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## Utilizing FIV (Feline Immunodeficiency Virus) to develop a novel animal model to study HIV (Human Immunodeficiency Virus)

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Microbiology and Immunology

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## Abstract

This project sought to perform the *in vitro* work needed to accomplish the long-term vision of harnessing the similarities between HIV (Human Immunodeficiency Virus) and FIV (Feline Immunodeficiency Virus) to develop an animal model whereby cats can be used to study HIV pathogenesis and therapeutics. We transfected CRFK (Crandell Rees Feline Kidney) fibroblasts with plasmids that could express human or feline CD4, CCR5, or both, and determined receptor surface expression through flow cytometry. We discovered that HIV envelope expressed on 293T can fuse with huCD4/huCCR5 on CRFK. These cat cell lines were also capable of supporting HIV infection. Additionally, we evaluated whether the yeast recombination system could be used to clone FHIV<sub>env</sub> chimeras wherein the HIV *env* is inserted into the FIV backbone in place of FIV *env*. In the future, FHIV<sub>env</sub> adapted to replicate in the cat cell lines produced herein, can be tested in *in vivo* in cats.

## Keywords

FIV, HIV, CD4, CCR5, Cell fusion, Envelope, Cat, FHIV, FHIV<sub>env</sub>

## Summary for Lay Audience

The lack of suitable, cost-effective animal models remains a challenge in studying HIV (Human Immunodeficiency Virus). Cats are natural hosts for FIV (Feline Immunodeficiency Virus), which is similar in structure and pathogenesis to HIV. This project's goal was to establish the *in vitro* work needed to accomplish the long-term vision of utilizing cats *in vivo* to study HIV therapeutics and vaccines. The *in vitro* work involves creating chimeric viruses as well as engineering the cell lines required for testing viral envelope interactions with entry receptors. These viruses and cell lines are also required for the infectivity assays. We sought to execute this through the creation of FHIV<sub>env</sub> chimeras composed of HIV envelope genes inserted into the FIV genome, in place of FIV envelope. We attempted several DNA cloning strategies to accomplish this. We also engineered CRFK (Crandell Rees Feline Kidney) Fibroblast cell lines to express human and feline CD4 and CCR5 and tested the receptor expression level on their surface. We were able to achieve higher human CD4/CCR5 expression than feline CD4/CCR5 expression on independent CRFK cell lines. Human CD4 and CCR5 are typically used by HIV envelope to enter and infect human cells and we found that not only can HIV envelope proteins from diverse HIV subtypes and strains bind to human CD4 and CCR5 expressed on cat cells, HIV-AD8 (a subtype B virus) can also use these receptors to enter and infect cat cells. In the future, receptor-envelope interactions can be tested between these diverse HIV envelopes and the engineered feline CD4/CCR5 CRFK. The envelopes that are capable of interacting with these feline receptors can be cloned into FHIV<sub>envs</sub>, following which infectivity assays can be conducted, where the chimeras can be used to infect feCD4/feCCR5 CRFK. The eventual goal is to test these chimeras *in vivo* in cats. If they are pathogenic, infectious, and replication-competent *in vivo*, cats can be utilized as model organisms to develop and test therapeutics and vaccine strategies targeting the HIV envelope.

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## List of Common Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
ART	Anti-retroviral Therapy
CCR5	C-C Chemokine Receptor Type 5
cDNA	Complementary DNA
CD4	Cluster of Differentiation 4
CD134	Cluster of Differentiation 134
Cplt	Complement
CXCR4	C-X-C Chemokine Receptor Type 4
DNA	Deoxyribonucleic Acid
FACS	Fluorescence Activated Cell Sorting
feCCR5	Feline C-C Chemokine Receptor Type 5
feCD4	Feline Cluster of Differentiation 4
FIV	Feline Immunodeficiency Virus
FHIV	Feline-Human Immunodeficiency Virus
FHIV <sub>env</sub>	Feline-Human Immunodeficiency Virus (envelope)
FMO	Fluorescence Minus One
HIV	Human Immunodeficiency Virus
huCCR5	Human C-C Chemokine Receptor Type 5
huCD4	Human Cluster of Differentiation 4
LTR	Long Terminal Repeat
MFI	Mean Fluorescence Intensity
MOI	Multiplicity of Infection
Nfl	Near-full-length

NRTI	Nucleoside Reverse Transcriptase Inhibitors
NNRTI	Non-nucleoside Reverse Transcriptase Inhibitor
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RRE	Rev Response Element
RT	Reverse Transcriptase
SIV	Simian Immunodeficiency Virus
SHIV	Simian-Human Immunodeficiency Virus

# CHAPTER 1: INTRODUCTION

## 1.1 Overview

Worldwide, at the end of 2018, 36.9 million people were living with the Human Immunodeficiency Virus (HIV), the majority of whom reside in sub-Saharan Africa. Almost one million individuals lost their lives within the same year due to HIV-related illnesses<sup>1</sup>. In Canada, by the end of 2014, 75,500 individuals were living with HIV and/or AIDS (Acquired Immune Deficiency Syndrome)<sup>2</sup>. These statistics reveal that HIV continues to be a global healthcare crisis.

Currently, anti-retroviral therapy (ART) regimens are widely used to control HIV replication and plasma viral load. However, these require strict adherence to the ART regimen and a lack thereof can promote viremia and increase the development of drug-resistant HIV populations<sup>3</sup>. Furthermore, access to treatment still remains a challenge due to stigma, gender disparities where women are disproportionately affected, and socioeconomic factors<sup>4</sup>. These drugs are also prohibitively expensive in developing countries<sup>5</sup>, which is even more problematic as these areas are where populations that are most vulnerable to the disease are situated. Due to this, research efforts have shifted to create vaccines, and have been undertaken globally to prevent and eradicate the infection. Despite these attempts, we do not currently have an effective vaccine to tackle HIV infections. Vaccine trials such as RV144 have shown promise<sup>6</sup> as the vaccine efficacy was close to 60% in the first year of administration. However, this decreased to 31.2% over the course of 3.5 years<sup>7,8</sup>. Therefore, further investigation needs to be undertaken to elicit an effective, durable, and long-lasting protective immune response. Thus, it is imperative to continue studying novel vaccine strategies.

A major barrier to generating a successful vaccine against HIV is the lack of ideal animal models. Prior studies have exploited animals ranging from rodents, such as ‘humanized’ mice and rats, to non-human primates including macaques and chimpanzees. The major issues with using these organisms are that they are extremely expensive to produce, obtain, and house<sup>9-11</sup>, and inoculation with HIV does not recapitulate the same extent of disease observed in humans.

Cats, on the other hand, are natural hosts for Feline Immunodeficiency Virus (FIV), which is very similar in genomic structure and pathogenesis to HIV<sup>12</sup>. Additionally, they provide a more cost-effective avenue than non-human primates. Our long-term vision is to establish a novel animal model that utilizes cats to study HIV pathogenesis and vaccine development. If successful, the novel system could be used in future to develop and test HIV vaccines using cats as model organisms. This project was geared towards generating and evaluating the cell lines and viruses to test the validity of this model *in vitro*.

## 1.2 Animal models for HIV research

### 1.2.1 Mouse models

The majority of small animal models have utilized “humanized” mice that are genetically immunocompromised and engrafted with human tissue. Variations of severe combined immunodeficiency (SCID) mice that lack functional B and T cells have been used<sup>9,13</sup>. One such variation makes use of *scid*-hu-PBL (hu-PBL-SCID) mice, where *scid* mice are injected with human peripheral blood lymphocytes (PBL), followed by intraperitoneal injection with HIV<sup>14</sup>. These mice can also respond to passive immunization with human antibodies, which confer protection upon being challenged with HIV-1<sup>15,16</sup>. This makes them good candidates to study the neutralizing antibody response during infections. Alternatively, SCID-hu-Thy/Liv mice produced by transplanting fetal human thymus and liver cells into SCID mice are capable of producing human hematopoietic progenitor stem cells and thymocytes, eventually leading to the circulation of human T cells in peripheral blood. When inoculated with HIV, they present rapid CD4+ T cell loss and increased viral loads, which is a hallmark of HIV infection in humans. Thus, the model can be used to study HIV tropism, cellular pathogenesis, and CD4+ T cell loss<sup>17</sup>. A disadvantage of both these mouse models is that they require direct injection of HIV and mucosal transmission cannot be tested<sup>9</sup>.

In addition to the two models above, NOD (Non-obese diabetic) *scid* mice that lack NK cells and proper complement activation can also be crossed with IL2rg<sup>-/-</sup> mice that contain a mutation in the interleukin-2 receptor common  $\gamma$ -chain to produce NSG

mice (NOD *scid* gamma) or NOG mice (NOD/Shi-*scid* IL2rg<sup>null</sup>). As the IL2rg protein is implicated in immune cell development, crossing IL2rg<sup>-/-</sup> and NOD *scid* mice results in mice that lack functional immune cells. NOD/SCID/IL2rg<sup>null</sup> mice can be transplanted with CD34+ hematopoietic stem cells from cord blood, which then produce human B cells and T cells<sup>18</sup>. NSG mice have successfully been infected with HIV and have also served as model organisms to test cART (combinational ART) to suppress plasma viremia, as well as HIV latency<sup>19</sup>.

Bone marrow-liver-thymus (BLT) mice are derived from NOD *scid* and NSG mice that are engrafted with fetal human thymus and liver cells. This is followed by a bone marrow graft through the introduction of CD34+ hematopoietic progenitor cells. These mice develop a thymus where human thymocytes are educated<sup>20</sup>. The advantages to this model are that T cells mature in the implanted thymus, mimicking T cell development in humans, and it is possible to infect them with HIV via mucosal routes<sup>9</sup>.

Mouse models are valuable as these organisms are easy to access due to being widely available. This allows for larger sample sizes that could produce more reliable statistical results. However, these models do not recapitulate a fully functional human immune system, making them less reflective of HIV pathogenesis in humans. Additionally, the possibility of studying tissue-specific infection and latent reservoirs is limited in these models, as human immune cells do not fully engraft in mouse organs. Lastly, producing these mice through surgical engraftment and maintaining them is very expensive<sup>9</sup>.

### 1.2.2 Non-human primate models: Simian Immunodeficiency Virus (SIV)

Several different Simian Immunodeficiency Virus (SIV) strains are endemic to non-human primates. Phylogenetic analysis has also revealed that HIV-1 is a direct descendant of SIV<sub>cpz</sub> that typically infects the chimpanzee species *Pan troglodytes troglodytes*<sup>9,21</sup>. HIV-2, on the other hand, is more closely related to SIV<sub>smm</sub> isolated from sooty mangabeys (Figure 1).

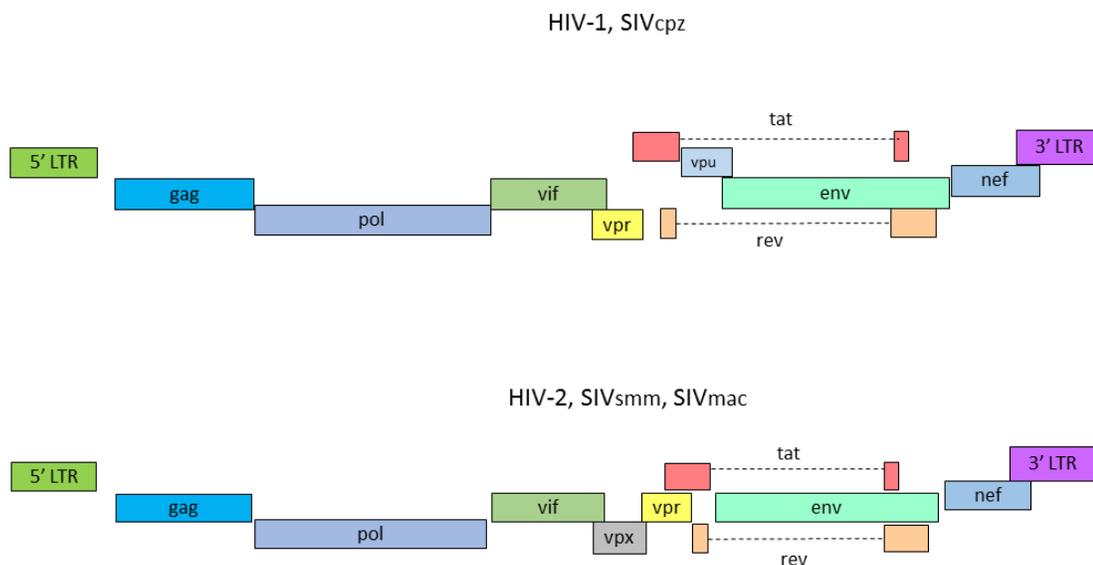


Figure 1. Comparison of SIV and HIV genomes. HIV-1 is a descendent of SIV<sub>cpz</sub> and the two share the same structural and accessory genes. HIV-2, SIV<sub>smm</sub> and SIV<sub>mac</sub> are closely related and share the same structural and functional genes. HIV-2 lacks the *vpu* accessory gene but instead encodes *vpx*, another accessory gene.

Upon inoculation with HIV-1, chimpanzees evolve similar symptoms to humans infected with this virus. These include the establishment of persistent infection, virus presence in peripheral blood, as well as cellular and humoral immune responses directed against HIV. Despite this, chimpanzees rarely develop AIDS<sup>21</sup>. A 1997 study by Novembre et al. described the development of AIDS in a chimpanzee infected with HIV-1. This was associated with the loss of CD4+ T cells, increased plasma viral loads, and opportunistic infections. However, this was the case with only one of the twelve chimpanzees that were inoculated with several strains of HIV-1 in the mid-1980s. Sequence analysis revealed that in the chimpanzee that developed AIDS, there was a drastic amount of divergence between the initial inoculation strain and the prevalent strain during acute infection. This was indicative of adaptive mutations that occurred in the virus over time, which also resulted in increased pathogenicity<sup>22</sup>. Nevertheless, HIV-induced pathogenesis and progression to AIDS in HIV-infected chimpanzees is a rare occurrence<sup>9,22,23</sup>, which reduces their suitability to serve as model systems for HIV study. In addition to this, other logistical reasons including cost, endangered status<sup>9,21</sup>, and

ethical considerations<sup>11</sup> pose challenges in utilizing them as model organisms.

The majority of primate species cannot be infected by HIV-1. Since SIV is closely related to HIV, it is instead used widely in studies involving primates to evaluate pathogenesis and immune responses upon infection. The advantage of using these organisms is that in addition to direct injection, they can be infected through vaginal or rectal routes that are comparable to sexual transmission or through oral routes, which models maternal transmission via breast milk in humans<sup>11</sup>. Physiologically, the structure of reproductive and gastrointestinal mucosa of macaques and humans are similar and serve as important sites for HIV infection and replication. The most common primate species used are rhesus macaques (*Macaca mulatta*), pig-tailed macaques (*Macaca nemestrina*), and cynomolgus macaques (*Macaca fascicularis*)<sup>9</sup>.

SIV infection has several similarities to HIV infection in humans including increased viral load and replication, some loss of lymphocytes, and immune activation. However, depending on the strain of SIV used, infection is not necessarily fatal. Natural macaque hosts do not have progressive loss of CD4+ T cells, chronic immune infection<sup>24</sup>, or total deterioration of lymph nodes<sup>9</sup>, indicating that these infections do not completely mimic HIV infection. There are certain differences between HIV and SIV at the genetic and structural level. For example, both SIV and HIV use CD4 (Cluster of Differentiation 4) as their primary receptors for entry. They also predominantly use CCR5 (C-C Chemokine Receptor Type 5) as their co-receptors. However, HIV can also gain the ability to utilize CXCR4 (C-X-C Receptor Type 4) as its co-receptor, later in infection; a phenomenon rarely seen in SIV infection<sup>9</sup>. CXCR4 and CCR5 are both G-protein coupled receptors that bind chemokines. CCR5 regulates the trafficking and effector functions of immature dendritic cells, macrophages, and T lymphocytes<sup>25</sup>. The interaction of CXCR4 with its ligand, CXCL12, is required in hematopoiesis for hematopoietic stem cell quiescence, hematopoietic stem cell retention in the bone marrow, and thymocyte trafficking in the thymus<sup>26</sup>. CD4 is primarily expressed on helper T cells and it plays a role in T cell signaling<sup>27</sup>.

In addition to studying HIV pathogenesis, non-human primate models have also been used to study lentiviral vaccines. SIV has served as a vaccine model for several

years<sup>28,29</sup>. In a recent example, Berry et al. (2015) demonstrated that inoculation with live-attenuated SIVmacC8 and SIVmacJ5 that are *nef*-disrupted and *nef*-intact, respectively, can prevent superinfection upon macrophage and neurotropic SIV challenge<sup>30</sup>. Following that, Shin et al. (2018) also constructed replication-incompetent recombinant rhesus monkey rhadinovirus containing the near full length SIV genome. Not only did they see SIV protein expression upon administration of this recombinant virus in macaques, they also observed some level of anti-SIV immune responses<sup>31</sup>. For their study, Hansen et al. (2013) utilized RhCMV/SIV vectors which are cytomegalovirus (CMV) vectors containing SIV genes. Macaques that were inoculated with these, followed by pathogenic SIVmac239 challenge demonstrated viral dissemination at first. However, over time, viral loads decreased. Tissues necropsied several weeks after the challenge had no levels of SIV DNA or RNA greater than background<sup>32</sup>.

Although various vaccines have demonstrated protection of macaques, there has been difficulty in isolating the immune effector variables or markers *in vitro* that are responsible for the protection observed *in vivo*, making it difficult pinpoint the exact mechanism of protection. Variation in host genetics that could contribute to conferring protection can also confound these analyses<sup>33</sup>. Vaccine studies as such require a large sample size to find the common host factors that are responsible for the defensive mechanism seen in vaccine protected animals.

The close phylogenetic relationship between humans and non-human primates, as well as HIV and SIV make macaques decent candidates for studying HIV pathogenesis and vaccine development. HIV and SIV share similar pathology, but they are not equivalent when comparing genetic structures. SIV<sub>mac</sub> and SIV<sub>simm</sub> lack the *vpu* (viral protein U) gene seen in HIV-1, and instead have a *vpx* gene (Figure 1). Although both function as antagonists against host restriction factors, Vpu downregulates CD4<sup>34</sup> and antagonizes host tetherin to promote viral progeny release<sup>35</sup>; whereas, Vpx counteracts SAM domain-and-HD domain- containing protein (SAMHD1) to inhibit it from reducing the concentration of dNTPs in the cell cytoplasm<sup>9</sup>. Thus, HIV-1 does not have a known mechanism for countering SAMHD1. Additionally, the open reading frames are organized differently in HIV-1 and SIV<sub>mac</sub>/SIV<sub>simm</sub> and they only have 53% identity in the arrangement of their nucleotides<sup>9</sup>. Moreover, protective immune responses generated

against SIV may not protect against HIV. Consequently, to continue to utilize non-human primates as model organisms to study HIV microbicides and for vaccine development, it became important to design a virus that was more genetically similar to HIV, but was still able to infect and replicate in macaques. This led to the establishment of the Simian-Human Immunodeficiency (SHIV) model.

### 1.2.3 Non-human primate models: Simian Human Immunodeficiency Virus (SHIV)

SHIV is a chimeric virus, created using components of SIV and HIV. Specifically, it uses the SIV backbone in which several SIV genes have been replaced with HIV counterparts (Figure 2). The benefit of this is that since the chimeric virus contains genes belonging to both SIV and HIV, it allows for the establishment of more potent microbicides and vaccines targeted at the HIV components, with the advantage of continuing to use macaques as the model organisms.

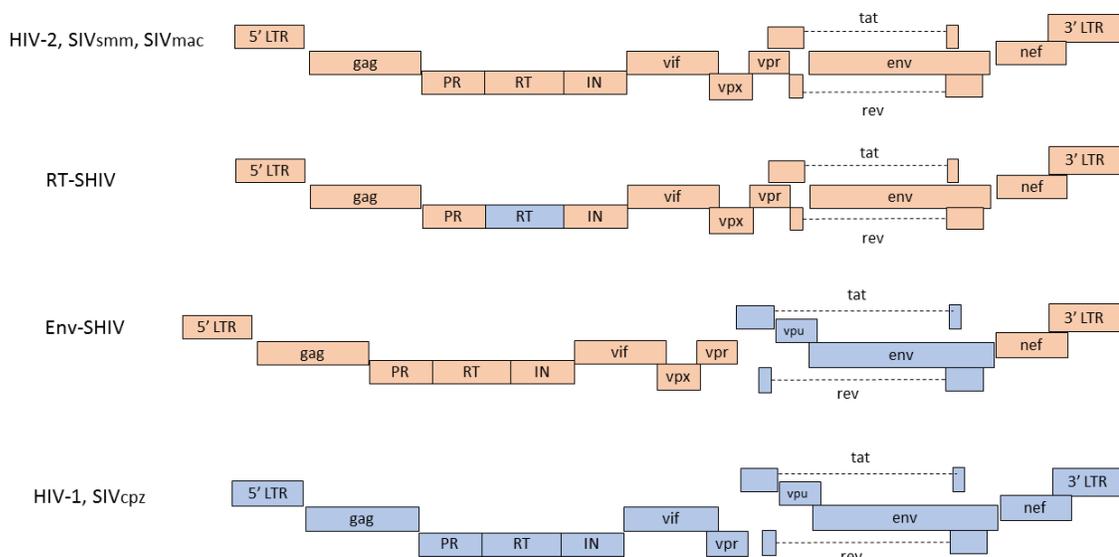


Figure 2. Representation of chimeric SHIVs composed of the SIV backbone wherein a variety of genes are replaced with their HIV counterparts. Blue boxes denote HIV genes. Pink boxes denote SIV genes.

Early versions of SHIV were comprised of the insertion of HIV *env* (envelope), *rev* (regulator of expression of viral proteins), *tat* (trans-activator of transcription), and *vpu* genes into the SIV backbone (Env-SHIV in Figure 2). These versions were unable to efficiently replicate and induce disease in macaques initially, but after several passages, they achieved replication and infection competence. Infection with these adapted virulent strains of SHIV induce rapid CD4+ T cell loss within a few weeks and cause AIDS within two years in macaques. They were also able to promote lymphoid and organ-specific deterioration<sup>36–38</sup>.

Another version called RT-SHIV (Figure 2) includes the HIV-1 reverse transcriptase (RT) portion of the *pol* gene., which made it a good candidate to study ART drugs that target this enzyme. This was especially important as SIV RT was insensitive to non-nucleoside reverse transcriptase inhibitors (NNRTIs)<sup>9,33</sup> such as efavirenz. However, some mutations seen in SIV and RT-SHIV infection of macaques due to prolonged treatment with zidovudine, nevirapine, efavirenz, lamivudine (3TC), emtricitabine (FTC), or tenofovir are reminiscent of those seen in HIV-1 and HIV-2<sup>33</sup>.

