript cDNA Synthesis Kit. The RT and No RT solutions were diluted and stored at -20. RT had a total volume of 200  $\mu$ L with 20  $\mu$ L of cDNA and 180  $\mu$ L of Deionized Water (DI) and No RT had a total volume of 100  $\mu$ L with 20  $\mu$ L cDNA and 80  $\mu$ L of DI.

### 2.2.3 Primer Selection

Primers for both PLAC<sub>1</sub> and CBY2's Endpoint RT-PCR amplification were designed with NCBI/Primer-BLAST. Three potential primers were selected for each, and ultimately Primer Seven was selected for CBY2 and Primer 10 for PLAC<sub>1</sub> (Table 2.1).

**Table 2.1. Primer Selection for PLAC<sub>1</sub> and CBY2** Primers were designed with NCBI/Primer-BLAST and the chosen primer is highlighted in green.

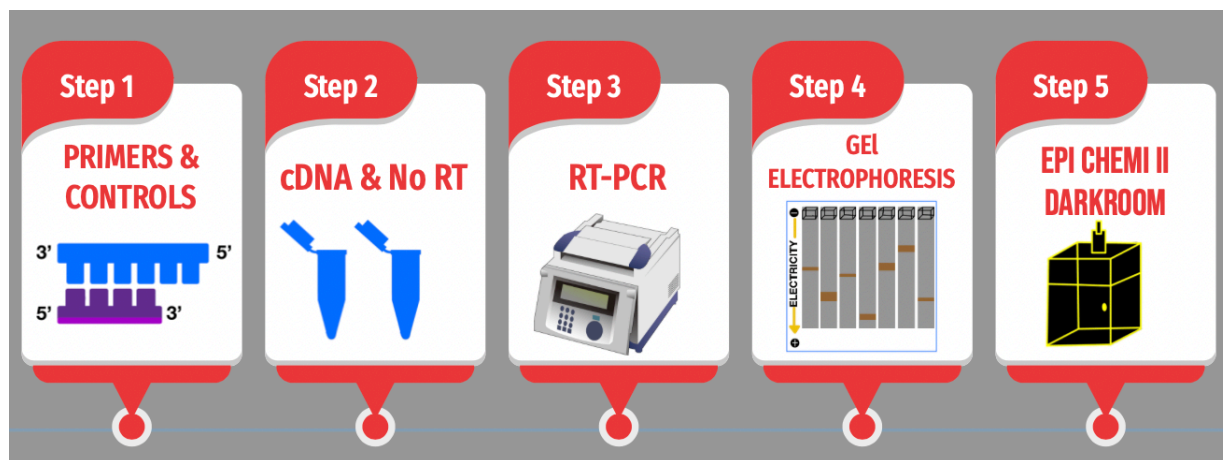
Gene	Primer number	Sequence	Annealing temperature	Ideal Cycling temperatures (NCBI)
CBY2	2	<b>Forward primer (F):</b> GGTGGGTGAACGAAAAC GC <b>Reverse primer (R):</b> GAGGATGCCCTGCTCTC ATC	<b>F:</b> 59.97 <b>R:</b> 59.96	<b>F:</b> 54.97- 56.97 <b>R:</b> 54.96 - 56.97

**Table 2.1. (continued)**

	5	<b>Forward primer (F):</b> TCGGTAACCTGTCTGGGTCT  <b>Reverse primer (R):</b> CATCTCCATTGGGCAGTGA	<b>F:</b> 59.89 <b>R:</b> 59.82	<b>F:</b> 54.89 – 56.89 <b>R:</b> 54.82 – 56.82
	7	<b>Forward primer (F):</b> CACTCCTACCCGCTGAATCG  <b>Reverse primer (R):</b> ATGCCCTGCTCTCATCATGG	<b>F:</b> 60.25 <b>R:</b> 59.89	<b>F:</b> 55.25 – 57.25 <b>R:</b> 54.89 – 56.89
PLAC <sub>1</sub>	3	<b>Forward primer (F):</b> CACATCACCAAGTGGAGGCT  <b>Reverse primer (R):</b>  ACCTGGGGATCACATGGACT	<b>F:</b> 59.96 <b>R:</b> 60.25	<b>F:</b> 54.96 – 56.96 <b>R:</b> 55.25 – 57.25
	4	<b>Forward primer (F):</b> TGACAGCGTCCTGATTCCTG <b>Reverse primer (R):</b>  AGATAGCCTTGGCACGGATG	<b>F:</b> 59.75 <b>R:</b> 59.89	<b>F:</b> 54.75 – 56.75 <b>R:</b> 54.89 –  56.89
	10	<b>Forward primer (F):</b> AGTTCACCTACCGCGTTACC <b>Reverse primer (R):</b>  CGGCCTCACTGGCTACTTTT	<b>F:</b> 59.76 <b>R:</b> 60.32	<b>F:</b> 54.76 – 56.76 <b>R:</b> 55.32 – 57.25

For Endpoint RT-PCR, all primers were tested using the Hotstar HiFidelity Polymerase Kit with Q solution (Figure 2.1). According to the manufacturer's instructions, each reaction was performed in 25  $\mu$ L reaction volumes, including 1  $\mu$ L of cDNA and 2.5  $\mu$ L of each forward and reverse primer. For PLAC<sub>1</sub> primers, placenta

tissue was used as the positive control and DI was used as the negative. For CBY2 primers, testis was used as the positive control, and DI was used as the negative.