The SHIV model has also lent itself to HIV vaccine research. Joag et al. (1998) utilized live attenuated vaccines constructed using SHIV deletions. One of these had deletions in SHIV *vpu*, and the other had deletions in SHIV *nef* and *vpu*, both of which are accessory genes that increase virulence. Upon challenge with pathogenic SHIV<sub>KU-1</sub>, 10 out of the 12 macaques that were vaccinated with either of the vaccines had reduced viral replication and demonstrated anti-viral immune responses. All the control organisms that were not vaccinated experienced CD4+ T cells loss and AIDS onset as early as 12 weeks after being inoculated with SHIV<sub>KU-1</sub><sup>39</sup>. This was a promising avenue for the potential of live-attenuated vaccines to confer resistance to HIV infection. More recently, Pauthner et al. (2019) demonstrated that macaques with a high titre of neutralizing antibodies, produced as a result of immunization with HIV envelope glycoprotein SOSIP trimers, showed protection against challenge with SHIV<sub>BG505</sub><sup>40</sup>, a type of Env-SHIV described in Li et al. (2016)<sup>41</sup>.

SHIV chimeras have also proved themselves as valuable in studying HIV pathogenesis and ART drug efficacy and vaccine strategies using non-human primates, primarily macaques, as model organisms. However, even with the replacement of certain genes in the SIV backbone with HIV, the chimeric virus infection does not completely mimic HIV infection in humans. This serves as a barrier, especially in developing vaccines, as they may confer resistance upon infection with the chimeric virus, but may be incapable of inducing the same response during HIV infection.

Apart from this, using non-human primates poses numerous difficulties. In the US, there has been a steady decline since 2008 in the number of non-human primates being imported from Asia for research purposes<sup>10</sup>. Lack of non-human primate usage can be attributed to economic, logistical, and ethical factors. These organisms are extremely expensive to obtain and accommodate, so studies are limited to small population sizes, which could reduce the statistical power of results<sup>11</sup>. In accordance with these reasons, it has become imperative to establish other animal models to study and develop HIV vaccines and anti-HIV drugs.

### 1.3 Feline Immunodeficiency Virus (FIV)

Feline Immunodeficiency Virus (FIV) was first described in 1987, with infection leading to AIDS in cats from which it was isolated<sup>42</sup>. Its similarity to HIV became obvious soon after that, and its potential to serve as a tool for learning more about HIV became evident<sup>43</sup>.

While many felid species including lions, bobcats, and pumas are infected with different species of FIV, only domestic cat FIV infection is known to cause overt disease<sup>43,44</sup>. Natural transmission of FIV occurs mainly through bites during fights or mating between cats via exposure to infected blood or blood-contaminated saliva. In addition to transmission via mucosal routes, vertical transmission has been reported to occur prenatally from infected mother to child, and postnatally through infected milk or colostrum<sup>45,46</sup>.

Experimentally, cats can be infected with FIV by injection, oral inoculation<sup>47</sup>, and via the intact vaginal or rectal mucosa<sup>48,49</sup>.

Furthermore, various strains of FIV that are pathogenic *in vivo*, have been successfully used to generate molecular clones that are replication-competent *in vitro*. These include the FIV subtype A molecular clones 34TF10 and PPR, which were derived from cats that displayed significant disease symptomology. 34TF10 was able to infect CRFK (Crandell Rees Feline Kidney Fibroblast) and G355-5 (feline astrocyte cell line), whereas PPR was able to infect feline peripheral blood leukocytes<sup>50</sup>.

Clone FIVC-36, a subtype C strain, was isolated from a cat that developed severe immunodeficiency disease, following which it was cloned. This molecular clone was able to infect feline peripheral blood mononuclear cells and primary T-cell lines. This clone was infectious and replication-competent *in vitro* and highly pathogenic *in vivo*. Sequence analysis revealed that this clone had a large amount of envelope sequence divergence from the two subgroup A strains – 76% from 34TF10 and 78% from PPR<sup>51</sup>. The highly pathogenic nature of FIVC-36 led us to utilize it in this project for our purposes.

### 1.3.1 Comparison of FIV and HIV: Disease progression

FIV infection in cats is very similar in pathogenesis to HIV in humans. Upon infection, cats undergo an acute phase of infection within the first few weeks where there is generalized lymphadenopathy, pyrexia, and anorexia. Circulating levels of FIV can be found within a few days of infection<sup>45</sup>.

The acute phase is followed by an asymptomatic phase or latent phase that is characterized by negligible viral titer and minimal clinical symptoms. This asymptomatic phase can last from several months to several years, similarly to HIV infection<sup>46</sup>. Like HIV, CD4+ T cell loss is a hallmark of FIV infection, and this decline can start occurring as early as 4-6 weeks post infection<sup>45</sup>. Transition into the symptomatic phase and eventually feline AIDS occurs through the reduction of antiviral responses and increased secondary infections, such as gingivitis, pneumonia and rhinitis<sup>45</sup>. Neurotropic strains can also infect central nervous system microglia, astrocytes, and macrophages, clinically manifesting as aggressive behaviour, facial twitching, and delayed auditory and visual evoke responses<sup>45</sup>. Infected cats also experience increased susceptibility to opportunistic infections<sup>46</sup>.

### 1.3.2 Comparison of FIV and HIV: Structure and genome

The FIV genome is approximately 9400 nucleotides long<sup>12</sup>, while HIV-1 is typically approximately 9200 nucleotides<sup>52</sup>. Both HIV and FIV share homology in their structural proteins but differ in their accessory proteins (Figure 3).

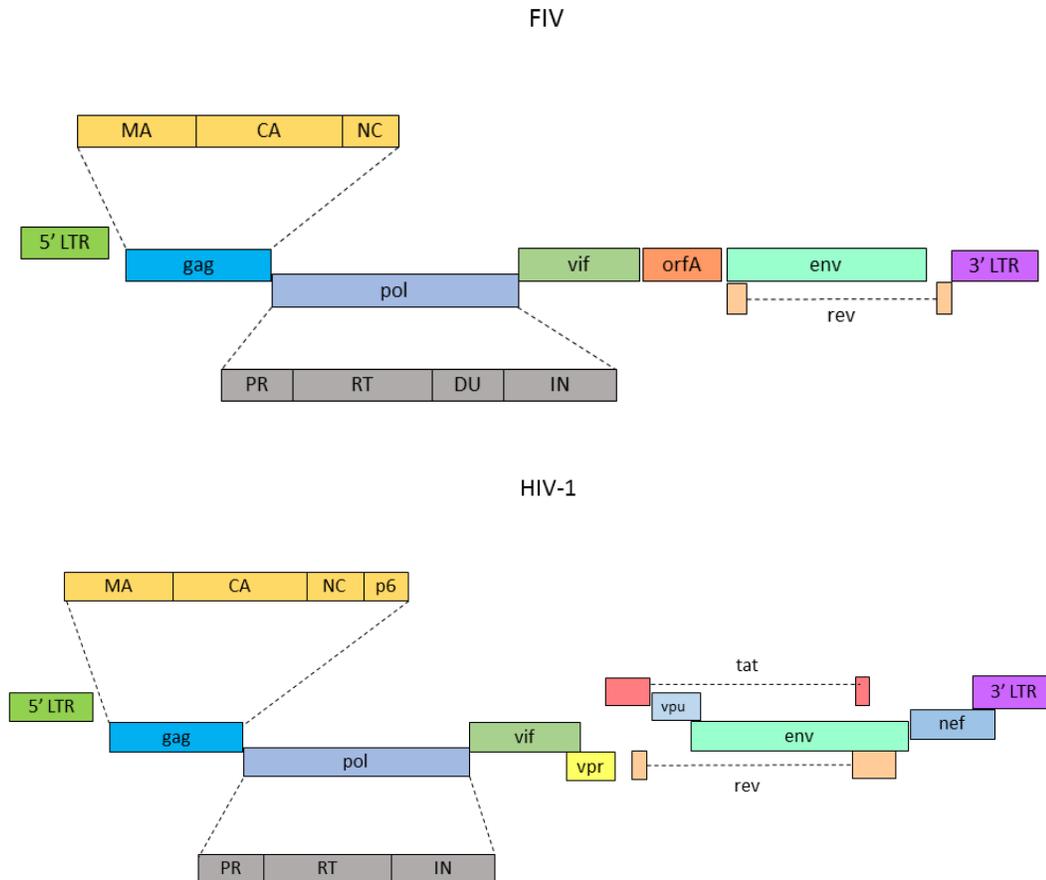


Figure 3. Genome organization of FIV and HIV-1. Both are flanked by LTRs and contain *gag*, *pol*, and *env*. The accessory genes *vif* and *rev* are shared by both the viruses. The accessory genes of *vpr*, *vpu*, and *nef* are only found in HIV, whereas FIV encodes the accessory gene *orfA*. FIV *pol* precursor additionally contains DU (dUTPase).

Like all retroviruses, the two viruses are flanked by long terminal repeats (LTRs), and contain the *gag*, *pol*, and *env* genes that code for structural proteins. The *gag* precursor encodes the p24 Capsid (CA), the p14 Matrix (MA), and p7 Nucleocapsid (NC)

in FIV and is cleaved by Protease. The corresponding counterparts in HIV are p24, p17, and p7, in addition to p6 that is implicated in the incorporation of Vpr into the virion<sup>53</sup>. The Gag proteins are necessary for assembly to form mature, infectious virion particles. The *pol* precursor is composed of Protease (PR), Reverse Transcriptase (RT), and Integrase (IN), with the addition of dUTPase (DU) in FIV<sup>12,43,45,46</sup>.

Structurally, the virion's capsid is surrounded by matrix proteins, whereas the nucleocapsid is tightly associated with the RNA genome. IN, PR, RT and dUTPase (FIV only) are packaged into the mature virion. PR is responsible for cleaving the Gag and Pol polyproteins. RT synthesizes cDNA from viral RNA. IN incorporates the cDNA into the host genome. dUTPase, a hallmark of FIV and equine infectious anemia virus (EIAV) is responsible for maintaining low levels of dUTP in the cell environment to prevent the mis-incorporation of uracil into DNA. This feature is not seen amongst primate lentiviruses<sup>43,46</sup>. Alternatively, HIV, but not FIV contains a trans-activator of transcription (Tat) that promotes RNA pol II transcription<sup>53</sup>.

The Rev protein regulates nuclear export of mRNA for the two viruses, and exon 1 of *rev* is located at 5' of the *env* gene in both. However, exon 2 of *rev* is located at the far 3' end of *env* in FIV, and within the transmembrane encoding region of *env* in HIV. Rev is implicated in the transport of unspliced or partially spliced, intron-containing RNA out of the nucleus using the CRM1 (exportin-1) pathway<sup>54</sup>. It does so by binding to the Rev Response Element (RRE) on the unspliced RNA<sup>12</sup>.

The *vif* accessory gene is present in both HIV and FIV, and responsible for countering APOBEC3 enzymes that serves as host restriction factors. Specific human and cat APOBEC3 proteins are responsible for cytidine deamination of the viral genome resulting in deleterious G-to-A mutations. Vif antagonizes this function by directing APOBEC3 to the proteasome for degradation, preventing its incorporation into the virion<sup>46</sup>. The Vif of pathogenic versions of FIV has also been shown to increase the replication rate of the virus *in vitro*<sup>55</sup>. FIV lacks the accessory genes *nef*, *vpu*, and *vpr* seen in HIV. These are mainly responsible for increasing HIV virulence and disrupting the action of host restriction factors. Nef is a multi-functional protein known to interact with several host receptors. The most notable of its functions are CD4 downregulation by

targeting the receptor to the endo-lysosomal pathway, which promotes envelope incorporation and budding<sup>53,56</sup>. It also promotes MHC-1 downregulation on the cell surface<sup>56</sup>, deterring immune surveillance. Vpu also decreases CD4 expression but unlike Nef that targets CD4 present on the surface, Vpu targets newly synthesized CD4 in the endoplasmic reticulum. Additionally, Vpu also antagonizes BST2 (tetherin), a host restriction factor that tethers viral particles to the membrane, and is implicated in enhancing viral particle release<sup>34</sup>. Vpr also has several functions including the promotion of cell cycle arrest in the G2 phase where the LTR promoter is most active, facilitation of the nuclear import of the pre- integration complex in non-dividing cells, and control of apoptosis<sup>57</sup>.

The accessory protein OrfA is unique to FIV. It has been implicated in downregulation of CD134 expression, as it is the primary entry receptor for FIV<sup>58</sup>, reminiscent of the downregulation of CD4 in HIV infection. It has also been implicated in altering the gene expression patterns of factors involved in post-transcriptional modifications, splicing machinery, and proteasome ubiquitination<sup>59</sup>. Although mechanistically different from HIV Tat, OrfA also regulates transactivation of mRNA synthesis<sup>60</sup>. Like HIV Vpr, FIV OrfA is implicated in inducing G2 cell cycle arrest<sup>61</sup>. The LTR is most active in the G2 phase. Lastly, OrfA has been implicated in viral infectivity as OrfA mutations can decrease infection of feline PBMCs<sup>62</sup>. These functions make OrfA an interesting, multifaceted accessory protein.

### 1.3.3 Comparison of FIV and HIV: Entry receptor usage and cellular tropism

FIV and HIV differ in their receptor usage. HIV utilizes CD4 as its primary receptor and either CCR5 or CXCR4 as its secondary receptor. FIV, on the other hand, utilizes CD134 as its primary receptor and CXCR4 as its co-receptor. As a consequence of this dependence on CD134 and CXCR4, FIV is not only tropic for T cells and macrophages, but also for B cells and CD8+ T cells<sup>12</sup>. Differences in HIV and FIV envelope are responsible for their differential cell tropism.

Furthermore, HIV envelope is a major target for therapeutic strategies aimed at the infection. The RV144 trial that demonstrated the most promise for an anti-HIV

vaccine targeted the HIV envelope. It consisted of immunization of the ALVAC (canarypox vector), with the VAX B/E gp120 protein vaccine booster to elicit an anti-gp120 antibody response. Glycan binding sites are of major importance as specific sites such as N-linked glycosylation epitopes are required for the binding of broadly neutralizing monoclonal antibodies<sup>63</sup>. Vaccine efficacy of the trial is linked to responses including the binding of IgG antibodies to variable loops 1 and 2, antibody-dependent cell cytotoxicity, CD4+ T cell responses, and Env-specific IgA responses, all of which target components of the envelope<sup>7</sup>.

Evidence as such has encouraged us to prioritize the importance of the HIV envelope in the process of establishing a novel animal model to study HIV.

#### 1.3.4 Comparison of FIV and HIV: Immune control of FIV infection

FIV immune responses mimic HIV responses to some extent. For instance, in both infections, the CD4:CD8 ratio decreases as the number of activated CD8+ cells increases. Additionally, T regulatory cells that are CD25+/CD4+ are responsible for downregulating CD8+ T cells that produce IFN- $\gamma$ . This is a potential immunosuppressive effect on CD8+ cells seen in both HIV and FIV<sup>12</sup>.

Furthermore, FIV-infected cats are less responsive to mitogen-induced lymphocyte blastogenesis with both T and B cell mitogens. Culturing of keyhole limpet hemocyanin with PBMCs resulted in a lower primary proliferative response in FIV-infected cats, compared to non-infected animals, which is indicative of a lack of a robust naive T cell response to antigens<sup>64</sup>. This was proposed as a mechanism for opportunistic infections to be able to manifest. Dysfunction of T cell proliferation is also seen with HIV<sup>65</sup>.

Grant et al. (2009) have also demonstrated that in addition to anti-SU antibodies, anti-CD134 autoantibodies were also produced in FIV-positive cats and their binding to CD134 induced the release of viral SU (surface component of Env) from the primary receptor, thereby blocking viral infection. This correlated with lower viral loads<sup>66</sup>. Similarly, anti-CCR5 autoreactive antibodies in HIV infection are implicated in downregulating CCR5 expression as an antiviral strategy<sup>67</sup>.

However, unlike HIV, a commercial vaccine for FIV called Fel-O-Vax-FIV® is available. It is a dual-subtype inactivated whole cell lysate virus composed of subtypes A (FIV<sub>Pet</sub>) and D (FIV<sub>Shi</sub>) FIV<sup>68</sup>. Although effective against some FIV subtypes, including subtypes D and F<sup>69</sup>, prior work has shown that the vaccine is variable in its efficacy against varying subtypes and strains, which also includes differences within homologous subtype challenge responses, depending on the neutralization tier that the challenge strain belongs to<sup>70</sup>. The tier system categorizes virus subtypes according to their sensitivity to neutralizing antibody responses. Tier-1 FIV are homologous to the vaccine strains and most-sensitive to neutralizing antibody responses. Tier-2 comprise FIV that have homologous subtypes to the vaccine strains, but are more resistant to neutralizing antibody responses. Tier-3 FIV are least sensitive to neutralizing antibody responses and are composed of subtypes that are different, i.e., heterologous to the vaccine strains<sup>70</sup>. Protection against tier-1 FIV<sub>Pet</sub> is strong; however, protection upon challenge with certain tier-2 and tier-3 subtype viruses is afforded to a lesser extent<sup>70</sup>. Passive antibody transfer studies have demonstrated that neutralizing antibody responses are not necessarily effective against heterologous subtype challenge, however, vaccine-induced T cell responses have been implicated in conferring protection in both homologous and heterologous challenges<sup>68</sup>. These most likely include T-helper 1 activity mediated by IL-2 and IFN $\gamma$ , and cytotoxic lymphocyte activity driven by the production of perforin<sup>69</sup>.

Furthermore, a case-controlled field study in Australia revealed a protective rate of 56% in vaccinated cats<sup>69</sup>, as opposed to the maximum protection rate of 31.2% for HIV-1, as seen in the RV144 trial<sup>63</sup>. In addition to antibody mediated cell cytotoxicity (ADCC), protection in the RV144 trial was also attributed to anti-V2(Env) CD4+ T cell immunity including CD4+ cytotoxic T lymphocytes and polyfunctional CD4+ T cells<sup>68</sup>. The vaccine protection linked to T cell responses in HIV vaccines is similar to what is observed with FIV vaccines. Based on this, lessons from FIV vaccine trials can supplement knowledge that can be used to improve HIV vaccine efficacy.

## 1.4 Project rationale

As outlined earlier, although mouse models and non-human primate models are effective and beneficial in studying HIV, there are several ethical, financial, and logistical issues that accompany utilizing these organisms. Cats, on the other hand, provide an avenue for a lower-cost model, as they are naturally infected by FIV. When considering genome structure, FIV and HIV share several similarities. Disease pathogenesis of FIV in domestic cats is also similar to HIV pathogenesis and AIDS progression in humans. Based on these similarities, cats serve as good candidates for being used as model organisms in studies aimed at developing HIV vaccines and therapeutics.

A limitation of the FIV model is that its cellular tropism and receptor usage differs from HIV. Due to this, HIV therapeutics targeted towards the envelope cannot be effectively tested in cats infected with FIV. An improved model would include a version of FIV that utilizes CD4 and CCR5 for entry into cat cells and thus has similar tropism to HIV.

When we compare their amino acid sequence, feline and human CD4 share 59% identity and 71% similarity (Figure 4a). When comparing CCR5 amino acid sequences, they share 76% identity and 86% similarity (Figure 4b). Percent identity here indicates the percentage of identical amino acids, while percent similarity accounts for the amino acids that are different, but share similar properties or characteristics.