**Figure 2.1. Experiment Design for Endpoint RT-PCR.** RT-PCR was used to amplify gene fragments.

Each reaction for PLAC<sub>1</sub> was put into a PCR Thermal Cycler (Eppendorf Mastercycler Nexus) using the program cycle PCR Apex 56 program (Table 2.2.) Each reaction for PLAC<sub>1</sub> was put into a PCR Thermal Cycler (Eppendorf Mastercycler Nexus) using the program cycle PCR Apex 55 program (Table 2.3). PLAC<sub>1</sub> reaction products were electrophoresed on a 2.5% agarose gel, and CBY2 reaction products were electrophoresed on a 1% agarose gel containing GelRed Nucleic Acid Stain at 130V for 45 minutes and visualized and photographed using an Epi Chemi II Darkroom.

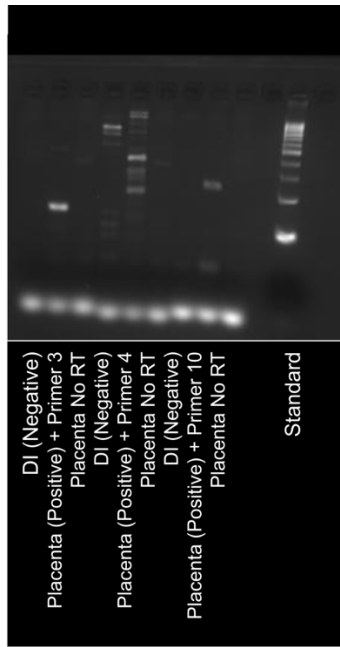
**Table 2.2. Cycler Conditions for PLAC1 RT-PCR** Temperatures for each stage were selected according to the Ideal Cycling temperatures listed in Table 2.1.

<b>PLAC1</b>	<b>Stage 1</b>	<b>Stage 2</b>	<b>Stage 3</b>	<b>Stage 4</b>	<b>Stage 5</b>
Temperature	95 degrees	95 degrees	57 degrees	72 degrees	72 degrees
Time	15 minutes	30 seconds	30 seconds	1 minute	5 minutes
Cycles	1	X 40	X 40	X 40	1

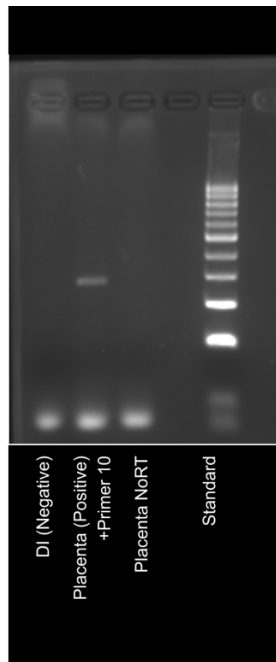
**Table 2.3. Cycler Conditions for CBY2 RT-PCR** Temperatures for each stage were selected according to the Ideal Cycling temperatures listed in Table 2.1.

<b>CBY2</b>	<b>Stage 1</b>	<b>Stage 2</b>	<b>Stage 3</b>	<b>Stage 4</b>	<b>Stage 5</b>
Temperature	95 degrees	95 degrees	55 degrees	72 degrees	72 degrees
Time	15 minutes	30 seconds	30 seconds	1 minute	5 minutes
Cycles	1	X 35	X 35	X 35	1

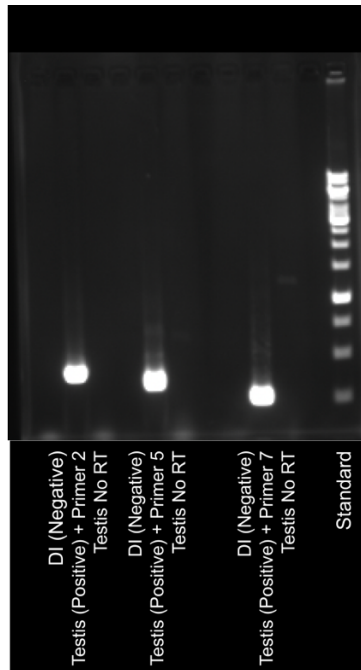
Initially, Primer Three was selected as the PLAC1 primer to test the normal canine tissue and lymphoma samples (Figure 2.2.). Upon further testing on lymphoma and tissue samples, the quality of Primer Three degraded. Ultimately, Primer 10 was determined to be more efficient at optimizing the PCR results (Figure 2.3.). Ultimately, for CBY2, Primer Seven was selected as the primer to test (Figure 2.4.)



**Figure 2.2. Expression of PLAC<sub>1</sub> Primer RT-PCR.** PCR results visualized and photographed using an Epi Chemi II Darkroom



**2.3. Expression of PLAC<sub>1</sub> Primer 10.** Primer 10 PCR results visualized and photographed using an Epi Chemi II Darkroom.