## a) CD4 alignment

```

Human MDYQVSSPIYDINYYTSEPCQKINVKQIAARLLPPLYSLVFIQGFVGNMLVILILINCKR 60
Cat MDYQATSPYYDIEYELSEPCQKTDVROIAARLLPPLYSLVFLSGFVGLLVVILINCKK 60
****.:** **:* ***** :*.*****: **** :*.*****:

Human LKSMTDIYLLNLAISDLFFLLVPPFAHYAAAQWDFGNTMCQLLTGLYFIFGFFSGIFFII 120
Cat LRGMTDVIYLLNLAISDLFFLLVPPFAHYAANGWVFGDMCKTVTGLYHVGYFVGGNFFII 120
*.:**.******:*.*.***** * *: *.:****.:*.* ****

Human LLTIDRYLAWHAFVFAKARTVTFGVVTSVITWVAVFASLPGIIFRISQKEGLHYTCSS 180
Cat LLTVDRYLAIVLAVFAVKARTVTFGAVTSAVTWAAAVVASLPGCIFRSQKEGSRFTCSP 180
**.:*****:* **.******:*.*.**.* **.* **.****** :.***

Human HFPYSQYQFWKNFQTLKIVILGLVLP LLVMVICYSGLKTLRCRNEKKRHRAVRLIFTI 240
Cat HFPSNQYHFKNFQTLKMTILGLVLP LLVMVICYSAILRTLRCRNEKKRHRAVKLIFVI 240
**.* **.******:*.*****:*.**.* **.* **.******:****.*.*

Human MIVYFLWAPYNIIVLLNTFQEFFGLNNCSSNRDQAMQVTETLGMTHCCINPIIYAFV 300
Cat MIGYFLWAPNNIVLPLSTFPEFGLNNCSSNRDQAMQVTETLGMTHCCINPIIYALV 300
** ***** **.* **.* *****:*****:*.

Human GEKFRNYLLVFFQKHIARFKCCCSIFQQEAPERASSVYTRSTGEQEISVGL 352
Cat GEKFRSYLLVFFQKHIAAFCKRCPVFGKALDRA-GCYTRSTGEQEISVGL 351
****.******: **.* **.* **.* *****

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## b) CCR5 alignment

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Human MNRGVVFRHLLLVQLALLPA-ATQGGKVVVGKKGDTVELTCTASQKKSIOFHMKNNSNQI 59
Cat MNQGVVFRHLLLVQLVMLKAAVPGKQEVVLGKAGGTAELPCQASQKQYMTFTWRLSSQV 60
**.:* *****:*. * . **.****** *.* **.* ***** : *.: *.:

Human KILGNQGS-FLTKGPKLNDRADSRRLWDQGNFPLIINKLIEDSDTYICEVEDQKEEV 118
Cat KILESQHSLLCLTGSSKLRTRFESKKILWDQGSFPLVIKSLQVADSGIYTCVENKKREV 120
**.* * : . * **.* * :*.: *****.***.*.*: **.* *****:*.**

Human QLLVFGLTANSD-----THLLQGQSLTTLTLESPPGSSPSVQCRRSPRGK 161
Cat ELLVFGLTAKVDPSSGSSSSSTSTSTSIYLLQGQSLTTLTLESPSSSNPSVQWKGPGNK 180
.******: * :*****.* **.* **.* **.*

Human NIQGGKTLVSVQLELQDSGTWCTVLQNKQKVEFKIDIVVLAFOKASSIVYKKEGEQVEF 221
Cat SKSGVHLSLSQLELQESGTCTCTVSQSKTLVFNTNIIVLVAFKVSNTVYAKEGEQVEF 240
.* * :*.*****:**.* **.* **.* **.* **.* **.* *****

Human SFPLAFTVEKLTGSGELMWQAERASSSKSWITFDLNKKEVSVKRVTDQPKLQMGKPLPL 281
Cat SFPLNFEDENLM--GNLRWKAEGAPSSLLWISFTLKNKQLSVKEVDPYSKLQMPDLSPLR 298
****.* **.* **.* **.* **.* **.* **.* **.* *****:***.* **.*

Human LTLPQALPQYAGSGNLTLEAKTGKLVHDEVNLMVVRATQLQKNLTCEVWGPTSPKLMLS 341
Cat FTLPNVLRSRYAGSGNLTLVLD--KGQLQEEVKLVVVRVTSQGNLTCVGLGPTSPETLS 356
.***.* * :*****.* * :*.*****:***.* **.* ***** **.* **.*

Human LKLENKEAKVSKREKAVIWLNPAGMWCQLLSDSGQVLLSNIKVLPTWST-PVQPMALI 400
Cat LKLKGAQKAVSKQKQKMRVEDAAGTWQCLLSHKDKVLLASKAEVLPVLTTRTWNLTI 416
***.: *****:*. * : **.* *****:***.* **.* **.* : *

Human VLGGVAGLLLIFGLGIFFCVRCRHRRAERMSQIKRLLSEKKTCCQPHRFQKTCSPI 458
Cat VLGGVGLVLYIGLWVYCCVKCNHRRRAERMSHIKRLLEKKTCCQSHRLQKTCNPI 474
****.* **.* **.* **.* ***** **.******:***.* **.*

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Figure 4. Alignment of cat and human amino acid sequences. a) CD4 alignment. Cat and human sequences share 59% identity and 71% similarity. b) CCR5 alignment. Cat and human sequences share 76% identity and 86% similarity. “\*” denotes conserved residues. “.” denotes residues with strongly similar properties. “:” denotes symbols with weakly similar properties.

Based on this level of similarity, we hypothesize that HIV envelope could potentially utilize the feline counterparts of CD4 and CCR5 to enter cat cells. Furthermore, chimeric viruses composed of both HIV and FIV components wherein the envelope gene in the FIV genome is replaced by the HIV envelope gene (called FHIV<sub>env</sub> from hereon) should be able to enter and infect cat cells. If these chimeras can replicate *in vivo*, HIV therapeutics and vaccines targeting envelope can be tested using cats.

Although HIV can use either CXCR4 or CCR5 as its co-receptor, we are choosing to focus on CCR5-utilizing strains. Prior work has demonstrated that CCR5-utilizing strains are more likely to establish acute infections, regardless of the route of transmission<sup>71</sup>. These CCR5-utilizing viruses are more likely to dominate and become the founder population when present alongside CXCR4-utilizing variants during infection transmission<sup>71</sup>. In addition to this, individuals that carry the  $\Delta 32$  mutation in CCR5 are still susceptible to CXCR4-utilizing variants; yet this occurs with a low frequency, suggesting that CCR5-utilizing variants are more likely to be transmitted<sup>72</sup>. Therefore, prophylactic HIV vaccines must be designed to prevent transmission of CCR5-utilizing strains and model systems for testing vaccines should focus on CCR5 tropic viruses. Thus, we aimed to make FHIV<sub>env</sub> chimeras utilizing *env* from CCR5-utilizing variants.

We sought to create these FHIV<sub>env</sub> chimeras to express HIV envelope. FHIV<sub>envs</sub> capable of adapting to utilize feline CD4 and CCR5 receptors to enter and infect cat cells *in vitro* will be tested in *in vivo* in cats in the future. Our long-term vision is to develop and isolate the FHIV<sub>envs</sub> capable of replicating *in vivo*, which will allow for the use of cats as animal organisms to test HIV vaccines and therapeutics targeting envelope. An advantage of this model is that all the accessory genes (excluding FIV envelope) are retained in the FHIV<sub>envs</sub>, which can allow the chimeric viruses to circumvent feline host-restriction factors. Using intact HIV to infect cats would not guarantee the evasion of feline restriction factors, which could potentially target HIV at different points in its life cycle.

This particular thesis' aim was to lay the groundwork for this long-term project by testing its validity *in vitro* in cat cells. Throughout this project, we worked with the highly pathogenic FIVC-36 clone. We attempted to utilize the yeast recombination strategy<sup>73</sup> to

make the plasmids required to produce FHIV $env$  chimeras, where HIV  $env$  would replace FIV  $env$  in the FIVC-36 backbone. For this, we started by testing whether infectious FIV could be produced from plasmid transfections of the vectors created using this cloning strategy. We tried several variations of the infection experiments with virus produced from these plasmids. We found little to no infectious FIV production. We also attempted alternate cloning strategies such as In-fusion cloning to make FHIV $envs$  but were not able to successfully generate them. The process of making the chimeras is currently ongoing in the Troyer/Arts Lab.

We additionally engineered CRFK cat cell lines expressing feline or human CD4/CCR5 (referred to as feCD4/feCCR5 or huCD4/huCCR5 CRFK hereafter), which were necessary for the *in vitro* part of this study. We utilized them in cell-to-cell fusion assays testing receptor-envelope interactions between diverse HIV envelope expressed on 293T cells and human or feline CD4 and CCR5 expressed on CRFK cells. Additionally, we utilized the cells expressing human CD4 and CCR5 in an infectivity assay with HIV.

In the future, these cell lines can be used in cell-to-cell fusion assays to test diverse HIV envelopes (from CCR5-utilizing strains) and isolate the subtypes and strains that are capable of interacting with feCD4 and feCCR5 expressed on cat cells. These particular HIV  $envs$  can then be pursued further in the FHIV $env$  cloning process. Following this, FHIV $envs$  can be utilized in infectivity assays using feCD4/feCCR5 CRDK to test their ability to replicate *in vitro*. The engineered huCD4/huCCR5 CRFK cell line can be used as the positive control for the cell-to-cell fusion and infectivity assays, as we expect HIV envelope to be able to bind these receptors to enter cells. Once replication-competent FHIV $envs$  are isolated from *in vitro* experiments, their ability to infect and replicate *in vivo* in cats can be tested. The steps required to accomplish the vision of this project are described in the flowchart in Figure 5.

Overall, this study provides an avenue for testing therapeutics or vaccines that target the HIV envelope, using a suitable and cost-effective animal model.

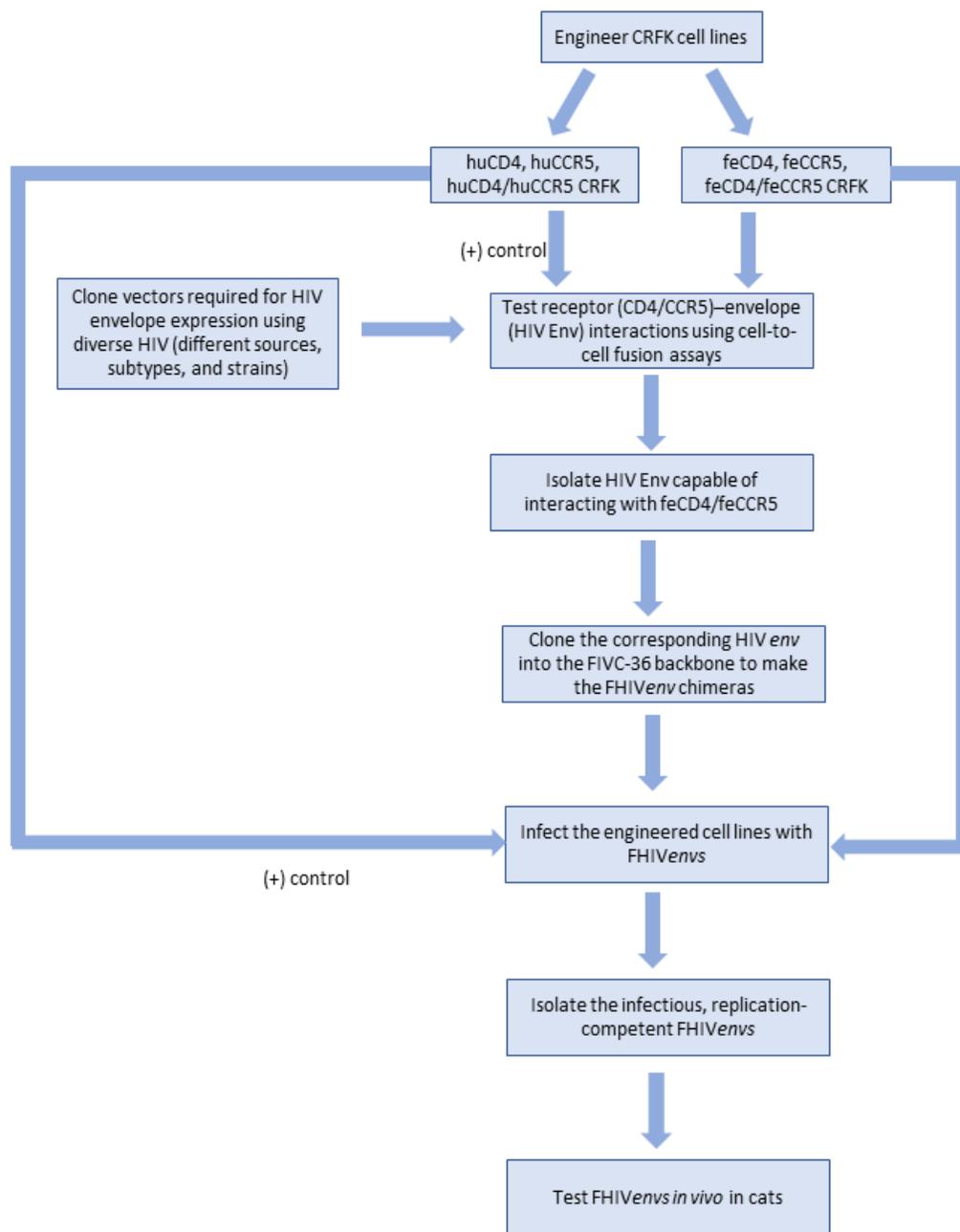


Figure 5. Flowchart detailing the project plan. Engineered huCD4/huCCR5 CRFK and feCD4/feCCR5 CRFK can be used in cell-to-cell fusion assays to isolate HIV envelopes (expressed on 293T from cloned vectors) from diverse subtypes and strains that can interact with these human and feline receptors. These *envs* can be cloned into the FIV backbone to make FHIV*envs*. The engineered cell lines can also be used in infectivity assays to test the capability of the FHIV*envs* to replicate *in vitro* in cat cells. These FHIV*envs* can then be pursued further to infect cat *in vivo*. The CRFK expressing huCD4/huCCR5 functions as the positive control for the fusion and infectivity assays as HIV envelope is tropic for these receptors.

## CHAPTER 2: MATERIALS AND METHODS

### 2.1 Yeast recombination and miniprep

The yeast recombination system was initially chosen to create the FHIV $env$  chimeras. Prior to this, the vectors produced from this cloning system had to be tested for their ability to produce infectious FIV after being utilized for transfections.

The yeast recombination system takes advantage of the yeast gap repair system where any double stranded DNA breaks can be repaired through the exchange of genetic material between damaged DNA and the intact DNA, if they share homology. The FIV genome of the virulent C36<sup>51</sup> strain was cloned into the pREC\_URA3 plasmid in place of URA3. This plasmid contains all the elements to promote replication in yeast including the yeast centromere (Cen6), autonomously replicating sequence (ARSH4),  $\beta$ -isopropylmalate dehydrogenase (LEU2) that maintains plasmid growth in LEU2 deficient yeast on Leucine drop-out plates<sup>73</sup>. This plasmid also contains the orotidine-5'-phosphate decarboxylase (ODCase) gene called URA3. In the presence of 5-fluoroorotic acid (5-FOA), URA3 would promote the reaction whereby a toxic by-product, 5-fluorouracil, is produced. Therefore, any yeast still carrying a URA3 containing plasmid post-transformation would be selected against on C-Leu+5-FOA plates.

The FIV genome had to be inserted into the pREC plasmid as two separate fragments. This is because the 5' and 3' LTRs are homologous and the co-existence of the two could result in either of them being recombined out during the homologous recombination process. Thus complement (cplt) extending from the 5' LTR to *gag* (Figure 6), and the near-full-length (nfl) extending from *gag* to the 3'LTR (Figure 8) were PCR amplified separately and inserted into the pREC backbone independently. The complement supplied the 5' LTR, whereas the nfl supplied the remainder of the genome. The nfl also contains the *env* sequence; it was synthesized with the intention of being used further to clone diverse HIV *env* in place of FIV *env*. An alternate version of the complement (cplt-rre) was also constructed to include the FIV Rev response element (RRE), an RNA secondary structure that promotes unspliced viral mRNA export through interaction with the FIV Rev protein (Figure 7). The nfl vector was transfected along with

the cplt or cplt-rre vector to produce FIV that was used for infections.

The FIV genome was PCR amplified using primers that contained 40-80bp overhangs that shared homology with regions in the pREC plasmid flanking URA3 (Table 2, primers 1 to 5 for nfl, primer 6 and 7 for cplt, primers 8 to 11 for cplt-rre). The pREC\_URA3 was digested overnight to linearize the plasmid using the restriction enzyme SbfI, for which a cut site is present in URA3. *Saccharomyces cerevisiae* was cultured overnight in liquid YPD media. The yeast was pelleted by centrifugation at 4000xg for 5 minutes, washed, and re-suspended in TE/LiAc solution. 3 µg linearized pREC\_URA3, 1 µg PCR product insert, and 5 µl salmon sperm carrier DNA were added to 50 µl yeast. Each reaction tube was mixed with 300µl PEG and incubated in a 30°C shaker for 1 hour. Following this, the yeast was shocked in a 42°C water bath for 15 minutes. Each reaction tube was centrifuged, after which the supernatant was discarded, and the yeast was resuspended in 100 µl sterile water and plated on C-Leu+5-FOA plates. The plates were incubated at 30°C for 2-5 days.

For plasmid preparation, single yeast colonies were taken from each plate and cultured overnight in C-Leu+5-FOA liquid media. The culture was pelleted by centrifugation and treated with 200 µl breaking buffer (10ml breaking buffer – 2ml 10% Triton X-100, 1ml 10% SDS, 200µl 5M NaCl, 100µl 1M Tris-Cl pH=8.0, 20µl 0.5M EDTA, 6.68ml sigma water) to lyse the cells, followed by the addition of 0.3 g acid-washed glass beads and 200 µL phenol/chloroform/isoamyl alcohol. This was vortexed and centrifuged, after which the aqueous phase was extracted. The DNA was ethanol precipitated and re-suspended in 20 µl sterile water. 10 µl of this was transformed into ElectroMAX Stbl4 bacterial cells. Single colonies were picked and screened via PCR and sequenced.

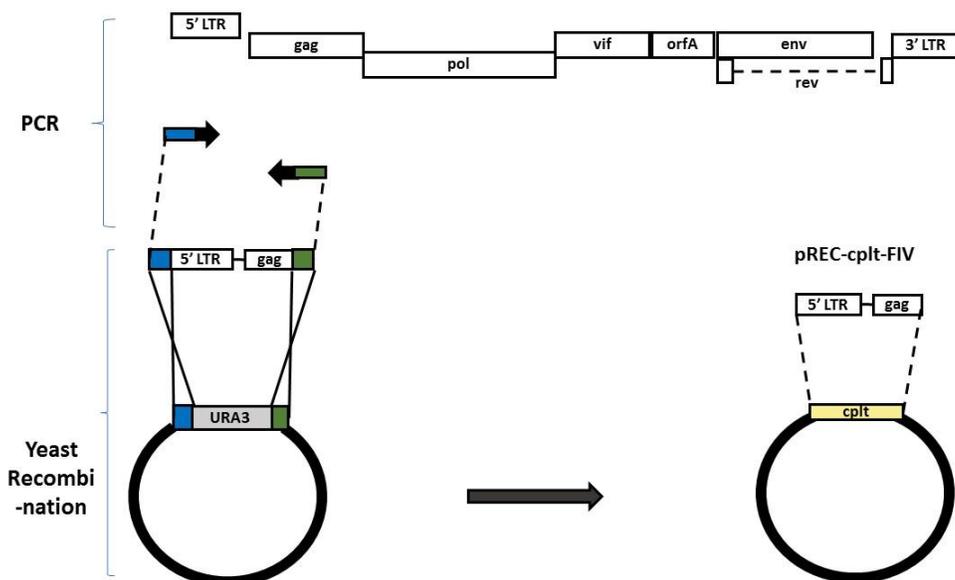


Figure 6. Yeast cloning strategy for creating the complementing plasmid that supplies the 5'LTR. For the original cplt vector shown here, PCR amplification of the FIV template was conducted to generate a fragment that included the entire 5' LTR and *gag* with primers containing 40bp overhangs that shared homology (blue and green boxes) with the pREC region flanking URA3 in pREC\_URA3. pREC\_URA3 cut with SbfI (which has a single cut site within URA3) was introduced with the PCR product into yeast, which facilitated recombination at sites that shared homology. The end product of this was pREC-cplt-FIV, where URA3 was replaced by the cplt fragment in the pREC\_URA3 backbone.

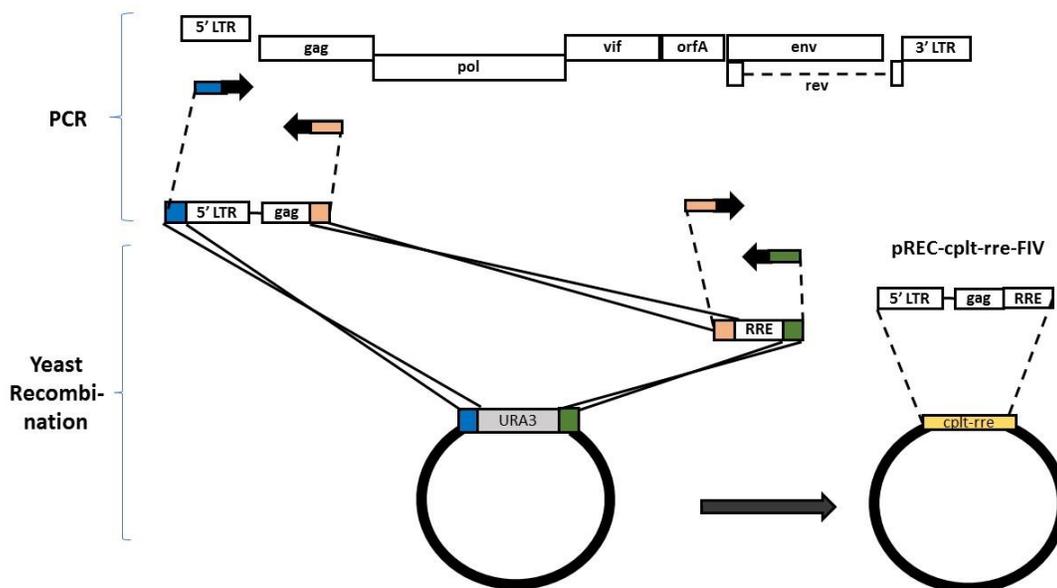


Figure 7. Yeast cloning strategy for creating the complementing plasmid that supplies the 5'LTR, altered to include the RRE. For the cplt-rre vector seen here, the position of the forward primer for PCR was changed such that it started at the R region of the 5' LTR of FIV and contained a 65bp overhang that was homologous to the pREC sequence (blue box). Additionally, the RRE was amplified from the backbone as a separate PCR product. The forward primer of this contained a 65bp overlap with the first half of the cplt-rre PCR fragment (pink box). The reverse primer contained a 60bp overhang that shared homology with pREC (green box). Introduction of the two PCR fragments with *Sbf*I-cut pREC\_URA3 resulted in recombination at three sites – two sites were at the ends where recombination with the pREC regions flanking URA3 occurred and the third site was where recombination between the two PCR fragments occurred. The end product of this was pREC-cplt-rre-FIV, where URA3 was replaced by the cplt-rre fragment in the pREC\_URA3 backbone.

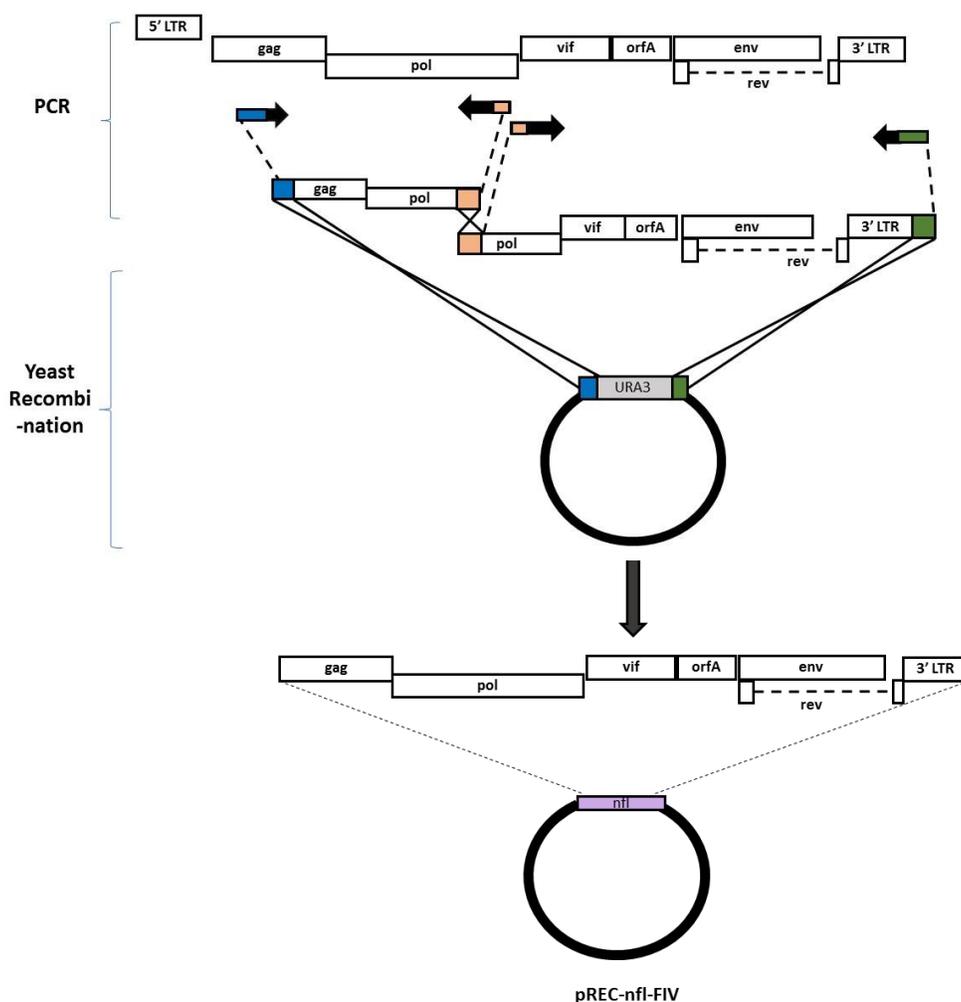


Figure 8. Yeast cloning strategy for the near-full-length (nfl) plasmid. The plasmid was cloned to include the FIV genome, excluding the 5' LTR. The nfl was introduced as two PCR fragments – the first fragment included the FIV sequences extending from the primer binding site to the middle of *pol*, while the second fragment had sequences extending from the middle of *pol* to the end of the 3' LTR. The first fragment's forward primer and the second fragment's reverse primer contained 65-80bp overhangs that were homologous to the pREC sequences flanking *URA3* in pREC\_URA3 (green and blue boxes). Introduction of the two PCR fragments with *Sbf*I-cut pREC\_URA3 resulted in recombination at three sites – two sites at the ends where recombination with the pREC region flanking *URA3* occurred, the third site where recombination between the two PCR fragments occurred (pink box). The end product of this was pREC-nfl-FIV, where *URA3* was replaced by the nfl fragment in the pREC\_URA3 backbone.

## 2.2 Cloning of CD4 and CCR5 plasmids

pBABE.huCCR5: The pBABE.CCR5 plasmid expressing human CCR5 as well as a gene for puromycin resistance was obtained from the NIH AIDS Reagent Program (Cat# 3331). The pBABE vector contains a retroviral promoter from which the transgene of choice is expressed. It is typically used along with an envelope-expressing plasmid to generate retroviral vectors. For our purposes, the plasmid was directly used for transfections to generate stable cells lines expressing CCR5 (discussed in Section 2.4 of the Methods).