**2.4. Expression of CBY2 Primer RT-PCR.** PCR results visualized and photographed using an Epi Chemi II Darkroom.

#### 2.2.4 Canine Tissue, Lymphomas & Cell Lines RT-PCR

Endpoint RT-PCR was performed on the normal canine tissue and lymphoma samples using the Hotstar HiFidelity Polymerase Kit with Q solution. (Figure 2.1) According to the manufacturer's instructions, each reaction was performed in 25  $\mu$ L reaction volumes, including 1  $\mu$ L of cDNA and 2.5  $\mu$ L of each forward and reverse primer. For PLAC<sub>1</sub>, placenta tissue was used as the positive control, and DI was used as the negative. For CBY<sub>2</sub>, testis was used as the positive control, and DI was used as the negative. Each reaction for PLAC<sub>1</sub> was put into a PCR Thermal Cycler (Eppendorf Mastercycler Nexus) using the program cycle PCR Apex 56 program (Table 2.2.). Each

reaction for PLAC<sub>1</sub> was put into a PCR Thermal Cycler (Eppendorf Mastercycler Nexus) using the program cycle PCR Apex 55 program (Table 2.3). PLAC<sub>1</sub> reaction products were electrophoresed on a 2.5% agarose gel, and CBY2 reaction products were electrophoresed on a 1% agarose gel containing GelRed Nucleic Acid Stain at 130V for 45minutes and visualized and photographed using an Epi Chemi II Darkroom.

### **2.2.5 Cell Lines Maintenance**

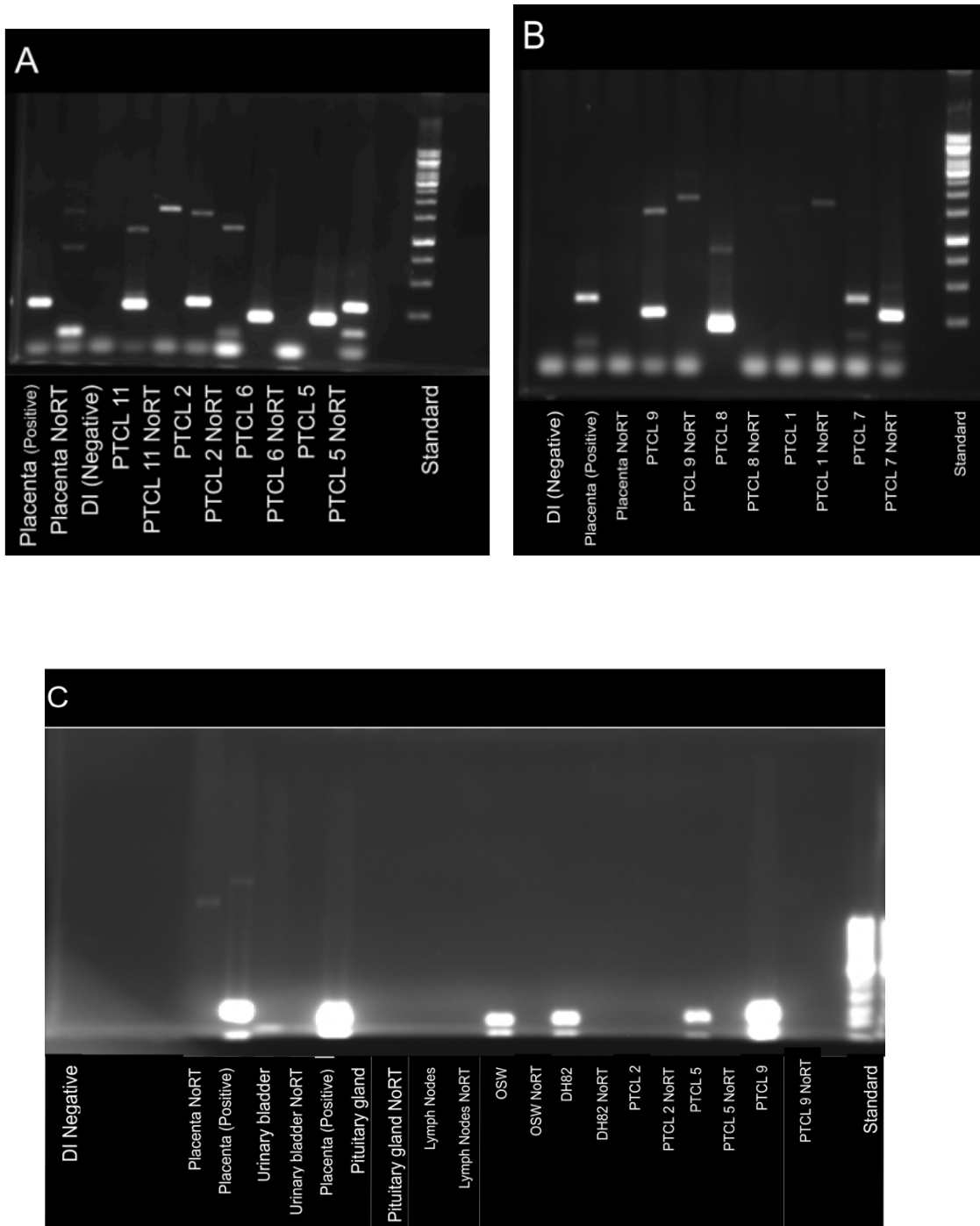
Once removed and thawed from the liquid nitrogen, each cell line was grown in their specific media solutions. The T-cell lymphoma line OSW was grown in Roswell Park Memorial Institute (RPMI) medium also containing 10% Fetal Bovine Serum (FBS), Penicillin/ Streptomycin (P/S) (100x), and L-glutamine in T25 Flasks. The canine HS cell line DH82 was grown in Dulbecco's Modified Eagle's Medium containing 15% FBS, and P/S in T75 Flask. Cell lines were incubated at 37 degrees and monitored until ready to be split. OSW was split every 3-4 days at 1:5 (cells: media) once the cell count reached about 1 million. Due to their adherent nature, DH82 was split every 6-7 days using trypsinization. Trypsin was added to cell culture for two minutes, the media solution was added then cells were split at 1:10 (cells: media).