pBABE.feCCR5: The huCCR5 gene was removed from the pBABE plasmid using New England BioLabs (NEB) restriction enzymes BamHI and Sall-HF, followed by gel extraction of the backbone with the QIAquick gel extraction kit. The feline CCR5 gene was amplified using the High Fidelity (HiFi) Platinum Taq (Thermo Fisher) protocol with primers containing overhangs overlapping with the restriction enzyme sites (BamHI and Sall) (Table 2, primers 22 and 23). Since the CCR5 gene is composed of a single exon with no introns, the coding sequence was amplified using laboratory cat blood DNA samples obtained from Colorado State University (CSU) as the template. PCR products were purified using the Agencourt AMPure XP PCR purification protocol. Products were ligated into the pBABE backbone using NEB's T4 DNA ligase protocol. After identifying successful recombinants by restriction analysis, clones were sequenced.

pBABE.huCD4: The pBABE.hygromycin empty vector was cut with NEB restriction enzymes Sall-HF and EcoRI-HF. huCD4 was PCR amplified from the T4-pMV7 plasmid obtained from the NIH AIDS Reagent Program (Cat# 158) using the HiFi Platinum Taq protocol with primers containing overhangs overlapping with the restriction enzyme sites (Sall and EcoRI) (Table 2, primers 24 and 25). The huCD4 product was ligated into the pBABE.hygromycin plasmid using NEB's T4 DNA ligase protocol. After identifying successful recombinants by restriction analysis, the clones were sequenced.

pBABE.feCD4: To amplify the feCD4 insert, Peripheral Lymph Node and Thymus RNA was obtained from a colony of lab cats at CSU. cDNA synthesis was conducted using the AccuScript Hi-Fi Reverse Transcriptase Kit, followed by nested PCRs using either the

HiFi Platinum Taq or Pfu Turbo DNA polymerase protocol (Table 2, primers 26 to 29). The same cloning strategy as huCD4 was used to insert feCD4 into the pBABE.hygromycin plasmid.

A total of seven feCD4 and seven feCCR5 PCR products, each from a different laboratory cat, as well as ligated plasmids containing the respective genes were sequenced at the Eurofins Genomics facility in Toronto ON and compared to cat sequences in GenBank. The samples with the highest identities to the consensus amino acid sequences for feCD4 and feCCR5 were used for stable cell transfections.

### 2.3 Cell culture of various cell lines

Three cells lines were maintained in media composed of Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), and 1X Penicillin-Streptomycin (referred to as DMEM complete hereafter): 1) 293T cells - human embryonic kidney cell line (ATCC CRL-11268), 2) CRFK cells (Crandell Rees Feline Kidney) - feline fibroblast cells line (ATCC CCL-94), 3) GFox cells – CRFK cells expressing feline CD134 and green fluorescent protein (kindly provided by John Elder, UCSD).

Cell lines generated via stable cell transfections were maintained in complete media along with drugs that maintained selection: 1) huCCR5 CRFK – DMEM complete with 5 µg/ml puromycin, 2) huCD4 CRFK – DMEM complete with 250 µg/ml hygromycin, 3) huCD4/huCCR5 CRFK – DMEM complete with 5 µg/ml puromycin and 250 µg/ml hygromycin, 4) feCCR5 CRFK – 5 µg/ml puromycin, 5) feCD4 CRFK – 250 µg/ml hygromycin, 6) feCD4/feCCR5 CRFK – 5 µg/ml puromycin and 250 µg/ml hygromycin.

U87.CD4.CCR5 (NIH AIDS Reagent Program Cat# 4035), a human glioblastoma cell line, was maintained in DMEM complete containing 1 µg/ml puromycin and 300 µg/ml geneticin.

The cells were passaged every 2-5 days. Since all of these were adherent cell lines, they were washed twice with phosphate-buffered saline (PBS), followed with trypsin/EDTA

treatment and incubation for 5 minutes. The cells were then resuspended in DMEM complete (plus drugs for cells maintained under selection conditions) and split either 1:10 or 1:20.

All the cell lines were incubated at 37°C at 5% CO<sub>2</sub>.

#### 2.4 Generation of CD4 and CCR5-receptor expressing stable cell lines

All transfections were conducted in 10 cm dishes with 3 million CRFK cells plated per dish and incubated overnight in DMEM complete. The media volume was adjusted to 7 ml DMEM/10% FBS the next day. 1 ml of the transfection reagent was prepared by mixing DMEM with 72 µl FuGENE6 and 24 µg of the plasmid encoding the receptor, which was added to the plated cells. 24 hours post-transfection, the media was changed to 15 ml DMEM complete (or any additional drugs for cells that were previously stably transfected and expressing a receptor). 48 hours post-transfection, the cells in the plate were harvested and split at ratios ranging from 1:2 to 1:20. At this point, the drug for which the transfected plasmid conferred resistance was added to the media. These were monitored for several days to weeks. Single colonies were then manually picked and passaged into individual wells of 96-well plates. The colonies that grew out in their respective wells were passaged further into larger flasks to be screened for receptor level expression. Table 1 lists the plasmids and cell types used for these transfections.

Table 1. Plasmids to stably transfect CRFK cells to express CD4 and CCR5 and the drug resistance they confer.

Cell line produced	Transfected cells	Transfection plasmid	Drugs for which resistance is conferred
huCCR5 CRFK	CRFK	pBABE.huCCR5	Puromycin
huCD4 CRFK	CRFK	Cloned pBABE.huCD4	Hygromycin
huCD4/huCCR5 CRFK	huCCR5 CRFK	Cloned pBABE.huCD4	Puromycin/ Hygromycin
feCCR5 CRFK	CRFK	Cloned pBABE.feCCR5	Puromycin
feCD4 CRFK	CRFK	Cloned pBABE.feCD4	Hygromycin
feCD4/feCCR5 CRFK	feCCR5 CRFK	Cloned pBABE.feCD4	Puromycin/ Hygromycin

## 2.5 Flow cytometry and Fluorescence Activated Cell Sorting (FACS)

The stable cell lines were tested by flow cytometry for receptor expression. Each cell layer was harvested using a cell scraper in order to leave surface receptors intact. The cells were stained with live-dead stain utilizing either BioLegend Zombie Violet (Cat# 423113) or Zombie Aqua (Cat# 423101). In some instances, Invitrogen normal mouse serum (Cat# 31881) was used to block interactions between the antibodies being used and non-specific targets.

The anti-human CCR5-PE antibody from either BioLegend (Cat# 359105) or BD Pharmingen (Cat# 550632) was used to detect huCCR5 expression. The anti-human CD4-BV711 antibody from BD Horizon (Cat# 563913) was used to detect huCD4 expression. feCCR5 was detected using a human/mouse/rat cross-reactive Novus Biologicals CCR5-PE antibody (Cat# FAB1802P). The CCR5 antibody is cross-reactive for human, mouse, and rat CCR5. feCD4 was detected using the feCD4-FITC antibody from Invitrogen (Cat# MA5-28776) or feCD4-FITC antibody from Bio-rad (Cat# MCA1346F). Staining and washes were conducted using flow cytometry buffer (PBS, 2% FBS, 0.5% Na-azide). After washing off the antibodies, the cells were fixed using the BD Cytofix/Cytoperm kit for fixation and permeabilization (Cat# 554714).

Flow cytometry was conducted using either the LSR II or FACSCanto machine at the Roberts Flow Cytometry Facility. Flow sorting was conducted using the FACSARIAIII. Data analysis was done using FlowJo v10 6.1.

## 2.6 RNA extraction

CRFK cells that were transfected with the feCD4 plasmid were lysed using Trizol (Thermo Fisher) and RNA was extracted using the Trizol manufacturer's protocol. The extracted RNA was treated with the Thermo TURBO DNA-free kit (Cat# AM1907) to eliminate any remaining DNA. This was followed by "RNA clean-up" purification using the QIAGEN RNeasy Mini Kit (Cat# 74104).

## 2.7 Transfections for virus production

Transfections were conducted in either 6-well plates or 12-well plates to make virus. When 6-well plates were used, 800,000 293T cells or CRFK cells were plated in 3 ml media and incubated overnight. One day later, the media volume was adjusted to 1 ml DMEM/10% FBS. 6 µg total plasmid was used for transfections. A double-stranded copy number calculator was used (<https://cels.uri.edu/gsc/cndna.html>) to calculate the amount of near-full-length (nfl) and complement (cplt or cplt-rre) plasmids (in ng) to add equivalent copies of the two plasmids. To make 1 ml of the transfection mix, DMEM was mixed with 18 µl FuGENE6 transfection reagent and incubated for 5 minutes. 6 µg total plasmid was added to this and incubated for 15 minutes. The transfection mix was added to the plated cells and incubated for 8 hours, following which the mix was replaced with 3 ml DMEM complete. 48 hours post-transfection, the virus-containing supernatant was collected and centrifuged at 1500xg for 10 minutes to remove cells and cellular debris. The virus-containing supernatant was aliquoted into 1 ml portions and placed at -80°C for long-term storage.

For smaller-scale transfection conducted in 12-well plates, 325,000 cells were plated overnight in 2 ml media. 1 day later, the media was replaced with 900 µl DMEM/10% FBS and 100 µl transfection mix (3 µg total DNA, 9 µl FuGENE6). Ratios ranging from 10:1 to 1:10 (calculated as ratio of µgs transfected) of nfl:cplt-rre were used for transfections, instead of equivalent copy numbers. 16 hours post-incubation, the media was replaced with 2 ml DMEM complete and collected 48 hours later.

## 2.8 Virus infections

Traditional: For FIV infections, 400,000 GFox cells (CRFK cells expressing CD134 – the primary receptor utilized by FIV for entry) were plated per well in a 6-well plate or 160,000 cells in a 12-well plate. 150-250 µl virus supernatant was mixed with DMEM complete and used to replace the plating media. 8-16 hours later, the media was replaced with fresh DMEM complete. Cultures were incubated for 7-10 days and monitored for syncytia formation, with 30 µl supernatant collected every 2-3 days for virus quantification by reverse transcriptase (RT) assay. AD8-HIV infection of

huCD4/huCCR5 CRFK (Colony M) was done using this method as well.

**Spinoculation:** Spinoculations are infections where virus and cells are incubated together and centrifuged for several hours, followed by plating onto a dish. 100,000 G<sub>1</sub> cells were aliquoted into 15 ml conical tubes. These were centrifuged at 300xg for 5 minutes, following which the DMEM media was removed. The cells were resuspended in 1 ml virus supernatant. These were “spinoculated” by centrifugation at 4000 rpm in the Sorvall Legend™ T / RT Centrifuge with a swinging bucket rotor (Model 7500 6434) containing round buckets (Order #: 7500 6441). This is approximately 3400xg. Spinoculations were conducted for 3 hours at 32°C. At the end of the spin, the supernatant was taken off and the cells were resuspended in DMEM complete and transferred into 6-well plates at a total volume of 3 ml per well. Cultures were monitored and maintained for up to a week, with 30 µl supernatant collected every 2-3 days for RT assay.

## 2.9 Reverse Transcriptase (RT) assay

10 µl of virus-containing supernatant was incubated with 20 µl RT buffer (5% 1M Tris, pH 7.5; 3.75% 2M KCl; 0.5% 1M MgCl<sub>2</sub>; 0.5% NP40; 50% Poly (rA)-p(dT) (1U/ml); 0.2% 1M DTT; 40.05% Sigma water) in a round-bottom 96-well plate for 20 minutes for detergent-mediated viral lysis to occur. A master mix of 0.04 µCi/µl radiolabelled solution was created using <sup>32</sup>P (10 µCi/µl) - radiolabeled TTP at the alpha phosphate position and RT buffer. 5 µl of this master mix was added to each well. The plate was incubated for 2 hours to overnight at 37°C. Following this, 10 µl of the contents of each well were spotted onto Whatman DE81 filter paper containing a 96-well grid. This was allowed to dry before washing to eliminate excess unincorporated <sup>32</sup>P. The mats were placed on a rocker and washed at least five times with 1X Saline Sodium Citrate (8.8 g/L Sodium Chloride, 4.4 g/L Sodium Citrate, pH 7.0) and two times with 85% ethanol for five minutes each. After the washes, the mats were dried by being placed on 65°C heat block for 10-15 minutes. They were then deposited into a plastic cover and placed facing a blanked phosphorimager screen inside a cassette overnight. The screen was imaged using the Amersham Bioscience Storm 820 Phosphorimager.

## 2.10 In-fusion cloning

As an alternative approach to clone the FHIV $env$  chimeras, the In-fusion cloning kit was used. This is because RT assay results revealed that the yeast cloning strategy did not produce infectious FIV particles (details discussed in the Results and Discussion sections). This cloning strategy can fuse DNA that share a 15bp overlap. For the FIV backbone, primers were designed to amplify around the FIV-C36 plasmid to include all components excluding the envelope (Table 2, primers 12 to 16). These primers were also positioned to include or exclude distinct portions of the FIV  $env$  sequences, in order to clone various diverse FHIV $env$  chimeras. To amplify the HIV envelope inserts, primers positioned on either sides of the envelope with 15bp overhangs (sharing homology with corresponding FIV sequences) were used for amplification (Table 2, primers 17 to 21). The PCR products were treated with the enzyme DpnI that cleaves methylated DNA and would theoretically cleave whole FIV plasmid. The PCR product of the HIV envelope was gel extracted using the Qiagen Gel Extraction kit. A reaction was set up including the insert, vector, and 5X In-fusion HD Enzyme Premix from Takara Bio (Cat# 102518) following the manufacturer's protocol in the In-fusion HD Cloning Kit User Manual. The reaction mix was transformed into Stellar cells supplied as part of the In-fusion kit. Several methods were attempted to eliminate carry through of whole plasmid including DpnI digestion, PCR purification, and gel extraction of the PCR products.

## 2.11 Cell-to-cell fusion assays

The assay is used to test fusion between cells due to receptor-envelope interactions. pREC-nfl-HIV is comprised of the near-full-length HIV genome under the control of the CMV promoter. This vector allows for the expression of HIV proteins but does not produce infectious virus particles as it lacks the 5' LTR. It was transfected into 293T, resulting in HIV envelope expression on the cell surface. Upon being incubated with cells expressing CD4 and CCR5 receptors (the receptors traditionally utilized by HIV envelope to enter and infect human cells), the two cell types should theoretically fuse due to receptor-envelope interactions, which can then be quantified as described below<sup>74</sup>. This system was used to test receptor-envelope interactions between HIV

envelope and human CD4/CCR5 or feline CD4/CCR5.

Two varying versions of the assay were used for this project – the luciferase system, and the  $\beta$ -galactosidase system. The former is dependent on luciferase expression from the pDM128fLuc plasmid, which is under the control of HIV Rev and Tat. The plasmid is transfected into the cells that express the receptors. If fusion occurs upon incubation of the receptor-expressing cells with the envelope-expressing cells, Rev and Tat produced in the pREC-nfl-HIV-transfected 293T should be able to drive the expression of luciferase in the CD4/CCR5 expressing cells<sup>75,76</sup>. The latter is a variation of the  $\alpha$ -complementation system. The  $\alpha$  and  $\omega$  portions of the  $\beta$ -galactosidase protein are enzymatically inactive independently, but active when expressed together<sup>77</sup>. The  $\alpha$  portion is transfected into the pREC-nfl-HIV-transfected 293T, and the  $\omega$  portion into cells expressing the receptors. If fusion occurs, the products of the two fragments can interact to produce the reconstituted product of the  $\beta$ -galactosidase gene<sup>77</sup>.

pREC-nfl-HIV samples included subtype A, B, C, and D samples that were cloned into the pREC backbone. Subtypes A, C, and D backbones were isolated from Uganda and Zimbabwe patient cohorts. Subtype B were isolated from America and Belgium patient cohorts. These had been cloned previously in the Eric Arts Lab. Table 4 contains a list of all the pREC-nfl-HIV plasmids used.

Envelope expression: 700,000 cells/well 293T were plated in 6-well plates and incubated overnight. Independent transfections were conducted to express envelopes from varying pREC-nfl-HIV. The  $\alpha$  portion of the  $\beta$ -galactosidase gene was additionally transfected into these when implementing the  $\beta$ -galactosidase system. No other vectors apart from the pREC-nfl-HIV plasmids were transfected when implementing the luciferase system.