## **2.3 Results**

### **2.3.1 Endpoint RT-PCR data for PLAC1 Expression**

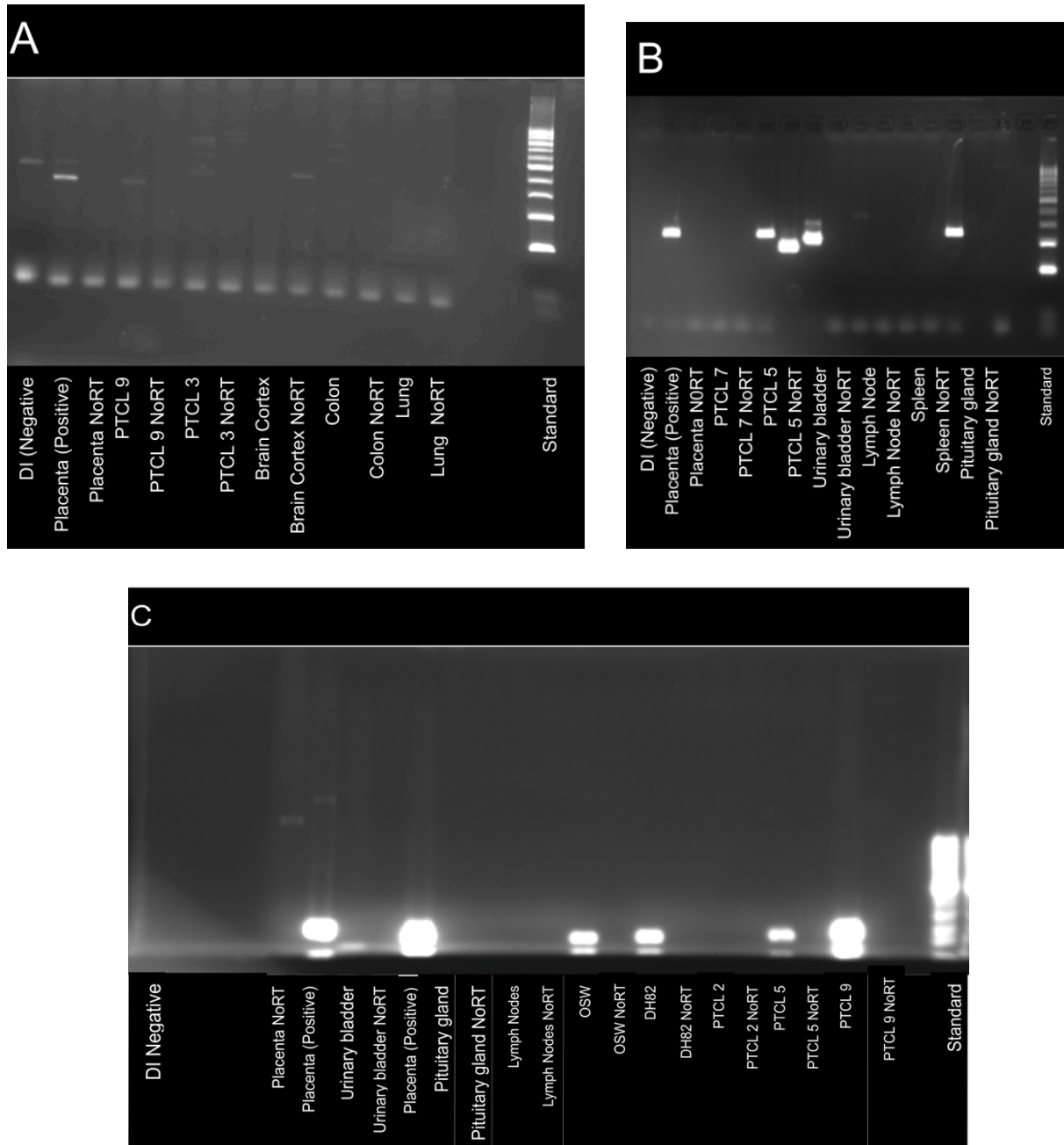
RT-PCR was performed on eight lymphoma samples, eight normal canine tissues – Placenta, Lung, Colon, Brain Cortex, Urinary Bladder, Lymph Nodes, Spleen, and Pituitary Gland – and two cell lines, canine HS cell line DH82 and T-cell lymphoma line OSW, to evaluate the expression of PLAC1. The positive control for PLAC1 was established as placenta, due to its primary expression in the placenta, and the negative control was DI water only.

Next, the results of the RT-PCR samples were visualized through gel electrophoresis and captured using low light Fluorescence Imaging. Through a course of three RT-PCR runs, expression of PLAC1 was evident in seven lymphoma samples – PTCL 3, PTCL 5, PTCL 6, PTCL 8, PTCL 9, PTCL 11, and PTCL 14 (Figure 2.5., Supplementary Table 1) PLAC1 was expressed in OSW and DH82 (Figure 2.5 C).



**Figure 2.5. Expression of PLAC1 Lymphoma RT-PCR.** A total of eight different samples were tested.

During the first two runs of RT-PCR, there was positive expression in three tissue samples – placenta, pituitary gland, and urinary bladder. (Figure 2.6A and 2.6B, Supplementary Table 2) After a third round of RT-PCR, only placenta tissue expressed PLAC1. (Figure 2.6C) Figures 2.5C and 2.6C are the same image as both canine normal tissues and lymphoma samples were run on the same gel.

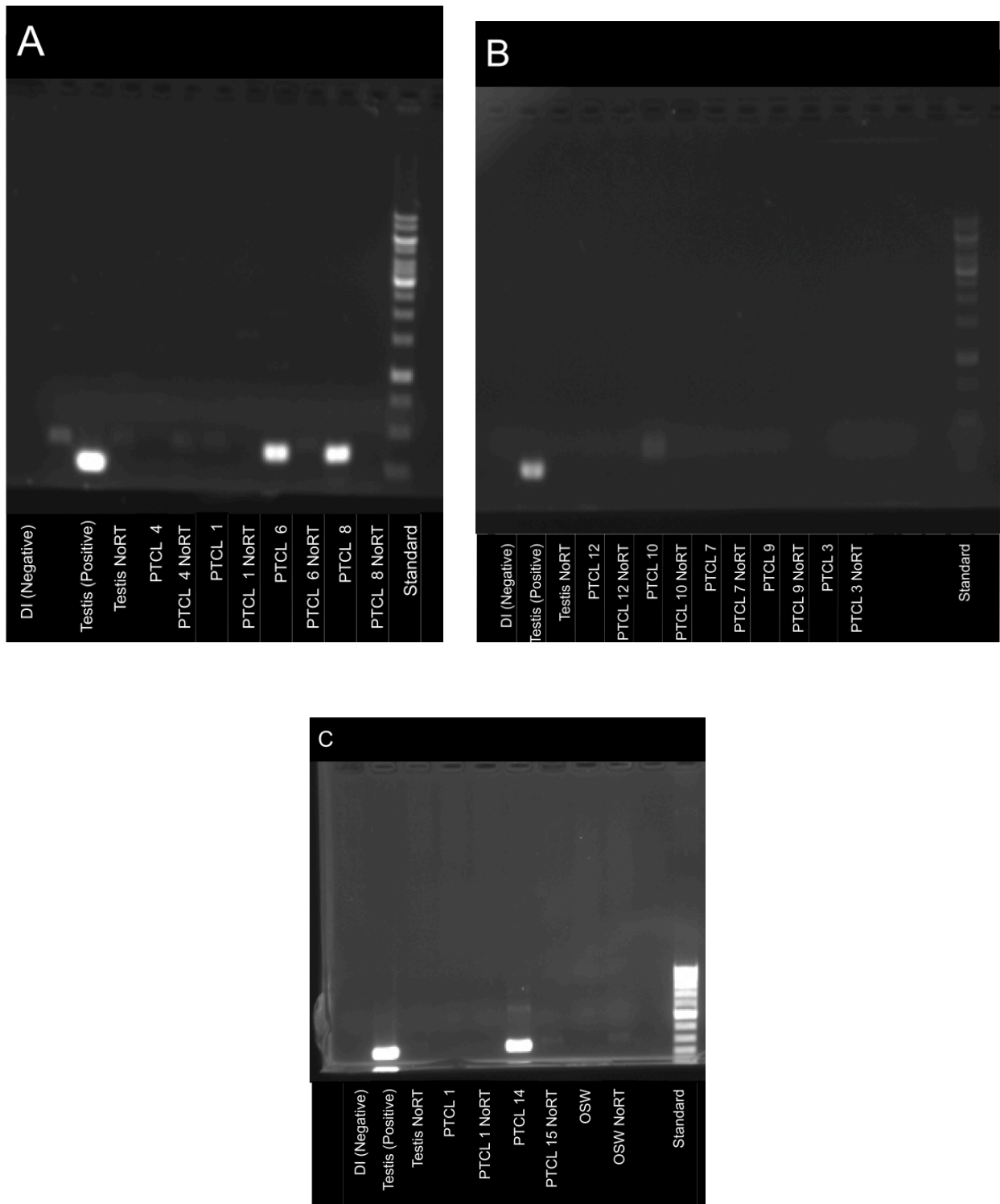


**Figure 2.6. Expression of PLAC<sub>1</sub> Normal Tissue RT-PCR.** A total of eight different tissues were tested - Placenta, Brain Cortex, Colon, Lung, Pituitary Gland, Urinary Bladder, Spleen and Lymph Nodes.