Receptor expression: The luciferase assay was first attempted with 293T cells that were triple-transfected with CD4 and CCR5 vectors to express the two receptors, along with the pDM128fLuc plasmid. In alternate versions of the assay, stable cell lines of CRFK and U87 already expressing CD4 and CCR5 were utilized and they were tested using the luciferase system as well as the  $\beta$ -galactosidase system. 600,000 cells/well U87.CD4.CCR5 and 500,000 cells/well huCD4/huCCR5 or feCD4/feCCR5 CRFK cells

were plated. These were transfected with the pDM128fLuc plasmid when the luciferase-detection assay was used. When the  $\beta$ -galactosidase system was used, these cell lines were transfected with the  $\omega$  fragment of the gene.

For transfections, the cells were plated and allowed to incubate overnight. The media in each well was replaced with 1.8 ml DMEM/1% FBS the next day. To make the transfection mix for each well, 12  $\mu$ l Polyethyleneimine (PEI) was mixed with Opti-MEM media to total a volume of 150  $\mu$ l and incubated for 5 minutes. A total of 4 $\mu$ g plasmid was mixed with Opti-MEM to total a volume of 150 $\mu$ l. The plasmid and transfection reagent media were mixed together and incubated for 20 mins. This was added to each well on the plate and incubated for 4 hours, after which the media was replaced with DMEM/10% FBS.

48 hours post-transfection, the cells were lifted from the plate as follows. Each well was washed thrice with PBS. 6.24 mM EDTA (500 $\mu$ l) was added to each well and incubated at 37°C for 10 minutes. Post-incubation, 1ml DMEM complete was added to each well and pipetted to break any cell clumps, following which they were counted using a hemocytometer. Cells of each well were centrifuged at 200xg for 5 minutes and resuspended to a concentration of 2 million cells/ml, of which 50 $\mu$ l of the cells (100,000 cells) expressing the receptors (and the pDM128fLuc plasmid for the luciferase system or the  $\omega$  plasmid for the  $\beta$ -galactosidase system) were added to independent wells in a round-bottom 96-well plate. These were mixed with 50  $\mu$ l of cells expressing a pREC-nfl-HIV plasmid (and the  $\alpha$  plasmid for the  $\beta$ -galactosidase system). The cells were pelleted to the bottom of the plate by pulse centrifugation for 5-10 seconds and incubated for 18-24 hours at 37°C/5%CO<sub>2</sub>. If fusion occurs, treatment of the mixed cells with either the luciferase or the  $\beta$ -galactosidase substrate should produce a luminescence signal. The luminescence level is a measure of the receptor-envelope interactions.

For measuring luminescence in the luciferase system, each well was treated with 100  $\mu$ l Britelite plus (Cat# 6066766), which contained a lysis buffer, as well as the substrate. The entire volume of each well was transferred into a black plate. Relative Light Units (RLU) were quantified using the Cytation 5 Imaging Reader.

For measuring luminescence in the  $\beta$ -galactosidase system, the cells were spun at

530xg for 4 minutes. The media was removed and 100µl lysis buffer was added to each well and spun at 600rpm for 5 minutes on an Eppendorf MixMate plate shaker (Cat# 2137900) to facilitate lysis. The plate was then incubated for 10 minutes at room temperature. It was centrifuged at 1200xg for 7 minutes. A volume of 10µl was transferred into a white plate and 100µl of the substrate, Galacto Star (appliedbiosystems Cat# T1012) diluted 1:50 in buffer, was added. This was incubated at room temperature in the dark for 50 minutes on a platform shaker, following with the reading was conducted. Relative Light Units (RLU) were quantified using the Cytation 5 Imaging Reader.

## 2.12 Graphing and statistical analysis

GraphPad Prism 8 was used to construct the graphs and conduct statistical analysis for all cell-to-cell fusion assay figures. A one-way ANOVA followed by Tukey's post-test was performed to compare the mean RLU for each full envelope to the mean RLU for SGΔenv. This was performed for huCD4/huCCR5 CRFK as well as U87.CD4.CCR5.

“\*” , p<0.05; “\*\*” , p<0.01; “\*\*\*” , p<0.001.

## 2.13 Alignments

Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) or BioEdit (for Windows 95/98/NT/XP) were used for sequence alignments. Percent Identity and Percent Similarity calculations were conducted using BLAST.

Table 2. List of primers used for PCR amplifications.

	Primer Name	Sequence	Purpose
1	pREC-FIVC-F3	TTGACGCAAATGGGCGGTAGGCG TGTACGGTGGGAGGTCTATATAA GCAGAGCTCTCTGGCTAACGTTG GCGCCCGAACAGGGACT	PCR amplification for yeast cloning – nfl
2	FIVC-4715R	GCATTATTGCTTTGACTGTGC	
3	FIVC-4650F	CAGGATTCCTTTGGGCACA	
4	pREC-FIVC-R3	GTTGGGATTCCATTTTAAATAAG GCAATAATATTAGGTATGTAGAT ATACTAGAAGTTCTCCTCGTGCG AAGTCTTCGGC	
5	pREV-FIVC-R4	GATTTTGATGTAATTGTTGGGATT CCATTTTAAATAAGGCAATAATA TTAGGTATGTAGATATACTAGAA GTTCTCCTCGTGCGAAGTCTTCGG C	
6	nfl-cplt-1F	TACGGTGGGAGGTCTATATAAGC AGAGCTCTCTGGCTAACTGGGAT GAGTATTGGGACCCTGA	PCR amplification for yeast cloning - cplt
7	nfl-cplt-2030-R2	AATAATATTAGGTATGTAGATAT ACTAGAAGTTCTCCTCGCAGGTC TTCTGTCTAATGTTGTAGTGG	
8	RRE-fwd	AGACATTGTTAGATTTATAAATT ATAATAAAGTGGGTACCACTACA ACATTAGACAGAAGACCTGGATG AGGAATTGTCACAAAATATG	PCR amplification for yeast cloning – cplt- rre
9	RRE-rvrs	GATTCCATTTTAAATAAGGCAAT AATATTAGGTATGTAGATATACT AGAAGTTCTCCTCGCTTTCCCTT ATAATACAGAGTTTATTAA	
10	cplt-r5-f	TTGACGCAAATGGGCGGTAGGCG TGTACGGTGGGAGGTCTATATAA GCAGAGCTCTCTGGCTAACGAGT CTCTCAGTTGAGGACTTTCG	
11	cplt-2030R	CAGGTCTTCTGTCTAATGTTGTAG TGG	
12	env-ext-5prime	TAACATTGTAACAAGTGTTATTT GCACA	PCR amplification of FIV backbone for In-fusion cloning
13	env-ext-3prime	TGGAATGGTAACCAATATCA	
14	env-int-5prime	GACTACTAAGGGTGGGAGTCT	
15	env-int-3prime-ct	TGCTTACCTACATTGCTTGATT	
16	env-int-3prime-rre	ATAGATGATGAGGAATTGTCACA A	

17	AD8-env-fivf	CCACCCTTAGTAGTCATGAGAGT GAAGGAGAAGTATC	PCR amplification of HIV envelope for In-fusion cloning
18	YU2-env-fivf	CCACCCTTAGTAGTCATGAGAGC GACGGAGAT	
19	hiv-env-fivct	CAATGTAGGTAAGCACTGCCTAA CTCTATTCATATAGAA	
20	AD8-env-fivrre	TTCCTCATCATCTATCTCAGCTAC TGCTATGGCTGT	
21	YU2-env-fivrre	TTCCTCATCATCTATCTCAGCTAC TGCTATAGCTGTG	
22	feCCR5-F1	CTTGGATCCCGTCGAGCAAAATG GATTAT	feCCR5 amplification
23	feCCR5-R1	CTTGTCGACGTACAAACACCCGG TCCA	
24	huCD4-F1	TATTGAATTCGCCACCATGAACC GGGGAGTCCCT	huCD4 amplification
25	huCD4-R1	TATTGTGCGACTCATCAAATGGGG CTACATGTCTTC	
26	feCD4-Fexternal	CTGCCTCAGCAAGGCCACA	feCD4 amplification
27	feCD4-Rexternal	AAGCTCCCCGGTCCTGTGC	
28	feCD4-F2	ATGCCTTACAGGCTCCTC	
29	feCD4-R2	AGCGGGAGTGAGTCCATCAT	

Table 3. List of plasmids used in this project

Plasmid	Purpose	Source
pBABE.huCCR5	Cell line generation transfection	NIH
pBABE.hygro	Cloning of feCD4 and huCD4 plasmids	Yong Gao Lab
pBABE.huCD4 (4)	Cell line generation transfection	Cloned
pBABE.feCCR5 (4657-3)	Cell line generation transfection	Cloned
pBABE.feCD4 (4370T)	Cell line generation transfection	Cloned
FIVC-36 plasmid	Virus production, yeast cloning	Elder Lab (UCSD)
pREC_URA3	Yeast cloning	Gao Lab
cplt	Virus production	Cloned
cplt-rre	Virus production	Cloned
nfl-3	Virus production	Cloned
nfl-6	Virus production	Cloned
pDM128fLuc	VERITROP assay	Arts Lab
pREC-nfl-HIV plasmids	VERITROP assay	Arts Lab
SG3 $\Delta$ env	VERITROP assay	Arts/Gao Lab
pCMV $\alpha$	VERITROP assay	Arts Lab
pCMV $\omega$	VERITROP assay	Arts Lab

## CHAPTER 3: RESULTS

### 3.1 Yeast cloning system to produce FIV particles

#### 3.1.1 Weak RT activity is detected with FIV produced from the two-plasmid system using cplt and nfl

The first step in producing recombinant FIV/HIV viruses was to generate yeast cloning vectors containing the complete FIV genome split into two parts – cplt and nfl (Figure 6 and 8) that can produce replication competent virus upon co-transfection of cells. The yeast cloning system was used to insert the FIV cplt and nfl fragments into the pREC backbone. The plasmids were verified by restriction digests and PCR screening post yeast-cloning, miniprep, and bacterial transformations. Additionally, each was thoroughly sequenced. Two nfl versions, nfl-3 and nfl-6, were chosen to proceed with, along with cplt-3. Sequencing showed that both nfl plasmids contained several sporadic point mutations distinct from the original FIV sequence (Figure 9). It is unknown whether these could impact the production of infectious virus particles, or effect replication competence and fitness. No such point mutations were seen in the cplt plasmid.

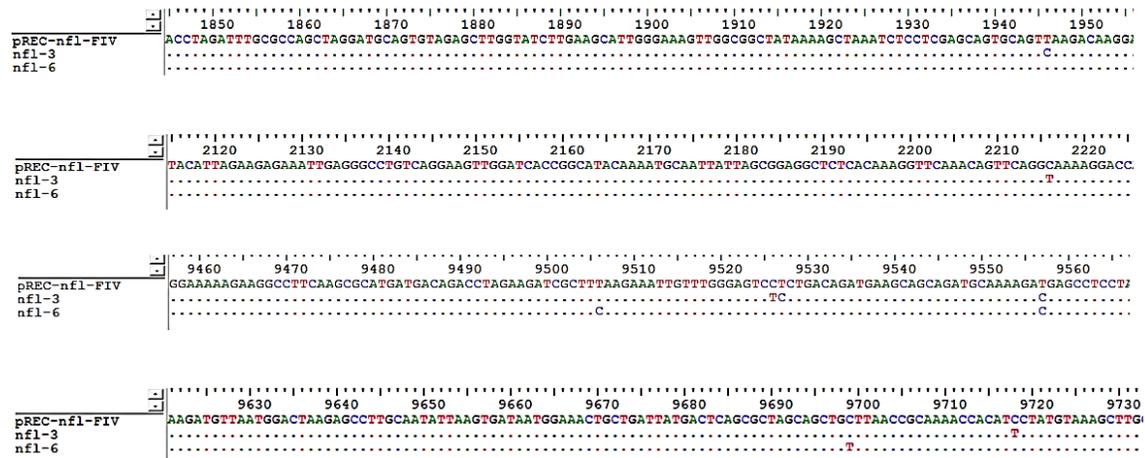


Figure 9. Point mutations seen in the two clones nfl plasmids. The first sequence represents the expected pREC-nfl-FIV. The second sequence represents nfl-3 and the third sequence represents nfl-6.

The two plasmids of *nfl* and *cplt* were transfected into CRFK cells and 293T cells to produce virus particles. Transfection supernatants containing virus were used to infect GFox cells (CRFK cells expressing the FIV receptor CD134) to evaluate viral replication. Activity of the reverse transcriptase enzyme in cell supernatant was used to quantify virus particles. The RT assay (Figure 10) demonstrated that there was stronger virus production from transfection of 293T cells in comparison to CRFK cells. Additionally, attempts to concentrate the virus using Amicon filters did not appear to increase virus concentration.

Furthermore, a strong positive signal indicative of virus replication was not observed post-infection for any virus produced using the two-plasmid system, and a faint signal is visible for the positive control. The virus for this positive control was made by transfecting the whole FIV-C36 plasmid into 293T cells. An additional positive control with FIV that was previously produced in the lab was used as the RT assay control.

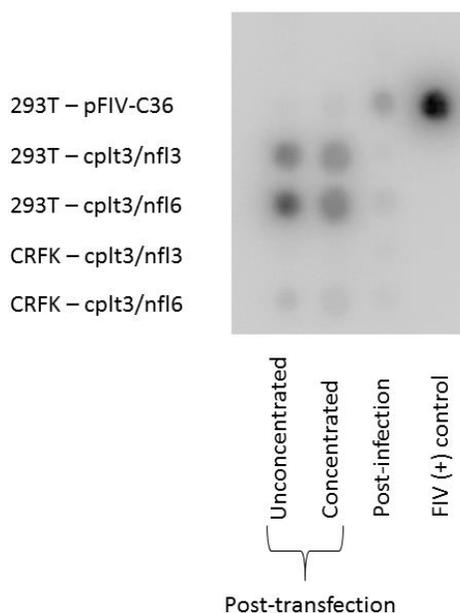


Figure 10. RT assay of FIV produced using the *nfl* and *cplt* plasmid cloned by the yeast complementation system. The assay was conducted on supernatants collected post-transfection (unconcentrated (lane 1) and concentrated (lane 2) supernatant), as well as 6 days post- infection (lane 3). The strong positive signal seen in lane 4 (FIV (+) control) is from FIV that was produced previously in the lab after several passages, to serve as a control.

### 3.1.2 Addition of the FIV RRE to the cplt vector does not improve virus production

According to previous data from the Eric Arts Lab, yeast cloning vectors using a different strain of FIV demonstrated that RNA was being transcribed from the cplt vector, however, it was not being exported to the cytoplasm (A. Moghadasi and E. Arts, unpublished data). Thus, it was hypothesized that the cplt RNA was hindered from being shuttled out of the nucleus as it did not contain the Rev Response element (RRE). FIV Rev protein binds the RRE and facilitate the transport of RNA out of the nucleus. Therefore, we concluded that cplt RNA was not reaching the cytoplasm to be packaged into new virus particles. In accordance with this, the cplt plasmid was altered to include the RRE (Figure 7).

The cplt-rre plasmid also contained a couple of point mutations not seen before (Figure 11).

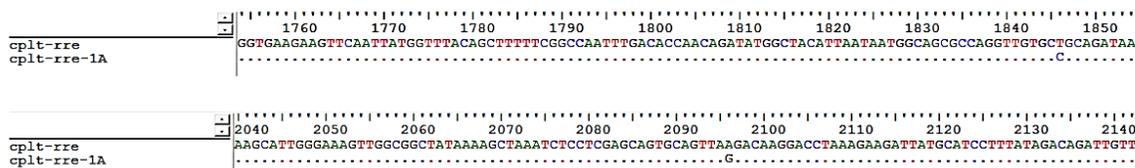


Figure 11. Point mutations seen in cplt-rre. The first sequence represents the expected cplt-rre sequence. The second sequence is of cplt-rre-1A, the clone that was pursued for transfections and infections.

Cplt-rre was transfected with either nfl-3 or nfl-6 into 293T cells. The virus was used to infect Gfox cells. The RT assay (Figure 12a) demonstrated that virus production post- infection did not increase, in contrast to the positive control. The virus harvested on day 9 post-infection was used to infect GFox cells for a second passage. This was monitored by RT assays (Figure 12b). Again, levels of RT activity did not demonstrate increases indicative of virus replication, in contrast to the FIV positive control.

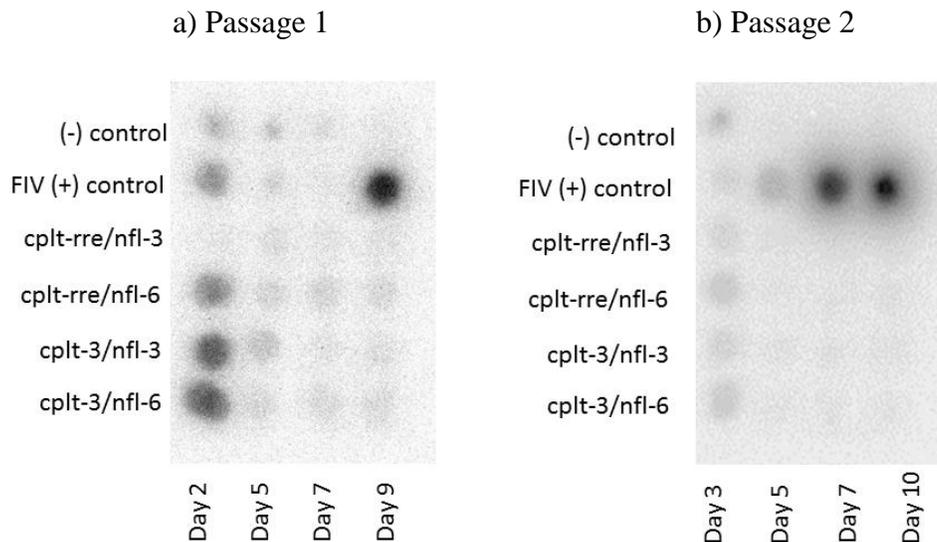
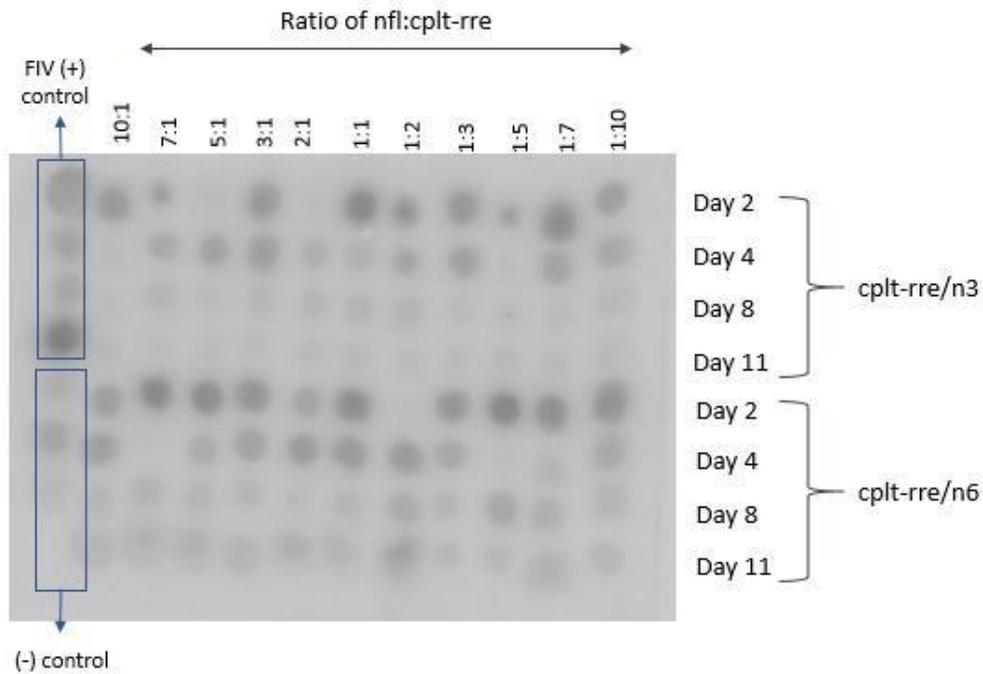


Figure 12. RT assay of Gfox infection with virus made using cplt-rre and nfl-3 or nfl-6. Virus-containing 293T transfection supernatant was used to infect GFox cells. a) Passage 1 was continued for 9 days, after which the virus was added to newly plated Gfox cells. b) Passage 2 of Gfox cells was continued for 10 days post-infection.

### 3.1.3 Altering the ratio of nfl to cplt during transfections produces FIV with weak RT activity

Furthermore, to attempt to produce infectious virus, the approach to control the ratio of cplt-rre to nfl plasmid during transfections was undertaken, as this was previously an effective strategy for HIV production in the Eric Arts Lab. The ratio range extended from 10:1 to 1:10 of nfl:cplt plasmid. This was done independently for nfl-3 and nfl-6. Supernatants were collected two days after transfection and used to infect GFox cells (Figure 13). The infection was continued for two passages where the virus harvested on day 11 of passage 1 was applied to GFox cells for a second passage. No detectable virus production was observed with either nfl-3 or nfl-6 ratio-controlled infections, in contrast to the FIV positive control which demonstrated increased RT activity during the first passage and strong positive results later in the second passage.

## a) Passage 1



## b) Passage 2

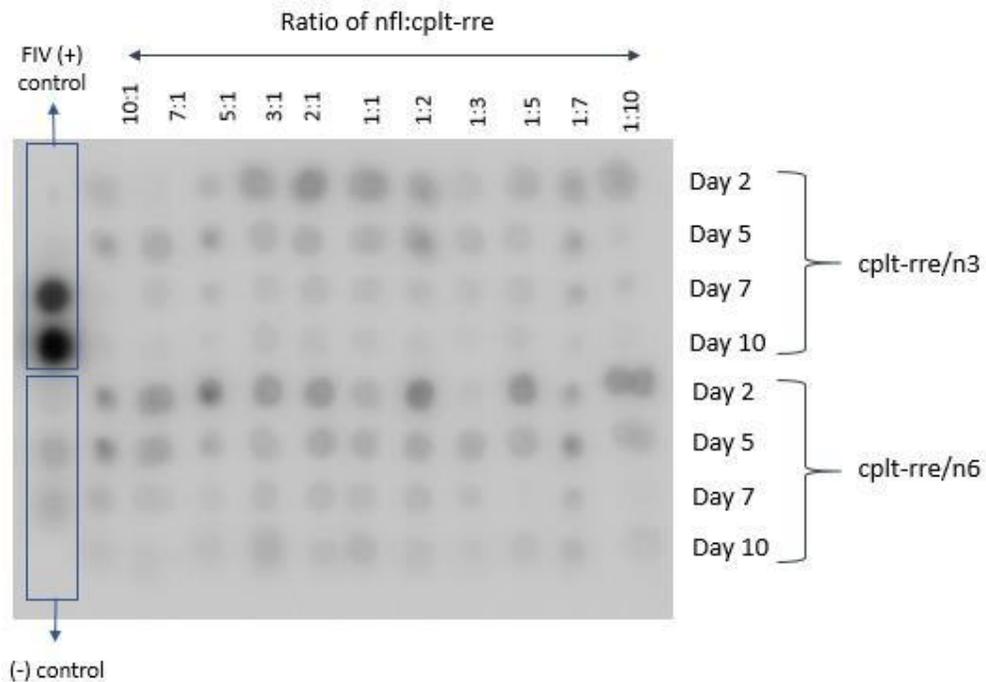


Figure 13. RT assays of Gfox infection with viruses made through ratio-controlled transfections. Ratios are indicative of nfl:cplt-rre a) Passage 1. b) Passage 2. Passage 1 ended on Day 11 and Passage 2 on Day 10.

### 3.1.4 Spinoculation of FIV on to GFox cells does not enhance infectivity

Spinoculations on GFox cells were done using selected viruses produced through ratio-controlled transfections. Spinoculations are centrifugal infections wherein the virus and cells are mixed together and centrifuged for several hours. Enhanced HIV infectivity has been demonstrated using spinoculations and the mechanism for this has been proposed to be due to increased viral deposition on the cell surface<sup>78</sup>. Thus, we attempted to use this protocol to test whether enhanced FIV infectivity was possible using virus produced from the two-plasmid system. The spinoculations were continued for two passages and were only performed using nfl-6, as it seems to produce virus with slightly higher RT activity than nfl-3, as seen in Figure 12. The positive control showed increasing RT activity during the first passage and strong signal starting at day 4 of the second passage. Spinoculations using virus produced from the two-plasmid transfection utilizing nfl-6 and cplt-rre did not result in Gfox infection (Figure 14).

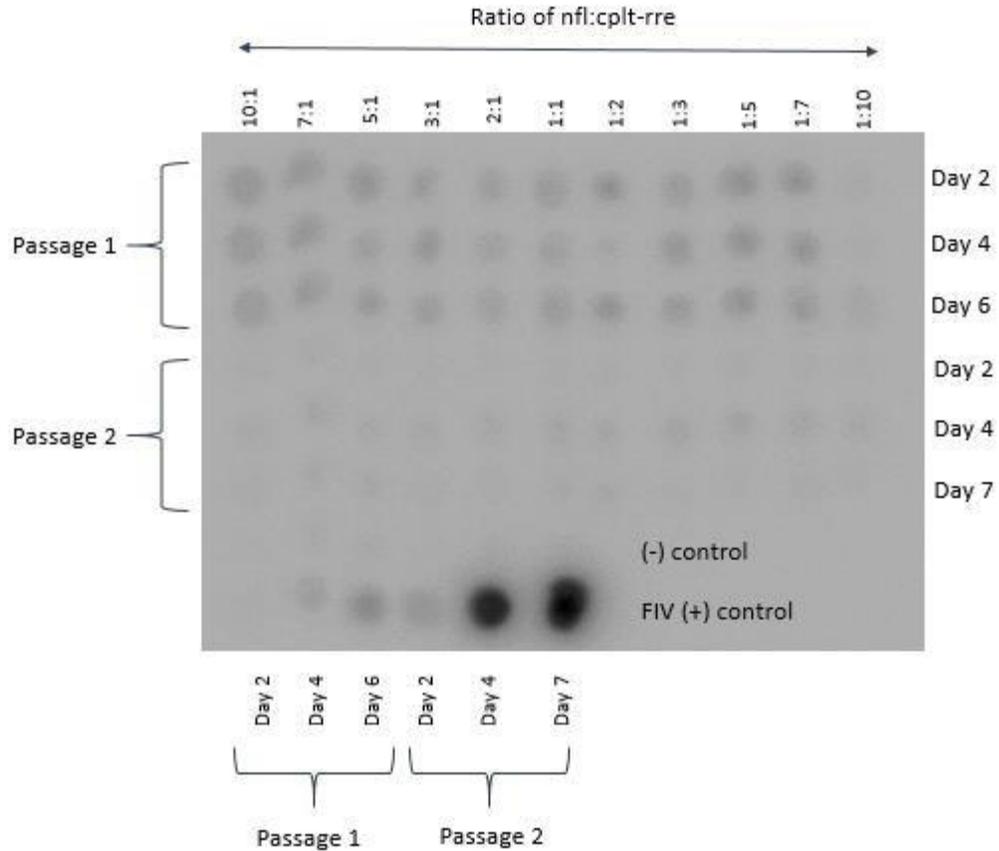


Figure 14. RT assay of spinoculations done with virus produced through ratio-controlled infections using cplt-rre and nfl-6. Supernatants of virus produced using 293T that were transfected with different ratios of nfl-6 and cplt-rre were used to spinoculate Gfox cells for Passage 1 that ended on day 6 post-infection. The supernatant of Passage 1 was applied on newly plated Gfox cells for a second passage that was continued for 7 days.

### 3.2 Sequencing lab cat feCD4 and feCCR5 to find the amino acid sequences with highest identity to the consensus sequence

feCD4 and feCCR5 vectors were cloned for CRFK transfection to generate stable cell lines expressing either of the receptors, in addition to a cell line expressing both. Prior to cloning feCD4 or feCCR5 into the pBABE plasmid, DNA and RNA samples from lab cats were obtained and sequenced. This was conducted because of the lack of available sequences in GenBank, as well as the expectation that diversity exists in both CD4 and CCR5, depending on the cat colony from which the samples are retrieved. Only one sequence was available through GenBank (AAB24450.1) for feline CD4 and only ten complete feline CCR5 sequences were available, which made it imperative to sequence several more samples.

For feline CCR5, twelve sequences, some of which were only partial, were available in GenBank, and seven more were sequenced from a lab cat colony. Sample 4657 (also referred to as 4657-3 in the Troyer Lab) was most identical to the consensus sequence (Figure 15) and was cloned into the pBABE.puro plasmid.

Sequencing seven cat samples from a lab cat colony at CSU revealed relatively low genetic diversity in feCD4, with only seven amino acid positions having polymorphisms. Sample feCD4 4370 (also referred to as 4370T in the Troyer Lab) was most identical to the consensus sequence (Figure 16), and it was cloned into the pBABE.hygro plasmid. Increased genetic diversity was seen for CCR5 between individual cats from the same colony, when compared to CD4.