### **2.3.2 Endpoint RT-PCR data for CBY2 Expression**

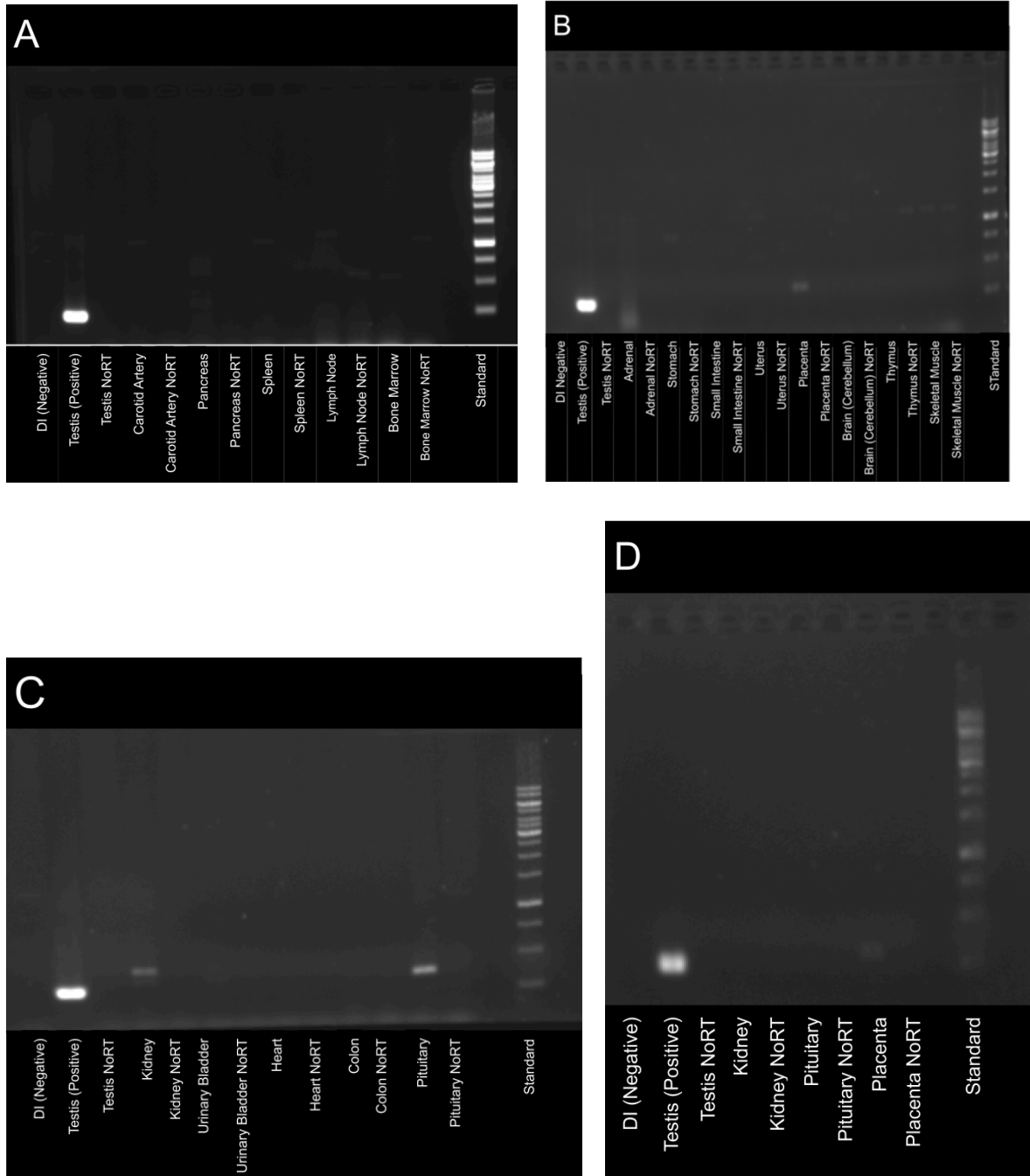
RT-PCR was performed on ten lymphoma samples, 19 normal canine tissues – Testis, Carotid Artery, Pancreas, Spleen, Lymph Nodes, Bone Marrow, Adrenal Gland, Stomach, Small Intestine, Uterus, Placenta, Cerebellum, Thymus, Skeletal Muscle, Kidney, Urinary Bladder, Heart, Colon, and Pituitary Gland – and one cell line, T-cell lymphoma line OSW, to evaluate the expression of CBY2. The positive control for CBY2 was established as testis, due to its involvement in the spermatogenesis, and the negative control was Deionized Water (DI) only.

Using low-light Fluorescence Imaging and gel electrophoresis, the RT-PCR samples were visualized. Through a course of three RT-PCR runs, there was CBY2 expression in three lymphoma samples – PTCL 6, PTCL 8, and PTCL 14 (Figure 2.7., Supplementary Table 3) CBY2 was not expressed in OSW (Figure 2.7 C).



**Figure 2.7. Expression of CBY2 Lymphoma RT-PCR.** A total of ten different samples were tested.

During the first two runs of RT-PCR, there was positive expression in three tissue samples – Testis, Placenta, Pituitary Gland, and Kidney (Figure 2.8, Supplementary Table 4). During a third round of testing, Pituitary Gland, Placenta, and Kidney were rerun. Pituitary Gland and Kidney were determined to be false positives and Placenta was a true positive (Figure 2.8D).



**Figure 2.8. Expression of CBY2 Normal Tissue RT-PCR.** A total of 19 normal canine tissues – Testis, Carotid Artery, Pancreas, Spleen, Lymph Nodes, Bone Marrow, Adrenal Gland, Stomach, Small Intestine, Uterus, Placenta, Cerebellum, Thymus, Skeletal Muscle, Kidney, Urinary Bladder, Heart, Colon, and Pituitary Gland– were tested.

## 2.4 Discussion

Other antigens have already been investigated, allowing for the creation of monoclonal antibodies (mAbs) to be used therapeutically in human cancer patients. For example, Alemtuzumab, a mAb specifically aimed to target CD52 antigen on T-lymphocytes, has been approved to treat several cancers, including T-cell prolymphocytic leukemia and cutaneous T-cell lymphoma. [17] Brentuximab vedotin, a mAb specifically aimed to target CD30, has been approved to treat classical Hodgkin Lymphoma. [18] Both of the drugs have shown efficacy against several T-cell tumors. On the other hand, there is a significant lack of mAbs when it comes to dogs. Tactress, a mAb that targets CD52, is one of the few US Department of Agriculture-approved mAbs for dogs but is no longer available [19] The exploration of potential new targets for CAR T-cells has allowed for the creation of a list of specific properties that make an ideal candidate for CAR Ts. They should be:

- 1) De-silenced in lymphomas
- 2) Unexpressed on normal T cells
- 3) Prevalent in PTCL's
- 4) Shared by cancer patients

With this understanding, a proper analysis of the results for PLAC1 and CBY2 was conducted.

Multiple lymphoma samples expressed CBY2, but due to placenta tissue repeatedly being expressed by CBY2, it was ultimately decided that it would not be the ideal candidate to move forward. PLAC1, on the other hand, was expressed by extensively more lymphoma samples and failed to be expressed by any other normal canine tissue samples. PLAC1 was determined to be the ideal candidate among the two. The next step would be to conduct antibody screening using flow cytometry. We need to conduct screening because:

1. Need to ensure that RNA was translated
2. Need to ensure that PTCLs make PLAC1 protein
3. Need to ensure that PLAC1 protein is on the surface of most or all of the cells

Screening is crucial because even if we see PLAC1 signal in the bulk RNA from the PTCLs, we don't know what percent of cancer cells express it on the surface. The amount of expression matters because if only 10% express it, then the other 90% are able to evade immunotherapy then the therapy won't be very effective.

This screening is easily done if you have an antibody. The first problem is that there isn't an anti-dog PLAC1 antibody available, but we were able to find one promising antibody against human PLAC1. Fortunately, with the human antibody, we were told what part of PLAC1 protein they immunized the rabbits. The immunogen comprises amino acids 22-84, so can compare that stretch between humans and dogs.

When the immunogen from the human protein was aligned with the canine ortholog, there were only four differences out of 62 amino acids, so 93% homologous. This similarity might be acceptable, but if any of those different amino acids are key to binding, it may not work at all. There is some risk in this experiment, but the payoff could be high:

**The pros of the approach are:**

- High similarity between human and dog PLAC<sub>1</sub>
- Polyclonal antibody – so really a mixture of antibody specificities – so even if one fails in the mix, another may work

**The cons of the approach are:**

- The antibody has been used for immunohistochemistry, not flow cytometry
- We don't have a true positive control – human placental tissue
- It is hard to obtain canine PTCL cells from patients

We have high hopes that this antibody will be able to detect PLAC<sub>1</sub>, but if not, there are multiple plausible reasons for this failure. First, the antibody against human PLAC<sub>1</sub> was not as good a fit as we thought it would be. In that case, we would try another antibody. Though we had other options, we believe the antibody we selected has the best chance of success. Another reason is that PLAC<sub>1</sub> is not on the surface as

we believed. If so, PLAC<sub>1</sub> cannot be used as a CAR T cell target. For the antibody to bind the antigen it is searching for, that antigen must be on the cancer cell's surface.