```

feCD4  MNQGVFRHLLLVQLVHLEAAVPGKKEVVLGKAGGTAELPCQASQKKYMTFTWRLSSQV 60
4370  MNQGVFRHLLLVQLVHLEAAVPGKKEVVLGKAGGTAELPCQASQKKYMTFTWRLSSQV 60
4376  MNQGVFRHLLLVQLVHLEAAVPGKKEVVLGKAGGTAELPCQASQKKYMTFTWRLSSQV 60
4385  MNQGVFRHLLLVQLVHLEAAVPGKKEVVLGKAGGTAELPCQASQKKYMTFTWRLSSQV 60
4362  MNQGVFRHLLLVQLVHLEAAVPGKKEVVLGKAGGTAELPCQASQKKYMTFTWRLSSQV 60
4381  -----XLVHLEAAVPGKKEVVLGKAGGTAELPCQASQKKYMTFTWRLSSQV 46
4305  -----XLLVLQLVHLEAAVPGKKEVVLGKAGGTAELPCQASQKKYMTFTWRLSSQV 51
4386  -----XLLQLVHLEAAVPGKKEVVLGKAGGTAELPCQASQKKYMTFTWRLSSQV 48
*****
feCD4  KILESQHSLLCTGSSKLRFECKKILHDQGSFPLVIKSLQVADSGIYCEVENKKREV 120
4370  KILESQHSLLCTGSSKLRFECKKILHDQGSFPLVIKSLQVADSGIYCEVENKKREV 120
4376  KILESQHSLLCTGSSKLRFECKKILHDQGSFPLVIKSLQVADSGIYCEVENKKREV 120
4385  KILESQHSLLCTGSSKLRFECKKILHDQGSFPLVIKSLQVADSGIYCEVENKKREV 120
4362  KILESQHSLLCTGSSKLRFECKKILHDQGSFPLVIKSLQVADSGIYCEVENKKREV 120
4381  KILESQHSLLCTGSSKLRFECKKILHDQGSFPLVIKSLQVADSGIYCEVENKKREV 106
4305  KILESQHSLLCTGSSKLRFECKKILHDQGSFPLVIKSLQVADSGIYCEVENKKREV 111
4386  KILESQHSLLCTGSSKLRFECKKILHDQGSFPLVIKSLQVADSGIYCEVENKKREV 108
*****
feCD4  ELLVFLGTAIKVDPSSGSGSSSSSTSTSIYLLQGGSLTLTLESPSSNPSVQIKGPGNK 180
4370  ELLVFLGTAIKVDPSSGSGSSSSSTSTSIYLLQGGSLTLTLESPSSNPSVQIKGPGNK 180
4376  ELLVFLGTAIKVDPSSGSGSSSSSTSTSIYLLQGGSLTLTLESPSSNPSVQIKGPGNK 180
4385  ELLVFLGTAIKVDPSSGSGSSSSSTSTSIYLLQGGSLTLTLESPSSNPSVQIKGPGNK 180
4362  ELLVFLGTAIKVDPSSGSGSSSSSTSTSIYLLQGGSLTLTLESPSSNPSVQIKGPGNK 180
4381  ELLVFLGTAIKVDPSSGSGSSSSSTSTSIYLLQGGSLTLTLESPSSNPSVQIKGPGNK 166
4305  ELLVFLGTAIKVDPSSGSGSSSSSTSTSIYLLQGGSLTLTLESPSSNPSVQIKGPGNK 171
4386  ELLVFLGTAIKVDPSSGSGSSSSSTSTSIYLLQGGSLTLTLESPSSNPSVQIKGPGNK 168
*****
feCD4  SKSGVHLSLSLQELQESGTCTCTVSQSQTLVFNNTNILLVLAFAKVSNTVYAKEGEQVEF 240
4370  SKSGVHLSLSLQELQESGTCTCTVSQSQTLVFNNTNILLVLAFAKVSNTVYAKEGEQVEF 240
4376  SKSGVHLSLSLQELQESGTCTCTVSQSQTLVFNNTNILLVLAFAKVSNTVYAKEGEQVEF 240
4385  SKSGVHLSLSLQELQESGTCTCTVSQSQTLVFNNTNILLVLAFAKVSNTVYAKEGEQVEF 240
4362  SKSGVHLSLSLQELQESGTCTCTVSQSQTLVFNNTNILLVLAFAKVSNTVYAKEGEQVEF 240
4381  SKSGVHLSLSLQELQESGTCTCTVSQSQTLVFNNTNILLVLAFAKVSNTVYAKEGEQVEF 226
4305  SKSGVHLSLSLQELQESGTCTCTVSQSQTLVFNNTNILLVLAFAKVSNTVYAKEGEQVEF 231
4386  SKSGVHLSLSLQELQESGTCTCTVSQSQTLVFNNTNILLVLAFAKVSNTVYAKEGEQVEF 228
*****
feCD4  SFPLNFEDELNGNLRWKAEGAPSSLLWISFTLKNKQLSVKVEDPYSKLQWIDSLPLRFT 300
4370  SFPLNFEDELNGNLRWKAEGAPSSLLWISFTLKNKQLSVKVEDPYSKLQWIDSLPLRFT 300
4376  SFPLNFEDELNGNLRWKAEGAPSSLLWISFTLKNKQLSVKVEDPYSKLQWIDSLPLRFT 300
4385  SFPLNFEDELNGNLRWKAEGAPSSLLWISFTLKNKQLSVKVEDPYSKLQWIDSLPLRFT 300
4362  SFPLNFEDELNGNLRWKAEGAPSSLLWISFTLKNKQLSVKVEDPYSKLQWIDSLPLRFT 300
4381  SFPLNFEDELNGNLRWKAEGAPSSLLWISFTLKNKQLSVKVEDPYSKLQWIDSLPLRFT 286
4305  SFPLNFEDELNGNLRWKAEGAPSSLLWISFTLKNKQLSVKVEDPYSKLQWIDSLPLRFT 291
4386  SFPLNFEDELNGNLRWKAEGAPSSLLWIS -XLLKNKQLSVKVEDPYSKLQWIDSLPLRFT 287
*****
feCD4  LPNVLRYAGSGNLTVLVDKQGLQEQVKLVWVRVTSQGNLTCVGLGPTSPELTSKLLK 360
4370  LPNVLRYAGSGNLTVLVDKQGLQEQVKLVWVRVTSQGNLTCVGLGPTSPELTSKLLK 360
4376  LPNVLRYAGSGNLTVLVDKQGLQEQVKLVWVRVTSQGNLTCVGLGPTSPELTSKLLK 360
4385  LPNVLRYAGSGNLTVLVDKQGLQEQVKLVWVRVTSQGNLTCVGLGPTSPELTSKLLK 360
4362  LPNVLRYAGSGNLTVLVDKQGLQEQVKLVWVRVTSQGNLTCVGLGPTSPELTSKLLK 360
4381  LPNVLRYAGSGNLTVLVDKQGLQEQVKLVWVRVTSQGNLTCVGLGPTSPELTSKLLK 346
4305  LPNVLRYAGSGNLTVLVDKQGLQEQVKLVWVRVTSQGNLTCVGLGPTSPELTSKLLK 351
4386  LPNVLRYAGSGNLTVLVDKQGLQEQVKLVWVRVTSQGNLTCVGLGPTSPELTSKLLK 347
*****
feCD4  GQAQVSKQQWVRVEDAEAGTQCLLSHKDVLVLLASKAEVLPVPLTRTITNLLTIVLGG 420
4370  GQAQVSKQQWVRVEDAEAGTQCLLSHKDVLVLLASKAEVLPVPLTRTITNLLTIVLGG 420
4376  GQAQVSKQQWVRVEDAEAGTQCLLSHKDVLVLLASKAEVLPVPLTRTITNLLTIVLGG 420
4385  GQAQVSKQQWVRVEDAEAGTQCLLSHKDVLVLLASKAEVLPVPLTRTITNLLTIVLGG 420
4362  GQAQVSKQQWVRVEDAEAGTQCLLSHKDVLVLLASKAEVLPVPLTRTITNLLTIVLGG 420
4381  GQAQVSKQQWVRVEDAEAGTQCLLSHKDVLVLLASKAEVLPVPLTRTITNLLTIVLGG 406
4305  GQAQVSKQQWVRVEDAEAGTQCLLSHKDVLVLLASKAEVLPVPLTRTITNLLTIVLGG 411
4386  GQAQVSKQQWVRVEDAEAGTQCLLSHKDVLVLLASKAEVLPVPLTRTITNLLTIVLGG 407
*****
feCD4  VLGVLVFIGLVVYCCVKWHRRRQAARMSHIKRLLEKKTCCQSHRLQKTCNPI* 474
4370  VLGVLVFIGLVVYCCVKWHRRRQAARMSHIKRLLEKKTCCQSHRLQKTCNPI* 474
4376  VLGVLVFIGLVVYCCVKWHRRRQAARMSHIKRLLEKKTCCQSHRLQKTCNPI* 474
4385  VLGVLVFIGLVVYCCVKWHRRRQAARMSHIKRLLEKKTCCQSHRLQKTCNPI* 474
4362  VLGVLVFIGLVVYCCVKWHRRRQAARMSHIKRLLEKKTCCQSHRLQKTCNPI* 474
4381  VLGVLVFIGLVVYCCVKWHRRRQAARMSHIKRLLEKKTCCQSHRLQKTCNPI* 449
4305  VLGVLVFIGLVVYCCVKWHRRRQAARMSHIKRLLEKKTCCQSHRLQKTCNPI* 453
4386  VLGVLVFIGLVVYCCVKWHRRRQAARMSHIKRLLEKKTCCQSHRLQKTCNPI* 452
*****

```

Figure 16. Feline CD4 alignment. The first input sequence was obtained from GenBank. The remaining seven were sequenced in this thesis. “\*” denotes conserved residues. “.” denotes residues with strongly similar properties. “:” denotes symbols with weakly similar properties.

### 3.3 Generation of cat cell lines expressing CD4 and CCR5

#### 3.3.1 Flow cytometry gating strategy to screen CD4 and CCR5 expression level

Flow cytometry was performed over the course of this aim to screen the level of CD4 and CCR5 expression on the cell surface of transfected CRFK. For our purposes, the cell population was first gated using the forward scatter and side scatter to eliminate any debris. A live/dead stain was utilized so that the live cells could be gated from the total population for analysis. Positive CD4 and CCR5 cells were evaluated from the live cells (Figure 17).

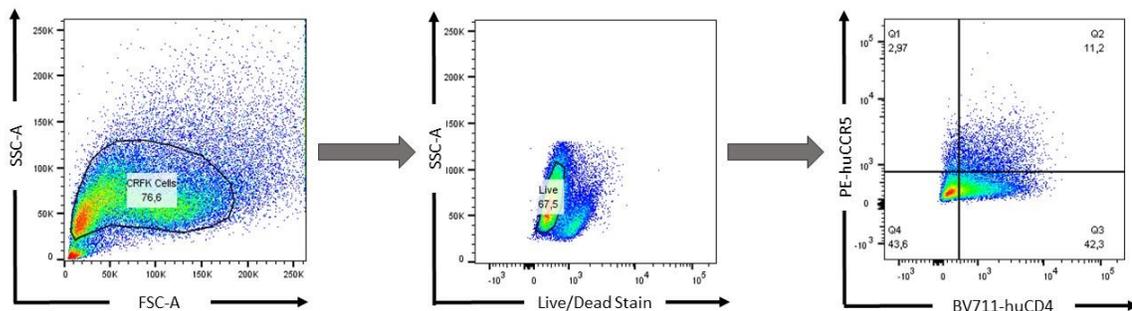


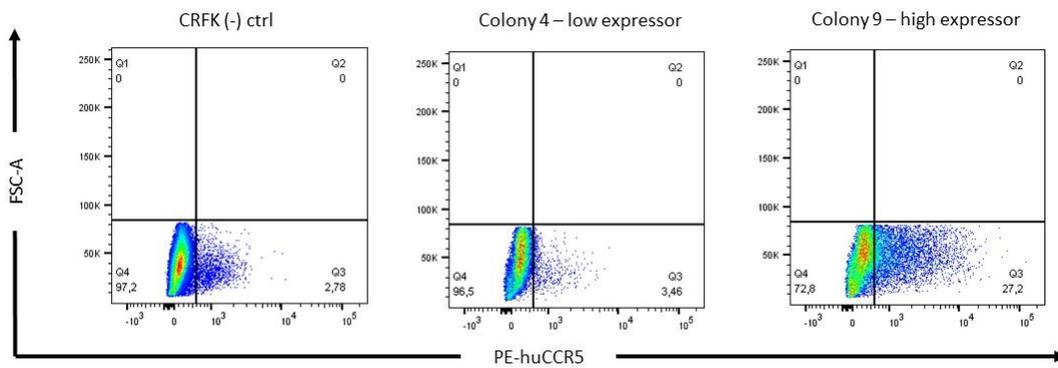
Figure 17. Representative figure of the gating strategy used to evaluate CD4 and CCR5 positivity on CRFK cells. SSC-A: Side Scatter (Area); FSC-A: Forward Scatter (Area).

### 3.3.2 CRFK cells were successfully stably transfected to express huCD4, huCCR5, or both

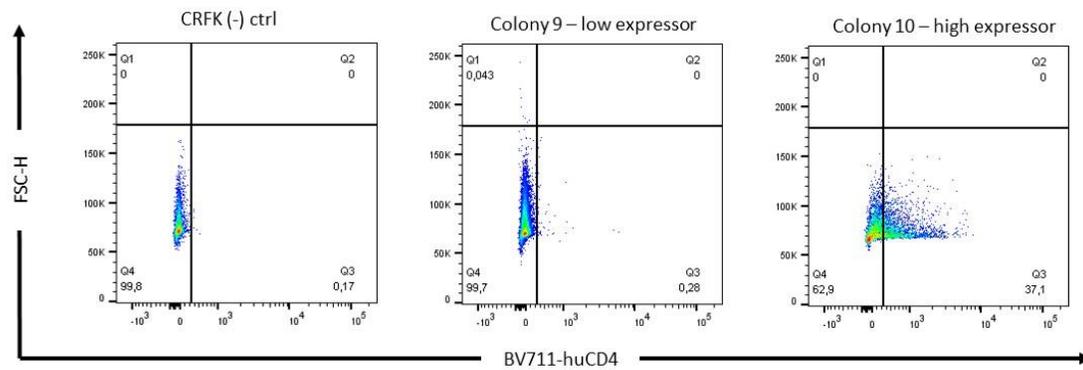
Cat cell lines expressing human CD4, human CCR5, or both were sought to be generated to conduct infection experiments and cell-to-cell fusion experiments. pBABE.huCCR5 and pBABE.huCD4 were transfected independently into CRFK cells and the cells were cultured with appropriate selective drugs: puromycin for CCR5 and hygromycin for CD4. Drug-resistant colonies were transferred to 96-well plates and grown under continued drug selection. Six and three colonies from the respective 96-well plates were expanded into larger cultures and tested for receptor expression by flow cytometry. Colony 9 had the highest level of huCCR5 expression with 27.2% of the live cells expressing the receptor (Figure 18a). Colony 10 had the highest level of huCD4 expression, with 37.1% of the live cells expressing the receptor (Figure 18b).

huCD4 CRFK (Colony 10) was used for pBABE.huCCR5 transfections to generate the huCD4 and huCCR5 double-positive cell lines. Nineteen colonies from the 96-well plate were passaged and grown in selection media containing puromycin and hygromycin. These were screened by flow cytometry (Figure 18c). The percentage of cells that were double-positive ranged <1% to 43.4% of the live cells, across various colonies. Colony M had the highest percentage of cells that were positive for huCD4 and huCCR5.

## a) huCCR5 CRFK



## b) huCD4 CRFK



## c) huCD4/huCCR5 CRFK

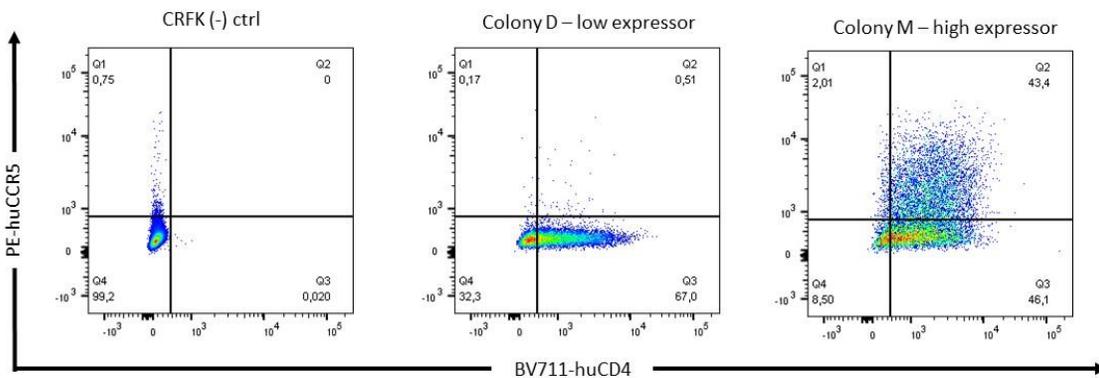


Figure 18. Flow cytometry screen of human receptor-expressing cell lines. a) huCCR5 CRFK. b) huCD4 CRFK. c) huCCR5/huCD4 CRFK. All cell lines screened are not included.

Colony M of huCD4/huCCR5 CRFK was pursued further for FACS to obtain a pure population. Figure 19 demonstrates the gating strategy used. Figure 19a and 19b represent the Fluorescence Minus One (FMO) controls. This control includes all the antibodies, except one. FMOs are used to ensure that fluorescence spillover from other fluorochromes into the given channel are not recorded as positives. They are useful in setting the gates to record true positives. Figure 19a and 19b are FMO controls for CD4 (stained with PE-CCR5 and viability dye only) and CCR5 (stained with BV711-CD4 and viability dye only), respectively. Figure 19c represents the gate for CD4<sup>+</sup>/CCR5<sup>+</sup> cells that were sorted out. Figure 19d represents an analysis of the purity of the cells post-sorting, which showed that there was 99.5% huCD4/huCCR5 double positivity. Flow cytometry was conducted in the same colony to compare the populations pre and post-sort (Figure 20a and 20b). After the cells were grown up and passaged for 8 days, flow cytometry was conducted on them again to check the level of double positivity (Figure 20b) and compared to the level of double positivity on the same cell colony before conducting FACS (Figure 20a). There was an overall shift in the entire population where a larger number of cells were double positive for CD4 and CCR5 and had higher MFIs (Mean Fluorescence Intensities). The MFI is indicative of the level of antibody expression.

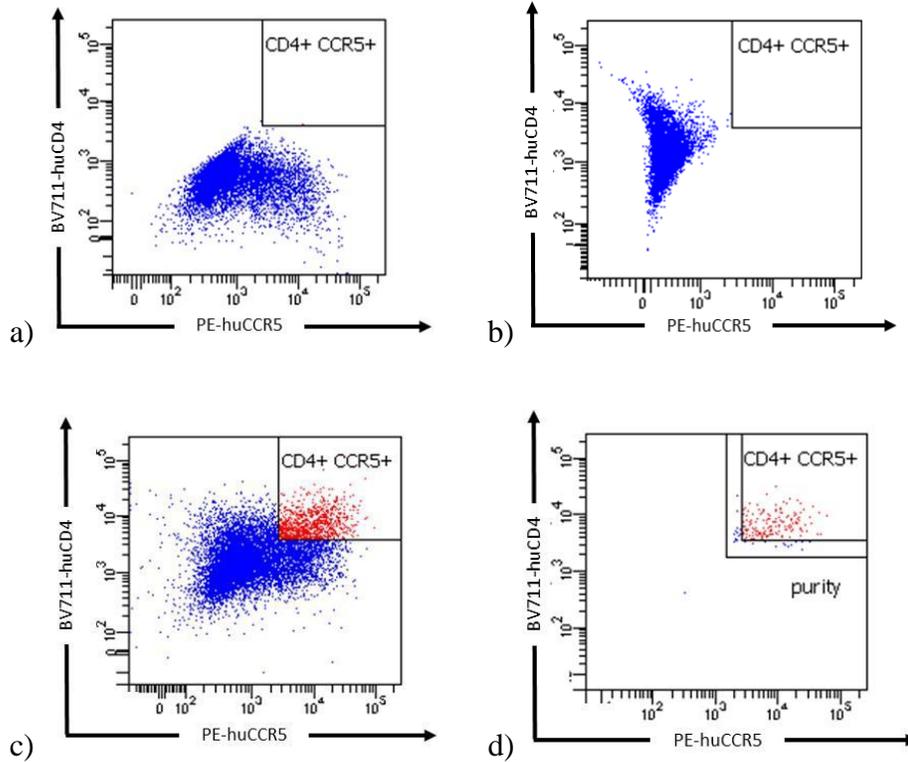


Figure 19. Fluorescence activated cell sorting to obtain double positive huCD4/huCCR5 cells. a), b), and c) represent the strategy to gate on the cells that are positive for huCD4 as well as huCCR5. a) CD4 FMO b) CCR5 FMO c) Gate for CD4+/CCR5+ double positive cells that were sorted out d) Test of double positive cells post-sort.

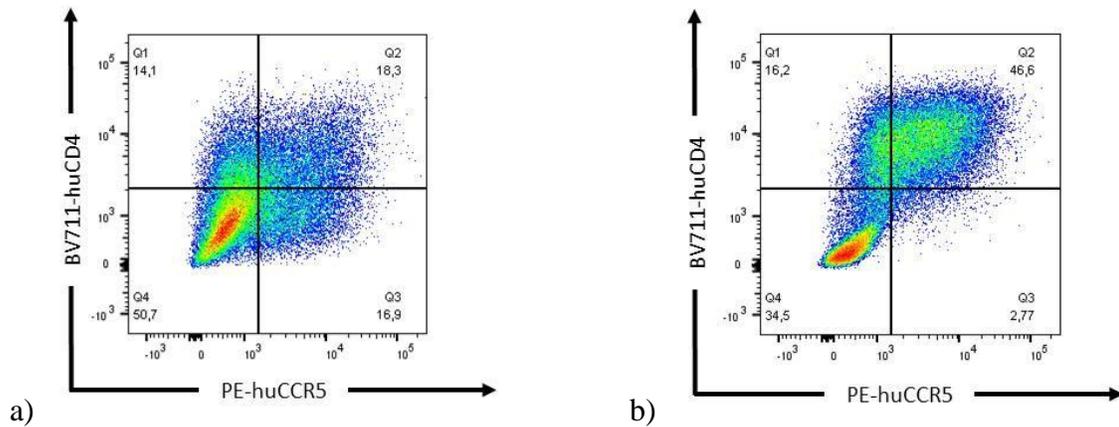


Figure 20. Flow cytometry conducted on huCD4/huCCR5 CRFK to compare the level of CD4 and CCR5 expression on Colony M before and after sorting a) Pre-sort b) Post-sort.

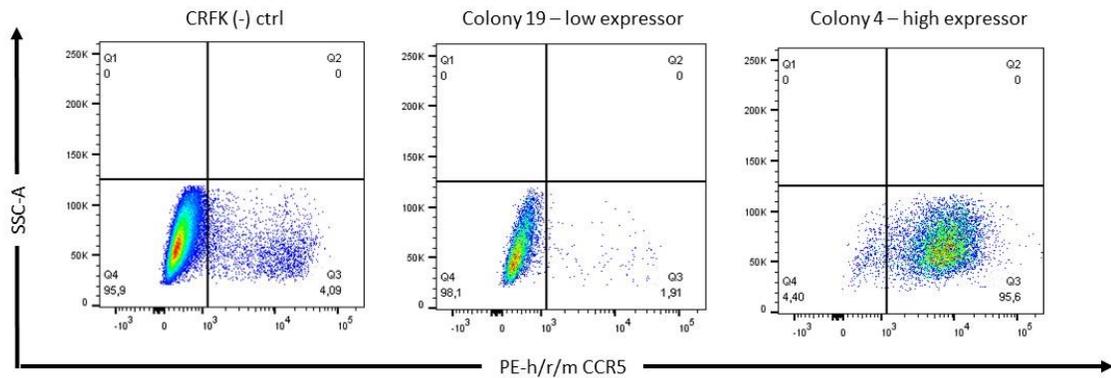
### 3.3.3 CRFK cells were successfully stably transfected to express feCD4, feCCR5, or both

Cat cell lines expressing feline CD4, feline CCR5, or both were sought to be generated to conduct infection experiments and cell-to-cell fusion experiments. CRFK is a cat cell line that does not express either of these receptors. They were transfected with the pBABE.feCCR5 (4657) plasmid that had the highest identity with the consensus feCCR5 sequence. Eighteen colonies were grown up from a 96 well plate, passaged for several weeks in media containing puromycin, following which they were screened by flow cytometry. Colony 4 had the highest level of feCCR5 expression, with 95.6% of the live cells expressing the receptor (Figure 21a).

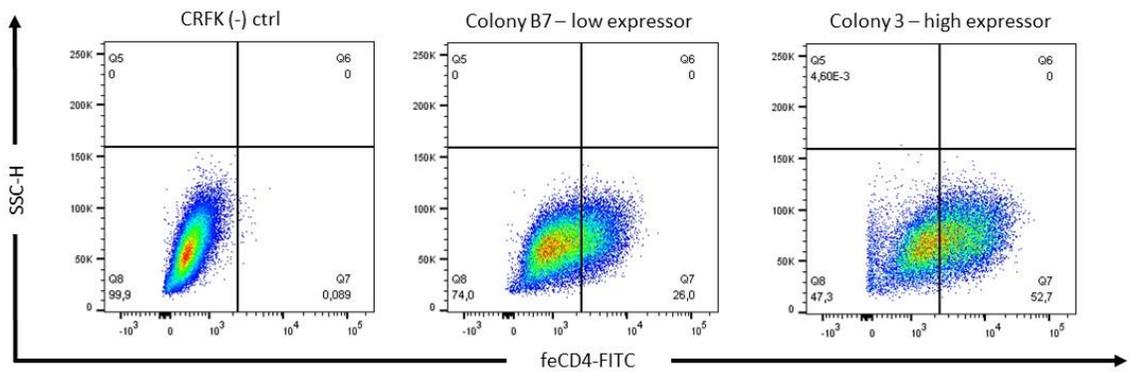
The pBABE.feCD4 (4370) was transfected into CRFK cells as it had the most consensus cat CD4 sequence. Thirteen single colonies were picked, passaged, and grown. Colony 3 had the highest level of feCD4 expression, with 52.7% of the live cells expressing the receptor (Figure 21b).

feCCR5 CRFK (Colony 4) was used for pBABE-feCD4 (4370) transfections to generate the double positive cell line, as it had the highest level of feCCR5 expression. Sixteen colonies were picked and passaged and screened by flow cytometry of feCD4 and feCCR5 expression. Colony 20 had the highest level of feCD4/feCCR5 expression with 8.59% of the cells expressing both receptors (Figure 21c).

## a) feCCR5 CRFK



## b) feCD4 CRFK



## c) feCD4/feCCR5 CRFK

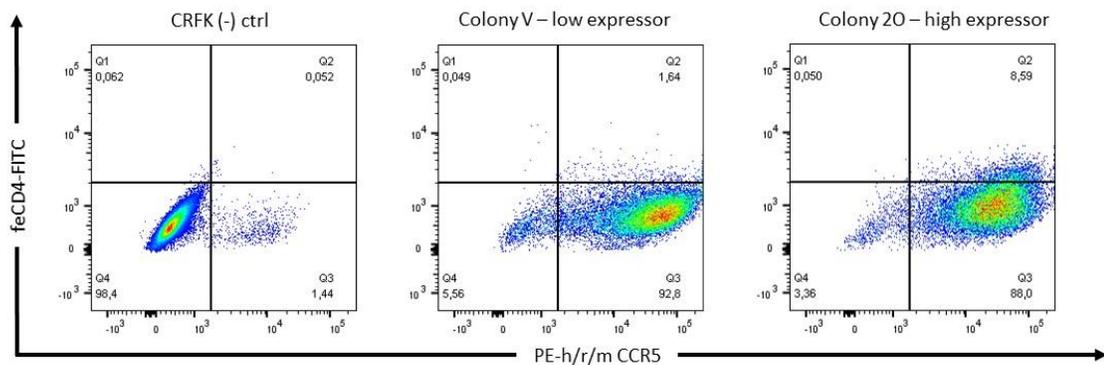


Figure 21. Flow cytometry screen of feline receptor expressing cells. a) feCCR5 CRFK b) feCCR5 CRFK c) feCD4/feCCR5 CRFK. The CCR5 antibody is cross-reactive for human, mouse, and rat CCR5.

### 3.4 huCD4/huCCR5 CRFK can support HIV infection

Colony M of huCD4/huCCR5 CRFK were infected with HIV-AD8, a subtype B virus to test for the presence and functionality of the two receptors on the cell surface. The RT assay (Figure 21a) conducted post-transfection of 293T cells with AD8 plasmids prepped from different bacterial colonies shows strong positivity, indicative of virus production. The RT activity was monitored on Days 2, 4, and 5, all of which showed a positive signal. The signals of AD8 (1) and AD8 (3) increased by Day 5, whereas AD8 (2) remained relatively unchanged from Day 4 (Figure 22a). The experiment was ended on Day 5, as there was a large amount of syncytia formation and the majority of the cell layer was obliterated (Figure 22c), whereas cell crowding is seen in the uninfected negative control (Figure 22b).

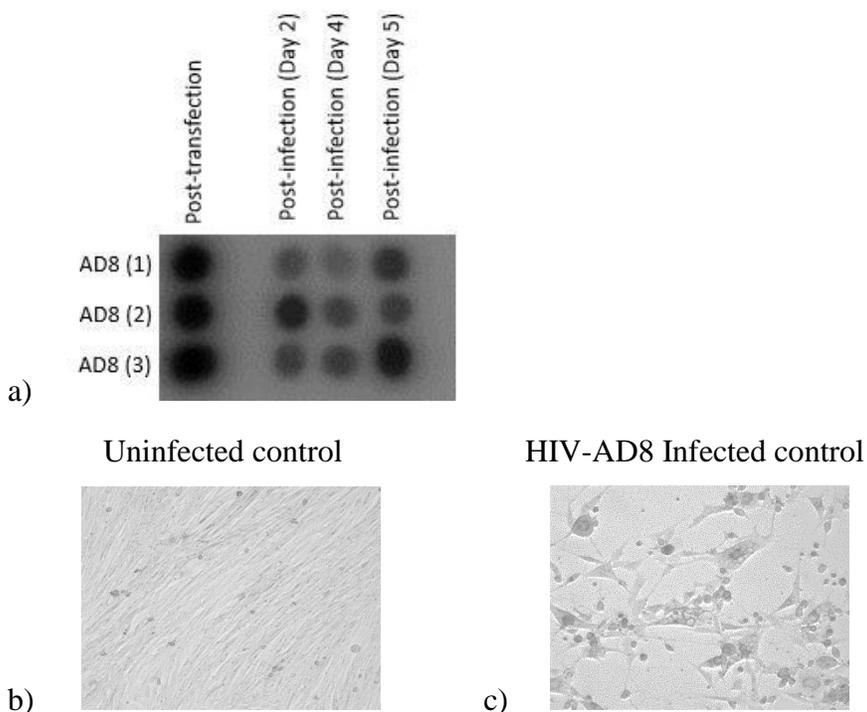


Figure 22. huCD4/huCCR5 CRFK (Colony M) infection with HIVAD8. a) RT assay post-transfection of 293T cells and post-infection of huCD4/huCCR5 (Colony M) CRFK. (1), (2), and (3) are AD8 plasmids prepped from different colonies. Limited sequencing results (not shown) show that no nucleotide differences exist between the three colonies. b) Representative image of the uninfected negative control on Day 5. c) Representative image of syncytia formation on Day 5 taken from the AD8 (2) well.

### 3.5 Cell-to-cell fusion assay experiments to test HIV envelope interactions with CD4 and CCR5 derived from humans

Fusion assays were developed by Eric Arts' laboratory to test receptor-envelope interactions. In their version of the assay, a cell line already expressing huCD4 and huCCR5 is incubated with another cell line expressing the HIV envelope. The level of fusion between the two can be detected either utilizing the luciferase assay or the  $\beta$ -galactosidase assay described in Section 2.11 of the Methods. In this project, the purpose of the assay was to test HIV envelope interactions with huCD4 and huCCR5, as well as feCD4 and feCCR5. Although cell lines expressing human CD4 and CCR5 were available, cell lines expressing feCD4 and feCCR5 was not available to us at first. To combat this, we instead sought to transfect the plasmids required for CD4 and CCR5 expression alongside the pDM128fLuc plasmid required for the luciferase system into cells. These could then be incubated with cells expressing the envelope to test for fusion.

As a proof-of-concept for the necessary three-plasmid transfection, 293T cells were triple-transfected with the huCD4, huCCR5, and pN1-GFP vectors to determine the level of triple-positivity using flow cytometry (Figure 23). Only ~7% of the GFP positive cells were double positive for CD4 and CCR5. Since 51.7% of live cells were GFP positive, only ~3.6% of the total live cells were triple-positive

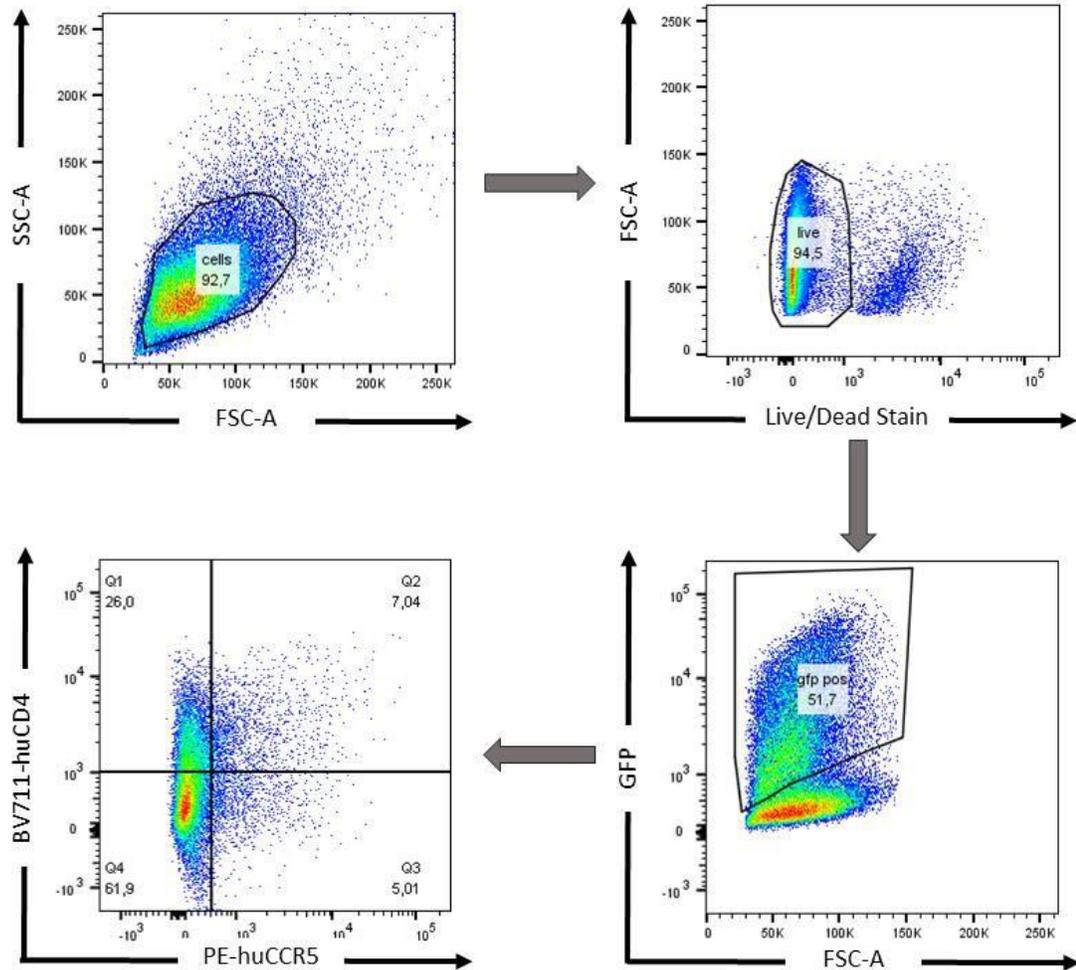


Figure 23. Flow cytometry on 293T cells triple-transfected with pN1-GFP, huCD4 and huCCR5. Gating strategy: cell population → live cells → GFP positive cells → huCD4/huCCR5 positive cells.

### 3.5.1 Triple transfection efficiency of 293T cells is too low to determine whether true receptor-envelope interactions are occurring when huCD4, huCCR5 and pDM128fLuc-transfected 293T are incubated with pREC-nfl-HIV-transfected 293T

The cell-to-cell fusion assay was conducted using 293T cells triple-transfected with the huCD4, huCCR5, and pDM128fLuc plasmid vectors. The pDM128fLuc plasmid encodes the luciferase gene whose expression is Rev and Tat dependent. The triple-transfected 293T were incubated with 293T cells expressing HIV envelope from the

pREC-nfl-HIV plasmid. Several pREC-nfl-HIV plasmids were obtained from Arts Lab, where they were cloned. A total of 45 plasmids were screened by restriction digestion using SacI and SacII; 15 of these were positive.

The HIV envelope expressed on the cell surface as a result of pREC-nfl-HIV transfection can mediate fusion with cell lines that express human CD4 and CCR5, upon being incubated with them, through receptor-envelope interactions. When cell-to-cell fusion occurs, the HIV Tat and Rev proteins expressed from pREC-nfl-HIV drive expression of luciferase via pDM128fLuc. In this case, the triple-transfected 293T (transfected with the huCD4, huCCR5, and pDM128fLuc vectors) were mixed 293T expressing HIV envelope. Individual pREC-nfl-HIV, each expressing a distinct envelope variant, were used for transfections. The subtypes of these envelope are listed in Table 4.

Table 4. List of all the IDs and the corresponding subtypes of the pREC-nfl-HIV plasmids. “U” indicates samples obtained from the Uganda cohort and “Z” indicates samples that were obtained from the Zimbabwe cohort. All the subtype B samples were obtained from American and Belgian cohorts.

pREC-nfl-HIV plasmid ID	Subtype
B2	B
B5	B
B9	B
B15	B
Q0	B
32U	D
38U	A
45U	A
82U	A
85U	A
42Z	C
95Z	C

As shown in Figure 24, a strong signal was observed in the positive control composed of cells where pDM128fLuc and pREC-nfl-HIV were co-transfected.

However, a positive signal was also seen in the negative control where triple-transfected cells were incubated with untransfected cells that do not express any envelope on their surface. A strong luminescence signal was also seen in the negative control where the cells triple-transfected with the plasmids for the receptors were incubated with the cells transfected with SG3 $\Delta$ env that contains a premature stop codon in *env* and thus should not express any envelope that could mediate fusion. Due to this, it can be concluded that the signals seen in the case of the test groups were not true positives and are a result of background level of luciferase activation. The proof-of-concept triple-transfection revealed that only ~3.6% of the cells could express the genes-of-interest from each plasmid. Thus, the lack of fusion seen here could be attributed to the scarcity of CD4 and CCR5 expression on the triple-transfected 293T cells.

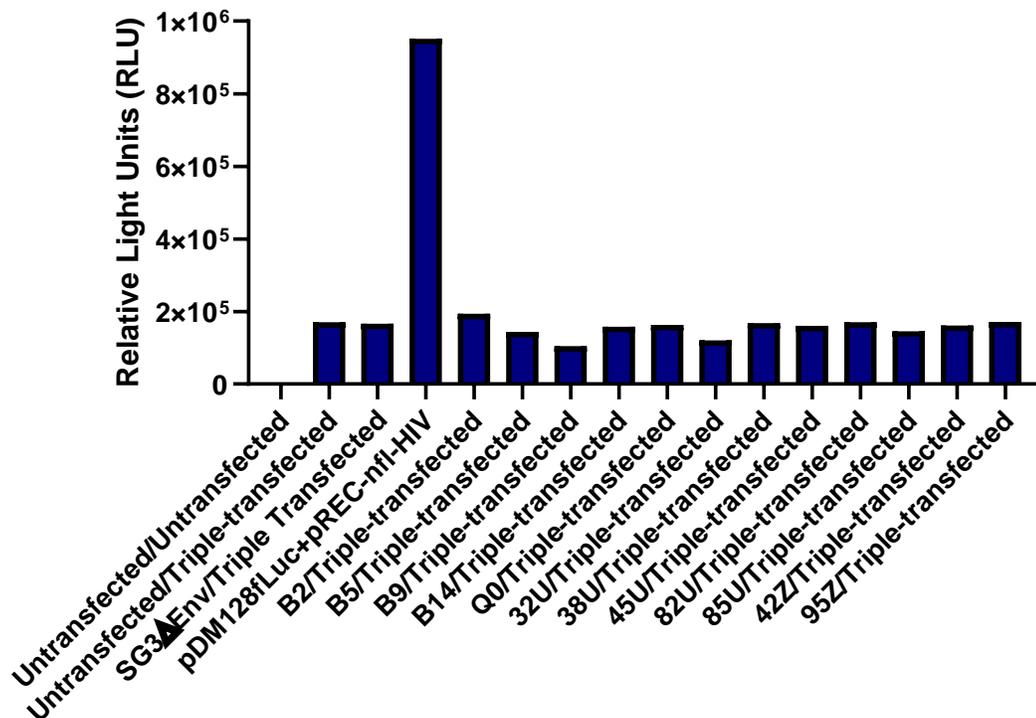


Figure 24. Cell-to-cell fusion assay (luciferase system) of 293T triple-transfected with huCD4, huCCR5 and pDM128fLuc, incubated with 293T cells transfected with varying version of the pREC-nfl-HIV plasmid for HIV envelope expression. The notation under each bar indicates the two cell types that were incubated together. pDM128fLuc and pREC-nfl-HIV were transfected into the same cell as a positive control. SG3Δenv is composed of the HIV backbone that contains a premature stop codon in *env*, thus the plasmid serves as a negative control. B2 through 95Z are distinct HIV strains with different envelope sequences.

### 3.5.2 The cell-to-cell fusion assay using luciferase detection produces high background luminescence

As the prior experiment demonstrated that triple-transfection resulted in low CD4 and CCR5 expression, the luciferase assay was repeated to test whether fusion is detectable in cells stably expressing the two receptors. The pDM128fLuc plasmid was transfected into either U87 or CRFK cells expressing human CD4 and CCR5, whereas the pREC-nfl-HIV plasmids were transfected into 293T cells. The U87.CD4.CCR5 and huCD4/huCCR5 CRFK were incubated with 293T expressing varying HIV envelope.

In the fusion assays with these cells, a strong signal was detected in the positive controls where pREC-nfl-HIV and pDM128fLuc were co-transfected for both the receptor expressing cell lines (Figure 25). In the case of the U87.CD4.CCR5 cells, a positive luminescence signal, indicative of fusion with the 293T cells expressing the envelope of varying HIV subtypes (32U through Q0 in Figure 25), was higher than negative controls in which 293T cells were untransfected or transfected with a non-functional envelope (SG3 $\Delta$ env). However, no drastic difference in luminescence was seen in these when compared to the negative controls (1.5-fold to less than 3-fold).

The background level of luciferase expression for the negative control (untransfected 293T) was over five times higher with CRFK cells in comparison to the U87 cells. This trend is observed across all the samples, where huCD4/huCCR5 CRFK had higher overall levels of luciferase expression in comparison to the U87.CD4.CCR5 cells. Luminescence levels for all treatments were similar for CRFK cells. Therefore, no conclusions can be drawn about whether the signal produced wherein the CRFK cells were incubated with 293T cells expressing HIV envelope was truly due to fusion or simply due to the background expression of luciferase.

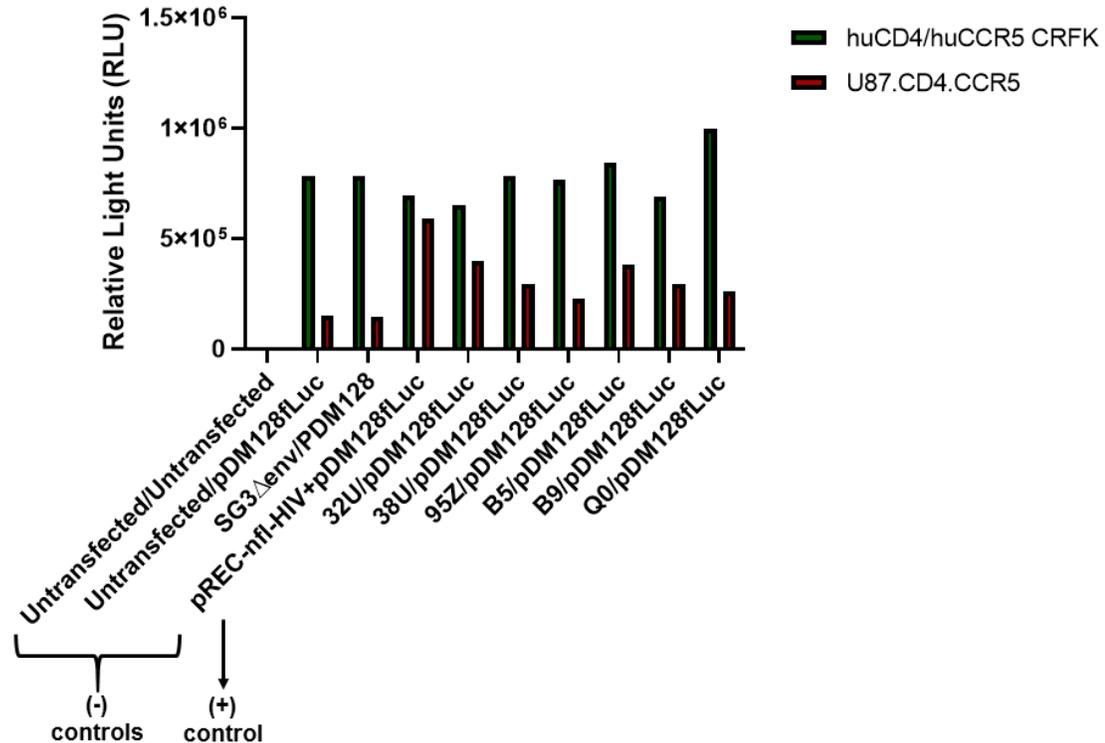


Figure 25. Cell-to-cell fusion assay (luciferase system) of CRFK or U87 cells expressing human CD4 and CCR5 receptors, incubated with 293T cells transfected with a varying pREC-nfl- HIV plasmid for HIV envelope expression. The notation under each bar indicates the two cell types that were incubated together. The first listed transfection status under each bar is of the 293T cells (expressing HIV envelope from distinct pREC-nfl-HIV) and the second listed transfection status is that of the CRFK/U87 CD4/CCR5 cells (transfected with pDM128fLuc). The positive control is comprised of U87.CD4.CCR5 or CRFK.huCD4.huCCR5 cells that were co-transfected with pREC-nfl-HIV and pDM128fLuc. Constructs 32U through Q0 indicate different pREC-nfl-HIV plasmids containing envelope sequences from different strains.

### 3.5.3 The cell-to-cell fusion assay with $\beta$ -galactosidase detection demonstrates human receptor-HIV envelope interactions

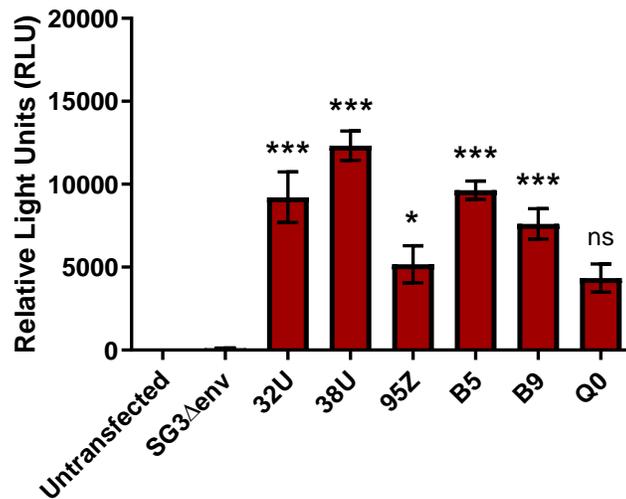
Since the luciferase system produced a high background level of luminescence, the  $\beta$ -galactosidase system was adopted to test for the presence of receptor-envelope

interactions. U87.CD4.CCR5 and huCD4/huCCR5 CRFK cells were transfected with the  $\omega$  plasmid of the  $\beta$ -galactosidase gene, whereas the 293T cells were transfected with a version of pREC-nfl-HIV, along with the  $\alpha$  plasmid of the  $\beta$ -galactosidase gene.

In the case of the U87.CD4.CCR5 cells, there was a significant difference between the luminescence level of the negative control (293Ts transfected with *env* mutant SG3 $\Delta$ env) and 293T cells expressing envelope from 32U, 38U, 95Z, B5 and B9 (Figure 26a). These samples serve as a positive control for the fusion assay itself as we expect HIV envelope expressed on 293T to interact with human CD4 and CCR5 expressed on U87 cells. This was shown by Weber et al. (2013)<sup>74</sup>.

The novel aspect tested here was the functionality of the assay with CRFK cat cells expressing human CD4 and CCR5 receptors. As seen in Figure 26b, there was a significant difference between the luminescence level of the negative control (SG3 $\Delta$ env) and three subtype B Envs (B5, B9, Q0). The level of fusion, as quantified by the RLU, was much greater between the subtype B Envs and huCD4/huCCR5 CRFK, than the subtype B Envs and U87 cells. Although not statistically significant, there was an increase in the mean RLU of all three of the non-B subtypes when compared to SG3 $\Delta$ env.

a) U87.CD4.CCR5 cells



b) huCD4/huCCR5 CRFK cells

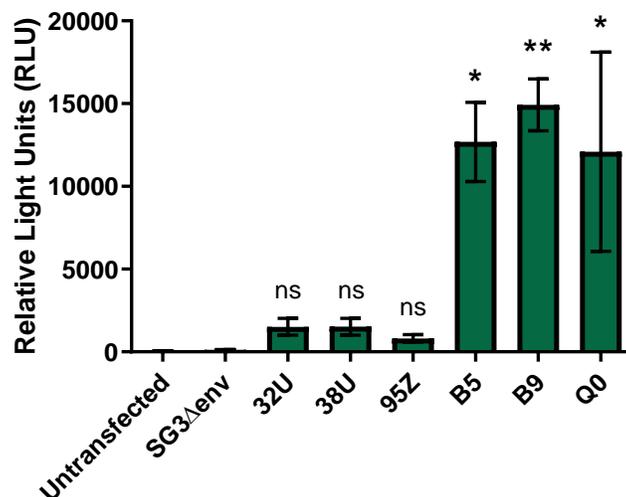


Figure 26. Cell-to-cell fusion assay of CRFK cells and U87 cells expressing CD4 and CCR5 with 293T cells expressing a distinct strain variant of Env. The assay was done using the  $\beta$ -galactosidase system. The CD4/CCR5 receptor-expressing cell lines were transfected with the  $\omega$  plasmid, whereas the 293T cells were transfected with the  $\alpha$  plasmid in addition to pREC-nfl-HIV. The X-axis labels represent the plasmids that the 293T cells were transfected with. a) U87.CD4.CCR5 fusion with 293T cells. b) huCD4/huCCR5 CRFK fusion with 293T cells.

n=3. “\*”, p<0.05; “\*\*\*”, p<0.01; “\*\*\*\*”, p<0.001. Error bars represent the standard error of the mean. A one-way ANOVA test followed by the Tuckey’s test was performed.

## CHAPTER 4: DISCUSSION

The long-term vision of this project is to establish a novel animal model whereby cats can be utilized to study HIV and develop vaccines and therapeutics against it. We are seeking to harness the similarities between HIV and FIV at the genetic level, as well as their comparable disease pathogenesis for our efforts. In particular, our aim is to generate FHIV $env$  chimeras (composed of HIV  $env$  inserted in the FIV backbone), that can infect and replicate in cats. Following this, therapeutics and vaccines targeting the HIV envelope can be tested in cats. The goal of this thesis was to lay the groundwork for this project, mainly through validating this concept *in vitro*.

We attempted to make use of the yeast cloning system to make the plasmids needed to produce FHIV $env$  chimeric viruses. Prior to this, we first had to test whether the system could be used to clone the plasmids required to generate infectious FIV particles. The yeast cloning system was used to construct two plasmids – a cplt supplying the 5' LTR, and the nfl supplying the remainder of the FIV genome, which were co-transfected to produce virus. We engineered these with the aim of eventually swapping the FIV  $env$  with HIV  $env$  in the nfl plasmid to make FHIV $envs$ . The FIV particles produced using this two-plasmid system were expected to have lower infectivity, as particles produced would contain either two copies of the nfl RNA, two copies of the cplt RNA, or a copy of each<sup>73</sup>. Infectious, replication-competent virus would only be produced in the third scenario, where both nfl and cplt are supplied. Thus, we expected longer infection periods and multiple passages on Gfox cells (which express CD134, the primary entry receptor for FIV) to see a positive signal on the RT assays of virus produced using the complementing system. We attempted to utilize several variations of the system including multiple passages of the virus on cells, cloning of the RRE into cplt and altering the ratio of nfl to complement. The rationale for using each of these is described in the Results.

The last variation we attempted were virus spinoculations. This is a process whereby the virus and the cells to be infected are centrifuged for several hours to facilitate virus deposition, which forces virus attachment to the cell surface<sup>78</sup>. Spinoculations have been previously used successfully for infections of viruses such as