## 2.5 List of Abbreviations

American Type Culture Collection	ATCC
Antibody-Based	Abd
Chibby Family Member 2	CBY2
Cyclophosphamide, doxorubicin hydrochloride, vincristine (Oncovin), and prednisone	CHOP
Deionized Water	DI
Fetal Bovine Serum	FBS
Histiocytic Sarcoma	HS
Hypoxanthine-guanine phosphoribosyl transferase	HPRT
Non-Hodgkin Lymphomas	NHL
Non-small cell lung cancer	NSCLC
Penicillin/ Streptomycin	P/S
Peripheral T-cell Lymphoma	PTCL
Placenta-specific protein 1	PLAC <sub>1</sub>
Receptor-activity-modulating protein	RAMP

Reverse transcriptase

RT

Reverse transcription-polymerase chain reaction

RT-PCR

RNA Sequencing

RNA-Seq

Roswell Park Memorial Institute

RPMI

## 2.6 References

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## 2.7 Supplementary Tables

**Supplementary Table 1. PLAC<sub>1</sub> Lymphoma RT-PCR runs.** A total of eight different samples were tested through three RT-PCR runs.

<b>Name</b>	<b>Run #1</b>	<b>Run#2</b>	<b>Run#3</b>
<b>PTCL 11</b>	Positive	Not Tested	Not Tested
<b>PTCL 14</b>	Positive	Not Tested	Not Tested
<b>PTCL 6</b>	Positive	Not Tested	Not Tested
<b>PTCL 5</b>	Positive	Positive	Positive
<b>PTCL 9</b>	Positive	Positive	Positive
<b>PTCL 8</b>	Positive	Not Tested	Not Tested
<b>PTCL 1</b>	Negative	Not Tested	Not Tested
<b>PTCL 3</b>	Not Tested	Positive	Negative
<b>PTCL 7</b>	Negative	Negative	Not Tested
<b>DH82</b>	Not Tested	Not Tested	Positive
<b>OSW</b>	Not Tested	Not Tested	Positive

**Supplementary Table 2. PLAC<sub>1</sub> Normal Tissue RT-PCR runs.** A total of eight different samples were tested - Placenta, Brain Cortex, Colon, Lung, Pituitary Gland, Urinary Bladder, Spleen and Lymph Nodes.

<b>Normal Tissue</b>	<b>Run #1</b>	<b>Run #2</b>	<b>Run #3</b>
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**Supplementary Table 2. (continued)**

<b>Placenta (positive control)</b>	Positive	Positive	Positive
<b>Brain Cortex</b>	Negative	Not Tested	Not Tested
<b>Colon</b>	Negative	Not Tested	Not Tested
<b>Lung</b>	Negative	Not Tested	Not Tested
<b>Urinary Bladder</b>	Positive	Positive	Negative
<b>Pituitary Gland</b>	Positive	Positive	Negative
<b>Spleen</b>	Negative	Not Tested	Not Tested
<b>Lymph Nodes</b>	Negative	Not Tested	Negative

**Supplementary Table 3. CBy2 Lymphoma RT-PCR runs.** A total of ten different samples were tested through two RT-PCR runs.

<b>Name</b>	<b>Run #1</b>	<b>Run#2</b>
<b>PTCL 13</b>	Negative	Not Tested
<b>PTCL 1</b>	Negative	Negative
<b>PTCL 14</b>	Not Tested	Positive
<b>PTCL 6</b>	Positive	Not Tested
<b>PTCL 8</b>	Positive	Not Tested
<b>PTCL 12</b>	Negative	Not Tested
<b>PTCL 10</b>	Negative	Not Tested
<b>PTCL 7</b>	Negative	Not Tested
<b>PTCL 9</b>	Negative	Not Tested

**Supplementary Table 3. (continued)**

<b>PTCL 3</b>	Negative	Not Tested
<b>OSW</b>	Not Tested	Negative

**Supplementary Table 4. CBY2 Normal Tissue RT-PCR runs.** A total of 19 normal canine tissues – Testis, Carotid Artery, Pancreas, Spleen, Lymph Nodes, Bone Marrow, Adrenal Gland, Stomach, Small Intestine, Uterus, Placenta, Cerebellum, Thymus, Skeletal Muscle, Kidney, Urinary Bladder, Heart, Colon, and Pituitary Gland– were tested through three runs.

<b>Tissue Name</b>	<b>Run #1</b>	<b>Run #2</b>	<b>Run #3</b>
<b>Testis (positive control)</b>	Positive	Positive	Positive
<b>Carotid Artery</b>	Negative	Negative	Negative
<b>Pancreas</b>	Negative	Negative	Negative
<b>Spleen</b>	Negative	Negative	Negative
<b>Lymph Nodes</b>	Negative	Negative	Negative
<b>Bone Marrow</b>	Negative	Negative	Negative
<b>Adrenal gland</b>	Negative	Negative	Negative
<b>Stomach</b>	Negative	Negative	Negative
<b>Small Intestine</b>	Negative	Negative	Negative
<b>Uterus</b>	Negative	Negative	Negative
<b>Placenta</b>	Weak Positive	Weak Positive	Weak Positive
<b>Cerebellum</b>	Negative	Negative	Negative
<b>Thymus</b>	Negative	Negative	Negative

**Supplementary Table 4. (continued)**

<b>Skeletal Muscle</b>	Negative	Negative	Negative
<b>Kidney</b>	Negative	Weak Positive	Negative
<b>Urinary Bladder</b>	Negative	Negative	Negative
<b>Heart</b>	Negative	Negative	Negative
<b>Colon</b>	Negative	Negative	Negative
<b>Pituitary Gland</b>	Positive	Weak Positive	Negative