murine coronaviruses<sup>79</sup>, Herpes Simplex Virus 1<sup>80</sup>, Hepatitis B Virus<sup>81</sup>, and HIV<sup>78</sup>. Additionally, spinoculations have also been shown to enhance FIV infection of monocyte-derived dendritic cells<sup>82</sup>. Thus, the protocol was utilized to infect Gfox cells with the virus produced from the ratio-controlled transfections of 293T cells with the *nfl* and *cplt-rre* plasmids. We found that FIV infectivity was enhanced in the positive control as positive RT activity was detected as early as Day 4 of the second passage (Figure 14). However, no such increase in activity was seen with the spinoculations of ratio-controlled *nfl-6/cplt-rre* viruses. Nevertheless, we were only able to observe weak RT activity from the FIV produced using the two-plasmid system.

The yeast cloning system has been used successfully to generate vectors for transfections to produce infectious HIV<sup>73,83</sup>, as well as SHIV<sup>76</sup>. It has also been implemented to produce infectious FIV particles (Moghadasi, S.A., Arts, E.J, unpublished data). However, this has only been the case with the 34TF10 strain, and not the FIV-C36 strain used here. Additionally, Sanger sequencing revealed several point mutations across the *nfl* vector. These mutations could have potentially resulted in decreasing the infectivity of the particles produced.

In-fusion cloning was additionally attempted to clone the HIV envelope into the FIV backbone, however, either the backbone plasmid of FIV, or insert plasmid of HIV carried through across the cloning reaction steps. This was the case even after gel extracting the PCR amplified fragments of either of the two and treating the product with DpnI, which should cleave any methylated DNA, thereby cleaving any carry-through plasmid. Several other strategies and modifications could be tested in the future that were not tested herein due to time constraints. Gibson cloning is a potential strategy that can be pursued<sup>84</sup>. The In-fusion cloning kit should not pose any issues with cloning large vectors; however, part of the issue here was PCR amplifying the large backbone around the FIV plasmid. The In-fusion cloning reaction should work with multiple fragments as well. A strategy can be implemented wherein the FIV plasmid is amplified as two separate fragments with the overlapping regions needed for In-fusion cloning.

In addition to generating infectious virus, another major aim of this project was to generate cat cell lines expressing human CD4 and CCR5, as well as feline CD4 and

CCR5, in order to test HIV Envelope and CD4/CCR5 receptor interactions through infections and fusion assays. CRFK cells were utilized as they are adherent, robust, and relatively transfectable.

Single-positive cell lines were generated to express either feCD4 or feCCR5. Colony 9, the colony with the greatest level of feCCR5 expression was used for transfections with feCD4 to generate double positive cell lines. However, only 8.6% of the total live cells of Colony 20 were positive for feCD4 and feCCR5, and this was the colony with the highest percentage of double-positive cells. This was the case even after several passages in double-drug (puromycin and hygromycin) media. To ensure that the feCD4 gene was in fact being expressed, RNA isolation using Trizol extraction was conducted 48 hours post- transfection of CRFK. This was DNase treated and used for cDNA synthesis, followed by PCR amplification with primers specific for the feCD4 gene. Results revealed that feCD4 was amplified, and the transfection was indeed successful (data not shown). feCD4 expression was also supported by the result that up to 52.7% of the cells in Colony 3 of feCD4 CRFK were positive for the receptor.

One possible explanation for obtaining a low level of feCD4/feCCR5 expression could be poor initial transfection efficiency of pBABE.feCD4 into the feCCR5 CRFK cell line. Another reason for this could be that the single colonies that were initially picked and grown for screening were low-expressors for feCD4 to begin with. Even after several passages in selection media, the level of feCD4 that the colony initially expressed would have remain unchanged, i.e., survival in drug-containing media would not necessarily guarantee high expression of feCD4 on the surface. To obtain a population with a higher level of double expression, Colony 3 of feCD4 CRFK, which was most positive for feCD4 by flow cytometry, could be transfected with the pBABE.feCCR5 plasmid. Furthermore, several colonies throughout these cell line generation experiments grew well in drug-containing media but showed poor expression of either receptors when screened by flow cytometry, indicating that the poor expressors were still acclimating to grow in the selection media. These colonies were passaged for up to 10 weeks before being screened. To avoid pursuing these colonies, flow cytometry can be conducted earlier in the process to eliminate any colonies that are low-expressors to begin with.

Only the colonies that demonstrate moderate to high expression can be grown and passaged, and re-screened by flow cytometry prior to sorting to achieve a pure population.

We successfully generated a huCD4/huCCR5 CRFK cell line in this project, which we pursued further for the cell-to-cell fusion assay as well an infectivity assay. The fusion assay has only been conducted using 293T cells and U87.CD4.CCR5 cells<sup>74,75</sup> in the past at the Arts Lab. Therefore, it was an important proof-of-concept experiment to demonstrate that the assay can be conducted using CRFK cells that stably express CD4 and CCR5 receptors. The luciferase system of cell-to-cell fusion assay produced too high background luciferase expression. Following this, the  $\beta$ -galactosidase system was implemented.

As seen in Figure 26, cell-to-cell fusion occurred in the case of huCD4/huCCR5 CRFK cells, as is the case with U87.CD4.CCR5 cells. There was a significant increase in mean RLU in all three of the non-B subtypes tested, and two of the subtypes B Envs tested in the case of the U87.CD4.CCR5 cells. In the case of the huCD4/huCCR5 CRFK, there was a significant increase in RLU of all the subtype B Envs. Although not significant, there was an increase in the mean RLU of the three non-B subtypes Envs, when compared to the negative control where SG3 $\Delta$ env was transfected. Interestingly, the level of fusion seen between the CRFK cells and subtype B Envs is a lot greater than the level of fusion seen between the U87 cells and subtype B and non-B Envs. Platt et al. (1997) have demonstrated that in cases where there is high-level expression of CD4, a low level of CCR5 is enough to obtain maximal infection. In cases where CD4 expression is low, a larger quantity of CCR5 is needed for maximal infection with macrophage-tropic HIV-1<sup>85</sup>. In contrast to this, Alexander et al. (2010) have demonstrated that in subtype C variants, high levels of CD4 and CCR5 are required for virus entry, and a decline in CCR5 was accompanied with a reduction in entry<sup>86</sup>. Lastly, Etemad et al. (2009) demonstrated that chronic subtype A Envs have an increased ability to replicate in cells with low CCR5 expression<sup>87</sup>. Although not much is known about the level of expression of either receptors on the cell surface of U87 cells, CD4 expression on the CRFKs is higher than CCR5, as seen in the flow cytometry performed post-sorting (Figure 20). This is most likely because the most positive huCD4-expressing CRFK

colony was used for huCCR5 transfections. If this is the case, it could potentially explain why Q0 Env, whose envelope is derived from a chronic virus, is able to fuse more efficiently with the CRFK cells.

This fusion assay was conducted using feCD4/feCCR5 (Colony 20), however, fusion was found to not occur (data not shown). This does not necessarily indicate that HIV envelope cannot bind to feline CD4 and CCR5. Much like in the case of triple-transfection of 293T cells (Figure 23), the lack of fusion events can most likely be attributed to the scarcity of feCD4/feCCR5 double-positive cells. We did discover that the CCR5 antibody that is cross reactive for human, mouse, and rat CCR5 was able to bind and detect feline CCR5 in the flow cytometry experiments, which could potentially be due to the sequence similarity of human and feline CCR5. It would therefore be plausible for HIV envelope to be able to bind feline receptors. In the future, the most positive feCD4 CRFK cell colony, Colony 3, can be used for transfections with feCCR5 to produce a cell line with higher double positivity.

The huCD4/huCCR5 CRFK (colony M) further demonstrated receptor positivity in infection experiments (Figure 22), where infection with HIV-AD8 resulted in strong, positive RT activity. The assay demonstrated that the RT activity of AD8 (1) and AD3 (3) increased on Day 5, whereas the activity of AD8 (2) remained relatively unchanged, as its signal is similar to that of Day 4. Munk et al. (2007) conducted a similar experiment where HIV-1<sub>NL-BaL</sub> was used to infect CRFK expressing human CD4 and CCR5<sup>88</sup>. Although capable of a first-round of infection (evident due to syncytia formation), no spreading infection was seen, as indicated by p24 ELISA levels equivalent to background levels. They attributed this lack of spreading to certain types of feline APOBEC3, including APOBEC3H and APOBEC3CH, which were packaged into the released virus particles, and elicited their effects by introducing G→A mutations in reverse-transcribed intermediates in the second round of infection. HIV Vif typically counteracts human APOBEC3, however, it is incapable of countering feline APOBEC3 similarly. Therefore, the RT positivity could be a result of the first round of AD8-HIV infection. Interestingly, they found syncytia formation on Day 3-4 of infection, but continued to monitor p24 levels by ELISA up to Day 12. Our experiment ended by Day 5, where we saw syncytia

formation, but the majority of the cell layer was destroyed, and so the virus had to be harvested. Part of this obliteration could be due to starting with too high of a virus input. Munk et al. (2007) infected their huCD4/huCCR5 CRFK with a low Multiplicity of Infection (MOI) of 0.05 to evaluate multiple cycles of infection. MOI is the ratio of virus to cells. The lack of difference seen between Day 4 and Day 5 of AD8 (2) infection in our case could be attributed to a halt of spread seen after the first round of infection. Although we did not control for MOI, it would be of interest to begin with a low MOI virus to infect huCD4/huCCR5 CRFK with HIV-AD8 and monitor this by RT assays with supernatants past Day 5 to see if RT activity decreases due to the halting of secondary spreading caused by feline APOBEC3. Nevertheless, the occurrence of any infection at all gave us confidence in the receptor expression of huCD4/huCCR5 on the CRFK cells.

Overall, our long-term mission is to generate a novel animal model whereby cats can be used to study HIV. The goal of this thesis was to establish the foundation for this project by making the FHIV*envs* and cell lines required for the *in vitro* experiments. Although the yeast cloning and in-fusion cloning strategies were ineffective, alternative cloning strategies can be implemented to generate the FHIV*env* chimeras. We successfully generated a huCD4/huCCR5 CRFK cell line expressing high levels of the two receptors. Although a feCD4/feCCR5 CRFK cell line was engineered, it only had low-level feCD4 expression. A cell line that has a greater level of feCD4 expression can be engineered using some of the strategies described above. Following this, cell-to-cell fusion assays can be conducted with the diverse pREC-nfl-HIV to isolate HIV envelopes that can utilize feline CD4/CCR5 to enter and infect cat cells. These HIV *envs* can be cloned into the FIVC-36 backbone, following which they can be used for infectivity assays with the CD4/CCR5-expressing CRFK cell lines to isolate the FHIV*envs* capable of replicating in cat cells *in vitro*. These can then be pursued for *in vivo* infection of cats. Overall, if successful, this project will allow for cats to be used as model organisms to test therapeutics and vaccines that target the HIV envelope. Their cost-effectiveness and suitability make cats good candidates for this purpose.

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### Publications

- 1) Hendrikse LD, Kambli A, Kayko C, et al. Identification of a novel gammaherpesvirus in Canada lynx (*Lynx canadensis*). *Viruses*. 2019;11(4):363. doi:10.3390/v11040363.
- 2) Cheng P, Kambli A, Stone J. Skeleton growth under uniformly distributed force conditions: Producing spherical sea urchins. *Int J Astrobiol*. 2017;16(4):343-348. doi:10.1017/S1473550416000409.